LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Egidijus Morkūnas

MULTIMODAL ANALYSIS OF INFLAMMATORY MEDIATORS AND microRNAs IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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Scientific Supervisor

Prof. Dr. Gediminas Kiudelis (Lithuanian University of Health Sciences, Medical and Health Sciences, Medicine – M 001).

Consultant

Dr. Greta Gedgaudienė (Lithuanian University of Health Sciences, Natural Sciences, Biology – N 010).

Dissertation is defended at the Medical Research Council of Lithuanian University of Health Sciences.

Chairperson

Prof. Habil. Dr. Limas Kupčinskas (Lithuanian University of Health Sciences, Medical and Health Sciences, Medicine – M 001).

Members:

Prof. Dr. Donatas Vajauskas (Lithuanian University of Health Sciences, Medical and Health Sciences, Medicine – M 001);
Assoc. Prof. Dr. Povilas Ignatavičius (Lithuanian University of Health Sciences, Medical and Health Sciences, Medicine – M 001);
Prof. Dr. Sonata Jarmalaitė (Vilnius University, Natural Sciences, Biology – N 010);
Prof. Dr. Joost P. H. Drenth (University of Amsterdam (the Netherlands), Medical and Health Sciences, Medicine – M 001).

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Address: Eivenių 2, LT-50161 Kaunas, Lithuania.

LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

Egidijus Morkūnas

DAUGIAPAKOPĖ UŽDEGIMO MEDIATORIŲ IR mikroRNR ANALIZĖ KEPENŲ LĄSTELIŲ KARCINOMA SERGANTIEMS PACIENTAMS

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Mokslinis vadovas

prof. dr. Gediminas Kiudelis (Lietuvos sveikatos mokslų universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Konsultantė

dr. Greta Gedgaudienė (Lietuvos sveikatos mokslų universitetas, gamtos mokslai, biologija – N 010).

Disertacija ginama Lietuvos sveikatos mokslų universiteto medicinos mokslo krypties taryboje:

Pirmininkas

prof. habil. dr. Limas Kupčinskas (Lietuvos sveikatos mokslų universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Nariai:

prof. dr. Donatas Vajauskas (Lietuvos sveikatos mokslų universitetas, medicinos ir sveikatos mokslai, medicina – M 001);
doc. dr. Povilas Ignatavičius (Lietuvos sveikatos mokslų universitetas, medicinos ir sveikatos mokslai, medicina – M 001);
prof. dr. Sonata Jarmalaitė (Vilniaus universitetas, gamtos mokslai, biologija – N 010);
prof. dr. Joost P. H. Drenth (Amsterdamo universitetas (Nyderlandai), medicinos ir sveikatos mokslai, medicina – M 001).

Disertacija bus ginama viešame medicinos mokslo krypties tarybos posėdyje 2025 m. balandžio 17 d. 16.00 val. Lietuvos sveikatos mokslų universiteto Slaugos fakulteto 114 auditorijoje.
Disertacijos gynimo vietos adresas: Eivenių g. 2, LT-50161 Kaunas, Lietuva.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AFP	–	alpha-fetoprotein
ALBI	–	albumin-bilirubin score
AUC	–	area under the curve
BCLC	–	Barcelona Clinic Liver Cancer
circRNA	–	circular RNA
DCP	–	des-γ-carboxyprothrombin
DNA	–	deoxyribonucleic acid
EGFR	–	epidermal growth factor receptor
ELISA	–	enzyme-linked immunosorbent assay
FABP	–	fatty acid-binding protein
GP3	–	glypican-3
HCC	–	hepatocellular carcinoma
IL	–	interleukin
LPS	–	lipopolysaccharides
MAFLD	–	metabolic dysfunction-associated fatty liver disease
mALBI	–	modified albumin-bilirubin score
MDS	–	multidimensional scaling
miRNA	–	microRNA
mRECIST	–	modified response evaluation criteria in solid tumors
NAFLD	–	non-alcoholic fatty liver disease
OS	–	overall survival
PCR	–	polymerase chain reaction
PFS	–	progression free survival
RNA	–	ribonucleic acid
ROC	–	receiver operating characteristic
ROS	–	reactive oxygen species
SIRT	–	selective internal radiation therapy
smRNA-seq	–	small RNA sequencing
TACE	–	transarterial chemoembolization
TGF	–	transforming growth factor
TLR	–	toll-like receptor
TNF	–	tumor necrosis factor
VEGF	–	vascular endothelial growth factor

INTRODUCTION

Liver cancer is one of the most frequently diagnosed cancers worldwide taking 7th place among all cancer sites, being surpassed only by breast, lung, prostate, skin, colon and stomach cancers. According to global cancer statistics, in 2020 liver cancer was newly diagnosed for 905,677 patients or almost 5% of new cancer cases of all sites. In the cancer-related death statistics liver cancer takes 2nd place worldwide. Only lung cancer is deadlier. In 2020 liver cancer caused death of 830,180 patients or more than 8% of cancer-related deaths of all sites [1]. Comparing incidence and mortality rates, liver cancer takes place as the primary cancer site with the worst overall prognosis as the number of cancer-related deaths almost reaches the number of new cases per year. Hepatocellular carcinoma (HCC) is the most common primary liver cancer. Unfortunately, according to the American predictions, the incidence of HCC is increasing and is predicted to continue to rise in the future [2]. This signalizes that there is still a need for far better diagnostic and therapeutic modalities for this disease.

HCC develops in the background of liver cirrhosis in more than 80% of cases [3]. The most common causes of chronic liver inflammation and cirrhosis globally as well as in Lithuania remain chronic viral infection (hepatitis C and B) and chronic alcoholic hepatic injury [4, 5], although, incidence of cirrhosis due to non-alcoholic fatty liver disease (NAFLD) or newly defined metabolic dysfunction-associated fatty liver disease (MAFLD) is increasing, especially in Western countries [6, 7].

All etiologic factors, being involved for a long time, induce chronic liver inflammation by interrupting the regulation of hepatic immune system. High regenerative capacity of the liver and induction of cell proliferation compensates the damage caused by chronic inflammation-induced liver cell death. This intensive regeneration is associated with the accumulation of reactive oxygen species (ROS) and DNA mutations which eventually leads to carcinogenesis and development of HCC. The tumor microenvironment and its pro-inflammatory elements, especially cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α), play a key role in this process [8–10]. Previous clinical and preclinical studies have already demonstrated that levels of IL-6 as well as IL-8 in blood increase in patients with HCC compared to cirrhotic patients without HCC [11–15].

The microenvironment of tumor or inflammatory liver tissue involves not only pro-inflammatory but also anti-inflammatory cytokines, such as IL-10 which role is to reduce pro-inflammatory immune responses, in the carcinogenesis process as well [16–18]. The higher severity of chronic liver

damage and liver cirrhosis, usually the higher IL-10 plasma levels are detected [19]. Several studies demonstrated that plasma IL-10 levels tend to be elevated in HCC patients mostly due to cirrhotic process rather than tumor load [20, 21].

The inflammation process in the liver is induced and kept also indirectly through other pathways. Kupffer cells within the liver are activated to release ROS and pro-inflammatory cytokines by various endotoxins, such as lipopolysaccharides (LPS), which blood levels increases in cirrhotic patients due to defective intestinal barrier [22, 23]. LPS, produced by gut bacteria, causes systemic and hepatic inflammatory response by stimulating the release of IL-8 and other proinflammatory cytokines via the toll-like receptor (TLR) pathway [24, 25], in this way inducing carcinogenesis. LPS also stimulates tumor cells to increase the production of vascular endothelial growth factor (VEGF) which promotes proliferation of HCC cells and angiogenesis within the tumor [26, 27].

As mentioned, increased gut permeability and bacterial translocation is associated with chronic liver damage and hepatocarcinogenesis. Fatty acid-binding proteins (FABP), which are small protein molecules released after the damage of enterocytes, could serve as the biomarker for gut permeability [28, 29]. Previously, FABP2 plasma levels were shown to be significantly elevated in cirrhotic patients and to have correlation with grade of portal hypertension [23].

The process of carcinogenesis is regulated not only by microenvironment but also by various genetic and epigenetic factors. MicroRNAs (miRNAs) are small non-coding RNA molecules controlling many gene targets and having potential influence on almost every genetic pathway. Deregulation of miRNAs could be associated with various types of cancers and may act as either tumor suppressors or promoters [30]. Several studies demonstrated a clear link between different miRNA molecules and carcinogenesis of HCC through different pathways [31, 32].

When HCC is already developed in the cirrhotic liver, it is crucial to detect it as early as possible as multiple radical therapeutical modalities, such as resection and ablation, could be applied with total elimination of the disease [33, 34]. In the later stages of HCC, usually radical therapeutical approaches are not possible, except for liver transplantation with very limited indications [35]. For more than ten years the main option for the first-line systemic treatment was sorafenib, an oral multi-target tyrosine kinase inhibitor, which was demonstrated to prolong survival in patients with HCC in advanced stages [36]. Despite novel immunotherapies that have displaced sorafenib in the first-line HCC treatment and ongoing clinical trials for other molecules [37], sorafenib will remain the important option, especially when

immunotherapy is unavailable, contraindicated, or ineffective. To improve efficacy of sorafenib therapy, several additional modalities for combination therapy, such as selective internal radiation therapy (SIRT), were investigated. Although the addition of SIRT, specifically ^{90}Y radioembolization, has not shown significant difference [38], further analysis has demonstrated positive effect of addition of SIRT to sorafenib on survival rates for selected patients with HCC [39].

Although recently the number of new molecules approved for the treatment of advanced HCC is increasing, none of them has specific targets or means to predict the efficacy or evaluate the prognosis of HCC patients. Therefore, there is a need for all kind of biomarkers – diagnostic, prognostic, predictive.

Alpha-fetoprotein (AFP) is the only biomarker used in everyday clinical practice in management and surveillance of HCC as well as in the selection of liver transplant recipients [40]. However, baseline AFP plasma levels are usually normal and may be elevated due to chronic viral hepatitis or other non-malignant reasons [41, 42].

Several other molecules, especially those having important role in hepatocarcinogenesis, have been investigated as potential HCC biomarkers. Inflammatory cytokines IL-6 and IL-8 have been shown to be related to sorafenib resistance [43–45]. Shao et al. demonstrated that pretreatment IL-6 levels correlate with HCC patients' survival after sorafenib treatment [46]. However, another study failed to detect prognostic value of pretreatment IL-8 levels for sorafenib-treated patients [47]. Both IL-6 and IL-8 have been shown to be able to predict response to treatment and survival in HCC patients after transarterial chemoembolization (TACE) [48]. Similarly, IL-6 and IL-8 were associated with overall survival after ^{90}Y radioembolization [49]. Serum IL-10 levels were demonstrated to be the independent prognostic factor for HCC patients [21]. Despite some significant findings, additional investigations are needed to understand better the role of IL-6, IL-8 and IL-10 for HCC patients.

Not only inflammatory cytokines but also other potential biomarkers for HCC diagnosis and prognosis were identified: molecular cellular markers (e.g., glypican-3 (GP3) [50, 51], des-γ-carboxyprothrombin (DCP) [52]), cancer stem cell markers (e.g., CD44 [53], CD90 [54]), non-cellular components (e.g., transforming growth factor-β (TGF-β) [55], VEGF [56]), miRNAs as well as circular RNAs (circRNAs) [57, 58], somatic genetic and epigenetic alterations [59].

Previous analyses of hepatic tissue and blood has revealed miRNA profiles as valuable biomarkers for early diagnosis for HCC, especially associated with chronic viral hepatitis B and C [60–62]. However, most of

studies analysed predefined miRNA panels or specific miRNAs by real-time polymerase chain reaction (PCR) [60–62]. As miRNA molecules are abundant in blood and their small size enables their analysis in different body fluids and tissues, miRNA could be a valuable marker [63]. As miRNAs take part in the carcinogenesis process, they could act as targets for future therapies. Recently more and more therapeutic miRNA-targeted approaches are investigated for various diseases, including HCC [64]. For all these reasons, comprehensive high-throughput studies identifying the complete miRNA profile with the best diagnostic and prognostic modalities are of high need and importance.

In the present study, we investigated biomarkers having the biggest potential for HCC diagnosis, prediction of treatment efficacy or survival. First, we analyzed inflammatory molecules with broader evaluation of IL-6 and IL-8. Further, we performed detailed analysis of miRNA transcriptome (miRNome) in HCC patients. As a result, we demonstrated significant and clinically valuable results revealing new potential diagnostic, prognostic and predictive biomarkers for HCC.

Aim and objectives

This study aimed to evaluate the diagnostic, predictive and prognostic value of blood plasma molecular biomarkers – inflammatory mediators and miRNA profiles – for HCC patients.

Objectives:

1. To investigate the value of blood plasma IL-6 and IL-8 levels before treatment for prognosis of survival for HCC patients and prediction of therapeutical efficacy in sorafenib-treated patients.
2. To investigate the value of blood plasma IL-6, IL-8, LPS and VEGF levels during treatment for prognosis of survival for HCC patients.
3. To investigate the diagnostic and prognostic value of blood plasma FABP2, IL-10 and LPS levels before treatment for HCC patients.
4. To investigate blood plasma miRNA profile and determine its value for diagnosis, prediction of therapeutical efficacy and prognosis of survival for HCC patients.

SCIENTIFIC NOVELTY

This study provides (1) detailed analysis of plasma IL-6 and IL-8 in HCC patients; (2) findings that demonstrate value of plasma IL-6 and IL-8 as the prognostic biomarker for HCC; (3) additional analysis of other inflammatory molecules – VEGF, LPS, FABP2, IL-10 – as potential plasma biomarkers for HCC; (4) differential plasma miRNA expression profiles in HCC patients and control cases and analysis of the most deregulated miRNAs; (5) detailed analysis of miRNA profiles comparing plasma samples before the initiation of HCC therapy, during or after the treatment and control plasma samples; (6) novel findings that reveal significant potential diagnostic, treatment-related and prognostic value of miRNA profiles for HCC patients.

Altogether, this was the first study to demonstrate the following: (1) clear prognostic value not only of blood plasma IL-6 and IL-8 levels before treatment but also of early reassessment of these molecules after starting HCC therapy, additionally showing predictive treatment-related value of IL-6 and IL-8 in sorafenib-treated patients; (2) value of potential inflammatory biomarkers – VEGF, LPS, IL-10, FABP2 – in HCC patients; (3) complete miRNome analysis and its diagnostic, prognostic and predictive value in HCC patients, highlighting hsa-miR-215-5p which could be identified as specific oncogenic miRNA with plasma expression levels related to HCC therapies and survival prognosis of HCC patients. All these new findings contribute to very important research on the best and most-effective approach for early diagnosis, prediction of survival and treatment efficacy in HCC in routinely minimally invasive blood test-based manner.

THE LAYOUT OF THE DISSERTATION

This thesis is prepared based on five articles (listed in the section "List of scientific papers"), all of them investigating the value of different molecules in blood for diagnosis and prognosis of HCC as non-invasive liquid biopsy-related biomarkers. The thesis is composed of two major parts: investigation of diagnostic, prognostic and treatment-related value of (1) inflammatory biomarkers; (2) miRNAs. The first part of the thesis is divided in two smaller parts: (1) investigation of the role of inflammatory cytokines IL-6 and IL-8; (2) additional investigation of the role of other inflammatory molecules, such as IL-10, FABP2, LPS, VEGF, in HCC. The results of four studies are published in peer-reviewed journals referred in Web of Science: Journal of Cancer Research and Clinical Oncology, EJNMMI Research, Medicina, Digestive Diseases. One publication is published in the peer-reviewed journal (Frontiers in Gastroenterology) which is not listed in the Clarivate Analytics Web of Science database. The layout of the thesis design and structure is presented in Fig. 1.1.

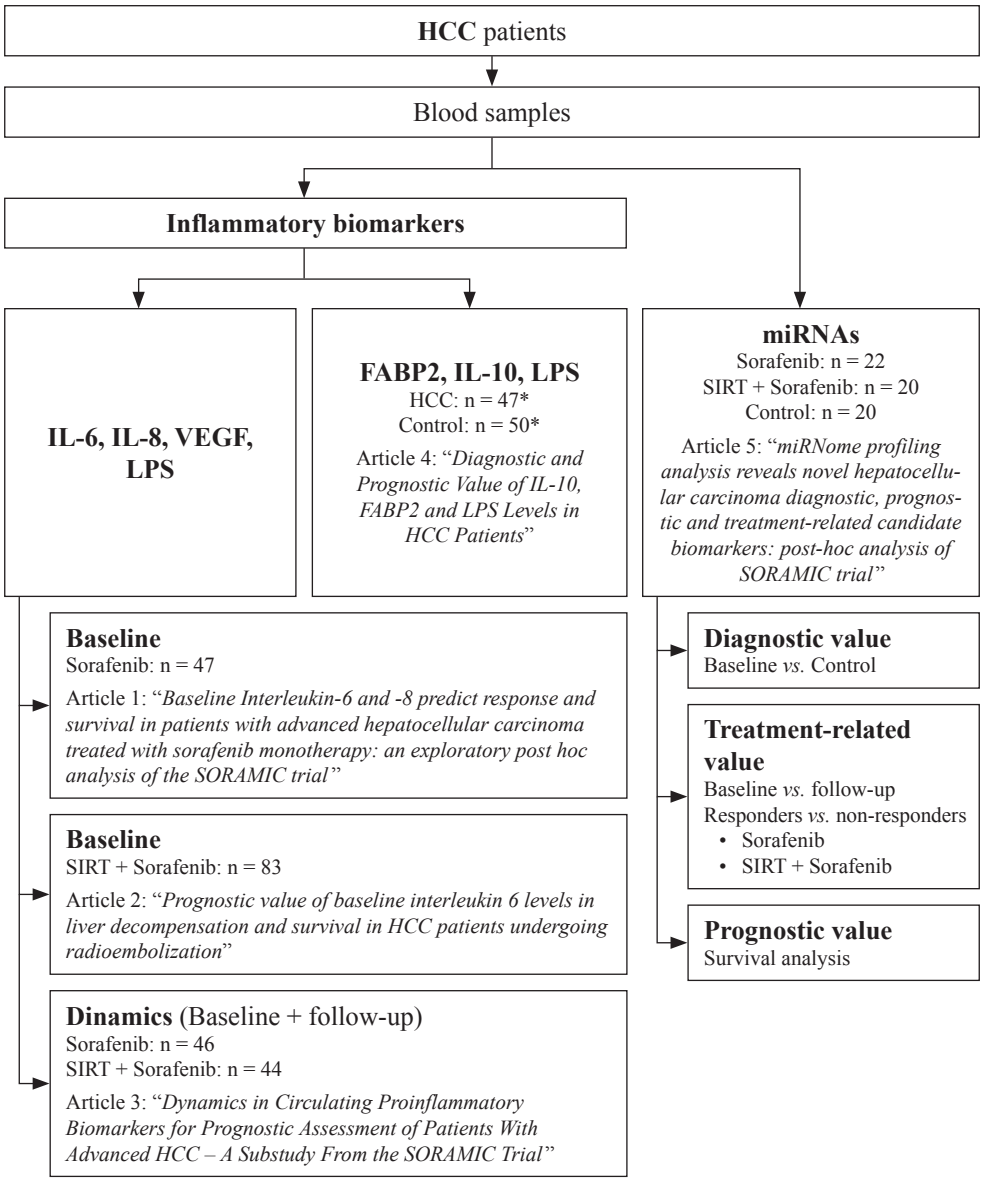


Fig. 1.1. *Structure of the thesis*

HCC – hepatocellular carcinoma, IL – interleukin, FABP2 – fatty acid-binding protein-2, LPS – lipopolysaccharides, VEGF – vascular endothelial growth factor, SIRT – selective internal radiotherapy. *IL-10 analysis included 45 HCC patients and 45 controls (due to insufficient amount of blood).

PhD CANDIDATE'S CONTRIBUTION

The contribution of author **Egidijus Morkūnas** in each of publications related to the dissertation (A1–A5 listed in Section "List of scientific papers") is presented below.

A1: was partially involved in generation of study concept and design of the research, contributed to performing ELISA tests, analysis, and interpretation of data, drafted and revised the manuscript, received the approval from the corresponding author and co-authors to defend this article in the dissertation.

A2: similarly to the first publication, was partially involved in generation of design of the research, performed ELISA tests to measure concentrations of interleukins, analyzed the data, drafted and revised the manuscript, received the full approval from the corresponding author to defend this article in the dissertation.

A3: were actively involved in technical part of the study by measuring concentrations of inflammatory molecules, analyzed and interpreted the data, drafted and revised the manuscript, received the approval from the corresponding author and co-authors to defend this article in the dissertation.

A4: conceptualized the study and its methodology, participated in the process of inclusion of HCC patients, collected the clinical data of participants, performed ELISA tests, analysed the data, drafted and prepared the final manuscript.

A5: participated in generation of study design and methodology, analyzed and interpreted the data, contributed in the drafting, editing and finalising the manuscript.

