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The Predictive Role of Serum C16 Ceramide and C24 Dihydroceramide in Patients with Hepatocellular Carcinoma

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ABSTRACT
Background: Ceramides are structural long-chain lipid molecules essential for cell architecture, with many biological functions involving cell proliferation, inflammation, apoptosis, and cell death. Ceramides have a diverse role in carcinogenesis depending on their chain length, metabolism, and localization. Disturbance in ceramide metabolism has been observed in many cancers such as head and neck, colorectal, lung, and liver. **Objective:** The current study aimed to use a high-performance liquid chromatography-tandem mass spectrometry analytical method for the measurement of C16 ceramide and C24 dihydroceramide in the serum of patients with hepatocellular carcinoma and chronic liver cirrhosis and to verify their potential role as markers of hepatocellular carcinoma.
Patients and methods: This case control study enrolled 100 patients diagnosed with hepatocellular carcinoma and chronic liver cirrhosis (n= 50 each) and 50 healthy subjects as a control group. Serum samples were subjected to separation of ceramides by ultra-performance liquid chromatography and quantitative detection and measurement by tandem mass spectrometry. The assay method allowed accurate measurement of C16 ceramide and C24 dihydroceramide at a serum concentration <2.5 ng/ml. **Results:** The calibration curves' linearity had regression coefficients >0.98, with intra-day and inter-day precision and accuracy ranging from 89%-102%. C16 ceramide and C24 dihydroceramide were significantly higher in hepatocellular carcinoma and chronic liver cirrhosis patients than in healthy subjects ($P<0.05$). Their serum levels were not different in liver cirrhosis and hepatocellular carcinoma ($P>0.05$) but showed an increasing trend to be higher in hepatocellular carcinoma. Both ceramides did not correlate with the clinical parameters of hepatocellular injury as Child-Pugh score, BCLC stage, or the burden of tumor of all $P>0.05$. **Conclusion:** Quantitative measurement of C16 ceramide and C24 dihydroceramide by tandem mass spectrometry is an accurate and precise method for laboratory workup in patients with chronic liver cirrhosis.
Keyword: C16 Ceramide, C24 Dihydroceramide, High-performance liquid chromatography-mass spectrometry, Hepatocellular carcinoma, Chronic liver Disease, Case control study, Menoufia University.

INTRODUCTION

The development of hepatocellular carcinoma (HCC) is a multistep, complex process associated with genetic modifications and genomic alterations presumably related to the underlying risk factors [1,2]. The risk factors for developing HCC vary with the geographic distribution, e.g., in Egypt and Japan are infection with hepatitis C virus (HCV), in China are hepatitis B virus (HBV) infection, in Europe and USA is infection with HCV, non-alcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD) [3].

Cirrhosis of the liver, consumption of aflatoxin B1, smoking, and diabetes mellitus are additional risk factors [1,2]. Patients who have underlying liver disease, disturbance in ceramide metabolism have been reported [4]. Ceramides (Cer) are a class of molecules with more than 200 species of different chain lengths identified in various tissues [5]. Ceramides can be synthesized de novo in the endoplasmic reticulum from serine and palmitoyl-Co A via a multistep process involving the action of several enzymes as serine palmitoyltransferase, dihydroceramide synthases, and dihydroceramide desaturase [6,7].

Sphingomyelinases, which are triggered by TNF-α, Fas ligands, and oxidative stress, may hydrolyze sphingomyelin in the endo-lysosome to generate ceramides [8]. The salvage process, also known as the sphingolipid-recycling pathway, is the third mechanism that leads to the production of ceramides. This pathway is more complicated and contains a number of enzymes, including ceramidases, dihydroceramide synthases, and sphingomyelinases. Complex sphingolipids are broken down in the lysosomes and plasma membrane into sphingosine, which can then be converted back into ceramide by ceramide synthases [9,10].

The pathway that is chosen to be engaged in order to produce ceramide may be influenced by a variety of stimuli, for example, heat shock proteins and certain chemotherapeutic drugs can both activate the de novo pathway [11]. Ceramides play a variety of biological roles in cellular differentiation, proliferation, trafficking, cell cycle arrest, autophagy, and apoptosis [12-14]. Depending on the length of their fatty acid chains, ceramides may either promote or prevent tumour growth [15]. In preclinical models, the ceramide pathways develop into a viable target for several anticancer medications due to their capacity to trigger apoptosis [6,16].

Long-chain ceramides have been linked to advanced carcinogenesis often, and it has been proposed that they may serve as cancer biomarkers for early diagnosis [16]. Depending on the fatty acyl chain they possess, the members of the ceramide and dihydroceramide families differ from one another. High serum concentrations of C16Cer, C18Cer, C18:1Cer,

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and C24:1Cer were also linked to advanced colorectal cancer. Serum levels of C16Cer were likewise linked to advanced metastatic breast cancer and tumors stages [17,18].

The aim of the current study was to verify a high-performance liquid chromatography-tandem mass spectrometry analytical method for quantitative measurement of C16 ceramide (C16Cer), and C24 dihydroceramide (C24DHC) in serum of patients with HCC and chronic liver disease (CLD) and verify their possible predictive potential of HCC.

PATIENTS AND METHODS

The study took place in the Department of Clinical Biochemistry and Molecular Diagnostics, National Liver Institute, from October 2019 to August 2021.

