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Original Research Article

Associations of systemic sphingolipids with measures of hepatic function in liver cirrhosis are related to cholesterol

Sabrina Krautbauer[a,c], Reiner Wiest[b], Gerhard Liebisch[c], Christa Buechler[a,*]

[a] Department of Internal Medicine I, Regensburg University Hospital, Regensburg, Germany
[b] Department of Visceral Surgery and Medicine, University Inselspital, Bern, Switzerland
[c] Institute for Clinical Chemistry and Laboratory Medicine, Regensburg University Hospital, Regensburg, Germany

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ABSTRACT

Lipoprotein particles are composed of various lipid classes including cholesterol and sphingolipids, and are low in serum of patients with liver cirrhosis. Hepatic decompensation is associated with a further decline of lipoproteins. Aim of the present work was to evaluate whether ceramide and sphingomyelin species are similarly changed in patients with liver cirrhosis and whether these variations are related to systemic cholesterol levels. In a cohort of 45 patients suffering from liver cirrhosis, cholesteryl ester species and subsequently total cholesterol were identified to be negatively associated with model of end stage liver disease (MELD) score. Indeed, the negative correlations of ceramide (Cer) and sphingomyelin (SM) species with MELD score, bilirubin and antithrombin 3 were non-significant after adjustment for cholesterol. Cer/SM ratios of species with identical acyl chains were not related to Child-Pugh or MELD score indicating that both lipids are comparably changed. Further, cholesterol levels and concentrations of all sphingolipids measured were similar in systemic, hepatic vein and portal vein blood. Cholesterol and distinct sphingolipids were similar before and 3 months after insertion of a transjugular intrahepatic portosystemic shunt while hexosylceramide 24:1 was significantly induced. It is concluded that analysis of distinct systemic sphingolipid species is not superior to measurement of cholesterol as non-invasive marker of hepatic injury in patients with liver cirrhosis.

1. Introduction

Liver cirrhosis is the final stage of chronic liver diseases caused by different pathogenic factors [1]. Child-Pugh score and model of end stage liver disease (MELD) score are widely used for prognosis in patients with liver cirrhosis. The Child-Pugh score includes ascites, hepatic encephalopathy, bilirubin, albumin and prothrombin time or international normalized ratio (INR). The MELD score is calculated from bilirubin, creatinine, and INR [2].

Hepatic dysfunction in liver cirrhosis is linked to disturbed lipid and lipoprotein metabolism [3]. Mass spectrometry techniques are currently applied with the aim to comprehensively measure lipids in biological systems [4,5]. These approaches revealed that several lipid species are changed in serum of patients with chronic liver disease [6–11]. Of note, these metabolites are associated with disease severity and/or secondary complications of liver cirrhosis [6–11].

The sphingolipid ceramide has gained much attention in hepatology. Ceramide de novo synthesis uses serine and palmitoyl-CoA. This lipid is also produced from sphingomyelin by sphingomyelinases or is derived through degradation of complex sphingolipids [12]. Absolute amount of ceramide is higher in the liver compared to fat depots, and hepatic and serum lipid compositions are very similar [13]. Analysis of systemic ceramide species is therefore a feasible approach for the identification of surrogate markers of liver dysfunction.

Acid sphingomyelinase is induced in serum of patients with chronic hepatitis C infection and patients with non-alcoholic fatty liver disease [14]. This enzyme hydrolyzes sphingomyelin in lipoprotein particles and/or the extracellular leaflets of cells and thereby may increase ceramide levels [4,12].

Biologic effects of ceramides depend on the length of the acyl chain. Long chain (C16-C20) and very long chain (C22-C24) ceramide species may even have opposing effects on biophysical properties of cell membranes and cell death [15,16]. Concordantly, very long chain ceramides protect from hepatic insulin resistance which is aggravated by long chain ceramide species [17].

In a cohort of excessive drinkers 31 patients with liver cirrhosis had increased systemic dihexosylceramide and trihexosylceramide species compared to the 28 non-cirrhotic patients, and the latter even correlated with MELD score [10]. Associations of serum sphingolipids with stage of liver fibrosis have been further identified in hepatitis C virus

* Corresponding author at: Department of Internal Medicine I, Regensburg University Hospital, D-93042 Regensburg, Germany.
E-mail address: christa.buechler@klinik.uni-regensburg.de (C. Buechler).

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(HCV) infected patients. Here, dihydroceramide (C18), sphingosine and sphinganine were increased while ceramide (C24) was lower in patients with severe fibrosis. Interestingly, these lipids were not associated with fibrosis stage in hepatitis B (HBV) caused liver injury indicating that disease etiology has to be considered when analyzing these lipid species [7]. Negative associations of C24 ceramide and sphingomyelin species with MELD score were nevertheless identified in patients with HBV cirrhosis in a separate investigation [11].

A further study shows that hexosylceramide C12 is increased in serum of HCV infected patients with severe fibrosis [18]. Various ceramide species including C24 were also analyzed in this cohort but were comparable between patients with and without severe fibrosis [18]. A prospective study including 244 patients with liver cirrhosis of different etiologies shows lower levels of several ceramide species (C18, C20, C24, C24:1) in patients with decompensated liver disease compared to those with compensated disease [8]. Lower long and very long chain ceramide species were further associated with the major complications of liver dysfunction, which are ascites, spontaneous bacterial peritonitis, hepatic encephalopathy and hepatorenal syndrome [8].