LIST OF SCIENTIFIC PAPERS

Publications related to the results of the dissertation listed in order of results description below:

A1: Öcal, Osman; Schütte, Kerstin; Kupčinskas, Juozas; **Morkūnas, Egidijus**; Jurkevičiūtė, Gabija; De Toni, Enrico N; Ben Khaled, Najib; Berg, Thomas; Malfertheiner, Peter; Klümpen, Heinz Josef; Sengel, Christian; Basu, Bristi; Valle, Juan W; Benckert, Julia; Gasbarrini, Aantonio; Palmer, Daniel; Seidensticker, Ricarda; Wildgruber, Moritz; Sangro, Bruno; Pech, Maciej; Ricke, Jens; Seidensticker, Max. Baseline Interleukin-6 and -8 predict response and survival in patients with advanced hepatocellular carcinoma treated with sorafenib monotherapy: an exploratory post hoc analysis of the SORAMIC trial. J Cancer Res Clin Oncol. 2022 Feb;148(2):475-485.

A2: Öcal, Osman; Kupčinskas, Juozas; **Morkūnas, Egidijus**; Amthauer, Holger; Schütte, Kerstin; Malfertheiner, Peter; Klümpen, Heinz Josef; Sengel, Christian; Benckert, Julia; Seidensticker, Ricarda; Sangro, Bruno; Wildgruber, Moritz; Pech, Maciej; Bartenstein, Peter; Ricke, Jens; Seidensticker, Max. Prognostic value of baseline interleukin 6 levels in liver decompensation and survival in HCC patients undergoing radioembolization. EJNMMI Res. 2021 Jun 2;11(1):51.

A3: Schütte, Kerstin; Kupčinskas, Juozas; **Morkūnas, Egidijus**; Öcal, Osman; Schinner, Regina; Seidensticker, Max; De Toni, Enrico N; Ben Khaled, Najib; Pech, Maciej; Palmer, Daniel; Berg, Thomas; Sengel, Christian; Basu, Bristi; Valle, Juan W; Benckert, Julia; Gasbarrini, Antonio; Sangro, Bruno; Malfertheiner, Peter; Ricke, Jens. (2022) Dynamics in Circulating Proinflammatory Biomarkers for Prognostic Assessment of Patients With Advanced HCC – A Substudy From the SORAMIC Trial. Front. Gastroenterol. 1:939192.

A4: **Morkūnas, Egidijus**; Vaitkevičiūtė, Evelina; Varkalaitė, Greta; Pilvinis, Vidas; Skiecevičienė, Jurgita; Kupčinskas, Juozas. Diagnostic and Prognostic Value of IL-10, FABP2 and LPS Levels in HCC Patients. Medicina (Kaunas). 2023 Dec 17;59(12):2191.

A5: **Morkūnas, Egidijus**; Vaitkevičiūtė, Evelina; Inčiūraitė, Rūta; Kupčinskas, Juozas; Link, Alexander; Skiecevičienė, Jurgita; Alunni-Fabbroni, Marianna; Schütte, Kerstin; Malfertheiner, Peter; Varkalaitė, Greta; Ricke, Jens. miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial. Dig Dis. 2024;42(4):336-348.

CONFERENCE PRESENTATIONS

1. Oral presentation. **E. Morkūnas**, J. Kupčinskas. *Hepatocellular carcinoma in Gastroenterology Department in Kaunas*. International Scientific Conference on Medicine 2021, April 23–24, Riga, Latvia (online).
2. Oral presentation. **E. Morkūnas**, *Hepatoceliulinės karcinomos molekulinis charakterizavimas*. Bioateitis: gamtos ir gyvybės mokslų perspektyvos. November 24, 2022, Vilnius, Lithuania. Best presentation award.
3. Poster presentation. **E. Morkūnas**, E. Vaitkevičiūtė, R. Inčiūraitė, J. Kupčinskas, A. Link, J. Skiecevičienė, P. Malfertheiner, G. Varkalaitė, J. Ricke. *miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*. EASL Liver Cancer Summit 2023, April 20-22, Estoril, Portugal.
4. Oral presentation. **E. Morkūnas**, E. Vaitkevičiūtė, R. Inčiūraitė, J. Kupčinskas, A. Link, J. Skiecevičienė, M. Alunni-Fabbroni, K. Schütte, P. Malfertheiner, G. Varkalaitė, J. Ricke. *miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*. European Bridging Meeting in Gastroenterology 2023. November 17–18, Budapest, Hungary.
5. Oral presentation. **E. Morkūnas**, E. Vaitkevičiūtė, R. Inčiūraitė, J. Kupčinskas, A. Link, J. Skiecevičienė, M. Alunni-Fabbroni, K. Schütte, P. Malfertheiner, G. Varkalaitė, J. Ricke. *miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*. ESDO Masterclass 2024, March 8-9, Warsaw, Poland.
6. Oral presentation. **E. Morkūnas**, E. Vaitkevičiūtė, R. Inčiūraitė, J. Kupčinskas, A. Link, J. Skiecevičienė, M. Alunni-Fabbroni, K. Schütte, P. Malfertheiner, G. Varkalaitė, J. Ricke. *miRNA profiles as hepatocellular carcinoma diagnostic, prognostic and treatment-related biomarkers*. International Health Sciences Conference for All 2024, March 25–26, Kaunas, Lithuania.

1. SUMMARY OF MATERIALS AND METHODS

1.1. Ethics

The post-hoc studies on inflammatory and miRNA biomarkers were part of SORAMIC clinical trial (EudraCT, 2009-012576-27, registered 9 April 2010; ClinicalTrials.gov, NCT01126645, registered 20 May 2010). The study on FABP2, IL-10 and LPS as well as inclusion of the control group into the miRNome study were approved by Kaunas Regional Biomedical Research Ethics Committee (Kaunas, Lithuania, No. BE-2-10, 27 December 2010; No. BE-2-31, 14 February 2015). All participants signed informed consent before the study. Study procedures were performed according to ethical principles of Declaration of Helsinki.

1.2. Study design and selection of patients

Studies on inflammatory molecules (IL-6, IL-8, LPS, VEGF) as well as study on miRNA profiles were performed as post-hoc analyses of plasma samples available from SORAMIC (SORAfenib in combination with local MICro-therapy guided by gadolinium-EOB-DTPA enhanced MRI) clinical trial, which was a prospective phase II trial investigating the additional benefit of radioembolization to sorafenib monotherapy for HCC patients. Subgroups of HCC patients were selected from the palliative arm of this clinical trial. The inclusion and exclusion criteria as well as treatment protocols were described previously [38].

The patients with HCC within the study on inflammatory molecules FABP2, IL-10 and LPS were enrolled retrospectively at the Gastroenterology Department of Lithuanian University of Health Sciences from June 2010 to May 2021. Both the control group within the study on inflammatory biomarkers FABP2, IL-10 and LPS as well as the control group of the miRNome study were consisted of individuals from Hospital of Lithuanian University of Health Sciences Kauno klinikos, aged 48–80 years, endoscopically diagnosed with diverticulosis of the colon and free of other chronic or malignant diseases.

1.2.1. Study population of studies on inflammatory biomarkers

The study on prognostic and treatment-related value of baseline IL-6 and IL-8 in sorafenib monotherapy-treated HCC patients involved 47 patients. The study on prognostic value of baseline IL-6 and IL-8 in SIRT-sorafenib combination therapy-treated HCC patients included 83 patients. Finally, the

study on prognostic value of early reassessment of inflammatory biomarkers involved 46 sorafenib monotherapy-treated HCC patients and 44 SIRT-sorafenib combination therapy-treated HCC patients with both baseline and follow-up blood samples available. All above mentioned HCC patient groups were included from the palliative arm of SORAMIC clinical trial. These subgroups of HCC patients were representative of the whole sorafenib-treated or SIRT-sorafenib combination therapy-treated patient cohorts respectively within this trial.

Detailed demographic and clinical characteristics of patients are described in the respective articles: "*Baseline Interleukin-6 and -8 predict response and survival in patients with advanced hepatocellular carcinoma treated with sorafenib monotherapy – an exploratory post hoc analysis of the SORAMIC trial*", "*Prognostic value of baseline interleukin 6 levels in liver decompensation and survival in HCC patients undergoing radioembolization*" and "*Dynamics in Circulating Proinflammatory Biomarkers for Prognostic Assessment of Patients With Advanced HCC*".

The study on prognostic and diagnostic value of baseline FABP2, IL-10 and LPS included 47 newly diagnosed HCC patients as the study group and 50 individuals as the control group. These groups were enrolled in Hospital of Lithuanian University of Health Sciences as described above.

Detailed characteristics of both study and control groups are described in the article "*Diagnostic and Prognostic Value of IL-10, FABP2 and LPS Levels in HCC Patients*".

1.2.2. Study population of miRNome study

In total 22 sorafenib monotherapy-treated HCC patients and 20 SIRT-sorafenib combination therapy-treated patients from the palliative arm of SORAMIC trial with both baseline and follow-up blood samples available were included in this miRNome study population group. Also, 20 healthy individuals were involved in the control group.

Detailed clinical characteristics of the study group as well as control group are described in the article "*miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*".

1.3. Blood plasma samples

For each HCC patient within these studies blood samples were obtained at the baseline, i.e. before initiation of any specific HCC treatment. In the studies where follow-up evaluation was planned, additional blood samples

were drawn 7 to 9 weeks after the initiation of treatment – either sorafenib monotherapy or SIRT-sorafenib combination therapy. Individuals from the control groups underwent the collection of blood at the time of inclusion to the studies. Plasma was isolated from the whole blood according to standard operating procedures and stored at –80°C.

1.4. Measurements of levels of inflammatory molecules

Enzyme-linked immunosorbent assay (ELISA) was used to measure plasma levels of IL-6, IL-8, IL10, LPS, VEGF, FABP2. The following ELISA kits were used in these studies: Human IL-6 Quantikine ELISA Kit (R&D Sys, Minneapolis, MN, USA; D6050), Human IL-8/CXCL8 Quantikine ELISA Kit (R&D Sys; D8000C), Human IL-10 Quantikine ELISA Kit (R&D Sys; D1000B), Human VEGF Quantikine ELISA Kit (R&D Sys; DVE00), Human FABP2/I-FABP Quantikine ELISA Kit (R&D Sys; DFBP20), Human LPS ELISA Kit (Cusabio, Houston, TX, USA; CSB-E09945h). Optical densities were measured using Tecan SUNRISE Microplate Absorbance Reader (Tecan Group Ltd.) at 450 nm and 570 nm (as reference) wavelengths. All sample processing and data analysis was performed according to manufacturer's protocols

1.5. Isolation of nucleic acids, small RNA-seq library preparation and next-generation sequencing

Previously prescribed methodology [65] was used for isolation of circulating miRNAs and preparation of sequencing libraries. Plasma circulating nucleic acids, including circulating miRNA fraction, was isolated using QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) according to the manufacturer's instructions. Small RNA sequencing (smRNA-seq) libraries were constructed using Illumina TruSeq Small RNA Sample Preparation Kit (Illumina, United States) according to the manufacturer's protocol. After the quantification and quality assessment, the small RNA-seq libraries were randomized and sequenced using Illumina HiSeq 2500 (1 × 50 bp single-end reads).

Detailed descriptions are presented in the article "*miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*".

1.6. Bioinformatics analysis

smRNA-seq data analysis was performed according to previously defined methodology [65]. nf-core/smrnaseq pipeline v.1.0.0 was used to perform read quality control, removal of 3' adapter sequences, mapping to mature and hairpin miRNAs (miRBase v.22.1 [66]), and GRCh37 human reference genome. Normalized counts were generated using the isomiRs package and differential expression analysis was carried out using the DESeq2 Bioconductor package v.1.32.0 [67]. The threshold for significant differential expression was Bonferroni adjusted p-value < 0.05 and absolute value of $\log_2$ fold change (FC) $|\log_2 FC| > 1$.

Detailed descriptions are presented in the article "*miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*".

1.7. Statistical analysis

Statistical analysis of data within these studies was performed using different software: for miRNome study – R (v. 4.1.0) and RStudio software (v. 1.4.1106), for studies on prognostic and predictive value of IL-6 and IL-8 – R (v. 3.5.0), for study on prognostic value of reassessment of inflammatory biomarkers – SAS (v. 9.4), for study on value of FABP2, IL-10 and LPS – IBM SPSS Statistics (v. 27.0).

The distribution of data was tested with Shapiro-Wilk normality test. Depending on the normality of the distribution of data, statistical significance was assessed by Student's *t*-test, Man-Whitney U test or non-parametric Wilcoxon rank-sum test. Correlations were evaluated with Chi-square and Fisher's exact tests. The receiver operating characteristic curve (ROC) analysis was performed to determine the cut-off values and to evaluate the diagnostic value. The Kaplan–Meier method and log-rank test were used to evaluate the prognostic value. Cox regression models were used to assess the effects of cofounding factors and to identify prognostic factors of OS. p values < 0.05 were considered statistically significant.

Detailed descriptions are presented in respective publications.

2. SUMMARY OF RESULTS

2.1. Analysis of inflammatory biomarkers

2.1.1. Role of baseline plasma IL-6 and IL-8

To determine the prognostic and treatment-related value of proinflammatory cytokines IL-6 and IL-8, blood samples of HCC patients before the initiation of active treatment (baseline) were collected under the frames of the SORAMIC clinical trial. Only sorafenib monotherapy-treated patients were included in this study.

ROC curve analysis identified cut-off value of 8.58 pg/mL for baseline IL-6 to be able to best predict survival in HCC patients (sensitivity 76.9%, specificity 69.3%). Similarly, the optimal cut-off baseline IL-8 value of 57.9 pg/mL was defined (sensitivity 68%, specificity 73.2%).

Survival analysis revealed significant median OS differences comparing low and high blood levels of both IL-6 and IL-8. Median OS for HCC patients with low IL-6 concentrations was 30.3 months while those with high IL-6 levels had significantly shorter OS of 10.3 months ($p < 0.001$) (Fig. 2.1). Patients with high baseline IL-8 levels (10.3 months) also survived significantly shorter than patients with low IL-8 levels (30.3 months, $p = 0.009$) (Fig. 2.2).

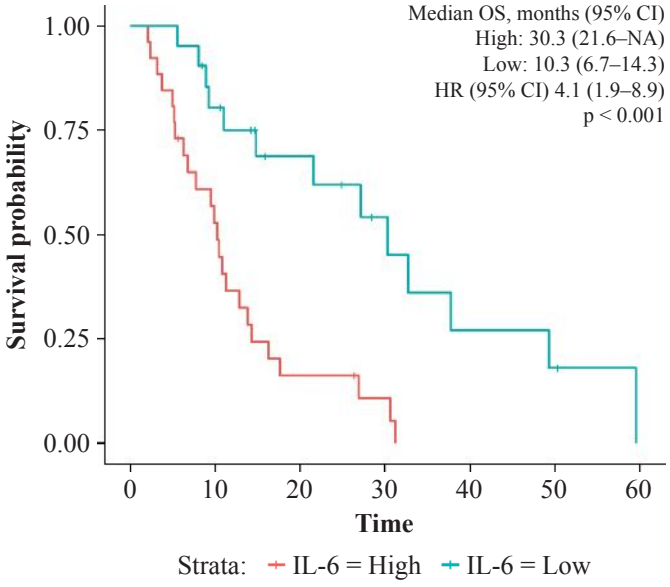


Fig. 2.1. *Kaplan–Meier curve showing overall survival of HCC patients grouped by baseline IL-6 levels according to cut-off value of 8.58 pg/mL*

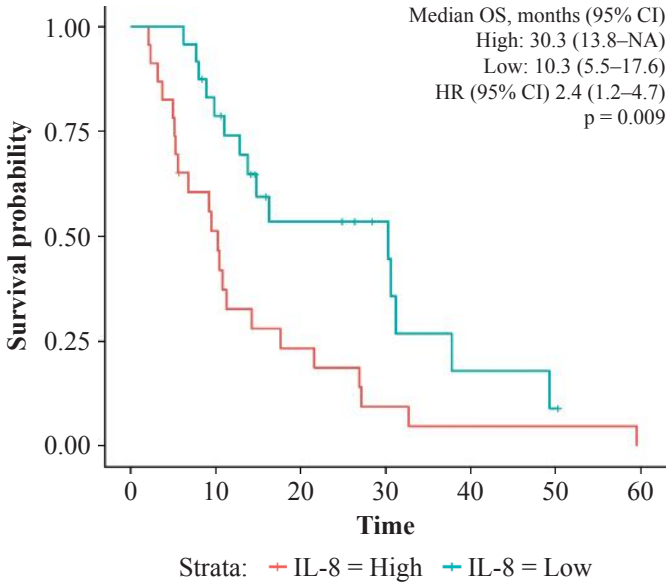


Fig. 2.2. *Kaplan–Meier curve showing overall survival of HCC patients grouped by baseline IL-8 levels according to cut-off value of 59.7 pg/mL*

Univariate analysis including various clinical and pathological parameters demonstrated that high baseline IL-6 levels were significantly associated with low albumin levels (< 36 g/L, p = 0.013), while high baseline IL-8 concentrations were associated with larger tumor diameter (p = 0.013).

Multivariate Cox regression analysis showed that both high baseline IL-6 (p = 0.017) and IL-8 (p = 0.044) levels were the only independent factors predicting worse overall survival rates.

To evaluate combined IL-6 and IL-8 prognostic value, interleukin score was created were score 0 signified that both IL-6 and IL-8 baseline levels were below cut-off values, score 1 meant that levels of one interleukin were above cut-off value and score 2 reflected that both IL-6 and IL-8 baseline levels were above cut-off values. Median OS was significantly different across different score groups – 37.7 months for score 0, 16.3 months for score 1 and 9.5 months for score 2 (p < 0.001) (Fig. 2.3).

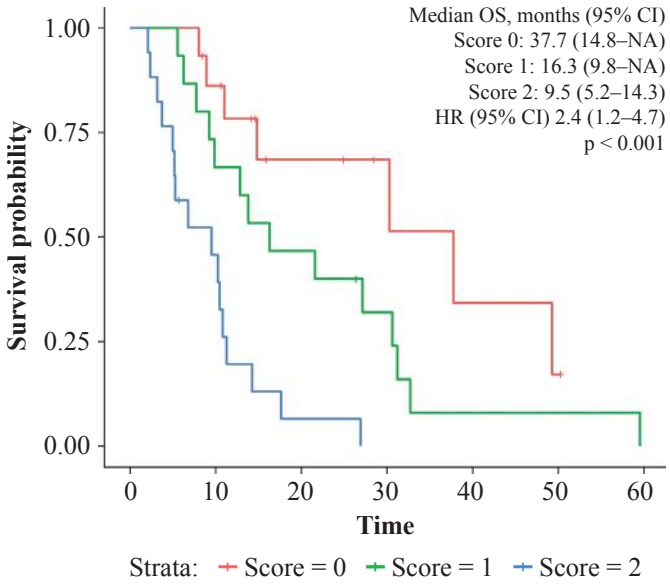


Fig. 2.3. *Kaplan–Meier curve showing overall survival of patients according to interleukin score*

Predicting sorafenib therapy efficacy, correlation analysis between interleukin levels and radiological response (according to mRECIST criteria) was performed. Both low baseline IL-6 and IL-8 levels were associated with significantly higher objective response rate comparing to higher than cut-off values of IL-6 and IL-8 levels (IL-6: 46.6% *vs.* 19.2%, p = 0.007; IL-8: 50.0% *vs.* 17.4%, p = 0.011).

For more details of the results regarding prognostic and predictive value of baseline IL-6 and IL-8 in sorafenib-treated HCC patients please refer to the article "*Baseline Interleukin-6 and -8 predict response and survival in patients with advanced hepatocellular carcinoma treated with sorafenib monotherapy – an exploratory post hoc analysis of the SORAMIC trial*".

To evaluate and compare the prognostic value of baseline IL-6 and IL-8 in HCC patients with different treatment modalities, blood samples were collected before the initiation of ^{90}Y radioembolization combined with sorafenib therapy also under the frames of the SORAMIC clinical trial. Similar but not exactly the same previously defined cut-off values of 6.53 pg/mL for IL-6 and 60.8 pg/mL for IL-8 were used (28283704).

As expected, median OS was significantly longer for HCC patients with low IL-6 levels comparing with high IL-6 levels (19.0 *vs.* 7.8 months, p < 0.001) (Fig. 2.4 A). The same tendency appeared for IL-8 levels – low concentration of IL-8 meant significantly longer survival (OS 16.0 vs. 8.4 months, p = 0.009) (Fig. 2.4 B).