The study enrolled three groups of patients:

The HCC group (n= 50) enrolled patients with HCC complicating chronic HCV infection. The diagnosis of HCC was established by detecting focal hepatic lesion(s) on ultrasound examination confirmed by arterial hyper-vascular focal lesions with rapid washout by tri-phasic spiral CT, MRI, and the finding of elevated serum AFP >200 ng/ml and or detection of HCC on histological examination of the liver biopsy.

The CLD group (n= 50) enrolled patients with liver cirrhosis secondary to previous HCV infection. The diagnosis of liver cirrhosis is based on finding the characteristic echogenic pattern of liver cirrhosis by ultrasound examination, fibro scan at ≥14.5 kilopascals, and positive anti-HCV antibody and HCV- RNA PCR tests.

The control group (n= 50) enrolled healthy subjects, matching the age and gender of the other groups with no clinical, laboratory, or imaging indication of liver cirrhosis or focal hepatic lesions. The control subjects were also free from apparent systemic diseases such as cardiovascular disease, obesity, chronic cholecystitis, drug abuse, and alcohol consumption.

HCV and HBV co-infection, metastatic liver tumors, obstructive gall bladder illnesses, comorbid renal or inflammatory ailments, and lipid-lowering medication use are all exclusion factors. None of the research participants had ever abused illegal drugs or used alcohol.

Blood samples were collected from patients and control subjects after overnight fasting (8-12h) under an aseptic venipuncture. Blood samples were divided into standard color-code tubes for serum separation, complete blood count, and blood chemistry analysis. The collected serum for C16 Cer and C24CDHC was stored at - 80°C until LC/MS analysis. The blood chemistry was examined using an automated biochemical analyzer (Beckman Coulter AU840, Ltd, USA), which also tested the liver function, renal function, serum cholesterol, and triglycerides. Auto-analyzer Abbott Architect with AFP III Gen kits (Abbott, Park, IL USA) were used to measure fetoprotein. CBC was performed by a semi-automated Sysmex analyzer (Siemens, Germany) and INR by (The Sysmex CA-600 Systems, Siemens, Germany).

Chemicals and reagents: Methanol, isopropanol, acetonitrile, chloroform, and formic acid were of HPLC grade, C16 ceramide external standard, and C24 dihydroceramide external standard and purchased from Sigma Chemical Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Type 1 water was used from a Millipore Milli-Q water purification system (Thermo Fischer, USA).

Standard and quality control used for UPLC-MS/MS method development: Matrix and standard preparation: A matrix-free endogenous lipid was prepared by extraction of lipids from serum by chloroform-methanol mixture as described by **Ferraz *et al.*** [19]. Briefly, 1ml of human pooled serum mixed with 10 ml of chloroform-methanol 2:1(v/v) was agitated manually for 20 seconds and then centrifuged at 2500×g for 10 min at 20C. The aqueous phase was collected and stored at – 20ºC. The lyophilized powders of C16 Cer and C24 DHC were dissolved in a solution of methanol: chloroform (1:1) at 1mg/ml. A working solution of each ceramide was prepared at 2000 ng/ml and ten-point standard solutions ranging from blank to 2000 ng/ml in the previously prepared matrix and used as external standard calibration points. Quality control (QC) samples were prepared as a low QC (LQC=250 ng/ml) and high QC (HQC=2000 ng/ml) using the same matrix and used to assess the intra-day and inter-days performance of the assay.

Standard Calibration curves of C16 Cer and C24DHC: Ten-point calibration curves (0, 12.5, 25, 50, 100, 125, 250, 500, 1000, 2000 ng/ml) were prepared by serial dilution into the prepared matrix (**Figure 1**)**.** Using weighted (1/x2) least square regression analysis, calibration curves were created by plotting the ratio of analyte to external standard peak regions against the matching analyte concentration. By calculating the slope, intercept, and coefficient of determination of the 1/ (concentration) 2 weighted regression line, the linearity of the ten-point calibration curve was examined. Six out of ten calibration points required to fulfil the acceptance requirement, which was set at 15% of the nominal concentration for a calibration curve. LQC and HQC sample performance was evaluated in triplicate to ascertain precision and accuracy. By determining the percent deviation (% dev.) from the nominal concentration, accuracy was assessed. The coefficient of variation (% CV) of replicates within each batch was calculated to determine precision. Precision and accuracy acceptance standards were set at 15%. Recovery was evaluated by comparing spiked and unspiked serum samples prepared from the same serum pool. The mean recoveries were 102 (SD 7) % for C16Cer and 101 (SD 9) % for C24DHC **(Table 1).**

Table (1): Performance of the optimized tandem mass spec method for the quantitative detection of C16 Cer and C24DHC.

Analyte	Actual (ng/ml)	Measured (ng/ml) M ± SD	Inter-days (n=10)		Intra-days (n=10)		Recovery (%) (ng/ml) M ± SD
			Precision CV%	Accuracy	Precision CV%	Accuracy	
C16 Cer	0	0.9 ± 0.05	1.68	102	2.04	102	---
	250	222 ± 34	15	89	8.95	93	98.3 ± 8.23
	2000	1705 ± 218	12.8	85	7.07	90	91.48 ± 3.56
C24DHC	0	1.19 ± 0.28	2.95	112	3.16	103	---
	250	226 ± 37	16.5	90.5	5.2	92.4	95.5 ± 3.16
	2000	1716 ± 195	11.4	85.8	7.9	89.4	101.37 ± 8.25

CV: coefficient of variation. C16 Cer: C16 ceramide, C24DHC: C24 dihydroceramide, CV: Coefficient of variance.

