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ORIGINAL ARTICLE

Metabolomic Analysis of Human Cirrhosis and Hepatocellular Carcinoma: A Pilot Study

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Abstract

Background Molecular changes in HCC development are largely unknown. As the liver plays a fundamental role in the body's metabolism, metabolic changes are to be expected.

Aims We aimed to identify metabolomic changes in HCC in comparison to liver cirrhosis (LC) patients, which could potentially serve as novel biomarkers for HCC diagnosis and prognosis.

Methods Metabolite expression from 38 HCC from the SORAMIC trial and 32 LC patients were analyzed by mass spectrometry. Metabolites with significant differences between LC and HCC at baseline were analyzed regarding expression over follow-up. In addition, association with overall survival was tested using univariate Cox proportional-hazard analysis.

Results 41 metabolites showed differential expression between LC and HCC patients. 14 metabolites demonstrated significant changes in HCC patients during follow-up. Campesterol, lysophosphatidylcholine, octadecenoic and octadecadienoic acid, and furoylglycine showed a differential expression in the local ablation vs. palliative care group. High expression of eight metabolites (octadecenoic acid, 2-hydroxybutyrate, myo-inositol, isocitrate, erythronic acid, creatinine, pseudouridine, and erythrol) were associated with poor overall survival. The association between poor OS and octadecenoic acid and creatinine remained statistically significant even after adjusting for tumor burden and LC severity.

Conclusion Our findings give promising insides into the metabolic changes during HCC carcinogenesis and provide candidate biomarkers for future studies. Campesterol and furoylglycine in particular were identified as possible biomarkers for HCC progression. Moreover, eight metabolites were detected as predictors for poor overall survival.

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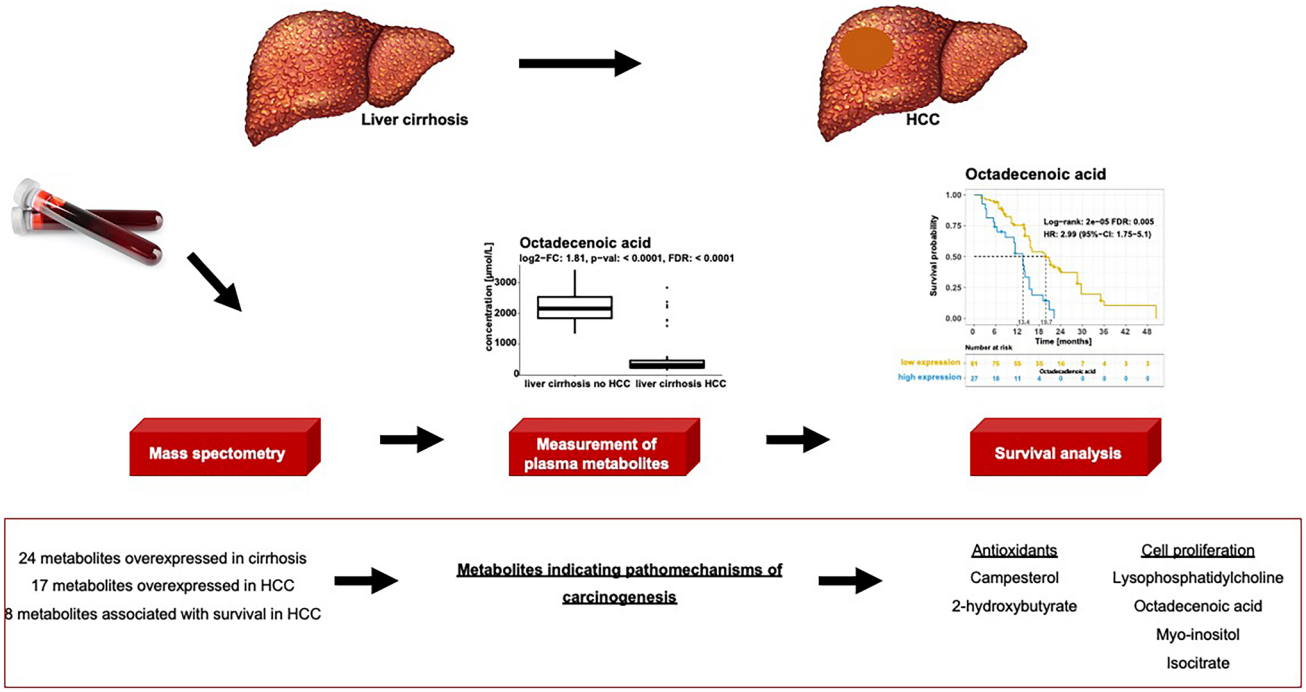
Graphical Abstract



Keywords Cancer biology · Liver cancer · Metabolomics · Metabolism

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies worldwide [1]. The majority of HCC develops in patients with underlying liver cirrhosis (LC) which provides the opportunity to closely monitor those patients at risk [2, 3]. However, there is still lacking knowledge regarding the pathomechanisms behind the development of HCC in LC patients. While a variety of studies have focused on immunological processes, transcriptomic dysregulation, and genetic alterations [4–6], the knowledge on metabolomic changes and their impact on HCC carcinogenesis are scarce. Yet, with the liver being the central organ for the metabolism of amino acids, carbohydrates, and lipids in the human organism [7], disruptions are to be expected in patients with chronic liver disease and HCC. Thus, metabolomic technologies might pose a promising tool to reveal distinctive metabolic pathways that are contributing to hepatocellular carcinogenesis. Those metabolomic patterns could possibly help to identify candidate biomarkers that can distinguish HCC and LC.

To this end, we used a metabolomic approach to identify metabolomic signatures in HCC patients in comparison to LC patients without HCC. Moreover, we aimed to evaluate differences in the metabolite expression between patients who, based on their tumor burden, received local ablation or a palliative therapeutic approach, and studied the impact of the identified metabolomic patterns on overall survival (OS) in HCC patients.

Materials and Methods

Patient Selection

EDTA plasma samples from 42 patients with HCC who had been recruited for the SORAMIC trial, a multicenter clinical trial for the treatment of HCC stage BCLC (Barcelona Liver Clinic Cancer) A, B, or C, were analyzed and compared to EDTA plasma samples from 32 patients with liver cirrhosis but without presence of HCC who were recruited at LMU Klinikum, Munich, Germany. Further details on the SORAMIC trial have previously been reported elsewhere [8]. Briefly, 529 patients were prospectively enrolled in 38 centers across Europe, out of whom 104 patients were allocated in the local ablation group receiving radiofrequency ablation (RFA) and sorafenib or RFA and placebo, while 425 patients were allocated in the palliative study group receiving SIR-Spheres® therapy (SIRT) and sorafenib or sorafenib alone. The study was approved by the

institutional review boards of all 38 participating centers. Written informed consent was obtained from all participants [8].