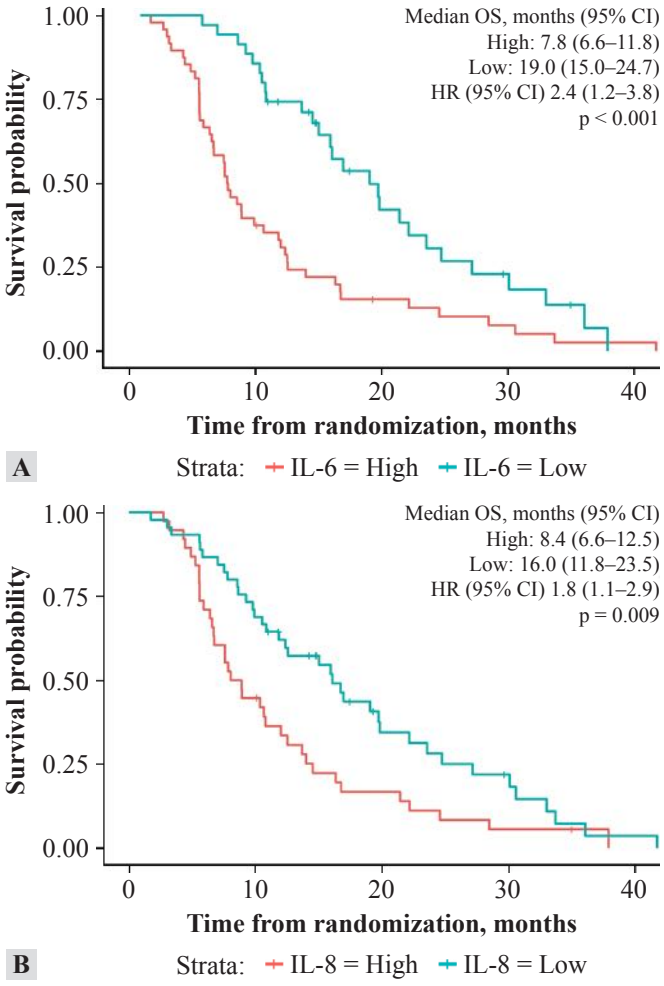


Fig. 2.4. *Kaplan–Meier curves showing OS of HCC patients grouped by baseline IL-6* ***(A)*** *and IL-8* ***(B)*** *according to cut-off values*

Univariate analysis for this cohort including different clinical parameters demonstrated that not only IL-6 and IL-8 levels had significant prognostic value. Background liver cirrhosis ($p = 0.032$), worse liver function (Child-Pugh class B *vs.* A, $p = 0.014$), lower albumin levels (< 36 g/L, $p = 0.024$), higher bilirubin levels (≥ 17 μmol/L, $p = 0.009$) and higher mALBI score ($p = 0.007$) were associated with worse OS of HCC patients. Multivariate analysis, however, revealed high IL-6 levels as the only independent prognostic factor for shorter OS.

Similarly, baseline blood IL-6 and IL-8 levels appeared to have prognostic value not only for OS but also for PFS. HCC patients with low baseline IL-6 (17.9 *vs.* 5.5 months, $p < 0.001$) and IL-8 (15.0 *vs.* 7.8 months, $p = 0.017$) concentrations had significantly longer PFS.

Prognostic value of both interleukins for time to liver dysfunction (defined as grade ≥ 2 increase of bilirubin concentration) was also evaluated. HCC patients with low baseline IL-6 levels had significantly longer time to liver dysfunction comparing to those with high baseline IL-6 levels (32.6 *vs.* 9.7 months, p = 0.003) (Fig. 2.5 A). Same tendency was shown for baseline IL-8 levels; however, the difference was not significant (Fig. 2.5 B). Multivariate analysis demonstrated that high baseline IL-6 levels (p = 0.016) as well as high baseline bilirubin levels (p < 0.001) were independent prognostic factors for liver dysfunction.

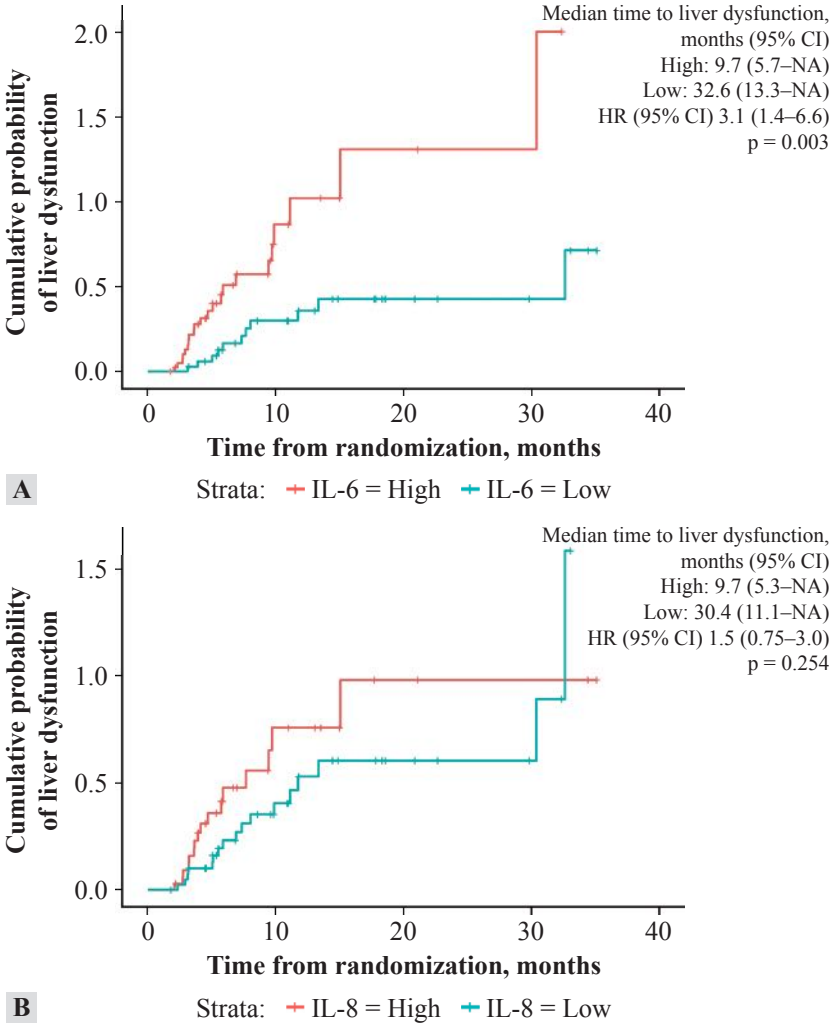


Fig. 2.5. *Kaplan–Meier curves showing time-to-liver dysfunction of HCC patients grouped by baseline IL-6* ***(A)*** *and IL-8* ***(B)*** *according to cut-off values*

For more details of the results regarding prognostic value of baseline IL-6 and IL-8 in ^{90}Y radioembolization and sorafenib combination-treated HCC patients please refer to the article "*Prognostic value of baseline interleukin 6 levels in liver decompensation and survival in HCC patients undergoing radioembolization*".

2.1.2. Prognostic value of early reassessment of proinflammatory biomarkers during HCC treatment

To evaluate whether changes in plasma levels of proinflammatory molecules, such as IL-6, IL-8, VEGF and LPS, during active treatment of HCC could add any prognostic value as potential biomarkers, plasma samples of HCC patients who were under sorafenib monotherapy or SIRT-sorafenib combination therapy, from two different time points (before and 7–9 weeks after the initiation of the treatment) were compared.

First, analysis demonstrated that both baseline (rho = –0.3024, p = 0.0038) and follow-up (rho = –0.3192, p = 0.0022) levels of IL-6 negatively correlated with OS.

ROC curve analysis identified a concentration of follow-up IL-6 of 24.18 pg/mL being able to best distinguish patients with OS longer and shorter than 12 months (sensitivity 42.9%, specificity 87.5%). Median OS of these two distinguished groups (patients with follow-up IL-6 levels lower and higher than 24.18 pg/mL) were 17.0 and 7.7 months accordingly (p < 0.0001) (Fig. 2.6 A). Median PFS also was significantly different in these two groups: 9.6 months for HCC patients with lower follow-up IL-6 levels *vs.* 5.0 months for those with higher levels (p = 0.0278) (Fig. 2.6 C).

ROC curve analysis revealed increase of IL-6 levels from baseline to follow-up time point in absolute numbers with cut-off value of 16.8 pg/mL to best distinguish HCC patients with OS shorter and longer than 12 months (sensitivity 38.1%, specificity 85.4%). Patients with increase in IL-6 levels of less than 16.8 pg/mL had significantly longer median OS of 16.3 months comparing to 8.9 months for those with greater increase of IL-6 concentrations during therapy (p = 0.0354) (Fig. 2.7 A).

HCC patients with baseline IL-6 levels more than 9.7 ng/mL had significantly worse prognosis when their IL-6 concentrations increased more than 16.8 pg/mL at the follow-up time point comparing with those with less pronounced increase: median OS of 6.8 months *vs.* 11.3 months accordingly. The best prognosis was shown for patients with low baseline IL-6 levels and less increase during HCC therapy (Fig. 2.8).

There was no significant correlation detected between OS and percentage change of IL-6 concentrations as well as between PFS and both absolute and percentage difference in IL-6 levels (Fig. 2.7 C).

Analysis of IL-8 levels demonstrated significant correlation between OS and both baseline (rho = –0.3913, p = 0.0001) and follow-up (rho = –0.4370, $p \leq 0.0001$) levels. Follow-up IL-8 concentration cut-off value of 75.25 ng/mL appeared to be the most suitable for survival prognosis with median OS of 28.5 months *vs.* 10.3 months for those with follow-up IL-8 levels below and above the threshold ($p < 0.0001$) (Fig. 2.6 B). However, this IL-8 cut-off level did not correlate with PFS (Fig. 2.6 D). Similarly, the absolute and percentage changes in IL-8 plasma levels did not have significant correlation neither with OS (Fig. 2.7 B) nor with PFS (Fig. 2.7 D).

Multivariate Cox regression analysis including various clinical HCC parameters revealed that HCC patients' OS is impacted by these independent factors: baseline levels of both IL-6 and IL-8 as well as liver function and portal vein infiltration status.

Both baseline (p = 0.0275) and follow-up (p = 0.0109) IL-8 levels as well as follow-up IL-6 levels (p = 0.0267) correlated with liver decompensation defined by increase of ALBI grade at the follow-up time point. However, there was no such correlation with baseline IL-6 levels (p = 0.2413).

Serum LPS and VEGF levels did not appear to have any correlation with survival of HCC patients in this cohort at baseline and follow-up levels as well as the difference during the therapy.

For more details of the results as well as tables and additional figures regarding prognostic value of reassessment of IL-6, IL-8, LPS and VEGF please refer to the article "*Dynamics in Circulating Proinflammatory Biomarkers for Prognostic Assessment of Patients With Advanced HCC*".

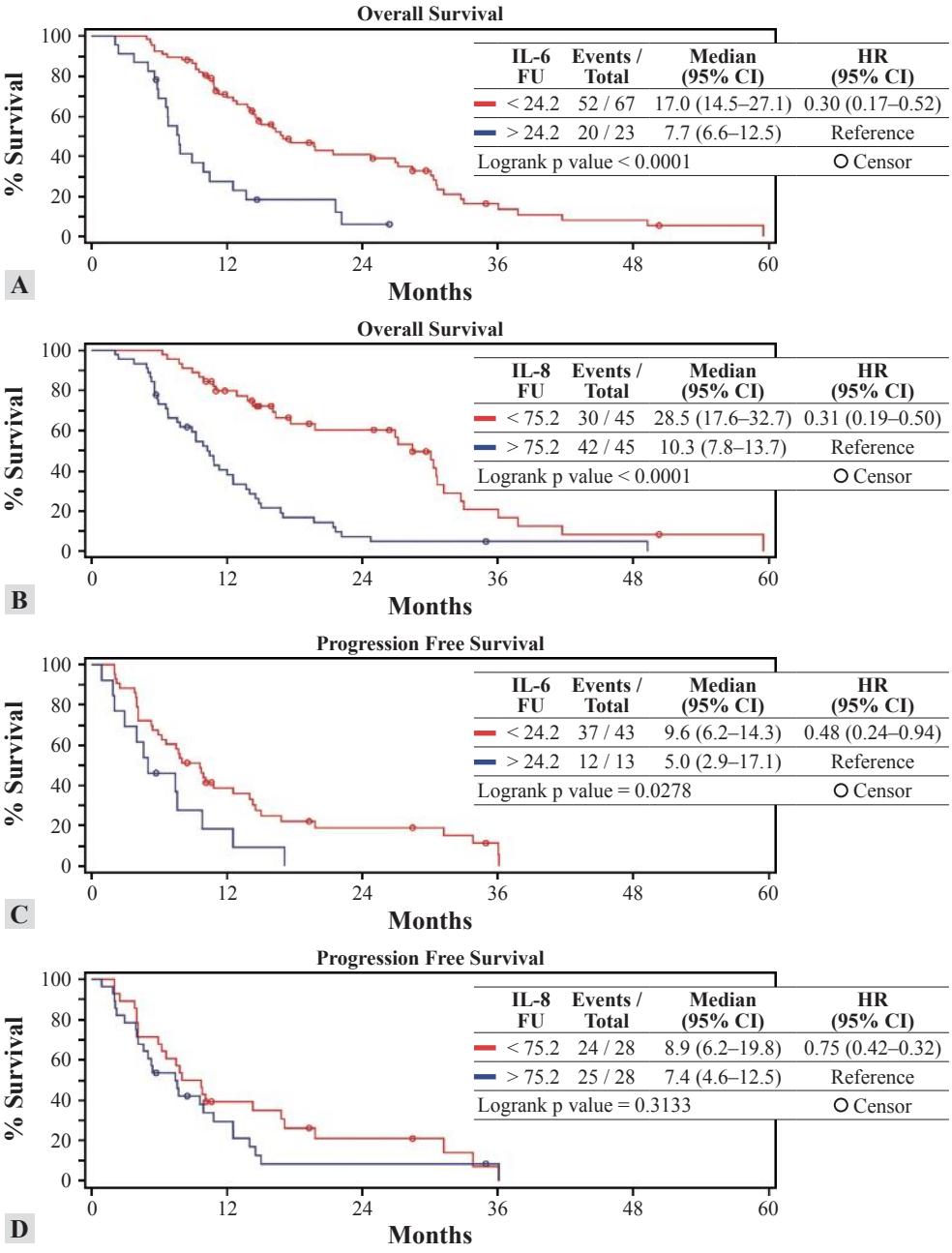


Fig. 2.6. *Kaplan-Meier curves grouped by follow-up plasma levels according to cut-off values* ***(A)*** *OS and IL-6, cut-off at 24.2 pg/mL,* ***(B)*** *OS and IL-8, cut-off at 75.2 pg/mL,* ***(C)*** *PFS and IL-6, cut-off at 24.2 pg/mL, and* ***(D)*** *PFS and IL-8, cut-off at 75.2 pg/mL*

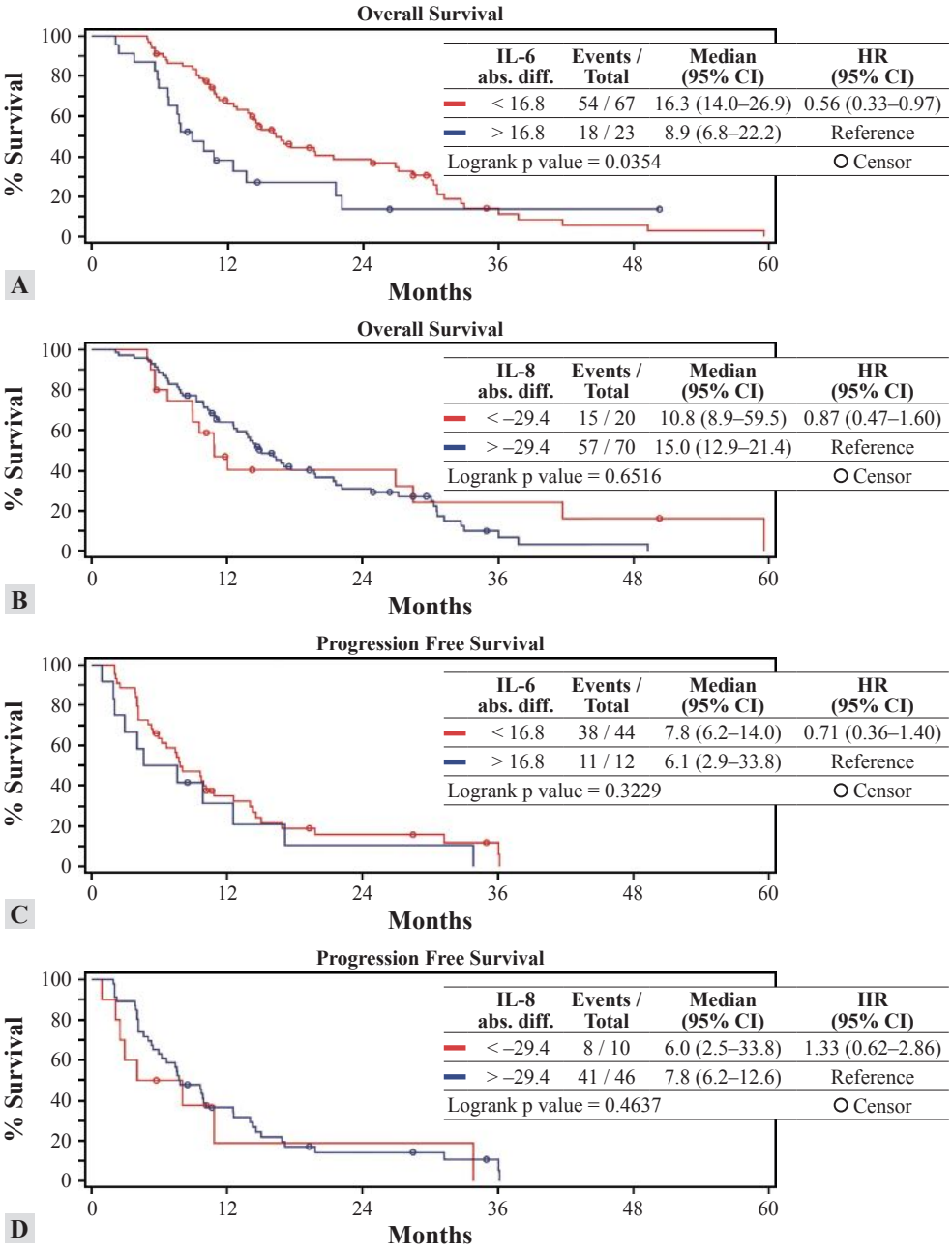
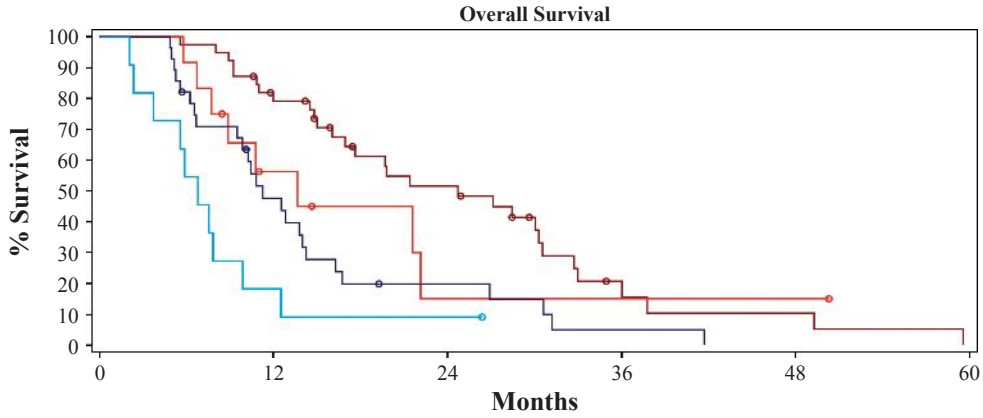


Fig. 2.7. *Kaplan-Meier curves grouped by absolute difference between baseline and follow-up levels according to cut-off values* ***(A)*** *OS and absolute difference in IL-6, cut-off at 16.8 pg/mL,* ***(B)*** *OS and absolute difference in IL-8, cut-off at –29.4 pg/mL,* ***(C)*** *PFS and absolute difference in IL-6, cut-off at 16.8 pg/mL and* ***(D)*** *PFS and absolute difference in IL-8, cut-off at –29.4 pg/mL*



IL-6 (BL and FU)	Events / Total	Median (95% CI)	HR (95% CI)
BL < 9.7; FU < +16.8	29 / 39	24.7 (17.6–32.7)	0.18 (0.08–0.38)
BL < 9.7; FU < +16.8	8 / 12	13.7 (8.9–NE)	0.27 (0.10–0.69)
BL < 9.7; FU < +16.8	25 / 28	11.3 (9.9–16.3)	0.43 (0.20–0.91)
BL < 9.7; FU < +16.8	10 / 11	6.8 (5.6–NE)	Reference
Logrank p value < 0.0001			O Censor

Fig. 2.8. *OS of different prognostic groups defined by cut-off levels of baseline IL-6 of 9.7 ng/mL and increase of IL-6 of 16.8 months*

2.1.3. Value of IL-10, LPS and FABP2

Besides significant prognostic value of proinflammatory cytokines IL-6 and IL-8, other molecules directly or not directly involved in chronic liver inflammation and HCC carcinogenesis processes were studied. We investigated diagnostic and prognostic value of anti-inflammatory cytokine IL-10, endotoxin lipopolysaccharide (LPS) and gut permeability marker fatty acid-binding protein 2 (FABP2) for HCC patients. For this purpose, blood samples were drawn from newly diagnosed HCC patients and healthy individuals (controls).