Sample preparation:

Based on **(Barr et al., 2010)** [20], Proteins were precipitated from defrosted sample (50 ul) by adding four volumes (200ul) of ice cold methanol in 1.5 ml microtube, then vortexed, centrifuged at 13500 rpm for 10 minutes, the supernatant was transferred into plate for injection.

Chromatographic separation and Tandem mass spectrometry analysis:

Chromatographic separation was carried out using a triple quadruple tandem mass spectrometer (ACQUITY UPLC H-Class. Waters Corporation, MA, USA) using an analytical column (ACQUITY UPLC BEH C18; 1.7 m; 2.1x100mm; Waters); and a column heater (ACQUITY UPLC BEH C18; maintains column at 50°C). With a gradient and a flow rate of 0.28 ml/min, standards of C16 ceramide (d18:1/16:0) and C24 dihydroceramide (d18:0/24:0) were eluted. Water/acetonitrile (80:20, v/v) mobile phase (A) contained 0.1% formic acid.

Isopropanol/acetonitrile (80:20, v/v) mobile phase (B) contained 0.1% formic acid. After injection, the samples were first eluted with 100% mobile phase A for 1 min, followed by a gradient of mobile phase (B) from 30% to 100% over the next 5.5 min, then mobile phase (A) from 70% to 100% over the next 3 min.

The injection volume was 3 ul, and the overall run time for the analysis was 9.5 min. Prior to injecting the next sample, the column was equilibrated with 100% (A) for 1.5 minutes. Multiple reaction monitoring (MRM) was used to measure analytes as the mass spectrometer ran in positive ESI mode. MassLynx software was used to acquire the data, and TargetLynx software was used to quantify it.