For the current analysis, samples from 29 patients (76%) from the palliative study group and 9 patients (24%) from the local ablation study group were selected (Fig. 1). Four HCC patients were excluded from the analysis due to HCC in a non-cirrhotic liver. First, samples from the baseline visit (BL), i.e., the time of inclusion in the SORAMIC trial before initiation of ablative or palliative treatment, were analyzed from every HCC patient ($n = 38$). In addition, analyses of samples from follow-up (FU) visits were conducted in $n = 32$ patients for FU 1, $n = 20$ for FU 2, $n = 6$ for FU 3, and $n = 12$ for a selected FU at a later time point than FU 3. Simultaneous analyses of blood sugar levels were performed to assure fasting status. Blood glucose levels of ≤ 6 mmol/l (108 mg/dl) for non-diabetic and of ≤ 7.6 mmol/l (137 mg/dl) for diabetic patients were chosen as cut-off levels defining the fasting status.

As a control group, 41 patients with liver cirrhosis but exclusion of HCC based on ultrasound, computer tomography, or magnetic resonance imaging (MRI) were recruited between January 2019 and March 2019. Nine patients (22%) were excluded due to high ($n = 5$) or unknown ($n = 2$) blood sugar levels at the time of blood sampling, liver-related death before the possibility of adequate imaging for HCC exclusion ($n = 1$), and presence of a metastasized gynecological malignancy ($n = 1$).

As quality controls, samples from 200 healthy blood donors provided by Metanomics Health were used. The pre-analytic quality of the samples was tested using MxP® Biofluids Quality Control, a metabolomics-based profiling assay providing information about presences of pre-analytical deviations and where they have occurred in the process. For this purpose, specifically developed and validated multiple metabolic biomarkers were measured in single readouts. By applying proprietary algorithms three different test results were calculated, which provided precise assessment of sample quality, and with this also suggestions for sample utilization.

The study protocol conforms to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Faculty of Medicine, LMU Munich (Project Number 18-536). Written informed consent was obtained from every patient who had been recruited for SORAMIC and as a LC control patient.

Mass Spectrometry Analysis

The analysis of the metabolomic markers was conducted by Metanomics Health. For the analysis of the metabolomic profile EDTA plasma was collected for every patient from the HCC and LC control group. MxP® broad profiling, which can detect 200 to 500 metabolites at the same time (i.e., amino acids, carbohydrates, energy metabolites, nucleobases, vitamins and co-factors, and lipids), was used as described previously in more detail [9]. In summary, samples were extracted by a proprietary method resulting in a lipid and a polar fraction, each of which was utilized for the further analyses. Then, two types of mass spectrometry

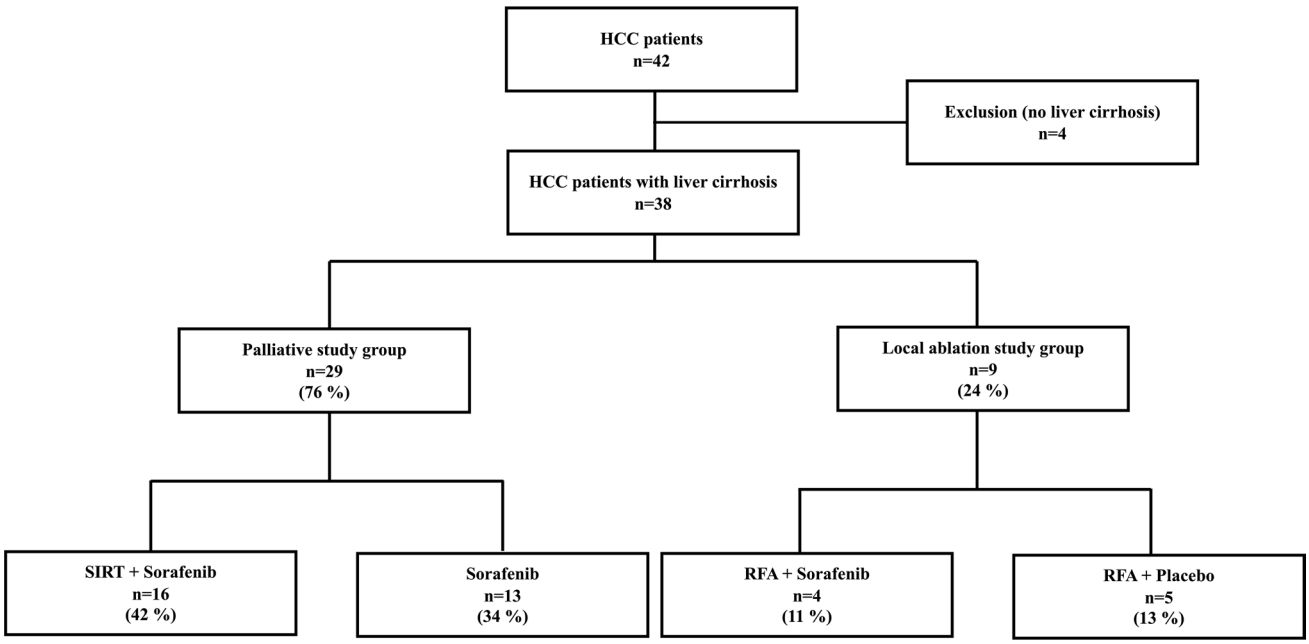


Fig. 1 Patient flowchart. Flowchart demonstrating the inclusion and exclusion as well as distribution of hepatocellular carcinoma (HCC) patients

analyses were applied: chromatography–mass spectrometry (GC–MS) and liquid chromatography–MS/MS (LC–MS/MS). For LC–MS/MS, a proprietary mass spectrometric detection technology was used which allows to target a high-sensitivity MRM (Multiple Reaction Monitoring) profiling in parallel to full screen analysis. A semi-quantitative approach was chosen with the comparison of the metabolites to control groups.

Statistical Analysis

Regarding clinical data, the statistical analyses were performed using SPSS (IBM, Armonk, New York, United States, version 27). Plasma concentrations in different subgroups were compared using p value ANOVA and p value t test. The level of significance was set at alpha < 0.05. Continuous variables are presented as median and range, and categorical variables as the number of patients and percentages. Categorical variables were compared by Chi-square tests, while continuous variables were compared by Mann–Whitney U tests.

Metabolic Data Analysis

Normalized metabolic measurements (Micromoles/L), as prepared by Metanomics Health, and clinical data were imported into R. Differential metabolite analysis was conducted using t-statistics from linear model fits as implemented in the R Limma library [10]. Raw p values were adjusted for multiple testing error by determining the Benjamini–Hochberg false discovery rate (FDR) [11]. The standard error of metabolite expressions was calculated per time point and given in each plot. Statistical significance was accepted for p values < 0.05 and FDR < 10%. Metabolites that showed statistical significance between samples from LC and HCC at BL were considered for time-course analysis. Longitudinal expressions were ordered from BL over first FU (FU 1), FU 2, to last FU (FU X). Metabolite expressions, dichotomized into high (≥ third quartile) and low (< third quartile), were considered as increasing/decreasing when increasing/decreasing over all time points and the absolute log2-ratio between of BL/last time point exceeded 1. An association of metabolite concentrations with overall survival was tested using univariate Cox proportional-hazard analysis with Log-Rank test p values as a measure of statistical significance and visualized using Kaplan–Meier plots. A multivariate analysis was conducted to control for potential confounding variables. Clinical variables with p values < 0.2 in the univariate analysis were included in the multivariate model. Log-rank test p values for the Cox proportional-hazard models and Wald test p values of covariates were reported.