Analysis revealed that levels of both IL-10 (9.94 *vs.* 4.89 pg/mL, $p < 0.001$) and FABP2 (2345 *vs.* 1327 pg/mL, $p = 0.026$) were significantly higher in HCC patients than in healthy individuals. However, difference of LPS levels in these two groups was not significant and was even higher in healthy controls (Table 2.1).

Table 2.1. *Plasma FABP2, IL-10 and LPS levels in HCC patients and healthy individuals*

	Controls (n = 50)	**HCC patients (n = 47)**	**p value**
FABP2	1327 (518.5–8388)	2345 (326.3–4587)	0.026
IL-10	4.89 (0.036–53) *	9.94 (0.04–564.1) *	< 0.001
LPS	56.38 (4.74–436.4)	51.95 (12.37–148.8)	0.263

Concentrations of FABP2, IL-10 and LPS expressed as median (min-max) (pg/mL). HCC – hepatocellular carcinoma; min – minimum value; max – maximum value; FABP2 – fatty acid-binding protein 2; IL – interleukin; LPS – lipopolysaccharide. * Due to insufficient amount of blood for analysis of IL-10, samples of 45 HCC patients and 45 controls were used.

ROC curve analysis suggested a cut-off value of 2479 pg/mL for FABP2 to best distinguish HCC patients with longer and shorter than median OS of 17 months (sensitivity 66.7%, specificity 55.6%, AUC 0.622). However, there was no significant difference between median OS of HCC patients with high and low FABP2 levels (Fig. 2.9).

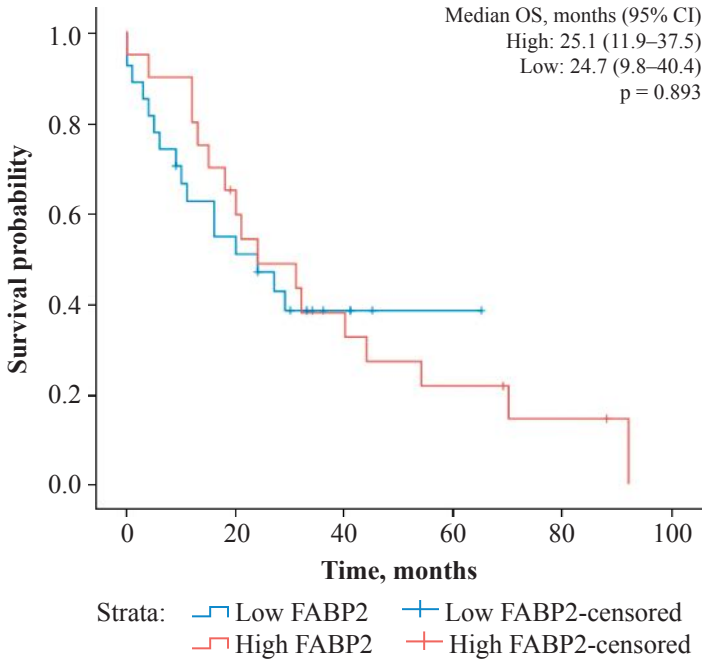


Fig. 2.9. *Kaplan–Meier curve showing OS of HCC patients grouped by baseline FABP2 according to cut-off value*

Cut-off values for IL-10 as well as LPS to significantly predict prognosis of HCC patients could not be identified. ROC curve analysis with different OS of 6, 12 and 24 months did not add any significant prognostic value for FABP2, IL-10 and LPS.

For more details on analysis of diagnostic and prognostic value of FABP2, IL-10 and LPS please refer to the article "*Diagnostic and Prognostic Value of IL-10, FABP2 and LPS Levels in HCC Patients*".

2.2. Analysis of miRNome profiles

To evaluate the potential role of miRNA molecules for diagnosis, prognosis and prediction of treatment response for HCC patients, plasma samples were collected from HCC patients at two different time points (before and 7–9 weeks after the initiation of HCC treatment) under the frames of above mentioned SORAMIC clinical trial. Both groups of HCC patients who underwent radioembolization and sorafenib combination therapy as well as sorafenib monotherapy were included. Blood samples were also drawn from healthy individuals as the control group.

2.2.1. Diagnostic value of miRNome

Plasma smRNA-seq was performed and miRNA expression profiles were determined for HCC baseline samples as well as control samples. In total 711 miRNAs were identified (annotated in miRbase v22.1). After data filtering and pre-processing 408 miRNAs were retained for further differential expression analysis.

Comparing HCC baseline and control plasma samples, 42 differentially expressed miRNAs were identified out of which 16 appeared to be up-regulated and 26 were down-regulated. The similarity of samples and overlap between two analyzed clusters were demonstrated by multidimensional scaling analysis (MDS) of normalized miRNA expression (Fig. 2.10 A). Top five up-regulated miRNAs were hsa-miR-576-5p, -200b-3p, -374a-3p, -140-5p, -1228-5p, and top five down-regulated miRNAs were hsa-miR-219a-2-3p, -218-5p, -129-5p, -132-3p, -3960 (Fig. 2.10 B).

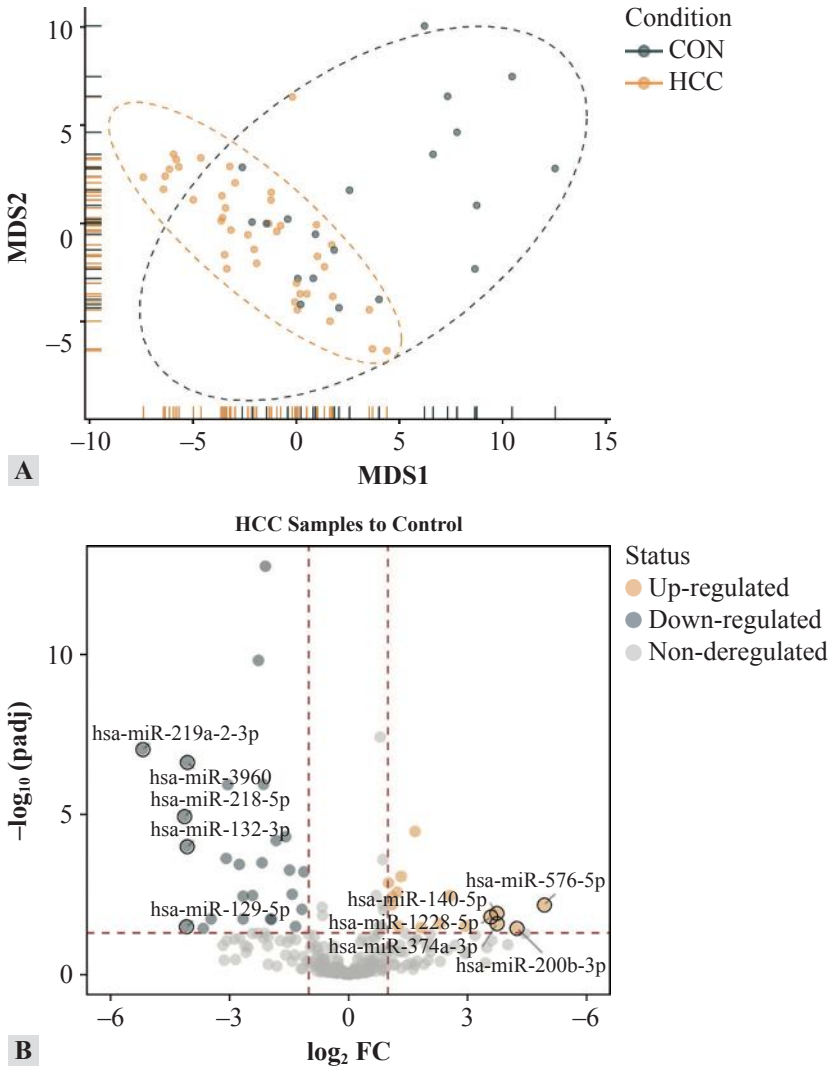


Fig. 2.10. (A) *Multidimensional scaling plot based on normalised expression values showing the similarity corresponding to control (n = 20) and HCC (n = 42) plasma samples;* ***(B)*** *Volcano plot showing the differentially expressed miRNAs between hepatocellular carcinoma and controls*

(A) Each dot corresponds to a sample, the centroid represents the group mean, and the ellipse presents the mean standard error (SEM) based confidence. The rug plots along the x and y axis show distributions of each dot's value; **(B)** The orange and dark grey dots represent the up-regulated and down-regulated miRNAs, the most deregulated miRNAs are highlighted. Dashed lines indicate p value < 0.05 and $|\log_2 FC| > 1$.

Correlation analysis between these deregulated miRNAs and main clinical parameters of HCC patients revealed that none of differentially expressed miRNAs had links with baseline plasma alpha-fetoprotein (AFP) levels as well as absolute and percentage difference in AFP levels. Only one deregulated miRNA (hsa-miR-142-5p) appeared to be able to distinguish different HCC stages (BCLC stages B and C).

To discriminate newly diagnosed HCC patients and healthy controls, analysis of normalized miRNA read counts were performed based on feature selection and random forest classificator algorithms. 10 miRNAs (hsa-let-7b-5p, hsa-let-7c-5p, hsa-miR-124-3p, hsa-miR-125b-5p, hsa-miR-219a-2-3p, hsa-miR-3960, hsa-miR-432-5p, hsa-miR-6734-5p, hsa-miR-7-5p, hsa-miR-95-3p) were confirmed as the important attribute. ROC curve analysis of these 10 miRNAs pointed out the best performance of hsa-let-7b-5p (AUC = 85.6%) and hsa-miR-3960 (AUC = 85.9%) (Fig. 2.11). Combination of all 10 miRNAs appeared to have even better performance with AUC of 92.68% when distinguishing HCC baseline and control groups.

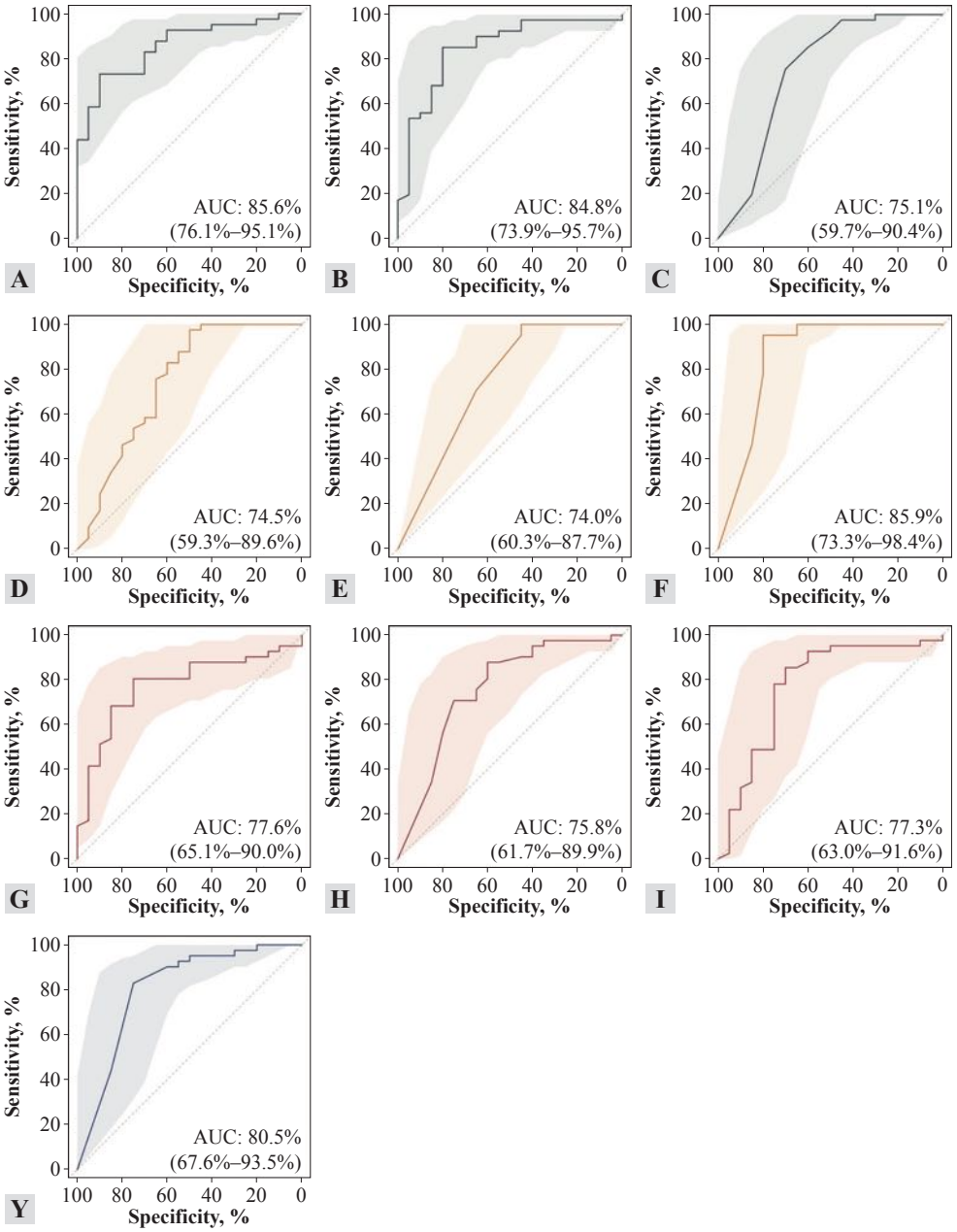


Fig. 2.11. *ROC curves for the discrimination performance of* ***(A)*** *hsa-let-7b-5p,* ***(B)*** *hsa-let-7c-5p,* ***(C)*** *hsa-miR-124-3p,* ***(D)*** *hsa-miR-125b-5p,* ***(E)*** *hsa-miR-219a-2-3p,* ***(F)*** *hsa-miR-3960,* ***(G)*** *hsa-miR-432-5p,* ***(H)*** *hsa-miR-6734-5p,* ***(I)*** *hsa-miR-7-5p,* ***(Y)*** *hsa-miR-95-3p between HCC baseline and control groups*

The shaded area represents a 95% confidence interval (CI). AUC – the area under the curve.

2.2.2. Treatment-related value of miRNome

To evaluate influence of HCC therapies on changes in miRNA profiles, HCC baseline plasma samples were compared with samples collected 7–9 weeks after the start of treatment in both sorafenib monotherapy and SIRT-sorafenib combination therapy groups.

When receiving sorafenib monotherapy, two miRNAs – hsa-miR-483-5p ($\log_2$ FC = 1.75; padj = 2.30×10^{-2}) and hsa-miR-23b-3p ($\log_2$ FC = 1.60; padj = 2.96×10^{-2}) – were found to be up-regulated while two other miRNAs – hsa-miR-215-5p ($\log_2$ FC = –3.65; padj = 7.72×10^{-8}) and hsa-miR-192-5p ($\log_2$ FC = –2.27; padj = 1.11×10^{-2}) – were down-regulated, comparing baseline and follow-up samples (Fig. 2.12 A). However, SIRT-sorafenib combination therapy appeared to have less influence – the only miRNA – hsa-miR-215-5p ($\log_2$ FC = –3.88; padj = 4.84×10^{-3}) – was deregulated (down-regulated) in this comparison group (Fig. 2.12 B).

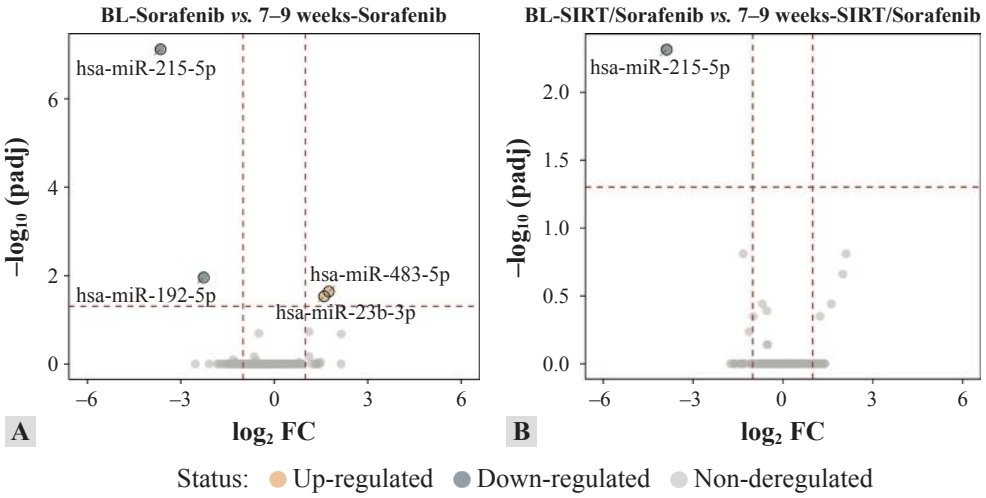


Fig. 2.12. *Volcano plot demonstrating differentially expressed plasma miRNAs when comparing HCC baseline and follow-up (7–9 weeks after initiation of treatment) plasma samples:* ***(A)*** *sorafenib monotherapy therapy group;* ***(B)*** *SIRT-sorafenib combination therapy group*

To determine if miRNA profiles could act as predictors of treatment efficacy, plasma samples of responders and non-responders (defined according to radiological mRECIST criteria) were compared in both treatment groups. Three miRNAs – hsa-miR-183-5p ($\log_2$ FC = 3.17, padj = 3.07×10^{-2}; hsa-miR-28-3p ($\log_2$ FC = 4.45, padj = 1.61×10^{-4}); hsa-miR-1246 ($\log_2$ FC = 4.53, padj = 2.26×10^{-3}) were found out to be significantly up-regulated comparing responders and non-responders to sorafenib monotherapy. ROC

curve analysis showed discrimination performance of AUC more than 75% for all three miRNAs: hsa-miR-183-5p – 75.5% (Fig. 2.13 A), hsa-miR-28-3p – 83.2% (Fig. 2.13 B), and hsa-miR-1246 – 78.6% (Fig. 2.13 C). However, no significantly deregulated miRNAs were detecting in response to SIRT-sorafenib combination therapy group.

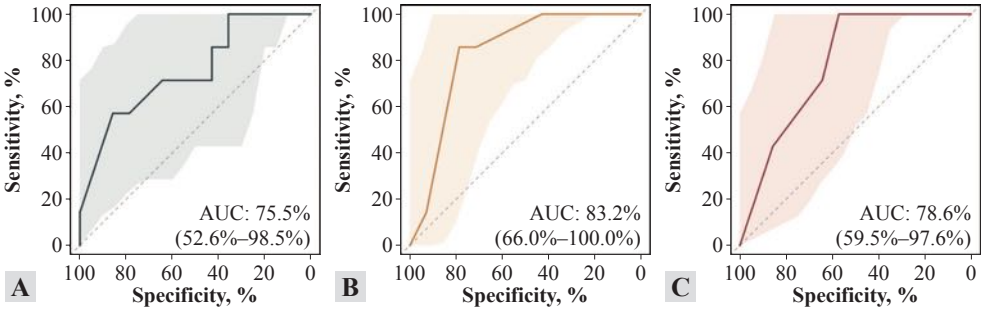


Fig. 2.13. *ROC curves for the discrimination performance of (A) hsa-miR-183-5p; (B) hsa-miR-28-3p; (C) hsa-miR-1246 between sorafenib monotherapy-treated responders and non-responders*

The shaded area represents a 95% confidence interval (CI). AUC – the area under the curve.

2.2.3. Prognostic value of miRNome

For survival analysis samples of HCC patients were grouped into three groups: (I) baseline, (II) follow-up for sorafenib monotherapy-treated patients, (III) follow-up for SIRT-sorafenib combination-treated patients. Analysis revealed two miRNAs – hsa-miR-215-5p ($\log_2$ FC = 3.39, padj = 1.06×10^{-2}); hsa-miR-25-5p ($\log_2$ FC = –6.05, padj = 1.06×10^{-2}) – to be significantly deregulated in Group II when comparing death and non-death outcomes. No miRNAs were found to be associated with survival in Group I and Group III.

HCC patients were grouped into two groups according to relevant miRNA cut-off expression level (above or below the median). Survival of these groups revealed that low hsa-miR-215-5p expression level is significantly associated (p = 0.031) with better survival prognosis of HCC patients with rather big difference in median OS (26.9 *vs.* 10.5 months) (Fig. 2.14).