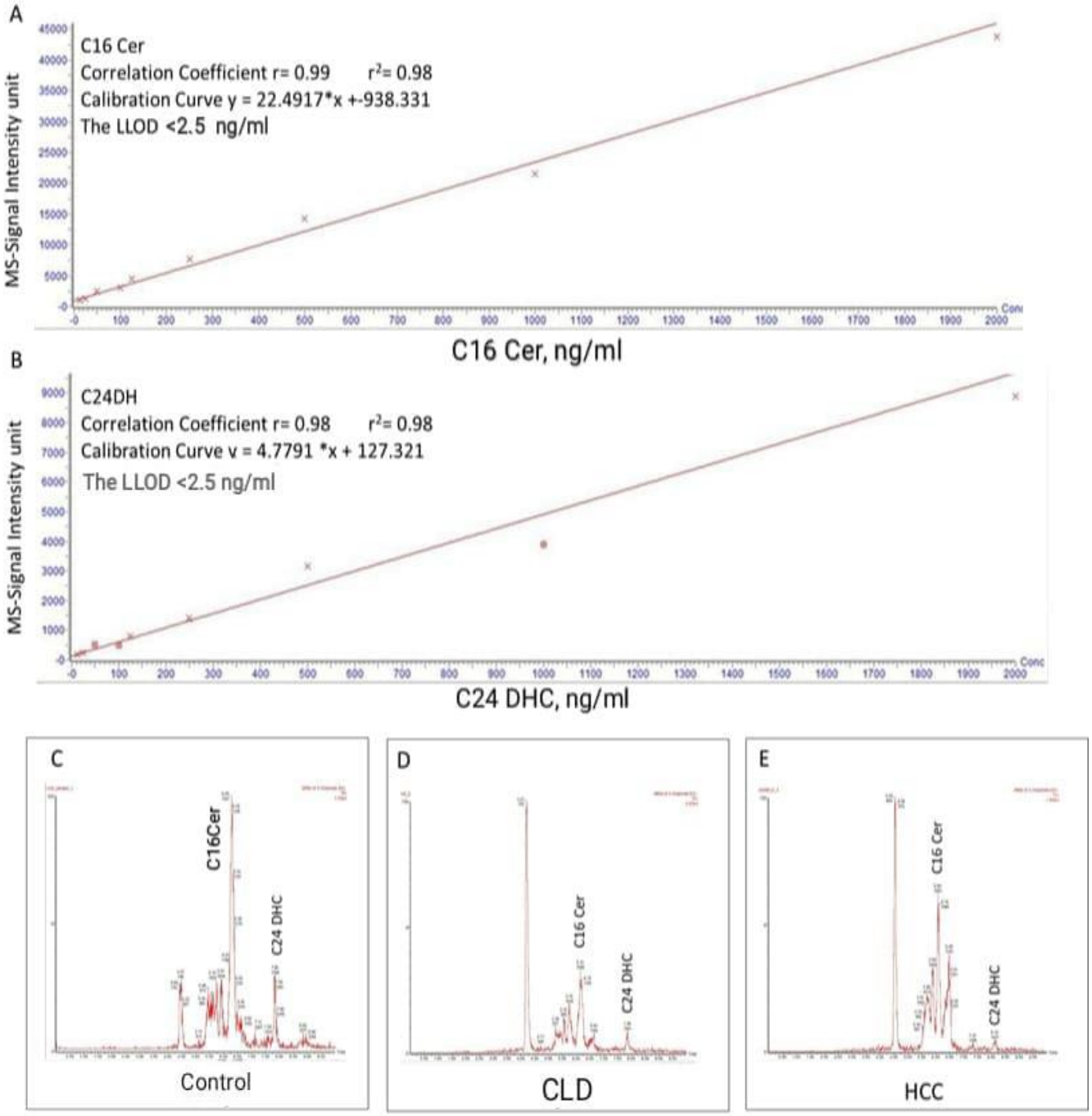


Figure (1): A-B linear regression curves of the measured and the expected concentration: analysis assessed by plotting the actual value of C16C and C24DHC standards, 10 serially diluted points from 2000 ng/ml to zero point. Coefficient r =0.99 and the r^2 =0.98. **C- E**; Chromatogram showing the identified MS-MS peaks of the C16Cer and C24DHC in control, HCC, and CLD samples. control, CLD: Chronic liver disease, HCC: Hepatocellular carcinoma. C16C: C16 ceramide, C24DHC: C24 dihydroceramide.

Ethics Consent:

This study was ethically approved by the Institutional Review Board of The National Liver Institute (IRB 00135/2019 INTM). Written informed consent was obtained from all participants. This study was executed according to the code of ethics of the World Medical Association (Declaration of Helsinki) for studies on humans.

Statistical Analysis:

IBM SPSS 23 was used to analyze the data (SPSS Inc., CA, USA). Qualitative data were defined as numbers and percentages. Quantitative data were tested for normality by Kolmogorov-Smirnov test. Normal distribution of variables was described as means and SD. The significance in multiple comparisons was determined using the non-parametric statistical Kruskal-Wallis test together with the Mann-Whitney test. To find a linear trend across the various groups, the linear contrast analysis, a one-way ANOVA and univariate analysis combination, was performed. The serum C16 Cer and C24DHC were evaluated for their sensitivity and specificity in identifying individuals with liver disorders using the receiver operating characteristic (ROC) curve. A significant-good or outstanding test to distinguish between two groups was defined as AUC ≥ 0.8 [21, 22]. P value ≤ 0.05 was considered to be statistically significant.

RESULTS

The clinical and demographic characteristics of the enrolled groups are summarized in **Table 2**. To reduce the impact of biological and lifestyle variables, patients were matched for age, gender, and body mass index (BMI). Age, gender, and BMI did not differ significantly across groups.

Table (2): The clinical and demographic characteristics of the enrolled groups (n= 50 each).

Variable	Control	CLD	HCC
Age, mean (SD) (P>0.05)	47.4 ± 10.4	46.4 ± 10.3	48.4 ± 9.7
BMI (kg/m^2) (P>0.05)	23 ± 2	23 ± 2	25 ± 1
Sex	N (%)	N (%)	N (%)
Male	23 (46)	26 (52)	43(86)
Female	27 (54)	24 (48)	7 (14)
HCV			
Yes	5 (10)	50 (100)	50 (100)
No	45 (90)	0 (0)	0 (0)
HFL			
Single	20 (40)	0 (0)	0 (0)
Multiple	30 (60)	0 (0)	0 (0)
DAAs			
Yes	---	13 (26)	21(42)
No	---	37 (74)	29(58)
DM			
Yes	0 (0)	14 (28)	16 (32)
No	50 (100)	37 (74)	34 (68)
HYP			
Yes	0 (0)	10 (20)	9 (18)
No	50 (100)	40 (80)	41 (82)
Child-Pugh			
A		31(62)	37(74)
B		12(24)	7(14)
C		7(14)	6(12)
BCLC staging			
A	---	---	7(14)
B	---	---	35(70)
C	---	---	8(16)

CLD: Chronic liver disease, HCC: Hepatocellular carcinoma. BMI: Body mass index, DM: Diabetes mellitus, HCV: Hepatitis C virus, HFL: Hepatic focal lesion, DAAs: Direct acting anti-virus, BCLC: Barcelona clinic liver cancer.

The mean serum levels of C16Cer and C24DHC in HCC and CLD were significantly higher than control group, with all *P*< 0.05. Both C16Cer and C24DHC levels were not significantly different in HCC and CLD, however, there was a trend of HCC > CLD > Control **(Table 3 and Figure 2).** The serum level of C16Cer and C24DHC were not correlated with each or with AFP in all groups **(Table 3).**

Table (3): Comparison and correlation analysis of the serum C16 Cer and C24DHC across the groups (n= 50 each)

ANOVA/	HCC	CLD	Control	P-value
C16 Cer (ng/ml) Mean ± SD	157.9 ± 38.2	124.8 ± 29.3	9.3 ± 2.3	P1 <0.05 P2 <0.05 P3 <0.05
C24DHC (ng/ml) Mean ± SD	300 ± 73.2	262.5 ± 63.21	15.9 ± 3.34	P1 >0.05 P2 <0.05 P3 <0.05