Histologic and Immunohistochemistry (IHC) Analyses

Tumor blocks, collected before therapy, were analyzed according to standard procedure. To this end, serial tissue sections (2 µm) were cut from formalin-fixed, paraffin-embedded tumor tissues, dewaxed, and rehydrated according to standard procedure (preheating at 60 °C; de-paraffinization in Neo-Clear, Merck KGaA, Darmstadt, Germany; rehydration in graded series of ethanol and distilled water). For the determination of necrotic areas, tissue slides were stained with hematoxylin and eosin (H&E) (Merck KGaA). For IHC analysis, the primary antibodies, anti-IDH1 (rabbit IgG polyclonal, dilution 1:100; Thermo Fisher Scientific, Darmstadt, Germany), or anti-LPCAT1 (rabbit IgG polyclonal, dilution 1:50, ThermoFisher Scientific) were applied overnight at 4 °C. Slides were incubated with secondary antibody (DAKO EnVision + System-HRP Anti-Rabbit; Agilent Technologies, Waldbronn, Germany) and a DAB substrate kit (DAB substrate kit, Cell Signaling Technology, Leiden, The Netherlands) was used as the chromogen. Sections were counter stained with Haemalaun (Roth, Karlsruhe, Germany) and mounted using a glycerine-gelatin (Roth) as mounting medium. Slides were analyzed using a Leica DM2500 microscope (Leica, Wetzlar, Germany) equipped with LAS version 4 software (Leica). The intensity of cytoplasmic staining was estimated using a four-point scale of 0, no detectable staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

All authors had access to the study data and then reviewed and approved the final manuscript.

Results

Clinical Characteristics of Patient Cohort

After exclusion of nine LC control subjects for reasons elaborated in the methods section and four HCC patients without LC, samples from 70 patients were available for analysis ($n = 38$ LC patients with HCC, $n = 32$ LC control patients; Fig. 1). Clinical characteristics of the HCC and LC control patients are summarized in Table 1. Sex distribution and body mass index (BMI) were comparable between both groups; however, median age of HCC patients was higher in comparison to LC controls (63 vs. 55 years, $p < 0.0001$). Alcohol as an underlying or contributing cause of LC was found in similar proportions of LC control and HCC patients (41% vs. 42%, ns). However, regarding other causes for LC we observed differences between both groups (Table 1). HCC was excluded in the LC control patients through MRI ($n = 15$, 47%), ultrasound (US; $n = 10$, 31%), or contrast-enhanced

Table 1 Clinical characteristics

	HCC $n=38$	LC $n=32$	p
Age (years)	63 (41–81)	55 (25–78)	**< 0.001**
Age ≥ 65 years	19 (50%)	7 (22%)	**0.015**
Sex			
Male	34 (90%)	27 (84%)	0.33
Female	4 (10%)	5 (16%)	
Diabetes mellitus type II	11 (29%)	8 (25%)	0.71
BMI (kg/m^2)	26.1 (20.1–40.6)	27.0 (18.6–36.4)	0.83
Etiology			
Alcoholic	13 (34%)	13 (41%)	**0.002**
Viral	15 (40%)	3 (9%)	
Alcoholic and viral	3 (8%)	0 (0%)	
MASLD	5 (13%)	2 (6%)	
Autoimmune	0 (0%)	4 (13%)	
Cholestatic	0 (0%)	6 (19%)	
Hereditary	1 (3%)	2 (6%)	
Other[1]	1 (3%)	2 (6%)	
Alcoholic LD	16 (42%)	13 (41%)	0.90
CPS	5 (5–7)	8 (5–10)	**< 0.001**
CPS			
CPS A	33 (87%)	10 (31%)	**< 0.001**
CPS B	5 (13%)	16 (50%)	
CPS C	0 (0%)	6 (19%)	
AFP (ng/ml)[2]	3.4 (1.2–17.4)	32.1 (3.0–18371.0)	**< 0.001**

Bold values indicate statistical significance ($p < 0.05$)

Continuous variables are presented as median and range; categorical variables are presented as number and percentage (n (%))

AFP Alpha-feto protein, *BMI* body mass index, *CPS* Child–Pugh score, *HBV* Hepatitis B virus, *HCC* hepatocellular carcinoma, *HCV* Hepatitis C virus, *LC* liver cirrhosis, *LD* liver disease, *MASLD* metabolic dysfunction-associated steatotic liver disease

[1]Other etiologies were chronic drug-induced liver injury in two control cases and cryptogenic liver cirrhosis in one HCC case

[2]AFP mean values for LC controls are composed of all available AFP values at the time of sampling ($n=17$) or at a later time point during follow-up ($n=9$), in total AFP values were available for 26 control cases.

computer tomography (CT; $n=7$, 22%). AFP values were significantly higher in HCC than in LC control patients (32.1 vs. 3.4 ng/ml, $p < 0.001$; Table 1). Table 2 shows the tumor characteristics in the local ablation group and palliative care group: Patients in the palliative care group had a significantly higher BCLC stage, significantly higher numbers of HCC lesions, and a higher maximal diameter of the largest lesion.

Table 2 Characteristics of HCC burden according to treatment group

	Local ablation $n=9$	Palliative care $n=29$	p
BCLC staging system			**< 0.001**
BCLC A	4 (44%)	0 (0%)	
BCLC B	5 (56%)	7 (24%)	
BCLC C	0 (0%)	22 (76%)	
Metastasis	0 (0%)	9 (24%)	0.10
Diffuse HCC lesions	0 (0%)	14 (48%)	**0.009**
Number of lesions	2 (1–4)	3 (1–22)	**0.013**
Maximal diameter of the largest lesion (mm)	25.0 (12.0 – 50.0)	52.0 (12.0–22.0)	**0.022**

Bold values indicate statistical significance ($p < 0.05$)

Continuous variables are presented as median and range; categorical variables are presented as number and percentage (n (%))

BCLC Barcelona liver clinic cancer, *HCC* hepatocellular carcinoma

Metabolomic Profile

Figure 2 shows the distribution of the 41 metabolites with significant different expressions in LC control patients without HCC ($n=32$) and LC patients with HCC ($n=38$). Octadecenoic acid, biliverdin, pantothenic acid, 1-methylhistidine, campesterol, pseudouridine, n-acetylcytidine, N,N'-dimethylarginine, sedoheptulose-7-phophatase, trans-4-hydroxyprolin, thymine, 7-methylguanosin, octadecadienoic acid, 2-hydroxyisobutyrate, trihexosylceramide, o-acetylcarnitine, citrate, 3-hydroxyisobutyrate, aspargine, lactosylceramid, phenylalanine, choline ether lipid, plasmenyl choline, and plasmanyl choline ($n=24$) were overexpressed in LC control patients while furoylglycine, glutamate, 2-phosphoglycerate, cholesteryl ester C20:5, hippuric acid, 1,5-anhydrosorbitol, docosahexaenoic acid, ergothioneine, guanine, cystathionine, total phosphatidylethanolamine, eicosanoic acid, phosphatidylethanolamine, lysophosphatidylethanolamine, adenine, diacylglycerol, and lysophosphatidylcholine ($n=17$) were overexpressed in patients with HCC. For a more detailed view of the differential expression of said metabolites, please also refer to the metabolite concentrations demonstrated in Online Resource 1, Suppl. Table 1 and to the box plots presented in Online Resource 2, Suppl. Figure 1.