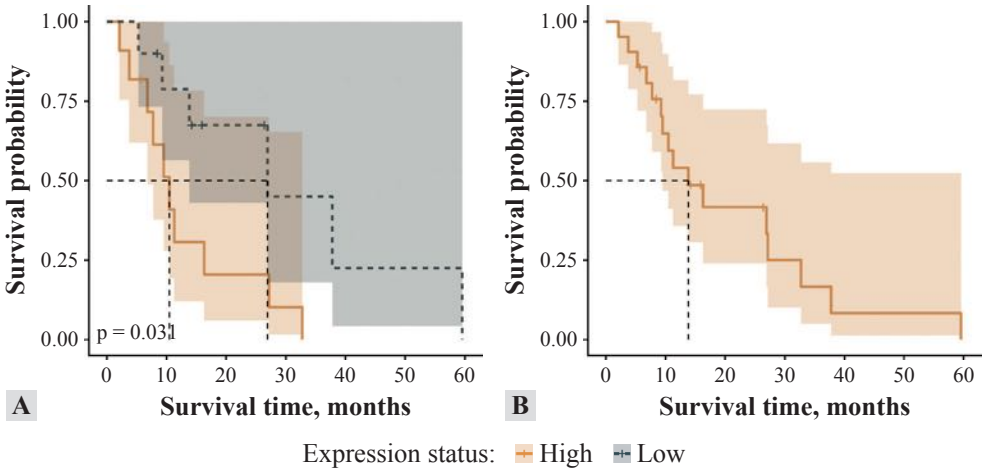


Fig. 2.14. *Kaplan–Meier survival analysis of HCC patients with low (dark grey line) and high (orange line) expression of plasma miRNA:* ***(A)*** *hsa-miR-215-5p;* ***(B)*** *hsa-miR-25-5p*

A 95% CI (estimated from a log hazard) is presented in the shadowed area. The tick marks indicate censored events, and the dashed line indicates the median survival time.

For more details on results from miRNome analysis, please refer to the article "*miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*".

3. DISCUSSION

The detection of biomarkers capable to distinguish patients with early HCC from cirrhotic patients as well as to reliably select HCC patients with the best predicted response to different therapeutic modalities and to predict the course of the disease and overall survival is of high priority as HCC remains one of deadliest cancer worldwide [1]. There is a wide variety of candidate biomarkers for HCC diagnosis, prognosis and prediction of treatment efficacy, having a role in carcinogenesis or progression of HCC and being shown to have some value for this purpose [68]. However, up until now only AFP is widely used in clinical practice, not for HCC diagnosis but only for surveillance or selection of liver transplant recipients [40]. Thus, there is a high need for complex studies investigating, identifying, and implementing HCC biomarkers, especially minimally invasive, without a need for liver biopsies. Inflammatory cytokines as well as miRNAs could be easily detected and evaluated in the blood and some evidence of relationship with HCC. This thesis presents a study on both inflammatory molecules and miRNAs as potential HCC biomarkers.

First, we performed analysis of baseline IL-6 and IL-8, defining them as prognostic biomarkers of overall survival in sorafenib monotherapy-treated HCC patients with cut-off values of 8.58 pg/mL and 57.9 pg/mL for IL-6 and IL-8, respectively. After adjusting for multiple factors in the multivariate analysis, baseline IL-6 and IL-8 remained the only independent prognostic factors for overall survival of HCC patients. Furthermore, the same cut-off values of both IL-6 and IL-8 were also able to distinguish responders and non-responders to sorafenib therapy according to follow-up radiological imaging. We also performed similar analysis of baseline IL-6 and IL-8 in radioembolization-sorafenib combination therapy-treated HCC patients, demonstrating that blood baseline IL-6 levels are independent prognostic factor for survival and liver dysfunction in these patients.

These findings were in accordance with previous studies that have demonstrated that higher IL-6 and IL-8 levels are associated with higher risk of developing HCC [69,70] as well as have correlation with advanced stages of chronic liver disease and worse liver function in HCC patients [15, 71–73]. Correlation between IL-6 and IL-8 levels and HCC therapies also was demonstrated before – lower IL-6 and IL-8 plasma levels were associated with better overall survival and better response to locoregional HCC therapies [49, 73, 74]. Differently from our study, Japanese study by Miyahara et al. has shown no correlation between baseline IL-8 and overall survival or response to sorafenib [47]. The only study before ours, demonstrating

prognostic value of IL-6 in HCC patients treated with sorafenib monotherapy, was performed by Shao et al. in an Asian cohort [46]. Different baseline IL-6 cut-off value of 4.28 pg/mL was found to predict survival of HCC patients. This discrepancy may be caused by differences between European and Asian patients, also having in mind different dominant etiologies of chronic liver disease in these cohorts (alcoholic liver disease in European cohort vs. viral hepatitis in Asian cohort). This also may signalize a need of different cut-off values for different populations. Our analysis has not revealed any significant influence of etiology of chronic liver disease to IL-6 and IL-8 values. IL-6 cut-off value defined in our study is in between previously described cut-off values for blood IL-6 to predict survival of HCC patients treated with radioembolization (6.53 pg/mL) [49] and chemoembolization (10 pg/mL) [73].

In the recent years, a few immunotherapy-related combination treatment regimens were approved for HCC treatment. Combination of atezolizumab with bevacizumab (Imbrave150) [75], tremelimumab with durvalumab (HIMALAYA) [76], camrelizumab with rivoceranib (CARES-310) [77], tislelizumab monotherapy (RATIONALE-301) [78] have demonstrated improved both overall survival and progression-free survival for patients with advanced HCC comparing to sorafenib. However, not all HCC patients are candidates for immunotherapy, e.g., organ transplant recipients, patients with chronic autoimmune diseases, patients with worse liver function (class B according to Child-Pugh classification), as they were not included in these clinical trials. Additionally, immunotherapy has its toxicities and is not cost-effective yet as compared with sorafenib [79]. Therefore, sorafenib will remain as the important treatment choice not only for the second line but also first line therapy, especially in countries with limited resources. Imbrave150 trial demonstrated that the survival rate at 12 months of patients treated with the combination of atezolizumab and bevacizumab was 67.2% while our analysis revealed that sorafenib-treated patients with low IL-6 or IL-8 levels had very similar survival rates at the same time point (66.6%). This illustrates the potential of IL-6 and IL-8 as valuable biomarkers for risk stratification and better selection of treatment approach in individualized manner.

Additionally, we performed analysis of early reassessment of blood IL-6 and IL-8 in HCC patients. This analysis suggested that repeated measurement of IL-6 during or after treatment (7–9 weeks after initiation of sorafenib with or without radioembolization) has prognostic value for these patients. An increase of 16.8 pg/mL or more from baseline IL-6 level is associated with worse prognosis of HCC patients' survival. High baseline IL-6 levels (above 9.7 pg/mL) and higher increase of IL-6 levels during 7–9 weeks' time (above 16.8 pg/mL) are associated with the worst outcomes with median OS of only

6.8 months. The best survival rate (median OS of 24.7 months) was achieved in patients with low baseline IL-6 levels (below 9.7 pg/mL) and lower increase of IL-6 at follow-up (less than 16.8 pg/mL). However, no significant similar value was detected for reassessment of plasma IL-8 levels. Previous studies also demonstrated similar tendencies. For instance, Wu et al. showed that reassessment of IL-6 levels after chemoembolization (rather than before treatment) better predicts tumor response in HCC patients [80].

Repeated measurements of both IL-6 and IL-8 concentrations had prognostic value for predicting future deterioration of liver function in HCC patients. This is of high importance in clinical practice as preserved liver function is the key factor for active HCC treatment. Reassessment of IL-6 and IL-8 levels may help to identify patients with HCC who are at risk for impairment of liver function and, thus, discontinuation of treatment not due to cancer progression but rather due to liver dysfunction. Analysis of baseline IL levels in radioembolization-sorafenib combination-treated patients also demonstrated similar value in predicting time to liver dysfunction – the higher baseline IL-6 concentration (also tendency but not significance for IL-8), the shorter time to liver impairment. These findings were in accordance with previous pre-clinical and clinical studies demonstrating the important role of both IL-6 and IL-8 in the progression of chronic liver inflammation as well as carcinogenesis [9, 81, 82].

VEGF, which promotes tumor angiogenesis, has been previously demonstrated prognostic value in HCC patients [83, 84]. Sorafenib, which has been the main therapeutical option for systemic HCC treatment for a long time, is multi-tyrosine kinase inhibitor and is targeted to block VEGF receptors as well [85]. Atezolizumab in combination with bevacizumab, binding to VEGF, at the moment is approved as the first line treatment for HCC. Therefore, blood VEGF levels seem as promising biomarker for HCC, already being demonstrated as having prognostic value in patients treated with loco-regional therapies, resection or transplantation [83]. Decrease in VEGF levels during sorafenib therapy was shown to be associated with better survival rates in patients with chronic viral hepatitis-related HCC [86]. However, in our study we did not observe any prognostic potential of blood VEGF levels for HCC patients. This might be explained by small patient numbers and relatively small fraction of patients with underlying chronic viral hepatitis.

It is previously described that LPS, which are cell wall components of Gram-negative bacteria in digestive tract, levels in systemic circulation correlate with the progression of chronic liver disease and liver dysfunction [87–90]. LPS take part in inducing proinflammatory tumor microenvironment as well as in promoting tumor progression [24, 25]. Our study, however, does not support using LPS levels measurements in the peripheral blood for

prognostic purposes in HCC patients. We found no significant difference in plasma LPS levels in HCC vs. healthy individuals. Possibly, LPS concentrations in portal system or liver tissue may have bigger value than systemic levels.

By causing bacterial translocation from the gut, increased intestinal permeability may be one of the most important factors in development of chronic liver injury. Recent studies have demonstrated that faecal levels of one of intestinal permeability markers – FABP2 – are significantly elevated in cirrhotic patients and correlate with disease severity [91]. Similar correlation was observed measuring FAPB2 levels in peripheral blood [23]. High FABP2 levels were also shown to be associated with higher rates of cirrhotic patients' mortality from variceal bleeding [92]. Up to now there were no studies investigating relationship between blood FABP2 levels and HCC. Our study revealed significantly higher FABP2 levels in HCC patients compared to healthy individuals. This might be associated with underlying liver cirrhosis rather that HCC itself. However, FABP2 measurements in plasma appeared to not be a suitable prognostic tool for HCC patients probably as this molecule is not specific for HCC and is not directly involved in hepatocarcinogenesis.

Similarly to FABP2, our study confirmed that plasma IL-10 levels are significantly elevated comparing to healthy individuals. This finding is in line with previous findings where higher IL-10 levels were shown to be associated with advanced stages of chronic liver disease while IL-10 polymorphisms also were demonstrated to have relations with both liver cirrhosis and HCC [19, 93]. Our analysis has not shown any prognostic potential of plasma IL-10. The detected increase in IL-10 levels in HCC patients most likely is associated with cirrhosis and chronic inflammation rather than tumor activity as IL-10 is well described as having a big role in inflammation processes [17].

To sum up, our study has demonstrated that baseline measurements of plasma IL-6 levels and in most cases IL-8 levels as well as early reassessment of these molecules have clear prognostic value being able to predict survival, liver dysfunction and response to treatment in HCC patients. However, other inflammatory biomarkers – IL-10, VEGF, LPS, FABP2 – have not shown significant value for HCC patients. Further broader investigations on these molecules are needed.

For a more detailed discussion on the value of inflammatory molecules, please refer to the papers "*Baseline Interleukin-6 and -8 predict response and survival in patients with advanced hepatocellular carcinoma treated with sorafenib monotherapy – an exploratory post hoc analysis of the SORAMIC trial*", "*Prognostic value of baseline interleukin 6 levels in liver decompensation and survival in HCC patients undergoing radioembolization*", "*Dynamics in Circulating Proinflammatory Biomarkers for Prognostic*

Assessment of Patients With Advanced HCC" and "*Diagnostic and Prognostic Value of IL-10, FABP2 and LPS Levels in HCC Patients*".

The second part of our study involved plasma miRNA profile or miRNome analysis in order to determine such profile and assess its diagnostic, prognostic and predictive value for patients with HCC.

This study revealed clearly different profiles of circulating miRNome in baseline plasma samples of treatment-naïve HCC patients comparing to plasma samples of healthy individuals. Some similarities and differences have been detected when comparing the most deregulated miRNAs from our analysis to previous findings in this field. hsa-miR-576-5p has been previously reported to be up-regulated in NAFLD [94], yet we have reported the link between deregulation of this miRNA and HCC for the first time. While Wu et al. have previously demonstrated a down-regulation of has-miR-200b-3p in HCC tissue [95], our findings indicate a significant up-regulation of this miRNA in HCC plasma samples compared to controls. Functional studies have identified the roles of hsa-miR-374a-5p and hsa-miR-140-5p in HCC *in vitro* [96, 97], yet up to our analysis there were no other studies reporting deregulation of these miRNAs in HCC patients. Cho et al. reported a decreased level of hsa-miR-1228-5p in cervical cancer [98], while Sonoda et al. associated this miRNA with cerebrovascular disorders [99]. Our study revealed an up-regulation of this miRNA in plasma from HCC patients for the first time. To our knowledge, down-regulation of hsa-miR-219-2-3p and hsa-miR-218-5p in plasma has not been previously reported in HCC patients. hsa-miR-219-2-3p has been implicated in gastric carcinogenesis, pituitary adenoma development and glioblastomas [100–102], while only in vitro studies have highlighted the role of hsa-miR-218-5p in regulating cell proliferation, metastasis, and invasion [103]. Consistent with our results, Shaker et al. demonstrated down-regulation of hsa-miR-129-5p in HCC patients' serum samples and its great performance in HCC discrimination analysis (sensitivity 100%, specificity 97.2%, AUC = 0.997) [104]. Functional studies have supported these findings showing the important role of hsa-miR-129-5p in proliferation, metastasis, and progression of HCC [105]. We observed reduced plasma levels of hsa-miR-132-3p. In contrast, previous work of Wen et al. and Yuan et al. reported opposite findings – increased expression of hsa-miR-132-3p in HCC cases [106, 107]. Although some studies suggest the potential roles of exosomal or circulating serum hsa-miR-3960 in diffuse large B-cell lymphoma or bladder cancer [104, 108], and hsa-let-7b-5p in acute myeloid leukaemia, skin melanoma and head and neck squamous cell carcinoma [109–111], there is no previously published data linking both hsa-miR-3960 and hsa-let-7b-5p with HCC. Thus, our study reports their association with this disease for the first time as well.

As AFP is the only approved and clinically used biomarker in HCC management, we checked if there is correlation between blood levels of AFP and newly identified differentially expressed miRNA profile. However, none of deregulated miRNAs exhibited a strong correlation with baseline AFP levels or changes (both absolute and percentage) in AFP levels. Previous research indicates that combining AFP levels with expression of certain miRNAs could enhance diagnostic capabilities [112]. Further investigations are needed to develop the best combinations of biomarkers for HCC.

Differential expression analysis revealed that only one miRNA (hsa-miR-142-5p) displayed deregulation that varied based on the HCC stage and could differentiate between BCLC stages B and C. Previously, only one study has established a connection between this miRNA and HCC [113]. However, its association with disease at different stages has not been previously analyzed.

Subsequently, we have identified several miRNAs such as hsa-miR-215-5p, -192-5p, -483-5p, -23b-3p whose expression patterns altered depending on different treatment strategies but irrespective of treatment response. The expression of miRNA hsa-miR-215-5p in plasma decreased 7–9 weeks after initiation of sorafenib monotherapy or combined SIRT-sorafenib treatment, while sorafenib monotherapy also impacted plasma levels of miR-192-5p, -483-5p, -23b-3p. These miRNAs have previously been associated with HCC [114–116]. However, only one study, consistent with our findings, demonstrated significant changes of miR-23b-3p one month after starting sorafenib therapy [117].

Analysis of plasma smRNA-seq data revealed associations between hsa-miR-183-5p, -28-3p and -1246 with HCC patients' response to sorafenib therapy. The important role of these miRNAs in HCC carcinogenesis were demonstrated in *in vitro* studies [118,119], however, their expression in relation with response to any therapies in HCC patients has not been previously reported. Nevertheless, one of these miRNAs – hsa-miR-1246 – was found to be differentially expressed after introducing sorafenib in colorectal cancer cell culture Caco-2 [120]. Although hsa-miR-28-3p has not previously been associate with HCC, we demonstrated its promising discriminatory ability with an AUC of 83.2% in distinguishing HCC patients who responded well to sorafenib therapy.

Finally, survival analysis revealed that hsa-miR-215-5p could be valuable not only as treatment-related but also as prognostic biomarker being associated with survival of HCC patients. Additionally, high expression level of hsa-miR-215-5p is able to predict significantly worse prognosis of HCC patients. While previous reports have suggested the potential diagnostic application of hsa-miR-215-5p for HCC patients [114], its association with patient survival has not been previously documented. Collectively, our data, along with previous findings, suggest that hsa-miR-215-5p functions as an oncomiR (miRNA associated with cancer) in HCC, responding to sorafenib treatment as well as predicting survival in these patients. Additional analysis of putative gene targets of hsa-miR-215-5p has identified genes, such as *NKAIN2*, *ZEB2, MFAP3*, *CCNT2, EREG*, *NIPAL1*, that could be potentially targeted by this miRNA (as per TargetScan 8.0 and miRDB [121]). This suggests potential involvement of hsa-miR-215-5p in signalling pathways including EGFR, angiogenesis, apoptosis, cadherin, Wnt, integrin, and TGF-beta (according to PANTHER 15.0 knowledgebase [122]), which have been implicated in cancer, including HCC [123]. Taken together, we have demonstrated that hsa-miR-215-5p could be a potential therapeutic target in HCC for future investigations.

For more details on discussion on results from miRNome analysis, please refer to the paper "*miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial*".

Despite important and promising results of this study, some limitations must be declared. First, analyses within this study includes rather small numbers of HCC patients. Therefore, the detailed analysis of subgroups of HCC patients, based on different etiologic factors, comorbidities, ethnicities, etc., was not performed and similar studies on larger cohorts are needed. Tumour tissue collection and analysis also were not included in this study. To minimize this limitation, open access data sets with previously performed miRNome analysis [124] was retained and compared to our findings in analysis of plasma samples. However, no significant overlapping was detected, probably due to different origin of the tissue or different ethnicity of involved HCC patients. Next, miRNA analysis was conducted using samples of circulating nucleic acids in total plasma since the isolation kits lacked technical procedures for the specific enrichment of small RNA molecules. Nonetheless, this yields valuable insights into the potential utility of multilayer molecular profiling from a single sample. Despite limitations, we believe that this study adds valuable data for further investigations and identification of reliable biomarkers for HCC.

In conclusion, first part of the study demonstrated that baseline plasma IL-6 and IL-8 levels as well as early reassessment of plasma levels of both ILs appear to have prognostic and predictive value for survival, liver dysfunction and response to treatment in HCC patients. Concluding our miRNome analysis in HCC patients, distinctively different miRNA profiles have been shown for HCC patients and healthy individuals. Several possible plasma miRNA biomarkers for HCC have been proposed in all diagnostic, treatment-related and prognostic dimensions. One miRNA – hsa-miR-215-5p – should be highlighted with ability to predict survival and being associated with HCC therapies.

CONCLUSIONS

1. Higher blood plasma IL-6 and IL-8 levels before HCC therapy were related to significantly worse survival in HCC patients treated with radioembolization-sorafenib combination as well as sorafenib monotherapy. Higher baseline plasma IL-6 levels were associated with shorter time to liver dysfunction in HCC patients treated with combination of radioembolization and sorafenib. Low baseline plasma levels of each IL-6 and IL-8 were related to higher objective response rate in sorafenib-treated HCC patients.
2. Higher plasma IL-6 and IL-8 levels at time point of 7 – 9 weeks after initiation of therapy as well as higher increase of IL-6 levels from baseline were related to significantly worse survival of HCC patients treated with sorafenib alone or in combination with radioembolization. Blood LPS and VEGF levels did not appear to have any prognostic value in HCC patients at baseline and follow-up time points as well as difference between them.
3. Baseline plasma FABP2 and IL-10 levels are significantly higher in HCC patients compared with healthy individuals. No prognostic value of baseline plasma FABP2, IL-10 and LPS levels were detected for predicting survival in HCC patients.
4. Distinctively different plasma miRNA profiles were revealed comparing HCC and control cases. Changes in hsa-miR-215-5p plasma expression levels are associated with both sorafenib monotherapy and combination of radioembolization and sorafenib as well as survival rates in sorafenib-treated HCC patients. miRNAs with response to treatment-dependent expression were identified, allowing predict survival prognosis for sorafenib-treated HCC patients.

