Diagnostic Value of Systemic Cholesteryl Ester/Free Cholesterol Ratio in Hepatocellular Carcinoma

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Abstract. Background: Hepatocellular carcinoma (HCC) most commonly occurs in the setting of liver cirrhosis which is characterized by low serum lipids. We hypothesized that composition of lipoproteins and consequently lipid species ratios are mostly unchanged in patients with cirrhosis compared to controls. This approach may be appropriate to identify lipid ratios altered in HCC irrespective of liver dysfunction. Patients and Methods: Lipids were measured in serum of 21 patients with HCC, 41 patients with liver cirrhosis and 22 controls. Ratios of lipids known to be changed in HCC tissues were calculated. Results: Ratios of polyunsaturated to monounsaturated lysophosphatidylcholine, ceramide/sphingomyelin and cholesteryl ester/free cholesterol were changed in HCC compared to both control cohorts. The latter was most suited to diagnosing HCC. Systemic ratios of these lipid classes were not associated with fibrosis, staging or grade in patients with HCC. Conclusion: The cholesteryl ester/free cholesterol ratio is comparable in controls and patients with cirrhosis, but is specifically increased in patients with HCC.

Viral infections, excessive alcohol intake, and non-alcoholic fatty liver disease (NAFLD) are the main pathologies of liver cirrhosis. Hepatocellular carcinoma (HCC) may arise in cirrhotic liver, but also occurs in non-cirrhotic NAFLD. The pathophysiology of non-cirrhotic HCC has not been clarified and surrogate markers to identify patients at risk for HCC development are not available. Patients with NALFD without cirrhosis are not screened regularly for HCC and may, therefore, be diagnosed with advanced-stage HCC (1, 2).

Chronic liver dysfunction is related to disturbed lipid metabolism, and consequently systemic lipid species are changed (3-5). Mass spectrometric techniques enable the measurement of various lipid species (6-9). Serum lipids can be easily analyzed and may evolve as non-invasive biomarkers for various diseases. A challenge is the identification of circulating lipids which are specifically changed in patients in order to establish biomarkers for liver fibrosis or HCC (3-5, 10). Identification of appropriate lipid biomarkers is further challenged by HCC arising in non-cirrhotic liver in those with NAFLD (1).

Lysophosphatidylcholine (LPC) is diminished in serum of patients with NAFLD and is negatively associated with Model of End-stage Liver Disease (MELD) score in cirrhosis (11, 12). Reduced LPC levels have been described in patients infected with chronic hepatitis B with liver cirrhosis. In serum of patients with HCC of different disease etiologies, concentrations of distinct LPC species are further reduced (4, 5). A combination of three metabolites, with one of them being LPC 22:5, was found to discriminate patients with HCC from those without HCC with a higher sensitivity and specificity than alpha-fetoprotein (13).

The sphingolipid ceramide induces apoptotic cell death, and therefore, is regarded to act as a tumor suppressor (14). Ceramide levels are indeed diminished in tumors and are markedly lower in HCC tissues (14, 15). Long-chain ceramides (C16-C20) induce apoptosis, while very long-chain ceramides (C22-C24) exert the opposite effect and even promote cell proliferation (16). This shows that...
Therefore, we measured different lipids in serum of patients with HCC, liver cirrhosis, and controls, and calculated the identification of lipids simply related to dyslipidemia. Considering that low systemic lipid levels are a feature of previous study (15). Experimental procedures were performed according to the guidelines of the charitable state-controlled foundation Human Tissue and Cell Research (19).

A recently performed analysis of lipid species by our group using paired samples of HCC tissues and adjacent non-tumorous tissues demonstrated that levels of various lipid species were altered in the tumors. Of note, ceramides were reduced and sphingomyelins (SMs) were increased in HCC tissue. Regarding phospholipids, we found raised levels of saturated and lower amounts of polyunsaturated species (PUFA) (15). Although not shown in our previous study (15), the quotients of the related lipid classes are obviously prominently changed especially when compared to the individual lipid classes.

This led us to hypothesize that the ratios of related lipid species may be altered in serum of patients with HCC. Considering that low systemic lipid levels are a feature of liver cirrhosis, calculation of ratios minimizes the identification of lipids simply related to dyslipidemia. Therefore, we measured different lipids in serum of patients with HCC, liver cirrhosis, and controls, and calculated the ratios of lipids identified to be differentially abundant in HCC (15).

### Materials and Methods

#### Patients

The patients with HCC included here were described in a previous study (15). Experimental procedures were performed according to the guidelines of the charitable state-controlled foundation Human Tissue and Cell Research (19).