The expression of the 41 metabolites identified to be differentially expressed in HCC and control patients was also evaluated in the subgroup of patients with alcoholic liver injury ($n=16$ LC controls, $n=13$ HCC patients). We observed a similar trend regarding the metabolite expression in LC controls vs. HCC patients as demonstrated in Suppl. Figure 2 (Online Resource 2). However, statistical

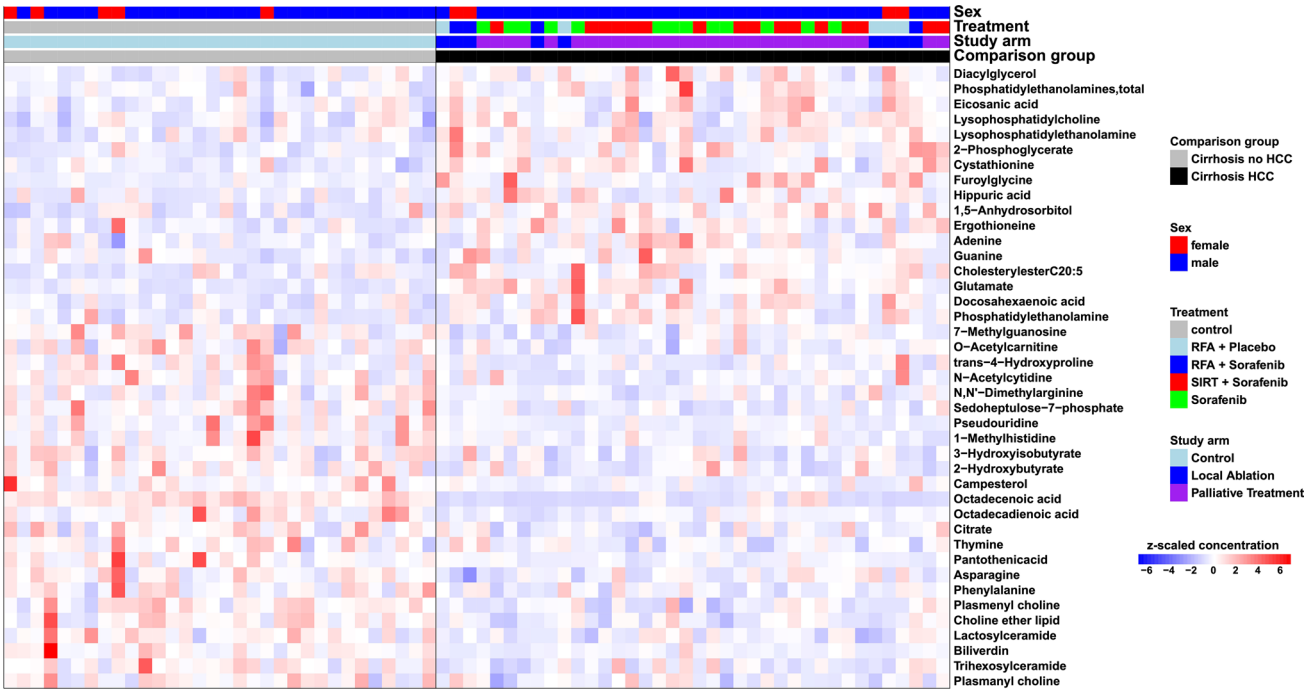


Fig. 2 Heat map of differential metabolite expression in liver cirrhosis and HCC. This heat map shows the differentially expressed metabolites compared between cirrhotic patients with and without hepatocellular carcinoma (HCC) with Z-scaled metabolite concentrations of the 41 metabolites statistically significantly and differentially expressed between patients with liver cirrhosis with and without HC. Blue colors indicate lower concentrations and red colors higher concentrations

significance was only observed for 23 of those 41 metabolites in this subgroup (Suppl. Table 2, Online Resource 1).

In order to exclude that a more severe liver disease in the LC controls had a significant influence on the metabolite expression, we further evaluated the metabolic signature in the patients with CPS A and B only. In this subgroup of HCC and LC patients, 40 of 41 metabolites remained statistically significant (Suppl. Figure 3, Online Resource 2).

Additionally, a correlation analysis between AFP values in the HCC patients with the 41 metabolites, which were differently expressed in HCC patients, was performed. No correlation was observed for the 41 metabolites differently expressed in control and HCC patients with continuous AFP values neither for the metabolites and AFP values categorized into low (< 20 ng/ml), medium (20–400 ng/ml), or high (> 400 ng/ml) levels (data not shown).

Differential Expression of Metabolites During Follow-Up of HCC Patients

In a next step, we evaluated the longitudinal expression of the metabolites in HCC patients throughout the FU in comparison to baseline. A statistically significant differential expression from baseline to the last FU was identified for 14 metabolites with the highest increase detected for o-acetylcarnitine, 2-hydroxybutyrate, biliverdin, phenylalanine, trans-4-hydroxyproline and pseudouridine, 2-phosphoglycerate, 3-hydroxyisobutyrate, furoylglycine, and citrate (Fig. 3).

Differential Expression of Metabolites in HCC Patients Receiving Local Ablation vs. Palliative Care

In addition to the time course analysis, a comparison of the metabolite expression between HCC patients who had been treated within the palliative group or the local ablative group was conducted. As demonstrated in Table 2, patients in the palliative care group suffered from a higher tumor burden and were classified into a higher BCLC stage. The analysis revealed a differential expression for campesterol, lysophosphatidylcholine, octadecenoic acid, octadecadienoic acid, and furoylglycine in the palliative vs. local ablation group as demonstrated in Online Resource 1, Suppl. Table 3. Campesterol, lysophosphatidylcholine, and furoylglycine were significantly higher in the local ablation group, while octadecenoic acid and octadecadienoic acid were expressed to a higher degree in patients from the palliative treatment group (Fig. 4; Suppl. Figure 4, Online Resource 2).

Prognostic Value of Metabolites in HCC Patients

Since a differential expression was observed between both treatment groups, we further investigated whether any of the baseline metabolites were prognostic regarding the OS of the HCC patients. Figure 5 shows the Kaplan–Meier analysis for the eight metabolites that at an individually defined cut-off level, with the threshold being the third quartile, a significantly lower OS was detected. As such, we found that a high expression of octadecenoic acid, 2-hydroxybutyrate, myo-inositol, isocitrate, erythronic acid, creatinine, pseudouridine, and erythrol at baseline was significantly associated with poor OS in HCC patients. Correlation of those metabolites with AFP values was evaluated. Interestingly, only octadecenoic acid was elevated in patients with high AFP > 400 ng/ml, whereas no correlation between 2-hydroxybutyrate, myo-inositol, isocitrate, erythronic acid, creatinine, pseudouridine, and erythrol and AFP categories (< 20 ng/ml, 20–400 ng/ml, and > 400 ng/ml) was observed (Online Resource 2, Suppl. Figure 5).