SANTRAUKA

Įvadas

Pirminiai kepenų navikai yra viena dažniausiai nustatomų onkologinių ligų pasaulyje, užimanti septintą vietą tarp visų vėžio lokalizacijų. Remiantis pasaulinės statistikos duomenimis, 2020 metais pirminis kepenų vėžys buvo naujai nustatytas 905 677 pacientams. Tais pačiais metais patvirtinti 830 180 su pirminiu kepenų naviku susijusių mirties atvejų, tai sudaro daugiau nei 8 proc. visų su vėžiu susijusių mirčių atvejų [1]. Lyginant naujai nustatytų atvejų ir mirčių statistikos duomenis, pirminis kepenų vėžys pasižymi blogiausia pacientų išgyvenamumo prognoze – metinis mirčių nuo šios ligos skaičius beveik siekia naujai nustatytų atvejų skaičių. Kepenų ląstelių karcinoma (KLK) yra dažniausias pirminis kepenų navikas. Deja, remiantis amerikiečių mokslininkų prognozėmis, daugelyje šalių sergamumas šia liga didėja ir numatoma, kad ateityje didės toliau [2]. Tai akivaizdžiai rodo gerokai geresnių diagnostikos ir gydymo galimybių poreikį KLK sergantiems pacientams.

Daugiau nei 80 proc. atvejų, KLK išsivysto, sergant kepenų ciroze [3]. Dažniausios kepenų cirozės priežastys daugumoje pasaulio valstybių, taip pat ir Lietuvoje, išlieka lėtiniai virusiniai hepatitai ir lėtinis alkoholinis kepenų pažeidimas [4, 5]. Vis dažniau nustatoma kepenų cirozė, sukelta nealkoholinės suriebėjusių kepenų ligos arba naujai apibrėžtos su metaboliniu sindromu susijusios suriebėjusių kepenų ligos, ypač Vakarų šalyse [6, 7].

Bet kuris ilgą laiką veikiantis etiologinis veiksnys sukelia lėtinį kepenų uždegimą, veikdamas kepenų imuninės sistemos reguliavimą. Didelis kepenų regeneracinis pajėgumas ir ląstelių proliferacijos aktyvinimas kurį laiką kompensuoja kepenų audinio pažeidimą, kurį lemia lėtinio uždegimo sukelta ląstelių mirtis. Šio proceso metu kaupiasi reaktyvieji deguonies radikalai, DNR molekulių mutacijos ir palaipsniui lemia karcinogenezės procesą ir kepenų ląstelių karcinomos vystymąsi. Svarbų vaidmenį šiame procese vaidina naviko mikroaplinka ir jos prouždegiminiai elementai, ypač citokinai, tokie kaip IL-1α, IL-1β, IL-6, IL-8 ir TNF-α [8–10]. Anksčiau atliktai ikiklinikinių ir klinikinių tyrimai patvirtina, kad interleukinų IL-6 ir IL-8 koncentracijos kraujyje didesnės KLK pacientų atveju, palyginus su ciroze sergančiais pacientais be KLK [11–15].

Uždegiminio ir navikinio kepenų audinio mikroaplinkoje svarbūs ne tik prouždegiminiai, bet ir antiuždegiminiai citokinai, pavyzdžiui IL-10 [16–18]. Kuo labiau pažengusi kepenų cirozė, tuo didesnės IL-10 koncentracijos nustatomos kraujo plazmoje [19]. Keletas tyrimų parodė, kad KLK pacientų atveju IL-10 koncentracijos plazmoje padidėjimas labiau susijęs su ciroze nei

su naviku [20, 21]. Uždegimo procesas kepenyse skatinamas ir palaikomas ir kitais mechanizmais. Kepenų cirozės atveju padidėjus žarnyno sienelių pralaidumui, kraujyje padidėja endotoksinų, pavyzdžiui, lipopolisacharidų (LPS), koncentracija [22, 23]. Šie endotoksinai skatina kepenų Kupferio ląsteles išskirti reaktyviuosius deguonies radikalus ir prouždegiminius citokinus, tokius kaip IL-8, šitaip prisidėdami prie karcinogenezės proceso [24, 25]. Veikiant LPS, navikinės ląstelės išskiria daugiau kraujagyslių endotelio augimo veiksnio (VEGF), šitaip skatinama KLK ląstelių proliferacija ir navikinė angiogenezė [26, 27]. Padidėjusį žarnyno pralaidumą kepenų cirozės atveju gali atspindėti specifiniai baltymai, pavyzdžiui, riebiąsias rūgštis sujungiantys baltymai (FABP), kurie išsiskiria suyrant enterocitams [28, 29]. Anksčiau atlikti tyrimai nustatė, kad FABP2 koncentracija kraujo plazmoje padidėja sergant kepenų ciroze [23].

Karcinogenezės procesas kepenyse valdomas ir įvairių genetinių ir epigenetinių veiksnių. MikroRNR (miRNR) yra mažos baltymų nekoduojančios RNR molekulės, turinčios įtaką daugeliui organizmo genetinių reguliavimo kelių. Įvairių miRNR dereguliacija gali būti susijusi su daugeliu onkologinių procesų, įskaitant KLK [30–32].

Kai KLK jau išsivysčiusi cirotinėse kepenyse, kuo anksčiau ją nustatyti, kadangi ankstyvose ligos stadijose galimas radikalus gydymas (pavyzdžiui, rezekcija ar abliacija) [33, 34]. Labiau pažengusiose ligos stadijose, dažniausiai radikalus gydymas nebeįmanomas. Daugiau nei dešimtmetį pirmojo pasirinkimo sisteminis gydymas buvo sorafenibas, daugelio taikinių tirozinkinazės inhibitorius, kurio veiksmingumas gydant pažengusios stadijos KLK buvo įrodytas klinikinių tyrimų metu [36]. Nepaisant to, kad šiuo metu veiksmingesnis imunoterapija paremtas gydymas tapo pirmojo pasirinkimo ir kad sukuriama ir patvirtinama vis daugiau veiksmingų molekulių [37], sorafenibas liks svarbus, ypač kai imunoterapija yra nekompensuojama, kontraindikuotina arba neveiksminga. Sorafenibo veiksmingumui pagerinti bandyta kartu skirti kitokį KLK gydymą, pavyzdžiui radioembolizaciją. Nors radioembolizacijos skyrimas kartu su sorafenibu neparodė reikšmingai geresnių rezultatų [38], detalesnė analizė išskyrė teigiamą terapinę naudą tam tikroms KLK sergančių pacientų grupėms [39].

Daugėjant naujų KLK gydymo galimybių, didėja biožymenų, galinčių padėti atrinkti pacientus, numatyti atsaką į gydymą, išgyvenamumo prognozę, poreikis. Iki šiol plačiai klinikinėje praktikoje naudojamas tik vienas KLK biožymuo – alfa-fetoproteinas (AFP). Visgi jo specifiškumas yra mažas – dažnai KLK atveju nustatoma normali AFP koncentracija, o padidėjimas stebimas ne onkologinių ligų atvejais [41,42].

Keletas su kepenų karcinogeneze susijusių molekulių buvo tyrinėtos kaip potencialūs biožymenys. Nustatyta, kad IL-6 ir IL-8 koncentracijos turi įtakos rezistentiškumui sorafenibui [43–45], kad IL-6 koncentracija prieš gydymą koreliuoja su pacientų, gydytų sorafenibu, išgyvenamumu [46], kad IL-6 ir IL-8 koncentracijos gali padėti numatyti pacientų prognozę po gydymo chemo- arba radioembolizacija [48, 49]. IL-10 koncentracija plazmoje taip pat pasižymėjo prognostinėmis savybėmis KLK sergantiems pacientams [21]. Ne tik uždegimo citokinai, tačiau ir nemažai kitokių molekulių (taip pat ir miRNR) pasižymi potencialių KLK biožymenų savybėmis [68].

Ankstesni tyrimai atskleidė miRNR profilio kepenų audinyje ir kraujyje svarbą ir vaidmenį ankstyvoje KLK, ypač susijusios su lėtiniais virusiniais hepatitais, diagnostikoje [60–62]. Tačiau dauguma autorių analizavo iš anksto nustatytus miRNR rinkinius arba specifines miRNR, atliekant realaus laiko PGR [60–62]. Kadangi miRNR molekulės mažos ir gausios kraujyje bei kituose audiniuose, įvairios miRNR ir jų raiškos pokyčiai gali būti labai naudingas biožymuo [63]. Kadangi miRNR svarbios karcinogenezės procese, jos gali būti terapiniai taikiniai. Su miRNR taikiniais susijusi taikinių terapija šiuo metu gana plačiai tyrinėjama, taip pat ir KLK atveju [64]. Dėl visų šių priežasčių išsamūs miRNR profilio tyrimai KLK diagnostikai ir prognozei vertinti yra itin reikalingi ir svarbūs.

Šiame tyrime vertinome didžiausią potencialą KLK diagnostikai, gydymo veiksmingumui ir pacientų išgyvenamumui turinčius biožymenis. Pirma, vertinome uždegimo molekules, analizuodami IL-6, IL-8, VEGF, LPS, FABP2 ir IL-10 vertę. Taip pat atlikome miRNR transkriptomo (miRNomo) analizę KLK sergančių pacientų kraujo plazmoje. Šis tyrimas leido pasiekti reikšmingų ir kliniškai vertingų rezultatų, atskleidžiant naujus diagnostinėmis, predikcinėmis ir prognostinėmis savybėmis pasižyminčius KLK biožymenis.

Darbo tikslas: įvertinti uždegimo mediatorių ir miRNR analizės tinkamumą kepenų ląstelių karcinomos neinvazinei diagnostikai, gydymo veiksmingumui bei ligos prognozei vertinti.

Darbo uždaviniai:

1. Įvertinti IL-6 ir IL-8 koncentracijų kraujo plazmoje prieš pardedant gydymą tinkamumą KLK sergančių pacientų išgyvenamumo prognozei ir gydymo sorafenibu veiksmingumui vertinti.
2. Įvertinti pakartotinio IL-6, IL-8, LPS ir VEGF koncentracijų kraujo plazmoje ištyrimo gydymo eigoje tinkamumą KLK sergančių pacientų išgyvenamumo prognozei vertinti.

3. Įvertinti FABP2, IL-10 ir LPS koncentracijų kraujo plazmoje prieš pradedant gydymą diagnostinę ir prognostinę vertę KLK sergantiems pacientams.
4. Ištirti miRNR profilį ir įvertinti jo tinkamumą KLK diagnostikai, gydymo veiksmingumui ir pacientų išgyvenamumo prognozei vertinti.

Mokslinis darbo naujumas ir aktualumas

Šiame tyrime pateikiama (1) išsami kraujo plazmos IL-6 ir IL-8 analizė KLK pacientams; (2) duomenys, pagrindžiantys IL-6 ir IL-8 koncentracijų kraujo plazmoje prognostinę vertę KLK sergantiems pacientams; (3) kitų uždegimo mediatorių – VEGF, LPS, FABP2, IL-10 – analizę, vertinant jų kaip KLK biožymenų tinkamumą; (4) miRNR profiliai KLK sergančių pacientų ir kontrolinės grupės asmenų kraujo plazmoje bei miRNR, pasižyminčių labiausiai pakitusia raiška, analizė; (5) išsami miRNR profilių analizė, lyginant kraujo plazmos mėginius prieš pradedant KLK gydymą, mėginius per ar po gydymo ir kontrolinės grupės mėginius; (6) naujus duomenis, parodančius potencialią diagnostinę, prognostinę ir su gydymu susijusią miRNR profilių vertę KLK sergantiems pacientams.

Apibendrinant, tai yra pirmasis tyrimas, kuriame parodoma: (1) aiški IL-6 ir IL-8 koncentracijų kraujo plazmoje prognostinė vertė ne tik tiriant prieš gydymo pradžią, bet ir pakartotinai tiriant gydymo metu ar po jo; šių molekulių predikcinė su gydymu sorafenibu susijusi vertė; (2) kitų uždegimo mediatorių – VEGF, LPS, IL-10, FABP2 – vertė KLK sergantiems pacientams; (3) pilna miRNR molekulių analizė ir jų diagnostinė, predikcinė ir prognostinė vertė KLK sergantiems pacientams, išskiriant hsa-miR-215-5p, kuri gali būti apibūdinama kaip specifinė su KLK susijusi miRNR, kurios raiškos lygis plazmoje susijęs su gydymu ir išgyvenamumo prognoze KLK sergantiems pacientams. Visi šie duomenys reikšmingai prisideda prie tyrimų, ieškant jautriausių ir specifiškiausių minimaliai invazinių kraujo biožymenų KLK diagnostikai, gydymo efektyvumo ir pacientų išgyvenamumo prognozei vertinti.

Autoriaus indėlis

Žemiau pateikiamas autoriaus Egidijaus Morkūno indėlis į su disertacija susijusias publikacijas (A1–A5, pateiktas skyriuje "*List of scientific papers*"):

A1: prisidėjo prie tyrimo idėjos ir dizaino kūrimo, atliko ELISA testus interleukinų koncentracijų nustatymui, atliko duomenų analizę ir interpretaciją, parengė pirminį publikacijos rankraštį, atliko rankraščio taisymus, gavo bendraautorių sutikimą naudoti publikaciją disertacijai.

A2: prisidėjo prie tyrimo dizaino kūrimo, atliko ELISA testus, duomenų analizę, parengė pirminį publikacijos rankraštį, atliko rankraščio taisymus, gavo bendraautorių sutikimą naudoti publikaciją disertacijai.
A3: aktyviai prisidėjo prie techninės tyrimo dalies, uždegimo molekulių koncentracijų kraujo plazmoje matavimo, atliko duomenų analizę ir interpretaciją, parengė pirminį publikacijos rankraštį, atliko rankraščio taisymus, gavo bendraautorių sutikimą naudoti publikaciją disertacijai.
A4: prisidėjo prie tyrimo idėjos ir metodologijos sukūrimo, dalyvavo KLK sergančių pacientų įtraukime į tyrimą, rinko klinikinius duomenis, atliko ELISA testus, analizavo duomenis, parengė pirminį ir galutinį publikacijos rankraščio variantą.
A5: aktyviai dalyvavo kuriant tyrimo dizainą ir metodologiją, analizavo ir interpretavo duomenis, didele dalimi prisidėjo prie pirminio publikacijos rankraščio rengimo, korekcijų ir galutinio rankraščio varianto rengimo.

Medžiagos ir metodai

Disertacijoje aprašomiems atliktiems tyrimams gauti atitinkamų institucijų bioetikos leidimai. Tiriamoji imtis detaliai aprašyta A1–A5 publikacijose.

Kiekvienam tyrime dalyvavusiam KLK sergančiam pacientui paimti kraujo mėginiai prieš pradedant bet kokį specifinį gydymą. Papildomi kraujo mėginiai paimti po 7–9 savaičių po KLK gydymo sorafenibu arba radioembolizacijos ir sorafenibo deriniu pradžios pacientams, kuriems buvo suplanuotas pakartotinis kraujo biožymenų vertinimas. Kontrolinės grupės asmenims taip pat atliktas kraujo mėginio paėmimas įtraukimo į tyrimą metu.

Uždegimo mediatorių – IL-6, IL-8, IL10, LPS, VEGF, FABP2 – koncentracijos kraujo plazmoje nustatytos naudojant šiuos su fermentais susietais imunosorbentinių tyrimų (ELISA) rinkinius: *Human IL-6 Quantikine ELISA Kit (R&D Sys)*, *Human IL-8/CXCL8 Quantikine ELISA Kit (R&D Sys)*, *Human IL-10 Quantikine ELISA Kit (R&D Sys)*, *Human VEGF Quantikine ELISA Kit (R&D Sys)*, *Human FABP2/I-FABP Quantikine ELISA Kit (R&D Sys)*, *Human LPS ELISA Kit (Cusabio)*.

Kraujo plazmoje cirkuliuojančios nukleorūgštys, įskaitant cirkuliuojančios miRNR frakciją, išskirtos naudojant komercinį rinkinį *QIAamp Circulating Nucleic Acid Kit (Qiagen)*. Mažųjų RNR sekoskaitos bibliotekos paruoštos naudojant komercinį rinkinį *Illumina TruSeq Small RNA Sample Preparation Kit (Illumina)* ir sekvenuotos naudojant *Illumina HiSeq 2500* platformą.

Atlikta sekvenuotų mažųjų RNR duomenų bioinformatinė analizė, remiantis anksčiau aprašyta metodologija [65]. Statistinė duomenų analizė atlikta naudojant skirtingą programinę įrangą: miRNR profilio tyrimui – *R (v. 4.1.0)* ir *RStudio software (v. 1.4.1106)*, prognostinę ir predikcinę IL-6 ir IL-8 vertę nustatančiam tyrimui – *R (v. 3.5.0)*, pakartotinio IL-6 ir IL-8 ištyrimo vertę nustatančiam tyrimui – *SAS (v. 9.4)*, FABP2, IL-10 ir LPS vertę nustatančiam tyrimui – *IBM SPSS Statistics (v. 27.0)*. Disertacijoje naudota metodologija detaliau aprašyta A1 – A5 publikacijose.

Rezultatai

Pirmoji disertacijos dalis apėmė tyrimus, analizuojančius uždegimo mediatorių koncentracijų kraujo plazmoje nustatymo vertę pacientams, sergantiems KLK.

Siekiant įvertinti prognostinę ar su gydymu susijusią uždegimo citokinų IL-6 ir IL-8 vertę, atlikta KLK sergančių pacientų kraujo mėginių, paimtų prieš pradedant KLK gydymą, analizė. Nustatytos skiriamosios IL-6 (8,58 pg/ml) ir IL-8 (57,9 pg/ml) koncentracijų kraujo plazmoje vertės, pasižyminčios geriausia KLK sergančių pacientų išgyvenamumo prognoze. Pacientai, kurių IL-6 arba IL-8 koncentracija kraujyje buvo žema, išgyveno reikšmingai ilgiau, nei pacientai, kurių atitinkami rodikliai buvo aukšti. Daugelio kintamųjų regresijos analizė parodė, kad didelė IL-6 ir IL-8 koncentracija prieš pradedant KLK gydymą yra vieninteliai nepriklausomi predikciniai blogos pacientų išgyvenamumo prognozės veiksniai. Nustatyta, kad suminė prognostinė IL-6 ir IL-8 koncentracijų kraujo plazmoje vertė yra dar didesnė. Jei abiejų interleukinų koncentracija kraujyje prieš pradedant KLK gydymą yra žema, pacientų išgyvenamumo prognozė reikšmingai palankesnė lyginant su pacientais, kuriems nustatytas vieno arba abiejų interleukinų koncentracijos padidėjimas.

Vertinant su KLK gydymu susijusią IL-6 ir IL-8 vertę, analizuota koreliacija tarp šių interleukinų koncentracijų KLK sergančiųjų kraujo plazmoje prieš pradedant gydymą ir radiologinio atsako, vertinant pagal mRECIST kriterijus. Ir žema IL-6 koncentracija, ir žema IL-8 koncentracija buvo susijusi su reikšmingai geresniu objektyvaus atsako dažniu, gydant KLK.

Panašūs rezultatai gauti, analizuojant prognostinę IL-6 ir IL-8 vertę prieš pradedant gydymą KLK sergantiems pacientams, gydytiems kitokiu gydymo režimu – radioembolizacijos ir sorafenibo deriniu. Šių pacientų bendrasis išgyvenamumas bei išgyvenamumas iki ligos progresavimo buvo reikšmingai ilgesni, jei IL-6 arba IL-8 koncentracija kraujo plazmoje buvo žemesnė nei anksčiau apibrėžta ribinė vertė [49]. Daugelio kintamųjų regresijos analizė

parodė, kad aukšta plazmos IL-6 koncentracija yra vienintelis nepriklausomas blogos išgyvenamumo prognozės veiksnys KLK sergantiems pacientams. Atliekant šių mėginių analizę, įvertinta ir abiejų interleukinų vertė, prognozuojant laiką iki kepenų dekompensacijos (apibrėžtos kaip ≥ 2 laipsnio bilirubino koncentracijos plazmoje padidėjimas). Rezultatai parodė, kad laikas iki kepenų dekompesacijos reikšmingai ilgesnis tiems KLK sergantiems pacientams, kurių IL-6 koncentracija plazmoje prieš pradedant gydymą buvo žema. Statistiškai reikšmingos atitinkamos IL-8 vertės negauta.

Siekiant įvertinti pakartotinio uždegimo molekulių – IL-6, IL-8, VEGF ir LPS – koncentracijų nustatymo kraujyje ir jų pokyčio KLK gydymo eigoje prognostinę vertę, analizuoti KLK sergančiųjų kraujo mėginiai, paimti prieš gydymą ir praėjus 7–9 savaitėms nuo gydymo sorafenibu arba radioembolizacijos ir sorafenibo deriniu pradžios.