In the present study ceramide and sphingomyelin lipid species were measured in serum of patients with mainly alcoholic liver cirrhosis. Here, we were interested whether changes in systemic ceramide and sphingomyelin species with identical acyl chains in patients with liver cirrhosis are similar (which indicates that synthesis/hepatic release is impaired) or opposing (which points to higher activity of sphingomyelinases). Considering that most sphingolipids are part of lipoprotein particles whose circulating levels are reduced in cirrhosis we also wondered if alterations of single species are related to cholesterol, a major constituent of lipoproteins [3,19,20].

2. Materials and methods

2.1. Transjugular intrahepatic portosystemic shunt (TIPS)

The cohort included forty five patients with liver cirrhosis and has been described in detail in previous studies [9,21]. Age of the patients was 54 (26–81) years. The median MELD score was 9 (6–21), nine of the patients were women and twelve had type 2 diabetes. Twelve patients had Child-Pugh score A, 16 patients B and 17 C. Six patients had no ascites, 13 little ascites, 4 modest ascites and 22 massive ascites. Nine patients did not have varices, seven had small varices and 29 had large varices. Etiology of liver cirrhosis was alcoholic in 38, hepatitis C infection in 3 and of distinct reasons in 4 patients. Transjugular intrahepatic portosystemic shunt (TIPS) (Viatorr-Stent, Putzbrunn, Germany) implantation has been described and was performed in the fasted state [22]. Complications indicating the insertion of the TIPS were variceal bleeding in 15 patients, hepatorenal syndrome in 1 patient, refractory ascites in 27 patients and other reasons in 2 patients. During this intervention, samples of the hepatic vein (HVS) not drained by the TIPS stent, of the portal vein (PVS) and of a peripheral vein (SVS) were obtained.

Blood was also collected immediately after TIPS implantation. Three months after this intervention, blood was drawn again, and samples of 23 patients were obtained.

Standard parameters such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the Institute for Clinical Chemistry and Laboratory Medicine (University Hospital of Regensburg).

The study was done in accordance with the Declaration of Helsinki and was approved by the ethical committee of the University Hospital of Regensburg. All patients provided written informed consent.

2.2. Quantification of lipids

Serum lipid species were measured by direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ion mode as already described [23,24]. Non-naturally occurring species were added as internal standards before lipid extraction. This was used to compensate for variations in the ionization efficiency and sample preparation. For calibration, defined quantities of the lipid species were added to serum and vacuum-dried.

Samples were dissolved in a mixture containing 10 mM ammonium acetate in methanol/chloroform = 3/1 (v/v) and injected with HTS PAL autosampler (Zwingen, Switzerland). A flow gradient was performed starting with a flow of 55 µl/min for 0.1 min followed by 30 µl/min for 1.0 min and an increase to 250 µl/min for 0.2 min using an Agilent 1100 binary pump (Waldbronn, Germany). The triple quadrupole mass spectrometer (Quattro Ultima, Micromass, Manchester, UK) was equipped with an electrospray ion source operated in positive ion mode.

For phosphatidylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LPC) a fragment ion of *m/z* 184 was used A neutral loss of 141 Da was used for phosphatidylethanolamine (PE) and of 277 Da for phosphatidylinositol (PI). Sphingomyelin species were assigned based on the assumption of a sphingoid base d18:1. Ceramides (Cer d18:1) and hexosylceramides were analyzed using a fragment ion of *m/z* 264 [4].

An acetyl chloride derivatization method was used to specifically convert free cholesterol to cholesteryl acetate [23]. Cholesteryl ester species were quantified by direct flow injection analysis using a fragment of *m/z* 369, selected reaction monitoring (SRM) and a precursor ion scan. Deuterated D(7)-free cholesterol and cholesteryl ester 17:0/22:0 were used as internal standards [23].

Self-programmed Excel macros were used to sort the results, calculate the ratios to the internal standards, generate calibration curves, and calculate quantitative values.

2.3. Statistics

Data are shown as box blots (IBM SPSS Statistics 21.0). Statistical differences were analyzed by two-tailed Mann-Whitney U Test. ANOVA with post hoc Bonferroni correction was done for multiple comparisons (IBM SPSS Statistics 21.0). A p-value < 0.05 were regarded as significant. Spearman correlation was calculated using the IBM SPSS Statistics 21.0 software. To address for multiple comparisons these p-values were adjusted by the false discovery rate.

3. Results

3.1. Association of ceramide and sphingomyelin species with severity of liver cirrhosis

To evaluate an association of ceramide and sphingomyelin species with liver cirrhosis severity, the significant different lipid species among patients with Child-Pugh score A and score C liver cirrhosis were identified. Regarding ceramide (Cer) species only Cer d18:1/23:0 ($p = 0.049$) was significantly reduced in patients with Child-Pugh score C (Fig. 1A and Suppl. Table 1). Cer d18:1/22:0 was higher in Child-Pugh B than C patients but did not differ in those with well-compensated and decompensated liver cirrhosis (Suppl. Table 1). Sphingomyelin (SM) 20:1, 22:0, 22:1, 23:0 and 23:1 were also significantly lower in patients with Child-Pugh score C than A (Fig. 1B, C and Suppl. Table 2A, B).

Cer and SM with a C23 acyl chain were both reduced in patients with Child-Pugh score C cirrhosis (Fig. 1A, B). Ratio of Cer d18:1/23:0 and SM 23:0 was not related to Child-Pugh score (Fig. 1D) showing that both lipid species are changed in equal measure.

3.2. Correlation of ceramide and sphingomyelin species with MELD score

Analysis of single ceramide species revealed that Cer d18:1/C23:0 and 24:0 were negatively associated with MELD score (Fig. 2A and

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