Influence of Treatment Response on Metabolic Signature

Of the 38 HCC patients included, detailed data on treatment response were available for 30 patients: 6 (20%) showed partial and 11 (37%) complete response, stable disease was seen in 7 (23%), while 6 (20%) HCC patients showed progressive disease. While complete response was associated with a tendency toward a higher probability of survival during the follow-up, there was no significant association between the treatment response groups and OS ($p = 0.38$; data not shown). With regards to the metabolite expression, no differential expression of the eight metabolites identified as having an association with OS was observed between the four response groups (Online Resource 2, Suppl. Figure 6).

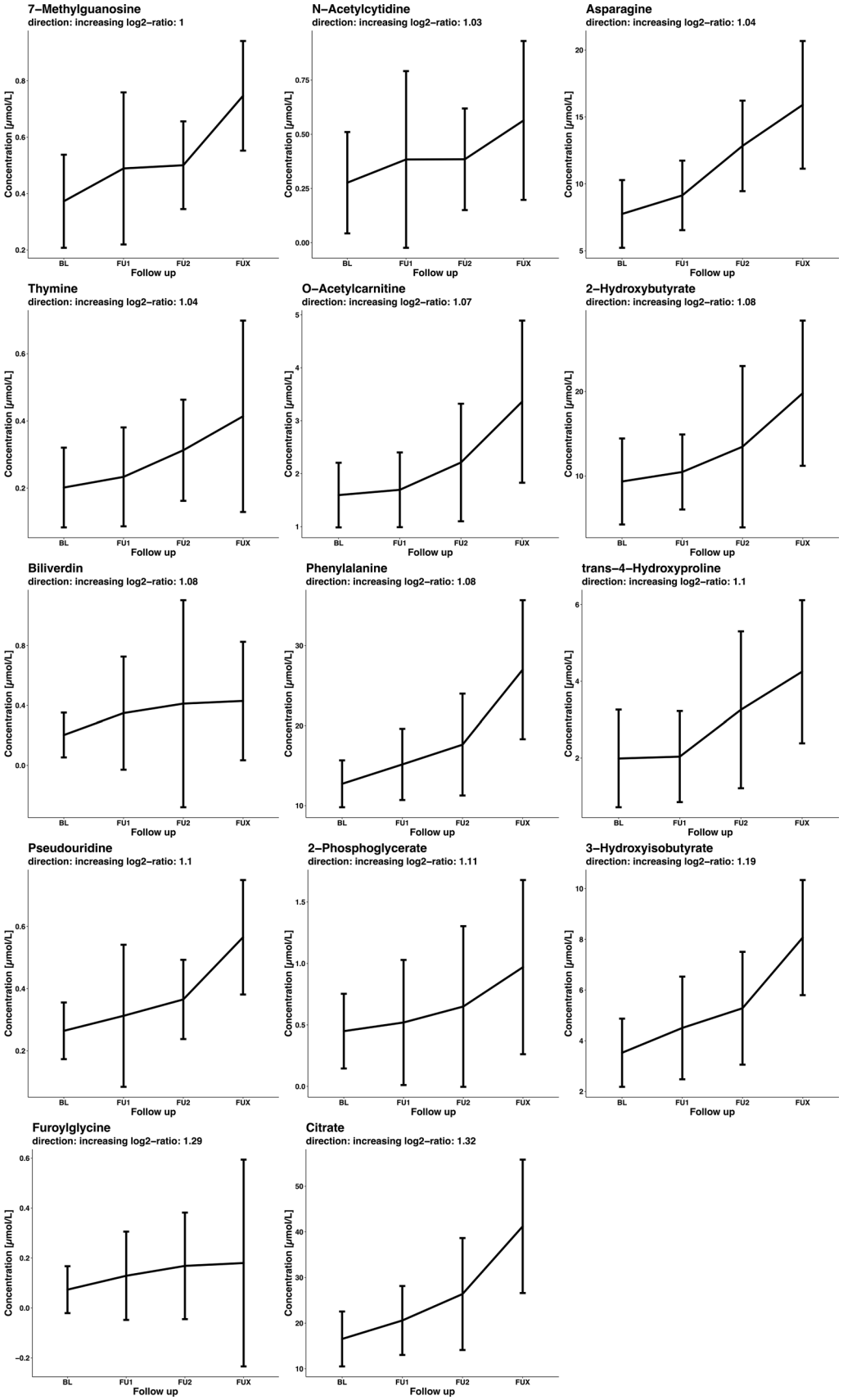
Since survival might be affected by many factors, e.g., tumor burden, severity of liver disease, treatment type, and treatment response, the prognostic value of metabolites was evaluated after adjustment for these possibly interfering co-variants. First, univariate analysis regarding the association between OS and BCLC stage, CPS, age, treatment group, and treatment response was performed. The only variable significantly associated with OS was CPS ($p = 0.041$). BCLC stage and treatment type showed a non-significant association ($p < 0.200$), while age and treatment response were not associated with OS and were therefore not included in the multivariate model (data not shown). Multivariate analysis then revealed that the association between OS remained statistically significant for octadecanoic acid and creatinine, in addition a trend toward an association was seen for isocitrate, pseudouridine, 2-hydroxybutyrate, and erythrol with p values < 0.2 (Online Resource 1, Suppl. Table 4). However, myo-inositol and erythronic acid were not independently associated with OS after adjustment for CPS, BCLC stadium, and treatment response (Online Resource 1, Suppl. Table 4).

Expression of Relevant Metabolic Enzymes in Tumor Tissue

To validate the source of the discovered metabolites, the expression's distribution of the two enzymes lysophosphatidylcholine acyltransferase 1 (LPCAT 1) and isocitrate dehydrogenase 1 (IDH1), playing key roles in the metabolism of two relevant markers identified in our study, were evaluated in liver tissues, including tumor and adjacent parenchyma, of selected patients. LPCAT1 catalyzes the reaction from lysophosphatidylcholine toward phosphatidylcholine, while IDH1 catalyzes the synthesis from isocitrate toward α-ketoglutarate (α-KG), and when overexpressed, both proteins are markers for bad prognosis in HCC. As such, LPCAT1 was stained in the tissue of two patients—patient 1 with an OS of only 7 and patient 2 with an OS of 13.4 months. As shown in Suppl. Fig. 7A and B (Online Resource 2), IHC analysis showed a strong cytoplasmic staining in the tumor from patient 1; on the contrary, no staining was observed in patient 2. Moreover, IDH1, was stained in the tissue of additional two patients—patient 3 with an OS of 14 and patient 4 with an OS of 24 months. As demonstrated in Fig. 7C and D (Online Resource 2), IDH1 was expressed to a higher degree in patient 3 when compared to patient 4. The presence of tumor necrosis was identified in less than 5% of the lesion in all analyzed slides (Online Resource 2, Suppl. Figure 7). In conclusion, IHC performed on tissues suggest a tumor origin of the metabolites.

Discussion

LC patients are at high risk for developing HCC and therefore require close surveillance. In order to better identify the patients most at risk of developing HCC, a more detailed understanding of the molecular changes in HCC patients is required. Most studies thus far have focused on immunological processes or genetic disturbances [4–6, 12]. However, with increasing knowledge of the importance of gut microbiota, obesity, and metabolic dysfunction-associated steatotic liver disease for HCC development [13–15], it becomes evident that metabolic changes also most likely have a high impact on HCC evolution. With this pilot study, we aimed to identify metabolomic markers in HCC patients which can distinct them from LC patients and further analyze whether these markers also have a prognostic relevance.