Pearson Correlation							
	R	**P***	**r**	**P***	**r**	**P***	
C16 Cer / C24DHC	0.074	0.612	0.171	0.234	-0.102	0.482	---
C16 Cer/AFP	0.040	0.784	-0.134	0.535	0.114	0.431	
C24DHC/AFP	0.219	0.127	-0.97	0.501	-0.254	-0.76	

ANOVA and Bonferroni test analysis of serum C16C and C24DHC across the different groups. *ANOVA*. P1: HCC vs. CLD, P2: HCC Vs Control, P3: CLD and Control *P** ≤0.05 indicates significant difference in the mean serum level between2 groups. Pearson' correlation analysis of C16 Cer with C24DHC in different groups. r= correlation coefficient, *P** ≤0.05 indicates significant correlation between groups, CLD: Chronic liver disease, HCC: Hepatocellular carcinoma. C16 Cer: C16 ceramide, C24DHC: C24 dihydroceramide.

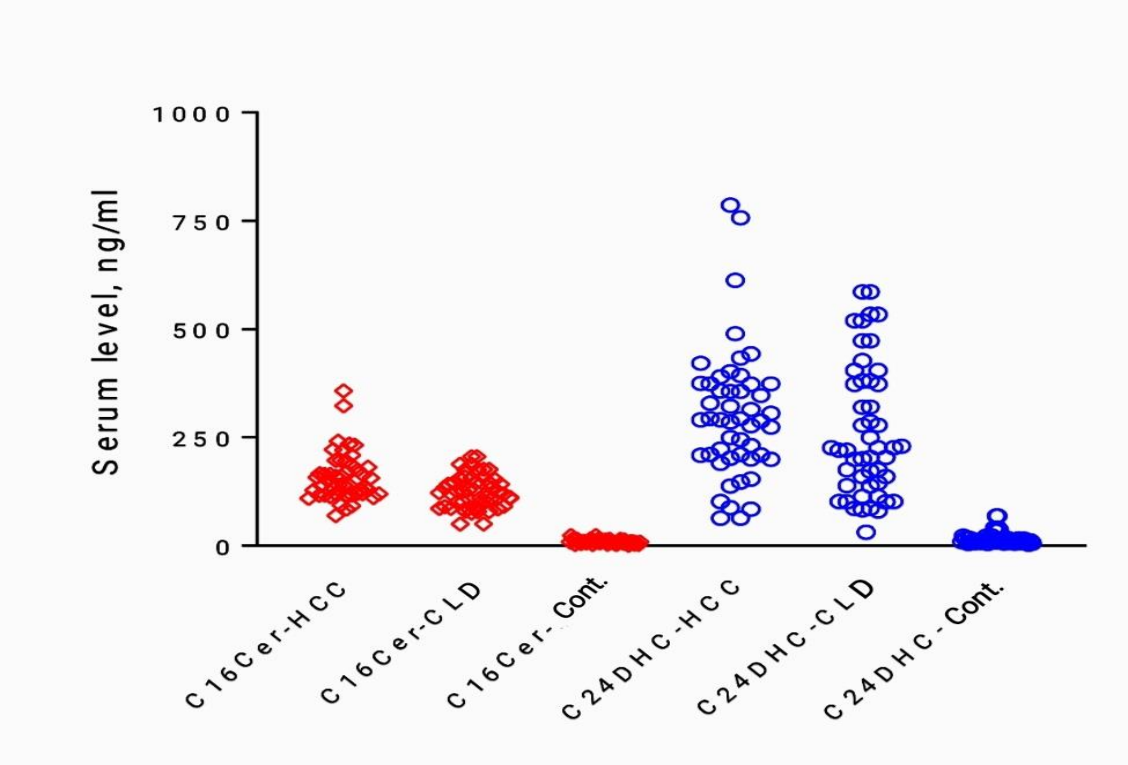


Figure (2): C16 Cer and C24DHC levels in different groups: Column scattered graph of the serum C16Cer and C24DHC in different groups. CLD: Cirrhotic liver disease, HCC: Hepatocellular carcinoma.