#### Quantification of lipids

Quantification of lipid species was carried out by direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ion mode as described previously (9). Non-naturally-occurring lipid species served as internal standards. Analysis was performed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a binary pump (Model 1100; Agilent, Waldbronn, Germany) and an autosampler (HTS PAL, Zwingen, Switzerland). For phosphatidylethanolamine (PE), phosphatidylcholine (PC), SM and lysophosphatidylcholine (LPC) a fragment ion of m/z 184 was used (7, 9). A neutral loss of 141 Da was used for phosphatidylethanolamine (PE) and of 277 Da for phosphatidylinositol (PI) (21). For phosphatidylserine (PS) a neutral loss scan of m/z 264 (22). Free cholesterol (FC), selectively derivatized, and cholesteryl ester (CE) were analyzed by a fragment ion of m/z 369 (6). Correction of lipid species isotopic overlap and data analysis were performed by self-programmed Excel Macros. Lipid species annotation was according to the published proposal for short-hand notation (23). Glycerophospholipid annotation assumes even-numbered carbon chains only.

#### Statistical analysis

Data are shown as box plots indicating median, lower and upper quartiles and range of the values. Statistical analysis was carried out using ANOVA with post-hoc Bonferroni correction, Mann–Whitney U-test, Receiver operating characteristics curve or Spearman correlation (SPSS Statistics 21.0; IBM Corp., Armonk, NY, USA). In order to account for multiple comparisons in correlation analysis, p-values were multiplied by 5. Youden’s index was calculated as: Sensitivity + Specificity –1. Values of p<0.05 were regarded as significant.

### Table I. Characteristics of the patient cohorts (median values and range).

<table>
<thead>
<tr>
<th></th>
<th>HCC</th>
<th>Cirrhosis</th>
<th>Controls</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/females, n</td>
<td>21/0</td>
<td>32/9</td>
<td>8/14</td>
<td>0.005*</td>
</tr>
<tr>
<td>Age, years</td>
<td>63 (47-84)</td>
<td>54 (26-81)</td>
<td>53 (21-88)</td>
<td>0.012‡, 0.041§</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28 (20-45)</td>
<td>26.0 (16.0-38.0)</td>
<td>25 (18.0-38)</td>
<td></td>
</tr>
<tr>
<td>T2D, n</td>
<td>11</td>
<td>12</td>
<td>3</td>
<td>0.01‡</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>42 (23-378)α</td>
<td>29 (2-84)</td>
<td>20 (12-40)</td>
<td>0.003§, 0.020§</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>32 (14-145)α</td>
<td>37 (4-108)</td>
<td>26 (16-48)</td>
<td></td>
</tr>
<tr>
<td>Bilirubin mg/dl</td>
<td>0.6 (0.2-2.5)α</td>
<td>1.3 (0.3-8.2)</td>
<td>0.5 (0.3-1.9)</td>
<td>&lt;0.001*, 0.002‡</td>
</tr>
</tbody>
</table>

ALT: Alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; T2D: type 2 diabetes. Significant differences: *Controls vs. patients with liver cirrhosis, ‡controls vs. patients with HCC, §patients with liver cirrhosis vs. patients with HCC. αData available only for 20 patients.
Results

Study cohort. Lipid species were analyzed in serum of 21 patients with HCC, 41 patients with liver cirrhosis and 22 controls (Table I). Measurements of lipid species in sera of patients were performed within one batch to avoid interassay variations. It is important to note that nine of the patients with HCC did not have liver fibrosis.

Phospholipid ratios. Impaired hepatic function in liver cirrhosis is associated with reduced levels of lipoproteins (24). These particles are composed of triglycerides, cholesterol, LPC, PC, PE, SM and ceramide (25). Therefore, changes of the absolute levels of lipid species may mostly be related to dyslipidemia in liver cirrhosis, while composition of lipoproteins, and consequently ratios of individual lipids or lipid classes may be unaltered (26).

Our recent study revealed reduced levels of PUFA-containing phospholipids and higher concentrations of saturated fatty acid (SFA)-containing phospholipids in HCC tissues when compared to non-tumorous liver (15).

Ratios of PUFA/SFA PE and PI in serum were, however, not different between the groups (data not shown). LPC PUFA/SFA ratios were significantly lower in cirrhosis and HCC compared to controls but were similar in HCC and cirrhosis (Figure 1A). The PC PUFA/SFA ratio was lowest in patients with liver cirrhosis. There was no difference between HCC and controls (Figure 1B). Only PC PUFA/SFA ratio negatively correlated with MELD score in patients with liver cirrhosis (Figure 1C and data not shown). Although MELD score for patients with HCC was not calculated, nine patients did not have liver fibrosis, indicating that the median MELD score was lower in this cohort. Thus, PC PUFA/SFA ratio appears to be associated with residual liver function.