7–Methylguanosine
direction: increasing log2–ratio: 1
N–Acetylcytidine
direction: increasing log2–ratio: 1.03
Asparagine
direction: increasing log2–ratio: 1.04
Thymine
direction: increasing log2–ratio: 1.04
O–Acetylcarnitine
direction: increasing log2–ratio: 1.07
2–Hydroxybutyrate
direction: increasing log2–ratio: 1.08
Biliverdin
direction: increasing log2–ratio: 1.08
Phenylalanine
direction: increasing log2–ratio: 1.08
trans–4–Hydroxyproline
direction: increasing log2–ratio: 1.1
Pseudouridine
direction: increasing log2–ratio: 1.1
2–Phosphoglycerate
direction: increasing log2–ratio: 1.11
3–Hydroxyisobutyrate
direction: increasing log2–ratio: 1.19
Furoylglycine
direction: increasing log2–ratio: 1.29
Citrate
direction: increasing log2–ratio: 1.32
Concentration [µmol/L]
Follow up
BL
FU1
FU2
FUX

◂**Fig. 3** Differential metabolite expression during follow-up. This figure demonstrates the concentrations of metabolites differentially expressed between cirrhotic patients with and without hepatocellular carcinoma (HCC) changing expression along follow-up. Shown are the top ten differentially expressed between cirrhotic patients with and without HCC (baseline) that incrementally change expression at each follow-up and which exceed an absolute log2-ratio between last follow-up and baseline of 1

We detected 41 metabolites with a significantly different expression in HCC patients when compared to LC controls, 24 were overexpressed in LC controls and 17 in HCC patients. Those metabolites were also similarly expressed when only LC control and HCC patients with underlying alcoholic etiology were regarded; however, statistical significance was only observed for 23 of 41 metabolites in this subgroup analysis. The lower rate of statistical significance is most likely due to the smaller sample size in the subgroup analysis with only 29 patients in total. The LC patients in our cohort showed higher CPS indicating more advanced liver disease severity. To exclude that CPS relevantly affected the metabolite expression, we evaluated the metabolic signature in HCC and LC patients with CPS A and B only. We could demonstrate that also in the subgroup of less severe HCC and control patients, 40 of 41 metabolites identified remained statistically significant.

In addition, eight metabolites (octadecenoic acid, 2-hydroxybutyrate, myo-inositol, isocitrate, erythronic acid, creatinine, pseudouridine, and erythrol) showed an association with OS in the HCC patients. This association remained statistically significant for octadecenoic acid and creatinine even when adjusted for BCLC stage, CPS, and treatment type, while a trend toward an association was observed for isocitrate, pseudouridine, 2-hydroxybutyrate, and erythrol after adjustment. The multivariate analysis is hindered by the small sample size; however, our results indicate that the metabolic signature was a relevant predictive feature for poor OS in HCC patients. While an association with outcome was clearly detected for those eight metabolites, treatment response was not significantly associated with OS either, although a trend toward a higher probability of surviving in patients with complete response was observed. It is unclear why better treatment response did not translate into a better OS in our HCC cohort. We speculate that this phenomenon could be due to the low sample sizes in the respective treatment groups. Nevertheless, the differential expression of metabolites in HCC and LC patients and the independent associations between some metabolites and OS in particular provide some interesting insights in the metabolic signature of HCC and possibly also the pathogenesis of HCC. Roughly, the metabolites identified in our study can be divided into biomarkers with antioxidant and with (anti-)proliferative properties.

Differential Expression of Metabolites with Antioxidant Properties

One example for an antioxidant metabolite is campesterol. Being a compound of guava seed oil, campesterol has been associated with antioxidant activity and hepato-protective effects via the reduction of peroxide-mediated oxidative stress [16]. Our analysis revealed that campesterol was expressed to a higher degree in LC controls than in HCC patients. Conversely, campesterol was found to be higher in patients from the local ablation group when compared to the palliative care group. In the SORAMIC trial protocol, the decision on whether a patient received local ablation rather than palliative care was based on the lower number of tumor lesions, smaller tumor size, and not having had any previous HCC therapy [8]. This is also highlighted by the significantly higher BCLC stage, higher number of tumor lesions, and larger maximal diameter which we saw in the palliative care group when compared to the local ablation group. Thus, patients from the palliative care group had more advanced malignant disease with a higher tumor burden. It can therefore be assumed that a lower expression of campesterol in the palliative care group at baseline indicates an inverse correlation with tumor burden, meaning that campesterol decreases with the development of HCC and with increasing HCC tumor burden. An explanation for the decrease in campesterol in HCC patients could be the enhanced oxidative stress and production of reactive oxygen species (ROS) in the HCC tumor environment resulting in the consumption of campesterol as an antioxidant metabolite.

Another metabolite with antioxidant potential and differential expression in our analysis was 2-hydroxybutyrate. We detected an increase in HCC patients from baseline to follow-up, indicating that 2-hydroxybutyrate was upregulated parallel to an increasing tumor burden. Interestingly, we also detected that high-expression levels of 2-hydroxybutyrate correlated with reduced OS pointing toward an association of 2-hydroxybutyrate with high tumor burden and increased mortality. This association could be explained by the higher oxidative stress which is caused by large tumor masses leading to the exhaustion of the antioxidant capacity of glutathione and therefore, the upregulation of 2-hydroxybutyrate as an additional antioxidative agent [17].

Differential Expression of Metabolites with Proliferative Properties

One metabolite detected in our analysis with a known proliferative function was lysophosphatidylcholine. This metabolite has been described to play an important role in cell proliferation, migration, and tumor angiogenesis [18].