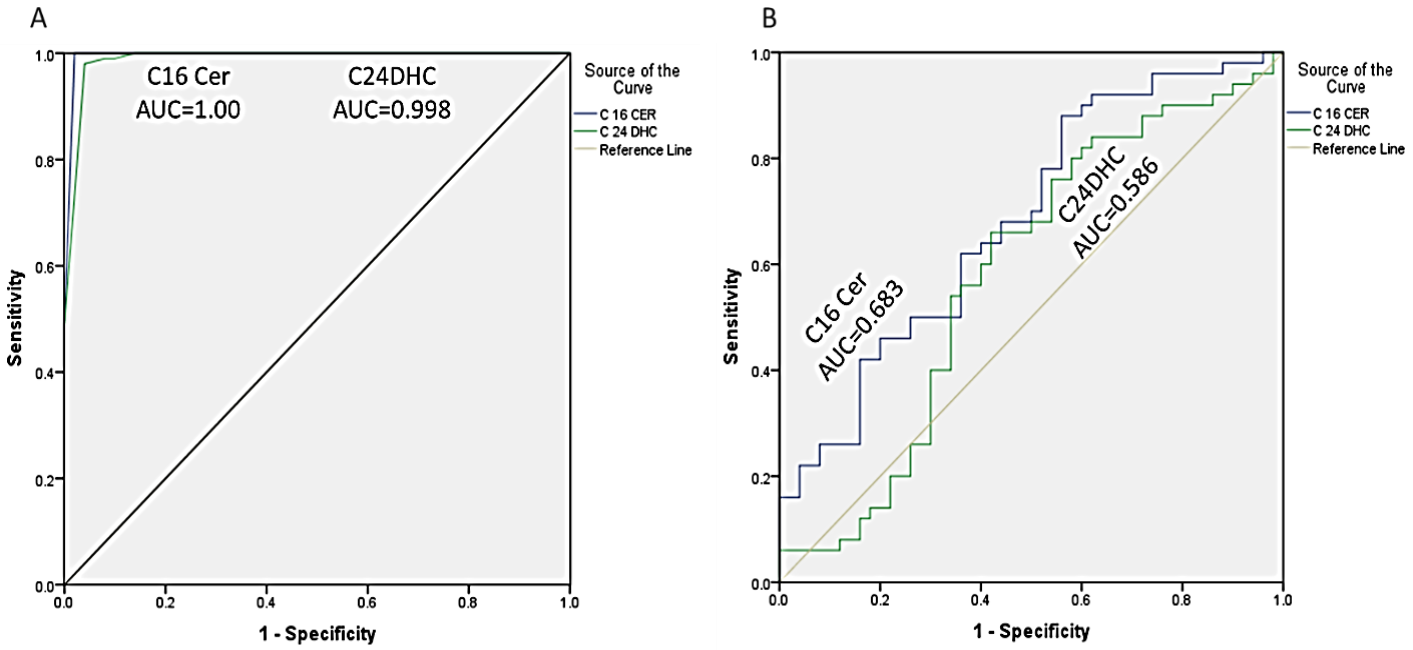
Table 4 presents Pearson's correlation analysis between serum C16Cer and C24DHC and the biochemical and hematological parameters in the patients with HCC and CLD. In the CLD group, C16Cer had a significant positive correlation with the liver function T. Bil, D. Bil, AST, and ALT; however, C24DHC had a significant positive correlation with ALP, Creat., Urea, Cholesterol, TriG, RBCs, and Hb. C16C or C24DHC had no significant correlation with the biochemical and the hematological parameters in the HCC group.

Table 4. Pearson's Correlation between C16 Cer, C24 DHC and biochemical and hematological parameters in HCC and CLD groups (n= 50)

Variable	HCC				CLD			
	C16C		C24DHC		C16C		C24DHC	
	r	P-value	r	P-value	R	P-value	r	P-value
AST (IU/L)	0.214	>0.05	0.097	>0.05	-0.44	<0.05	0.199	>0.05
ALT (IU/L)	-0.103	>0.05	0.161	>0.05	-0.424	<0.05	0.207	>0.05
GGT (IU/L)	0.019	>0.05	0.014	>0.05	-0.245	>0.05	0.086	>0.05
ALP (IU/L)	0.253	>0.05	-0.006	>0.05	-0.035	>0.05	- 0.294	<0.05
T.Bil (mg/dl)	0.245	>0.05	0.001	>0.05	-0.319	<0.05	-0.123	>0.05
D.Bil (mg/dl)	0.187	>0.05	-0.064	>0.05	-0.336	<0.05	-0.10	>0.05
T.P (gm/dl)	0.1	>0.05	- 0.066	>0.05	0.048	>0.05	- 0.07	>0.05
Alb (gm/dl)	0.053	>0.05	0.021	>0.05	-0.19	>0.05	0.183	>0.05
Chol (mg/dl)	0.164	>0.05	0.082	>0.05	-0.077	>0.05	0.315	<0.05
TriG (mg/dl)	0.188	>0.05	0.214	>0.05	-0.247	>0.05	0.304	<0.05
LDL (mg/dl)	0.172	>0.05	0.144	>0.05	0.105	>0.05	0.255	>0.05
HDL(mg/dl)	-0.138	>0.05	0.224	>0.05	-0.16	>0.05	0.057	>0.05
Urea(mg/dl)	-0.028	>0.05	0.011	>0.05	0.228	<0.05	-0.403	<0.05
Crt. (mg/dl)	0.119	>0.05	0.174	>0.05	0.183	>0.05	-0.304	<0.05
RBCs ($\times 10^6/mm^3$)	0.05	>0.05	-0.138	>0.05	0.23	>0.05	0.462	<0.05
WBCs($/mm^3$)	0.066	>0.05	-0.067	>0.05	-0.109	>0.05	-0.035	>0.05
HB (gm/dl)	-0.132	>0.05	-0.043	>0.05	-0.182	>0.05	0.315	<0.05
INR	0.131	>0.05	- 0.207	>0.05	0.162	>0.05	- 0.151	>0.05
Plet ($\times 10^3/mm^3$)	- 0.187	>0.05	-0.15	>0.05	-0.236	>0.05	0.133	>0.05

CLD: Chronic liver disease, HCC: Hepatocellular carcinoma. C16C: C16 ceramide, C24DHC: C24 dihydroceramide, r Pearson' correlation coefficient, p $\leq$0.05 indicates significant correlation. AST: aspartate transaminase, ALT: alanine transaminase, GGT: Gamma-Glutamyl Transferase, ALP: alkaline phosphatase, TBil: total bilirubin, DBiL: direct bilirubin, TP: total protein, Alb: albumin, AFP: alpha-fetoprotein, Chol: cholesterol, TriG: triglyceride, LDL: low-density lipoprotein, HDL: High-density lipoprotein, Crt: creatinine, Hb: hemoglobin, WBCs: white blood cells, INR international normalization ratio, Plat: platelet.