Levels of mono-unsaturated fatty acids (MUFA) in PC and PE were recently identified to be higher in human HCC tissues (17). Therefore, PUFA/MUFA ratios of the phospholipids measured were also calculated. PE PUFA/MUFA was highest in the controls but not different between patients with cirrhosis and those with HCC (Figure 1D). PI PUFA/MUFA was similar in patients with HCC and those with cirrhosis, with the latter displaying lower levels than those of the controls (Figure 1E). LPC PUFA/MUFA was reduced in patients with HCC and those with cirrhosis compared to controls, and was in fact lowest in cirrhosis (Figure 1F). PC PUFA/MUFA was specifically reduced in cirrhosis (Figure 1G).

A decreased PC/PE ratio in the liver is linked to hepatocyte ballooning and inflammation (27). While analyzing our samples, we did not find any difference in the PC/PE ratio in the three cohorts (Figure 1H).

Cholesterol ratios. Cholesterol production is induced in HCC tissue and higher serum cholesterol has been described in patients with HCC (17, 28). Total cholesterol was not different between the groups studied herein and FC was even reduced in serum from patients with HCC compared to controls (data not shown), suggesting that the CE to FC ratio is increased in HCC. Indeed, this ratio was similar in the control group and patients with liver cirrhosis but was significantly raised in those with HCC (Figure 2A).

Sphingolipid ratios. In HCC tissues, the ceramide level is low and sphingomyelin is increased, indicating lower activity of sphingomyelinase (15). In serum of patients with HCC, long- (C16-C20) and very long-chain (C22-C24) ceramides are even induced, while data on sphingomyelin were not available in this study (10). Ceramide/SM ratio was higher in HCC and similar in the control cohorts and patients with liver cirrhosis (Figure 2B).

Because long- and very long-chain ceramide have opposing functions regarding cell death, this ratio was also calculated. The quotient was highest in patients with liver cirrhosis but similar in controls and those with HCC (Figure 2C). Positive correlation of long-/very long-chain ceramide with MELD score shows that this ratio is associated with the extent of liver injury (Figure 2D).

Correlations with age, body mass index, bilirubin and aminotransferases. Correlations of lipid ratios with age, body mass index, bilirubin and aminotransferases were calculated separately for the three groups. Here, PC PUFA/SFA ratio (r=−0.535, p<0.001) and PC PUFA/MUFA ratio (r=−0.505, p=0.005) were negatively correlated with serum bilirubin in the cohort of patients with liver cirrhosis.

Lipid analysis in males. Females were not included in the HCC cohort study. However, LPC PUFA/SFA, LPC PUFA/MUFA, ceramide/SM and CE/FC ratios did not differ in the 14 female and the eight male controls (Figure 2E and F) and data not shown). The nine female and the 32 male patients with cirrhosis had similar ratios of these lipid classes (data not shown).

In order to further exclude whether HCC-associated changes were related to gender, calculations were carried out using data obtained from serum from males only. In principle, results were comparable to the distribution seen for the whole cohort. Ceramide/SM and CE/FC ratios were higher in male patients with HCC compared to patients with cirrhosis and controls (Figure 3A and B). LPC PUFA/SFA ratio steadily declined from controls to cirrhosis to HCC and was significantly lower in HCC compared to controls (Figure 3C). LPC PUFA/MUFA was lowest in patients with cirrhosis but was also reduced in patients with HCC compared to controls (Figure 3D). Receiver operating characteristic curve analysis of lipid ratios to detect HCC in the whole cohort revealed an area under the curve (AUC)
of 0.543 for LPC PUFA/MUFA, of 0.746 for ceramide/sphingomyelin and of 0.866 for CE/FC ratio (Figure 4A). In the male group, the corresponding AUCs were 0.606, 0.774 and 0.878, respectively (Figure 4B). This shows that the CE/FC ratio is best suited to HCC diagnosis. The optimal cut-off value for the CE/FC ratio for the whole cohort was 2.46 and identified HCC with a sensitivity of 76.2% and a specificity of 90.3%.

Association with staging, differentiation grade and fibrosis in HCC. In the 21 patients with HCC, neither systemic ceramide/SM nor LPC PUFA/MUFA or CE/FC ratios were related to T-stage or differentiation grade (Figure 5A-D and data not shown).

Of the 21 patients with HCC, 9 did not display liver fibrosis. Ceramide/SM, LPC PUFA/MUFA and CE/FC ratios were comparable in these groups, showing that altered levels are not related to liver fibrosis (Figure 5E and F, and data not shown).
Discussion

This analysis revealed that CE/FC ratio is increased in serum from patients with HCC when compared to controls and patients with liver cirrhosis. The LPC PUFA/MUFA ratio was found to be lowest in cirrhosis, but also reduced in patients with HCC when compared to controls. The ceramide/SM ratio was highest in HCC, but levels did not significantly differ from those of controls when calculations were carried out for males only. Although this does not exclude the possibility that the ceramide/SM ratio is indeed elevated in patients with HCC, changes in the CE/FC quotient were found to be most pronounced. Receiver operation characteristic curve analysis revealed that this ratio is best suited to diagnosing HCC.