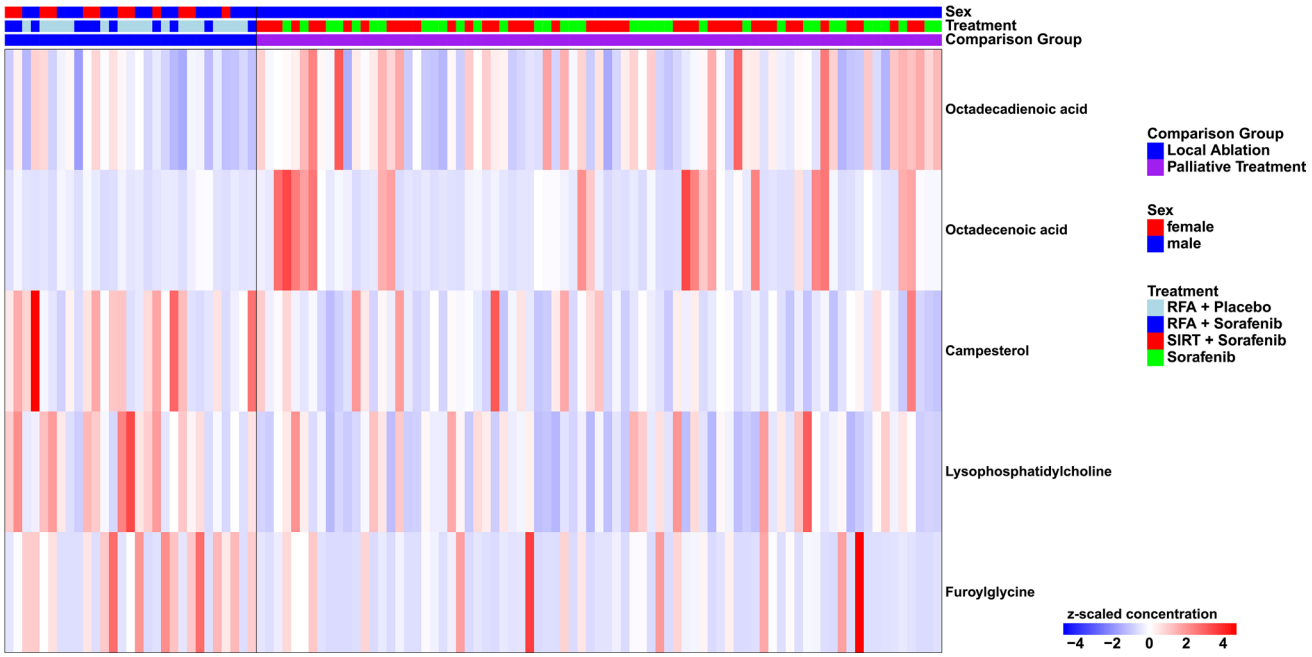


Fig. 4 Heat map of differential metabolite expression in the ablative and palliative care group. The heat map of shows the differential expression of metabolites between hepatocellular carcinoma (HCC) patients treated by ablative therapy and HCC patients treated in a palliative setting. Z-scaled metabolite concentrations of the 5 metabolites were statistically significantly differentially expressed. Blue colors indicate lower concentrations and red colors indicate higher concentrations

We found that lysophosphatidylcholine was expressed to a higher degree in HCC patients when compared to LC controls, which might represent enhanced tumor angiogenesis symbolizing the characteristic hyper-vascularization of HCC lesions. However, we observed lower baseline levels of lysophosphatidylcholine in the palliative care group when compared to the local ablation group. A possible explanation for the lower expression of lysophosphatidylcholines in patients with more advanced tumor stages might be that the upregulation of the metabolic activity and cell proliferation only occurs at earlier tumor stages and therefore, a higher expression of lysophosphatidylcholines is only found at the beginning of HCC development. Conversely, it is known that metabolic pathways are upregulated in HCC lesions to provide necessary energy for tumor growth [19]. At later HCC stages, pronounced changes in the metabolic activity with a shift to anaerobic conditions are therefore expected [20], which can explain the decrease of metabolites upregulated at earlier stages of tumor development. In order to better define if lysophosphatidylcholines actually play a role in the carcinogenesis of HCC, staining of LPCAT1, the key enzyme that catalyzes the reaction of lysophosphatidylcholine toward phosphatidylcholine, in the tumor tissue of two of the patients included in this study was performed. Interestingly we found a higher expression of LPCAT1 in tissue from the patient that presented with a lower OS. Thus, it can be hypothesized that upregulation of LPCAT1 in advanced HCC leads to lower levels of lysophosphatidylcholines underlining our findings of lower lysophosphatidylcholine levels correlating with more advanced tumor stages. Our histopathological observations are also in line with data from the protein atlas showing that LPCAT1 correlates with lower OS in HCC patients [21, 22]. In conclusion, lysophosphatidylcholines seem to play a crucial role in HCC development and progression, which therefore can be potentially used as a biomarker for HCC diagnosis and surveillance under therapy.

Two other metabolites that are known to play a role in cell proliferation showed differential expression in our analysis, i.e., octadecenoic acid and octadecadienoic acid. Both were expressed to a higher degree in patients from the palliative care group indicating a correlation with more advanced tumor stages. In line with this, we observed a significant association between a high expression of octadecenoic acid and reduced OS in HCC patients, which remained significant even after adjusting for BCLC stage, CPS, and treatment type. Thus, correlation between high expression of octadecenoic acid and tumor progression must be assumed. Contradicting those results, previous studies have proposed anti-carcinogenic properties for octadecenoic acid [23, 24]. As such, it has been demonstrated that octadecenoic acid induced dose-dependent anti-proliferative effects on various tumor cell strains, including HCC cell strain [24]. Similar cytotoxic effects have been described for octadecadienoic