To assess the sensitivity and specificity of serum ceramide to distinguish healthy subjects from disease patients (CLD and HCC) series of ROC curves analyses were performed. ROC curves analysis of C16Cer and C24DHC in healthy versus diseased (CLD+HCC) revealed Cer16 and C24DHC had AUC 1.0 and 0.99, with a significant ability to separate the healthy from the diseased group. The sensitivity and specificity of C16Cer (98, 94) and of C24DHC (96, 94) at the cut-off 120ng/ml determined by Youden's index **(Figure 3A).** ROC curves analysis in patients with HCC versus patients with CLD showed C16Cer and C24DHC had an AUC of 0.638 and 0.583 with poor ability to separate the CLD from the HCC patients. The sensitivity and specificity of C16Cer and C24DHC were (88, 44) and (66, 58) at cut-off 112 ng/ml and 230 ng/ml. **(Figure 3 B).**



	AUC	Cutoff (ng/ml)	Sen.	Spe.	CI
C16Cer	1.000	77.3	100%	100%	1.0 – 1.0
C24DHC	0.998	76.9	99.5%	100%	0.998-1.0

	AUC	Cutoff (ng/ml)	Sen.	Spe.	CI
C16Cer	0.683	112.9	88%	44%	0.58 - 0.79
C24DHC	0.586	230	66%	58%	`0.47- 0.7

Figure (3): Diagnostic performance of C16Cer and C24DHC in the differentiation of patients with HCC or CLD from healthy subjects: A) A ROC curve of C16 Cer and C24DHC in Control vs. CLD+HCC, **B**) A ROC curve of C16Cer and C24DHC CLD vs. HCC. AUC: area under the curve. Sen: sensitivity, Spe: Specificity, C16 Cer: C16 ceramide, C24DHC: C24 dihydroceramide, CLD: Chronic liver disease, HCC: Hepatocellular carcinoma.

To examine if the tumor mass and the extent of the HCC would compromise the ability of liver tissue to produce ceramide, the interaction of the serum C16Cer and DHC24 with the clinical parameter as Child-Pugh, BCLC stage, and the number of the focal lesion was evaluated. C16Cer and C24DHC have no significant difference among different Child-Pugh classifications and BCLC stages in the HCC group. The number of focal lesions did not significantly affect the serum ceramide level in HCC patients, with all p >0.05 **(Table 5)**. Similarly, the use of DAAs and the presence of associated chronic diseases such as DM and hypertension did not affect the serum level of C16Cer or C24DHC in either CLD or HCC group.

Table (5): The interaction of the serum C16 Cer and C24DHC with the clinical parameters of HCC and CLD.

Variable	HCC				CLD			
	C16C(ng/ml) Median (IQR)	P-value	C24DHC (ng/ml) Median (IQR)	P-value	C16C (ng/ml) Median (IQR)	P-value	C24DHC (ng/ml) Median (IQR)	P-value
Child-Pugh								
A	139 (118-165)	>0.05	293 (200-382)	>0.05	119 (91-146)	>0.05	225 (172-373)	>0.05
B	170 (150-196)		290 (209-347)		442 (97-188)		226 (110-380)	
C	168 (119-225)		296 (185-335)		109 (86-146)		113 (80-533)	
BRACA								
A	139 (134-157)	>0.05	285 (208-356)	>0.05	---			
B	150 (116-185)		290 (153-374)					
C	154 (121-217)		305 (215-460)					
HFL								
Solitary	134 (117-164)	>0.05	286 (210-425)	>0.05	---			
Multiple	153 (120-196)		292 (197-361)					
DM								
Yes	145 (129-200)	>0.05	279 (200-373)	>0.05	158 (140-188)	<0.05	226 (101-373)	> 0.05
No	144 (117-182)		351 (226-374)		111 (87-128)		203 (140-398)	
DAAs								
Yes	139 (124-167)	>0.05	274 (204-364	>0.05	112(100-167)	>0.05	199 (128-226)	>0.05
No	150 (118-190)		293 (200-364)		125(88-154)		249 (138-404)	

CLD: Chronic liver disease, HCC: Hepatocellular carcinoma. C16C: C16 ceramide, C24DHC: C24 dihydroceramide, association. DM: Diabetes mellitus, DAAs: Direct acting anti-virus, BCLC: Barcelona clinic liver cancer, HFL: Hepatic focal lesion. P ≤0.05 indicates significant relation by nonparametric Kruskal Wallis, and chi square.