CE was found to be modestly higher in HCC tissues compared to adjacent non-tumorous tissues, while FC was not changed, suggesting that their ratio is increased in the tumor (15). Cellular cholesterol synthesis is enhanced in liver cancer cells, and sterol regulatory element-binding transcription factor 2 and low-density lipoprotein receptor expression are induced (29). Higher endogenous cholesterol biosynthesis and uptake of low-density lipoprotein particles are essential for cell proliferation and survival (30). Blocking acyl-CoA cholesterol acyltransferase-1 suppresses proliferation and induces apoptosis of cancer cells, and this is attributed to the cytotoxic effects of free cholesterol (31). Although a higher CE/FC ratio is therefore supposed to promote cancer growth (31), it was not found to be changed with differentiation grade and T-stage in the present cohort. The number of patients enrolled was, however, very small and further studies are needed to exclude any such association. An increased CE/FC ratio in HCC may possibly be used as a biomarker to diagnose HCC in asymptomatic patients.

The lipid profile of patients with liver cirrhosis and HCC is highly similar (32). Compared to healthy controls most of lipid levels are low in patients with liver cirrhosis and HCC (32). Negative associations of blood total cholesterol levels and MELD score have been described (33). Therefore, differences in lipid concentrations mostly reflect the degree of liver dysfunction rather than being specifically changed in patients with HCC.

This does not apply to PC PUFA/SFA and long-/very long-chain ceramide ratios which are specifically altered in
patients with liver cirrhosis. Both of these ratios correlate with MELD score, demonstrating that the distribution of these lipid classes is associated with hepatic dysfunction. Thus, equilibrium of distinct lipid classes is obviously changed in liver cirrhosis and further studies are needed to evaluate whether this is of any pathophysiological or diagnostic value.

A further issue is the identification of HCC in the non-cirrhotic liver of patients with NAFLD. Such patients more often have dyslipidemia and an elevated serum cholesterol level (34). Hypercholesterolemia has been also described in HCC with steato-hepatitic features (35). This suggests that most lipids are concordantly changed in patients with dyslipidemias and therefore the ratios of different lipids should be mostly unaffected.

Recently, we showed reduced levels of PUFA phospholipids and induced concentrations of saturated phospholipid species in HCC tissues (15). An independent study described that levels of MUFA phospholipids are higher in HCC tissue compared to the adjacent liver (17). PUFA phospholipid to saturated phospholipid quotient is also lower in patients with alcoholic liver cirrhosis when compared to healthy controls (36). Indeed, the respective LPC and PC PUFA/SFA ratios and PUFA/MUFA ratios are significantly reduced in the patients with liver cirrhosis compared to controls. This does not apply to PE and PI PUFA/SFA ratios, which are comparable in the three groups. Of note, only the LPC PUFA/MUFA ratio is specifically changed in serum of patients with HCC. Lower levels of LPC have been identified in serum from patients with HCC but the respective ratios have not been calculated (37). Receiver operating characteristic curve analysis revealed a low AUC for the LPC PUFA/MUFA ratio, which is therefore unsuitable as a non-invasive marker for HCC diagnosis.

A recent study showed higher levels of long- and very long-chain ceramides in the serum of patients with HCC as compared to patients with cirrhosis, while data on SMs were not provided (10). In that study, patients with HCC had a significantly lower MELD score and concordantly, fewer patients had Child-Pugh C cirrhosis (10). Considering that serum long- and very-long chain ceramides decrease with increasing severity of liver cirrhosis (3), a higher ceramide level in patients with HCC (10) may at least in part reflect less severe liver injury.

The ceramide/SM ratio was similar in controls and patients with liver cirrhosis in the present cohort, indicating that both lipid classes are similarly reduced in these patients. The quotient of these two lipid classes nevertheless increased in patients with HCC, showing that the proportion is specifically changed in patients with tumor.

Only the CE/FC quotient was increased in both cancer tissue (15) and serum, while further lipid ratios were not concordantly changed. This excludes the notion that serum
lipids specifically associated with HCC are directly or inversely related to the altered lipidome in liver tumors.

In summary, the present study suggests that the CE/FC ratio is specifically increased in patients with HCC and may become a useful diagnostic marker.

Acknowledgements

This study was supported by the Stiftung für Pathobiochemie und Molekulare Diagnostik and partly by the German Research Foundation (BU 1141/13-1). The technical assistance of Jolante Aiwanger, Simone Düchtel and Doreen Müller is greatly appreciated.

References