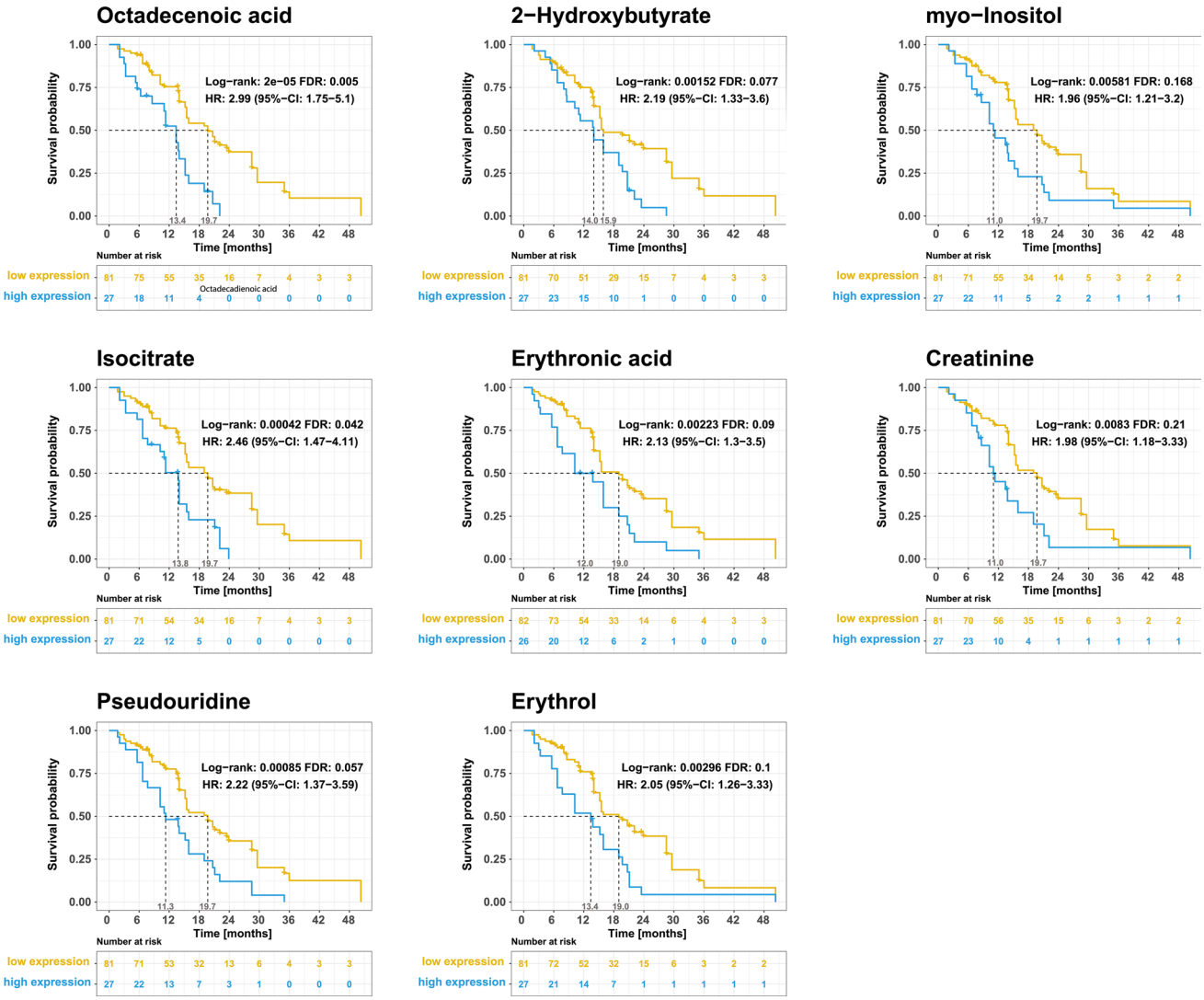


Fig. 5 Metabolites predicting overall survival (OS) in hepatocellular carcinoma (HCC) patients from blood. The Kaplan–Meier plots show metabolites determined in blood before treatment (baseline) in HCC patients that predict OS. Log-rank *p* values and false discovery rates are given. Patients were split into a high-expression and low-expression group, while the third quartile of each expression vector was used as cut-off (high: > cut-off, low: ≤ cut-off)

acid [25]. In vitro cytotoxicity assays, for example, have shown high anti-cancer activity of the extracts of Scenedesmus obliquus against human hepatocellular carcinoma (HepG2) cell lines with octadecadienoic acid being one of the major compounds responsible for the bioactivity [26]. Furthermore, an induction of apoptosis through the increase of ROS production and consecutive alteration of mitochondrial signaling pathways has been demonstrated for the extract of Typhonium giganteum Engl., an herb traditionally used in Chinese medicine for anti-cancer treatment, with octadecadienoic acid again being one of the major four compounds [27]. Thus, it remains unclear from the comparison of our and previous results whether octadecenoic and octadecadienoic acids correlate with the HCC tumor burden. Further studies are needed to evaluate the role of these two molecules in HCC carcinogenesis and their possible application as prognostic biomarkers.

A correlation between higher expression and reduced OS was also observed for myo-inositol, a metabolite which is associated with cell proliferation as well. Myo-inositol is required as a substrate for the synthesis of inositol triphosphate (PI3) and is consecutively involved in the phosphatidyl inositol 3 kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) (PI3K-AKT-mTOR) signaling pathway. The PI3K-AKT-mTOR pathway is associated with cell growth, proliferation, and survival and has been shown to be one of the most frequently upregulated signaling pathways in human tumors [28]. Thus,

an increased expression of myo-inositol might be indicative for an activation of the PI3K-AKT-mTOR pathway and consecutively for tumor growth, which could explain the observation of reduced OS in the case of high myo-inositol expression in our HCC cohort. Future studies should focus on the evaluation of the association between tumor growth and activation status of the PI3K-AKT-mTOR pathway as well as with myo-inositol plasma concentrations.

Additionally, high expression of isocitrate was associated with reduced OS. Isocitrate is a substrate of IDH1 and IDH2 both of which promote the synthesis of α-KG [29]. IDH2 expression in particular has been shown to be inversely correlated with prognosis of HCC patients: Low IDH2 levels are predictive for metastasis and poor survival [30]. Thus, the observed association of higher expression of isocitrate with reduced OS might be indicative for a suppressed state of IDH1 and 2 resulting in increased levels of their substrate isocitrate that then in turn would also be associated with poor OS. However, when tissue staining was performed for IDH1 in the tumor tissue of two patients, it was seen that the patient with longer OS actually had suppressed levels of IDH1 when compared to the patient with a drastically lower OS, which indicates that higher IDH1 expression correlates with poor outcome. This contradictory observation can be explained considering that in several type of cancers, including HCC, IDH1 is frequently mutated with an arginine residue substitution in position 132 (R132) [31, 32]. This mutation confers a gain of function, with IDH1 displaying a higher affinity for nicotinamide adenine dinucleotide phosphate (NADPH) than for isocitrate and with the consequent production of different oncometabolites with high pro-tumorigenic effect [33]. Therefore, we hypothesize that a higher level of isocitrate correlates with a lower OS because IDH1 might be carrying the R132 mutation and therefore is unable to metabolize its substrate, although IDH1 is still highly expressed in the tissue. Additional experimental work will be necessary to prove this hypothesis.

Differential Expression of Metabolites with Yet Unknown Properties

Some of the metabolites that showed differential expression in HCC and/or a correlation with OS in our analysis have unknown functions in the human body and cancer development. Furoylglycine, for instance, was expressed to a higher degree in HCC vs. LC control patients and was increased in HCC patients from baseline to the last follow-up, indicating a correlation with both HCC development and progression. This is an interesting finding, since the correlation of furoylglycine with tumor burden and/or prognosis in HCC patients has, to the best of our knowledge, not been reported before. In parallel with the above-mentioned findings for lysophosphatidylcholine, we detected a lower expression of furoylglycine in patients within the palliative care group vs. the local ablation group. Once again, this could represent a reduced metabolic activity in HCC patients at more advanced stages of tumor development. The function of furoylglycine in humans has not been elucidated in full detail thus far. Some studies have described furoylglycine as a possible biomarker for coffee consumption [34]. However, a role of furoylglycine in tumor development and growth is has not been discussed thus far. Thus, furoylglycine could be a promising biomarker that should be elaborated on in more detail in further studies.

We also observed that creatinine, an important marker for kidney function, was predictive for survival in HCC patients with a higher expression of creatine correlating with reduced OS. Interestingly, the association between OS and creatinine remained statistically significant even after adjusting for confounding factors (CPS, BCLC stage and treatment type), indicating that high creatinine is an independent risk factor for worse survival in HCC patients.

Moreover, a correlation between poor survival and high expression of erythronic acid and erythrol were observed. Erythrol is a decomposition product of erythritol, which has been identified as a liquid metabolic biomarker for lung cancer [35] and lymphoma [36]. For erythronic acid, it has been shown that its upregulation is a sign of deficient transaldolase activity, which in turn can result in mitochondrial dysfunction [37] and possibly impaired liver function [38]. Thus, the association seen between enhanced expression of erythronic acid and poor OS in HCC patients might represent transaldolase deficiency with consecutive disruption of mitochondrial function, a phenomena regularly observed in patients with advanced HCC [39]. In conclusion, our results indicate both, erythrol and erythronic acid, might serve as distinctive prognostic markers in HCC which should be investigated in more detail in future studies.

Our study has limitations, for instance, the relatively small number of patients, the lack of more longitudinal data, and the non-standardized time points of the follow-up visits in HCC patients. Further, HCC patients and LC control patients were not matched regarding age, etiology, nor severity of liver disease adding possible confounding variables. Nevertheless, we could identify metabolites with differential expression in HCC and LC patients, which give further insight into HCC carcinogenesis and the importance of metabolic changes during cancer evolution. Furthermore, we could also detect various metabolites with prognostic potential in HCC. Thus, in addition to revealing disturbances in signaling pathways, our findings also provide possible candidate biomarkers for HCC diagnosis and/or prognosis. Our results should therefore stimulate further studies in particular regarding possible pathogenic mechanisms,

novel therapeutic approaches, and clinical application of potential biomarkers.

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Declarations

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