DISCUSSION

The study assessed the serum level of the long-chain C16Cer and the very long-chain C24DHC in CLD complicating chronic HCV infection and using ultra-performance liquid chromatography-tandem mass spectrometry in HCC using a metabolomics approach. The high sensitivity of the UPLC-MS allowed accurate determination of C16Cer and C24DHC at a serum concentration as low as 2.5ng/ml. The serum levels of C16Cer and C24DHC were significantly higher in CLD and HCC patients rather than healthy subjects, and had a trend to be higher in HCC but without discriminative ability between HCC and CLD. **Grammatikos *et al.*** [23] found that C16Cer and C24DHC were significantly higher in the serum of patients with HCC than in cirrhosis. Similarly, **Haberl *et al.*** [24], found ceramide levels were induced in the tumor tissues. However, **Krautbauer *et al.*** [25], found (Cer) was markedly reduced in HCC tissues.

Previous studies identified a significant decrease in C24Cer in chronic HCV infection compared to healthy individuals, HCV patients with severe liver fibrosis, and patients with NAFLD [26,27]. In the same context, some studies found very long-chain C24Cer significantly reduced in patients with HCV-induced liver cirrhosis relative to cirrhosis induced by other causes. Others showed no variations in C24Cer levels in patients with cirrhosis brought on by persistent alcoholism or persistent HBV infection [28]. It is quite clear that the discrepancies could be partially due to the diversity of the etiology of cirrhosis and HCC, in our study was mainly due to HCV, others had etiology related to alcohol abuse and HBV [29].

Recent research indicates that serum ceramides are linked to indicators of liver damage in those with chronic HCV infection [30]. This study found no correlations between C16Cer and C24DHC with the laboratory liver function test that reflects the severity of the disease or serum cholesterol and triglycerides in either CLD or HCC. Similarly, **Grammatikos *et al.*** [26,27] found that Long-chain C16 and the C24 very long-chain ceramides did not correlate with liver function, cholesterol or triglyceride in chronic HCV patients. C16Cer and C24DHC did not correlate with clinical parameters of hepatocellular injury. Child-Pugh score constitutes the standard to estimate the severity of liver cirrhosis, similarly to the stage of HCC, and the number of the hepatic focal lesion indicates the severity of the disease [22].

The increase of serum C16 Cer and C24DHC levels was not associated significantly with the Child score or the stage of HCC, thus, these metabolites do not have the potential to predict the progress of liver cirrhosis to HCC. These results are in agreement with **Grammatikos *et al.*** [23], who did not find significant variations in serum sphingomyelins among the different BCLC stages in HCC patients. Patients with Child-A cirrhosis showed considerably greater quantities of long and very long-chain ceramide than patients with Child-C cirrhosis, according to research by **Grammatikos *et al.*** [31]. The current investigation discovered an increase in the blood levels of C16Cer and C24DHC in diabetic individuals related with CLD illness and HCC, which is in agreement with **Grammatikos *et al.*** [31] increased plasma ceramides have been linked to type 2 diabetes in obese people, and they strongly correlate with insulin resistance and plasma TNF-α.

The increased tissue flow of long-chain FFAs and promotion of TNF-mediated insulin resistance may be the causes of the higher plasma ceramide levels in obese people with insulin resistance. Ceramides may boost TNF-production through a positive feedback loop, worsening insulin resistance in those who already have it [32]. Ceramide and chronic hepatic inflammation are related, because ceramide can trigger TNF-α, which may start ceramide formation in hepatocytes. This increased ceramide then regulates cytokine release through its own feedback mechanism. As a result, a vicious loop might start, amplifying the negative metabolic consequences. Ceramide could thus affect the chronic liver through its interaction with cytokines [32,33].

Currently, DAAs therapy is highly effective, and sustained virological response is possible in almost all patients. This study found neither association between DAAs therapy and C16Cer nor C24DHC level. Recent studies showed that successful DAAs treatment was associated with a decrease in the serum concentrations of various sphingosines, and patients with liver cirrhosis and treatment failure have lower serum ceramide levels [29]. As the liver serves as the primary site for the synthesis and uptake of ceramides, the association between low serum ceramide levels and treatment failure may simply be the result of liver cirrhosis [29].

As a result, additional research is required to examine the hepatic effects of ceramide disequilibrium in CLD and HCC. Despite the fact that patients were matched based on demographic traits and concurrent cancers other than HCC were disregarded in order to control for potential confounding factors, other illnesses like diabetes, obesity, metabolic syndrome, cardiovascular disease, and gastrointestinal microbiota are linked to ceramide metabolism. As a result, the interpretation of the profiles of these ceramides is made more difficult by the conjunction of these disorders and liver cirrhosis.

A limitation of the study is that it did not include a group of patients with chronic HCV patients without cirrhosis or a group of HCC patients without cirrhosis. The comparison of HCV-only versus HCV-cirrhosis and HCC-only versus HCC with cirrhosis would allow unmasking the confounding effect of cirrhosis on the metabolic disturbances in ceramides.

CONCLUSION

UPLC-MS/MS allowed accurate quantification of C16 Cer and C24DHC at a serum concentration as low as 2.5ng/ml. The serum levels of C16 Cer and C24DHC were significantly higher in CLD and HCC patients than in healthy subjects showing a trend to be higher in HCC without discriminative ability between the two diseases. Neither C16 Cer nor C24DHC correlated with the clinical parameters of hepatocellular injury such as Child-Pugh score, BCLC stage of HCC, or the burden of the tumor HCC. Therefore, their value as potential markers for HCC is limited and equivocal, and further prospective studies are required before any implementation as biomarkers of clinical significance.

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