Analizės rezultatai parodė, kad abiejuose laiko taškuose tiek IL-6, tiek IL-8 koncentracija kraujo plazmoje neigiamai koreliavo su KLK sergančių pacientų išgyvenamumu. Nustatytos skiriamosios IL-6 (24,18 pg/ml) ir IL-8 (75,25 ng/ml) koncentracijų vertės pakartotinio ištyrimo metu gydymo eigoje, pasižyminčios geriausiomis prognostinėmis savybėmis. Didesnės nei ribinė vertė IL-6 ir IL-8 koncentracijos plazmoje susijusios su reikšmingai blogesniu KLK sergančiųjų išgyvenamumu. Ši tendencija taip patvirtinta statistiškai IL-6 (bet ne IL-8) atveju, vertinant pacientų išgyvenamumą iki KLK progresavimo. Taip pat įrodyta IL-6 pokyčio gydymo metu prognostinė vertė. KLK sergančių pacientų, kuriems plazmos IL-6 koncentracija 7–9 gydymo savaičių laikotarpiu padidėjo daugiau nei 16,8 pg/ml, išgyvenamumo prognozė reikšmingai blogesnė. Pacientams, kurių pradinė IL-6 koncentracija mažesnė nei 9,7 ng/ml, o 2 mėn. gydymo laikotarpiu šis rodiklis nedidėjo daugiau nei 16,8 pg/ml, stebėta geriausia išgyvenamumo prognozė. Taip pat nustatyta, kad pradinė bei pakartotinė IL-8 ir pakartotinė IL-6 koncentracijos pasižymėjo reikšminga koreliacija su kepenų funkcijos dekompensacija. Reikšmingos koreliacijos tarp LPS ir VEGF koncentracijos kraujyje prieš gydymą ar jo metu ir KLK pacientų išgyvenamumo nestebėta.

Su IL-6 ir IL-8 susijusių analizių rezultatai neprieštaravo ankstesnių kitų autorių publikacijų rezultatams ir reikšmingai juos papildė, pademonstruodami potencialią šių dviejų interleukinų vertę kaip biožymenų, padedančių patikimai vertinti KLK sergančių pacientų išgyvenamumo, kepenų funkcijos dekompensacijos ir gydymo veiksmingumo prognozę.

Kitų uždegimo molekulių – IL-10, FABP2 ir LPS – analizė parodė, kad KLK sergančiųjų kraujo plazmoje prieš pradedant gydymą nustatomos reikšmingai didesnės IL-10 ir FABP2 (bet ne LPS) koncentracijos lyginant su sveikais asmenimis. Deja, skiriamųjų šių molekulių koncentracijų verčių, pasižyminčių prognostinėmis savybėmis, nustatyti nepavyko. Neigiami rezultatai IL-10, FABP2, LPS ir VEGF atveju gali būti iš dalies paaiškinami. FABP2 ir IL-10 vaidina svarbų vaidmenį lėtinio kepenų uždegimo vystymosi ir progresavimo procese, dėl to yra susiję su kepenų ciroze, bet ne su hepatokarcinogeneze tiesiogiai [17, 23, 91]. LPS svarbus lėtinio uždegimo ir naviko progresavimo kepenyse procesuose [24, 25]. Tikėtina, kad LPS koncentracijos matavimas vartų venos kraujotakos sistemoje turėtų didesne vertę nei periferinio kraujo. VEGF, kuris skatina naviko kraujagyslių proliferaciją, kelių ankstesnių tyrimų buvo patvirtintas, kaip potencialus prognostinis žymuo KLK atveju, kai lėtinis kepenų pažeidimas buvo sukeltas virusinių hepatitų [83, 84, 86]. Mūsų tyrimo atveju, pacientai, sergantys virusiniais hepatitais, sudarė mažą imties dalį. Taigi, mūsų tyrimo duomenimis, IL-10, VEGF, LPS ir FABP2 koncentracijų plazmoje matavimai neturi reikšmingos vertės KLK sergantiems pacientams.

Antroji disertacijos dalis apėmė miRNR profilių arba miRNomo kraujo plazmoje analizę, siekiant nustatyti šį profilį ir įvertinti jo diagnostinę, prognostinę ir predikcinę vertę KLK sergantiems pacientams.

Lyginant KLK sergančiųjų kraujo plazmos mėginius prieš pradedant gydymą ir kontrolinės grupės mėginius, iš viso nustatytos 42 skirtingos raiškos miRNR, iš jų 16 – didesnės raiškos, 26 – mažesnės raiškos. Labiausiai padidėjusia raiška pasižymėjusios miRNR buvo hsa-miR-576-5p, -200b-3p, -374a-3p, -140-5p, -1228-5p, o labiausiai sumažėjusia raiška – hsa-miR-219a-2-3p, -218-5p, -129-5p, -132-3p, -3960. Lyginant šiuos rezultatus su ankstesnėmis kitų autorių atliktų tyrimų rezultatais, nustatyta ir skirtumų, ir panašumų. Nemaža dalis miRNR, turinčių sąsajų su KLK, mūsų tyrimo metu identifikuotos pirmą kartą.

Koreliacijos analizė tarp šių pakitusia raiška pasižymėjusių miRNR ir pagrindinių KLK klinikinių rodiklių, parodė, kad nei viena iš šių miRNR nėra susijusi su AFP koncentracija prieš pradedant gydymą arba jos pokyčiu gydymo eigoje. Vienintelė pakitusios raiškos miRNR – hsa-miR-142-5p – gebėjo patikimai atskirti skirtingas KLK stadijas (BCLC B ir C). Ankstesnis tyrimas parodė, kad tam tikrų miRNR ir AFP derinys gali sustiprinti diagnostinį KLK potencialą [112], tačiau reikia daugiau ir detalesnių tyrimų. miRNR sąsaja su skirtingomis KLK stadijomis anksčiau nebuvo tirta.

Siekiant atskirti pacientus, sergančius naujai diagnozuota KLK, ir sveikus kontrolinius asmenis, atlikta normalizuotų miRNR nuskaitymų skaičiaus analizė, remiantis funkcijų pasirinkimu ir atsitiktiniais *forest* klasifikatoriaus algoritmais. 10 miRNR buvo patvirtintos kaip svarbios: hsa-let-7b-5p, hsa-let-7c-5p, hsa-miR-124-3p, hsa-miR-125b-5p, hsa-miR-219a-2-3p, hsa-miR-3960, hsa-miR-432-5p, hsa-miR-6734-5p, hsa-miR-7-5p, hsa-miR-95-3p. ROC kreivės analizė išskyrė hsa-let-7b-5p ir hsa-miR-3960 kaip pasižyminčias geriausiu našumu (AUC 85,6 proc. ir 85,9 proc. atitinkamai). Visų dešimties miRNR derinys pasižymėjo dar geresniu potencialu atskirti KLK sergančius pacientus ir sveikus asmenis (AUC 92,68 proc.).

Tyrimo metu nustatyta, kad, skiriant gydymą sorafenibu, dvi miRNR (hsa-miR-483-5p, hsa-miR-23b-3p) pasižymėjo padidėjusia raiška, o kitos dvi (hsa-miR-215-5p, hsa-miR-192-5p) – sumažėjusia raiška, lyginant kraujo mėginius prieš gydymą ir praėjus 7–9 savaitėms po gydymo pradžios. Gydymo radioembolizacijos ir sorafenibo deriniu atveju, nustatyta tik viena miRNR (hsa-miR-215-5p), kurios raiška buvo pakitusi (sumažėjusi) gydymo eigoje. Ankstesnių tyrimų metu nustatyta šių miRNR sąsaja su KLK [114–116], tačiau gydymo metu iki šiol buvo aprašyti tik miR-23b-3p raiškos pokyčiai [117].

Įvertinti, ar miRNR profilis yra tinkamas KLK gydymo veiksmingumui numatyti, buvo palyginti pacientų, kuriems gydymas lėmė radiologinį atsaką (remiantis mRECIST kriterijais) ir kuriems toks atsakas negautas, kraujo plazmos mėginiai. Trys miRNR (hsa-miR-183-5p, hsa-miR-28-3p, hsa-miR-1246) pasižymėjo padidėjusia raiška lyginant šiuos pacientų, gydytų sorafenibu, mėginius. ROC kreivės analizė parodė, kad visų trijų miRNR atvejais AUC yra daugiau nei 75 proc. Lyginant pacientų, gydytų radioembolizacijos ir sorafenibo deriniu, kraujo mėginius, pakitusios raiškos miRNR nenustatyta. Šių trijų miRNR ryšys su KLK iki šiol aprašytas tik *in vitro* tyrimuose [118, 119], o jų raiškos pokyčių sąsaja su KLK gydymu iki šiol nebuvo tirta.

Išgyvenamumo analizė išskyrė dvi miRNR – hsa-miR-215-5p ir hsa-miR-25-5p – kurių raiška reikšmingai skyrėsi, lyginant išgyvenusių ir mirusių KLK sirgusių pacientų, gydytų sorafenibu, pakartotinius kraujo plazmos mėginius. Tolesnė analizė parodė, kad sumažėjusi hsa-miR-215-5p raiška yra reikšmingai susijusi su geresniu KLK sergančių pacientų išgyvenamumu. Ankstesnėse kitų autorių publikacijose aprašytas šios miRNR diagnostinis potencialas KLK atveju, tačiau jos ryšys su pacientų išgyvenamumu anksčiau nebuvo tirtas.

Nepaisant reikšmingų šio tyrimo rezultatų, svarbu paminėti ir keletą darbo trūkumų. Visų pirma, šioje disertacijoje aprašytos analizės buvo atliktos, vertinant nedideles KLK sergančių pacientų imtis, dėl to neatliktos detalesnės įvairių KLK sergančiųjų grupių (pavyzdžiui, skirtingų etiologinių veiksnių, etninių grupių, gretutinių ligų) analizės. Navikinio audinio medžiagos ištyrimas taip pat neįtrauktas į šį tyrimą. Siekiant sumažinti šį trūkumą, mūsų atliktos miRNR analizės rezultatai palyginti su anksčiau atliktos KLK navikinio audinio miRNR analizės rezultatais [124], tačiau reikšmingų sutapimų nestebėta greičiausiai dėl skirtingos tiriamosios medžiagos kilmės ir pacientų populiacijų skirtumų. Taip pat, miRNR analizė atlikta, naudojant cirkuliuojančių nukleorūgščių mėginius bendroje kraujo plazmoje, nes izoliavimo rinkiniuose trūko techninių procedūrų, skirtų specifiniam mažųjų RNR molekulių praturtinimui. Nepaisant to, šis tyrimas svariai prisideda prie minimaliai invaziniu būdu atliekamų biožymenų geresnei KLK diagnostikai ir pacientų prognozei vertinti nustatymo ir sukūrimo.

Apibendrinant mūsų atliktas analizes, galime patvirtinti potencialią IL-6 ir IL-8 vertę kaip biožymenų, padedančių patikimai vertinti KLK sergančių pacientų išgyvenamumo, kepenų funkcijos dekompensacijos ir gydymo veiksmingumo prognozę. Nustatėme, kad miRNR profiliai reikšmingai skiriasi, lyginant naujai diagnozuota KLK sergančius pacientus ir sveikus asmenis. Parodėme, kad tam tikros miRNR molekulės gali būti svarbios kaip biožymenys KLK diagnostikai, pacientų išgyvenamumui ir gydymo veiksmingumui vertinti. Išskirtina viena miRNR (hsa-miR-215-5p), kuri geba prognozuoti pacientų išgyvenamumą ir yra reikšmingai susijusi su KLK gydymu.

Išvados

1. Didesnės IL-6 ir IL-8 koncentracijos prieš gydymą sorafenibu arba radioembolizacijos ir sorafenibo deriniu susijusios su reikšmingai blogesniu pacientų išgyvenamumu. Didesnė IL-6 koncentracija plazmoje prieš gydymą radioembolizacijos ir sorafenibo deriniu taip pat susijusi su trumpesniu laiku iki kepenų funkcijos dekompensacijos. Maža pradinė IL-6 ir IL-8 koncentracija susijusi su reikšmingai geresniu objektyvaus atsako dažniu gydant sorafenibu.
2. Pakartotinis IL-6 ir IL-8 koncentracijos plazmoje įvertinimas turi papildomą prognostinę reikšmę KLK sergantiems pacientams, gydytiems sorafenibu arba radioembolizacijos ir sorafenibo deriniu. Didesnės IL-6 ir IL-8 koncentracijos kraujo plazmoje, praėjus 7–9 savaitėms nuo gydymo pradžios, ir reikšmingesnis IL-6 koncentracijos

padidėjimas gydymo metu susiję su blogesniu pacientų, gydytų sorafenibu arba radioembolizacijos ir sorafenibo deriniu, išgyvenamumu. LPS ir VEGF koncentracijos kraujyje prognostinės reikšmės KLK sergantiems pacientams nenustatyta.

3. FABP2 ir IL-10 koncentracijos kraujo plazmoje naujai nustatyta KLK sergantiems pacientams yra reikšmingai didesnės nei sveikų asmenų. Kraujo plazmos FABP2, IL-10 ir LPS prognostinės vertės šiems pacientams nenustatyta.
4. Nustatyti skirtingi kraujo plazmos miRNR profiliai, lyginant KLK ir kontrolinės grupės mėginius. Identifikuoti potencialūs kraujo plazmos miRNR biožymenys KLK diagnostikai, pacientų išgyvenamumo prognozei ir gydymo veiksmingumui vertinti. Sumažėjusi hsa-miR-215-5p raiška susijusi su KLK gydymu sorafenibu ir radioembolizacijos bei sorafenibo deriniu, taip pat su geresniu sorafenibu gydytų KLK pacientų išgyvenamumu.

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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Baseline Interleukin-6 and -8 predict response and survival in patients with advanced hepatocellular carcinoma treated with sorafenib monotherapy: an exploratory post hoc analysis of the SORAMIC trial

Osman Öcal[1] · Kerstin Schütte[2] · Juozas Kupčinskas[3] · Egidijus Morkunas[3] · Gabija Jurkeviciute[3] · Enrico N. de Toni[4] · Najib Ben Khaled[4] · Thomas Berg[5] · Peter Malfertheiner[4] · Heinz Josef Klümpen[6] · Christian Sengel[7] · Bristi Basu[8] · Juan W. Valle[9] · Julia Benckert[10] · Antonio Gasbarrini[11] · Daniel Palmer[12] · Ricarda Seidensticker[1] · Moritz Wildgruber[1] · Bruno Sangro[13] · Maciej Pech[14] · Jens Ricke[1] · Max Seidensticker[1]

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Abstract

Purpose To explore the potential correlation between baseline interleukin (IL) values and overall survival or objective response in patients with hepatocellular carcinoma (HCC) receiving sorafenib.

Methods A subset of patients with HCC undergoing sorafenib monotherapy within a prospective multicenter phase II trial (SORAMIC, sorafenib treatment alone vs. combined with Y90 radioembolization) underwent baseline IL-6 and IL-8 assessment before treatment initiation. In this exploratory post hoc analysis, the best cut-off points for baseline IL-6 and IL-8 values predicting overall survival (OS) were evaluated, as well as correlation with the objective response.

Results Forty-seven patients (43 male) with a median OS of 13.8 months were analyzed. Cut-off values of 8.58 and 57.9 pg/mL most effectively predicted overall survival for IL-6 and IL-8, respectively. Patients with high IL-6 (HR, 4.1 [1.9–8.9], $p < 0.001$) and IL-8 (HR, 2.4 [1.2–4.7], $p = 0.009$) had significantly shorter overall survival than patients with low IL values. Multivariate analysis confirmed IL-6 (HR, 2.99 [1.22–7.3], $p = 0.017$) and IL-8 (HR, 2.19 [1.02–4.7], $p = 0.044$) as independent predictors of OS. Baseline IL-6 and IL-8 with respective cut-off values predicted objective response rates according to mRECIST in a subset of 42 patients with follow-up imaging available (IL-6, 46.6% vs. 19.2%, $p = 0.007$; IL-8, 50.0% vs. 17.4%, $p = 0.011$).

Conclusion IL-6 and IL-8 baseline values predicted outcomes of sorafenib-treated patients in this well-characterized prospective cohort of the SORAMIC trial. We suggest that the respective cut-off values might serve for validation in larger cohorts, potentially offering guidance for improved patient selection.

Keywords Hepatocellular carcinoma · Sorafenib · Interleukin · Response

Introduction

Hepatocellular carcinoma (HCC) develops mostly on the background of chronic inflammation of the liver (El-Serag 2012). Cytokine signaling, including interleukins (IL), plays an intrinsic role in regulating this inflammatory process, and levels of various cytokines have been shown to be increased in patients with HCC compared to cirrhotic patients (Kakumu et al. 1993; Naugler et al. 2007; Porta et al. 2008; Bergmann et al. 2017).

Sorafenib, a multitarget tyrosine kinase inhibitor, has been shown to improve survival in patients with advanced HCC (Llovet et al. 2008). However, therapy benefit is not uniform for each patient. Several biomarkers have been investigated to predict the efficacy of sorafenib in HCC patients (Llovet et al. 2012). Additionally, after a long time with sorafenib being the only systemic treatment option for HCC, several first- and second-line therapies emerged (Bruix et al. 2017; Abou-Alfa et al. 2018; Kudo et al. 2018), and recently, atezolizumab–bevacizumab combination has been shown to be superior to sorafenib in the first-line setting (Finn et al. 2020). However, despite these advances,

✉ Max Seidensticker
max.seidensticker@med.uni-muenchen.de

Extended author information available on the last page of the article

sorafenib will undoubtedly continue to be an important treatment option, especially where atezolizumab–bevacizumab is unavailable or contraindicated, and find its new role in the complex HCC treatment algorithm. This situation intensified the need for additional biomarkers of sorafenib benefit. A few preclinical studies have shown that IL-6 and IL-8 are related to sorafenib resistance (Kahraman et al. 2019; Lai et al. 2019; Li et al. 2020). However, a study that evaluated the prognostic role of multiple biomarkers in HCC patients treated with sorafenib showed that baseline IL-8 values failed to detect treatment benefit (Miyahara et al. 2011). On the contrary, another study that investigated IL-6 in an Asian HCC cohort showed that pretreatment IL-6 values with a cut-off of 4.58 pg/mL are correlated with overall survival after sorafenib, with high pretreatment levels associated with a poor prognosis (Shao et al. 2017).

SORAfenib in combination with local MICro-therapy guided by gadolinium-EOB-DTPA-enhanced MRI (SORAMIC, EudraCT 2009-012576-27, NCT01126645) is a prospective, phase II, randomized, controlled study in HCC patients with three study arms. In the palliative arm of the study, HCC patients were randomized to sorafenib treatment either alone or combined with Y90 radioembolization (RE), and the addition of RE treatment failed to improve survival compared to sorafenib monotherapy (Ricke et al. 2019). This exploratory post hoc analysis of the palliative arm of the SORAMIC trial aimed to explore the predictive value of baseline IL-6 and IL-8 in patients with advanced HCC receiving sorafenib monotherapy.

Materials and methods

Study population

This post hoc analysis was a substudy of the palliative arm of SORAMIC, a prospective, randomized-controlled phase II trial exploring the additional benefit of RE to sorafenib treatment. We selected a subgroup of patients in the palliation arm receiving sorafenib monotherapy only, to eliminate potential effects of other therapies used within the trial (radioembolization). SORAMIC was conducted in 38 centers in Europe and Turkey. The study protocol was approved by the competent authorities as well as the institutional review board, and all patients gave written informed consent.

The inclusion and exclusion criteria for the SORAMIC trial have been described previously (Ricke et al. 2019). In summary, patients aged 18–85 years with a diagnosis HCC in intermediate stage (Barcelona Clinic Liver Cancer [BCLC] stage B, not eligible for TACE) or advanced stage (BCLC C), adequate liver reserve (Child–Pugh scores A to B7), an Eastern Cooperative Oncology Group performance status (ECOG PS) ≤ 2 were eligible. Patients with extrahepatic disease were recruited as long as the disease was liver-dominant

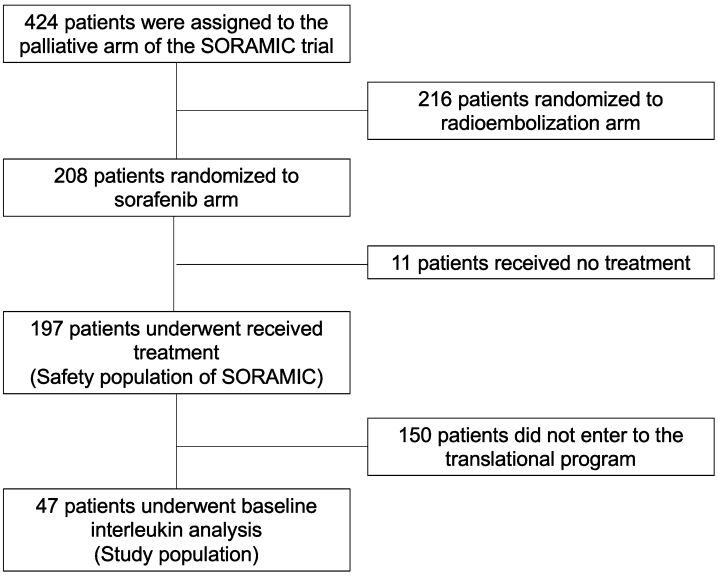

Fig. 1 Consort diagram

Table 1 Patient characteristics

	Number	%
All cohort	47	100
Gender (male)	43	91.4
Race (White)	39	82.9
Liver cirrhosis (yes)	41	87.2
HCC etiology		
Hepatitis B	4	8.5
Hepatitis C	9	19.1
Hepatitis C	9	19.1
Alcohol	23	48.9
ECOG PS		
0	36	76.5
1	11	23.4
Child Pugh score		
A	41 (87.2)	87.2
B	6 (12.7)	12.7
BCLC stage		
B	11 (23.4)	23.4
C	36 (76.5)	76.5
	Median	IQR
Age (years)	66	60.5–72.5
Albumin (g/dL)	38	33–40.7
Total Bilirubin (μmol/L)	15.2	11–21.2
AFP (ng/mL)	108.7	10–1374
IL-6 (pg/mL)	9.7	4.5–17.5
IL-8 (pg/mL)	56.3	34.6–172.9

and lungs were not involved. Inclusion into this substudy required the availability of a blood sample before the initiation of sorafenib treatment to measure IL-6 and IL-8 values as part of the translational program of the SORAMIC trial.

Of the 208 patients randomized to sorafenib monotherapy in the palliative arm of the SORAMIC trial and 197 patients received sorafenib within the trial. Of these 197 patients, 47 (23.8%) were included in the translational program (study population), and baseline blood samples were available for IL assessment (Fig. 1). There was no significant difference in baseline characteristics between the patients who entered the translational program and the rest of the patients who received sorafenib within the trial (Supplementary Table 1). Baseline characteristics of the study population are listed in Table 1. Forty-one (87.2%) patients had underlying liver cirrhosis. Whereas 23 (48.9%) patients had alcoholic liver disease (two without cirrhosis), 4 (8.5%) had hepatitis B (one without cirrhosis), and 9 (19.1%) had hepatitis C. Thirty-six (76.5%) patients had advanced HCC (BCLC C), and 41 (87.2%) had well-preserved (Child–Pugh A) liver function.

Patients were randomized in an 11:10 ratio to receive either combination of RE and sorafenib or sorafenib. Patients in the sorafenib arm were started sorafenib treatment after randomization with the starting dose of 200 mg twice daily. If tolerated, the dose was escalated to 400 mg twice daily (target dose) after 1 week. Treatment was continued until tumor progression or the emergence of a drug-related adverse event requiring discontinuation.

Blood samples were obtained before the initiation of the treatment and were stored deep frozen at study core facility and analyzed centrally using Human IL-6 Quantikine ELISA Kit (R&D Sys, Minneapolis, MN, USA; D6050), and Human IL-8/CXCL8 Quantikine ELISA Kit (R&D Sys; D8000C), following the manufacturer's instructions. Using enzyme-linked immunosorbent assays, serum levels of the IL6 and IL8 were measured.

As a secondary endpoint, in patients with follow-up imaging available for centralized image analysis, follow-up images were evaluated according to modified Response Evaluation Criteria in Solid Tumors (mRECIST) by a board-certified radiologist specialized in gastrointestinal imaging who was blinded to all the clinical information (Llovet and Lencioni 2020).

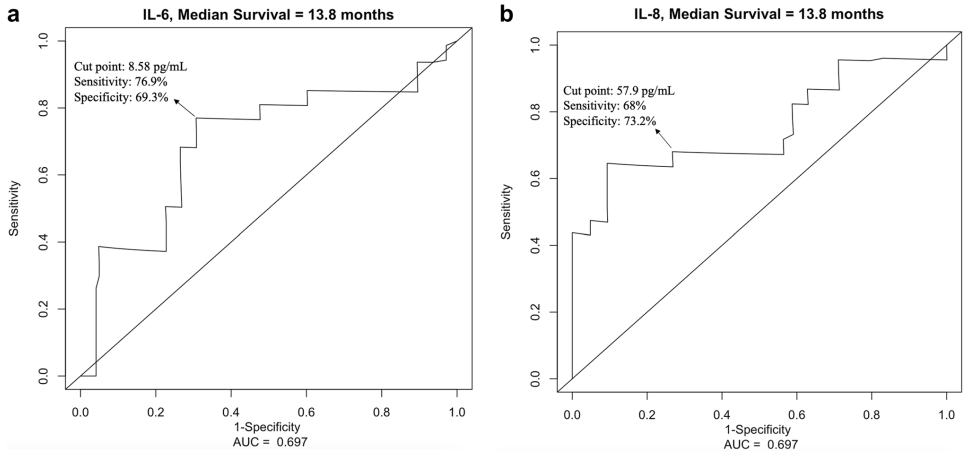

Fig. 2 The receiver-operating characteristics (ROC) curve showing the sensitivity and specificity of various cut-off values of baseline: **a** interleukin (IL)-6 and **b** IL-8 levels to analyze the overall survival

Statistical analysis

All statistical analyses were performed using R statistical and computing software, version 3.5.0 (http://www.r-project.org). Categorical variables were reported as counts and percentages, and continuous variables as means and standard deviations. Correlations were evaluated with Chi-square and Fisher's exact tests, and a *t* test was used to compare two groups. We used the receiver-operating characteristic (ROC) curve to determine the cut-off values for IL-6 and IL-8 that could produce the highest sensitivity and specificity to predict individual survival shorter than the median overall survival. The Kaplan–Meier method was used for estimates of overall survival, and the log-rank test was used to compare survival groups. Cox regression models were used to assess the effects of cofounding factors on overall survival. Variables with a p value of < 0.1 in the univariate analyses were analyzed in multivariate Cox regression models to explore prognostic factors of overall survival.

Results

By the end of the study, 37 (78.7%) patients had deceased, and the median OS in the subset of patients included in this biomarker analysis was 13.8 months.

Using ROC curve analysis, a cut-off value of 8.58 pg/mL for IL-6 was determined to have the highest sensitivity (76.9%) and specificity (69.3%) to predict survival in these patients (Fig. 2a), whereas an optimal cut-off value of 57.9 pg/mL was defined for IL-8 for a sensitivity of 68% and a specificity of 73.2% (Fig. 2b). Altogether, 26 (55.3%) patients had IL-6 values higher than 8.58 pg/mL, and 23 (48.9%) patients had IL-8 values higher than 57.9 pg/mL. Comparison of baseline characteristics of each subgroup according to IL levels is summarized in Table 2.

Univariate analysis of clinical and pathological variables conducted by stratifying patients according to these cut-off values showed that high baseline IL-6 was associated with albumin values of < 36 g/L ($p = 0.013$), whereas high baseline IL-8 was associated with larger maximum tumor diameter ($p = 0.013$). In addition, although the difference was not significant, there were more patients with high IL-6 in patients with larger tumors and ECOG 1; and high IL-8 in patients with total bilirubin ≥ 17 µmol/L.

The median overall survival of patients with low IL-6 was 30.3 months (CI 95% 21.6—NA), while patients with high IL-6 had a median overall survival of 10.3 months (95% CI 6.7–14.3; $p < 0.001$; Fig. 3). Similarly, patients with low IL-8 (30.3 [95% CI 13.8—NA] months) had significantly longer overall survival than patients with high IL-8 (10.3 [95% CI 5.5–17.6] months; $p = 0.009$; Fig. 4).

Besides IL-6 and IL-8 values, baseline albumin value ≥ 17 g/L ($p = 0.008$) and tumor diameter ≥ 65 mm ($p = 0.021$) were associated with overall survival, whereas there was a trend for better survival in patients with total bilirubin < 17 g/L ($p = 0.058$) and portal vein invasion ($p = 0.099$). There was no correlation between underlying liver disease and overall survival. Multivariate Cox regression analysis revealed that baseline high IL-6 (HR, 2.99

Table 2 Comparison of baseline characteristics of patients according to IL values

	Overall ($n=47$)	IL 6 high ($n=26$)	IL 6 low ($n=21$)	p	IL 8 high ($n=23$)	IL 8 low ($n=24$)	p
Gender (male)	43 (91.4)	22 (84.6)	21 (100)	0.117	20 (86.9)	23 (95.8)	0.347
Age (≥ 65 years)	28 (59.5)	16 (61.5)	12 (57.1)	0.760	14 (60.8)	14 (58.3)	0.859
Race (White)	39 (82.9)	23 (88.4)	16 (76.1)	0.437	19 (82.6)	20 (83.3)	> 0.99
ECOG PS							
0	36 (76.5)	17 (65.4)	19 (90.5)	0.080	17 (73.9)	19 (79.2)	0.670
1	11 (23.4)	9 (34.6)	2 (9.5)		6 (26.1)	5 (20.8)	
Liver cirrhosis (yes)	41 (87.2)	22 (84.6)	19 (90.5)	0.678	19 (82.6)	22 (91.6)	0.415
HCC etiology							
Hepatitis B	4 (8.5)	3 (11.5)	1 (4.7)	0.617	2 (8.6)	2 (8.3)	> 0.99
Hepatitis C	9 (19.1)	4 (15.3)	5 (23.8)	0.486	6 (26.0)	3 (12.5)	0.286
Alcohol	23 (48.9)	13 (50.0)	10 (43.4)	0.871	11 (47.8)	12 (50.0)	0.881
Previous TACE	15 (31.9)	7 (26.9)	8 (38.0)	0.414	6 (26.1)	9 (37.5)	0.401
Diffuse disease (≥ 10 lesion)	32 (68)	19 (73.0)	13 (61.9)	0.414	17 (73.9)	15 (62.5)	0.401
Median (mean) target lesion size, mm	53 (61.9)	62.5 (71.0)	47 (50.6)	0.084	68 (76.5)	49.5 (47.9)	**0.013**
Portal vein infiltration	28 (59.5)	16 (61.5)	12 (57.1)	0.760	16 (69.5)	12 (50.0)	0.171
Extrahepatic spread	5 (10.6)	3 (11.5)	2 (9.5)	> 0.99	3 (13.0)	2 (8.3)	0.666
Child–Pugh score							
A	41 (87.2)	21 (80.7)	20 (95.2)	0.204	18 (78.3)	23 (95.8)	0.097
B	6 (12.7)	5 (19.2)	1 (4.8)		5 (21.7)	1 (4.2)	
BCLC stage							
B	11 (23.4)	4 (15.3)	7 (33.3)	0.180	4 (17.4)	7 (29.2)	0.493
C	36 (76.5)	22 (84.6)	14 (66.7)		19 (82.6)	17 (70.8)	
Beyond up-to-7 criteria	42 (89.3)	24 (92.3)	18 (85.7)	0.644	21 (91.3)	21 (87.5)	> 0.99
Total bilirubin ≥ 17 µmol/L	15 (31.9)	11 (42.3)	4 (19.0)	0.120	10 (43.4)	5 (20.8)	0.095
Albumin < 36 g/L	16 (34.0)	13 (50.0)	3 (14.2)	**0.013**	10 (43.4)	6 (25.0)	0.181
AFP ≥ 400 ng/mL	17 (36.1)	10 (38.4)	7 (33.3)	0.731	9 (39.1)	8 (33.3)	0.848
Objective response	16 (38.0)	5 (19.2)	11 (46.6)	**0.007**	4 (17.4)	12 (50.0)	**0.011**

Bold type indicates statistical significance

[95% CI 1.22–7.3]; $p=0.017$) and high IL-8 (HR, 2.19 [95% CI 1.02–4.7]; $p=0.044$) values were the only independent predictors of shorter overall survival (Table 3).

The estimated rates of survival at 6 months and 12 months were 95.2% and 66.6%, respectively, in patients with IL-6 < 8.58 pg/mL and 100% and 66.6% in patients with IL-8 < 57.9 pg/mL.

Patients were scored according to IL levels as follows: both IL-6 and IL-8 lower than cut-off values (score 0), one of IL-6 or IL-8 higher than the cut-off (score 1), and both higher than the cut-off (score 2). Fifteen patients had score 0, 15 had score 1, and 17 had score 2. While the median OS of patients with score 0 was 37.7 (CI 95% 14.8—NA) months, score 1 was 16.3 (CI 95% 9.8—NA) months, and score 2 was 9.5 (CI 95% 5.1–14.3) months (Fig. 5).

For 42 (89.3%) patients, follow-up images were available. Response assessment according to mRECIST revealed an objective response in 16 (38.0%) patients. Patients with IL-6 values lower than cut-off had a significantly higher objective response rate than patients with IL-6 ≥ 8.58 pg/mL (46.6% vs. 19.2%, $p=0.007$). Similarly, low IL-8 values were also significantly associated with a higher objective response rate (50.0% vs. 17.4%, $p=0.011$).

Discussion

In the presented exploratory study, we define baseline levels of IL-6 and IL-8 as prognostic biomarkers of overall survival in patients with advanced HCC undergoing sorafenib treatment by identifying cut-off values of 8.58 pg/mL and 57.9 pg/mL for IL-6 and IL-8, respectively. Baseline IL-6 and IL-8 levels with respective cut-off values remained the only independent predictors of overall survival after adjusting for multiple prognostic factors in the multivariate analysis. In addition, these cut-off values were also associated with objective response in follow-up imaging.

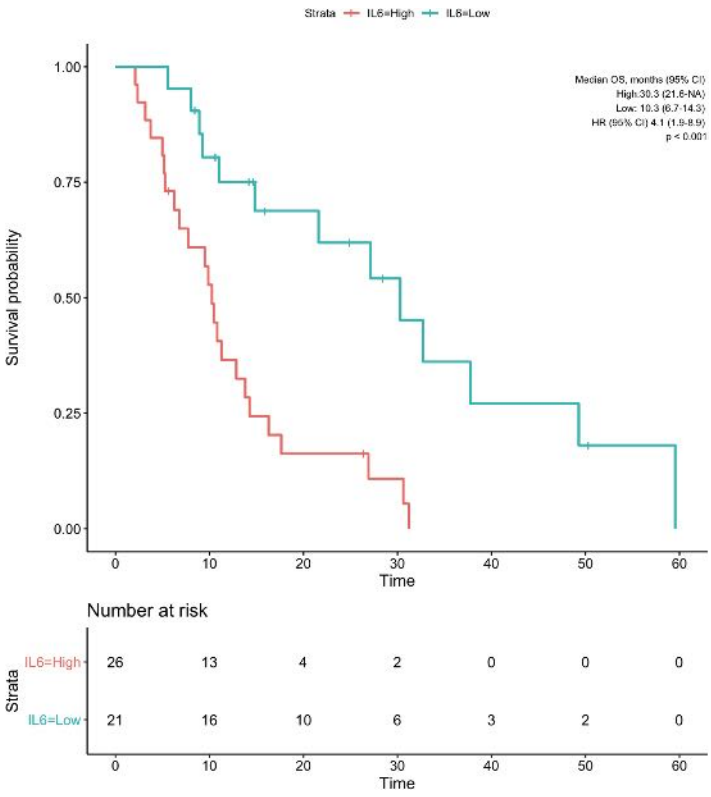

Fig. 3 Kaplan–Meier curve showing overall survival of patients grouped by baseline IL-6 values according to cut-off of 8.58 pg/mL

Previous studies have shown that higher IL-6 and IL-8 levels are associated with increased HCC risk in patients with chronic liver disease (Wong et al. 2009; Chien et al. 2011) and correlated with advanced disease stages (Sanmamed et al. 2014; Wang et al. 2016; Sun et al. 2019) and worse liver function in patients with HCC (Chan et al. 2012; Jang et al. 2012). High baseline IL-6 and IL-8 values have been also shown to correlate with treatment response and overall survival in patients who received minimally invasive locoregional therapies (Jang et al. 2012; Carpizo et al. 2014; Seidensticker et al. 2017). A study that investigated the prognostic role of baseline IL-8 under sorafenib treatment in a Japanese HCC cohort, in which 86.7% of the patients had viral hepatitis, showed no correlation between baseline IL-8 levels and treatment response or survival (Miyahara et al. 2011). A single study in the literature explored IL-6 as a predictor in HCC patients receiving sorafenib (55 and 73 patients in exploration and validation cohorts) showed a cut-off value of 4.28 pg/mL could predict survival (HR, 2.5 [1.3–5.0], $p = 0.005$) in an Asian cohort (Shao et al. 2017). Most patients (98.1%) in this study had viral hepatitis. The application of this cut-off value to our cohort failed to detect a survival benefit (data not shown). ROC analysis of the SORAMIC cohort revealed the cut-off value of 8.58 pg/mL for IL-6 with a sensitivity of 76.9% and specificity of 69.3% to predict individual survival longer than the median survival of the cohort. This discrepancy may represent the differences between Asian and Western cohorts and the need for different cut-off values for each. For example, while the most common underlying etiology was the alcoholic liver disease with 48.9% of the patients, and the rate of viral hepatitis was 27.6% in our cohort; the vast majority of patients (86.7–98%) in previously

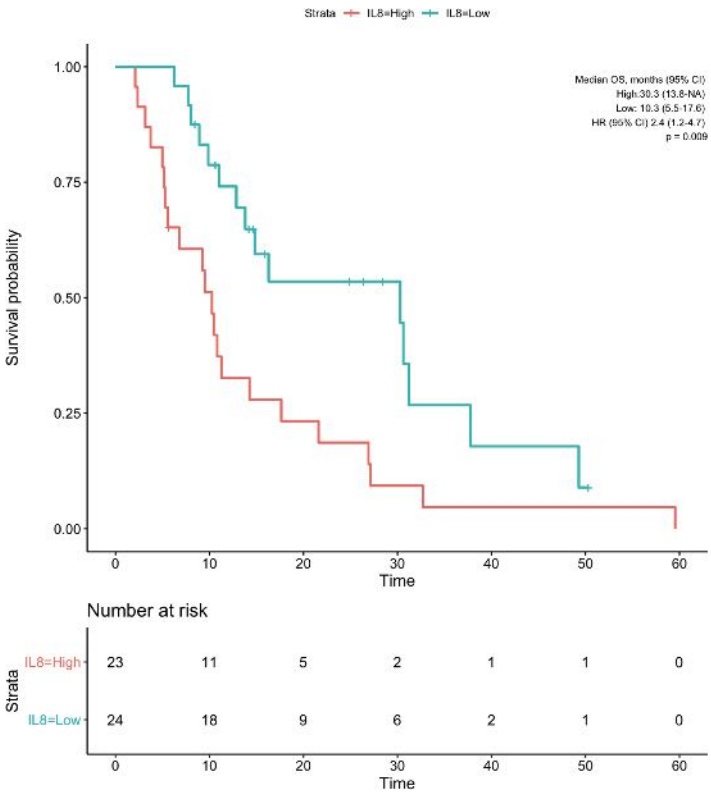

Fig. 4 Kaplan–Meier curve showing overall survival of patients grouped by baseline IL-8 values according to cut-off of 59.7 pg/mL

mentioned Asian cohorts had viral hepatitis (Miyahara et al. 2011; Shao et al. 2017). However, testing for the influence of etiology of underlying disease in our analysis did not identify a significant difference in IL values. This might be the result of small numbers and a combination of different causative factors. The cut-off value described in our study is in the range of previously reported cut-off values for IL-6 to detect survival benefit of HCC patients who underwent transarterial chemoembolization (10 pg/mL) and radioembolization (6.53 pg/mL) (Jang et al. 2012; Seidensticker et al. 2017). Furthermore, the identified cut-off values for IL-6 and IL-8 were also correlated with objective response during follow-up (according to mRECIST), demonstrating the capacity of baseline IL-6 and IL-8 as potential prognostic biomarkers.

IL-6 is a pro-inflammatory cytokine and induces the production of acute-phase reactants in the liver. It is also associated with cell proliferation, resistance to apoptosis and chemotherapeutics, and metastasis (Naugler et al. 2007; Schmidt-Arras and Rose-John 2016). IL-8 is a macrophage-derived cytokine that induces tumor angiogenesis and recruitment of immunosuppressive cells to the tumor (Koch et al. 1992; Fousek et al. 2020). Preclinical studies have shown that IL-6/STAT3 signaling contributes to sorafenib resistance in HCC cell lines, and blockage of IL-6 increases cytotoxicity of sorafenib (Niu et al. 2018; Lai et al. 2019; Li et al. 2020). Similarly, inhibition of IL-8 signaling reduces stem cell population in HCC and increases sorafenib sensitivity of tumor cells (Kahraman et al. 2019). Although both IL-6 and IL-8 are related to sorafenib resistance, mechanisms of action are through different pathways. The difference in the mechanisms of IL-6 and IL-8 was partially represented by our cohort. Whereas IL-6 was associated with albumin values and

Table 3 Univariate and multivariate analyses of factors associated with overall survival

Parameter	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
High IL-6	4.1 (1.9–8.9)	**< 0.001**	2.99 (1.22–7.3)	**0.017**
High IL-8	2.4 (1.2–4.7)	**0.009**	2.19 (1.02–4.7)	**0.044**
Sex (male vs. female)	0.79 (0.28–2.3)	0.667		
Age (≥ 65 vs. < 65 years)	1.1 (0.55–2.1)	0.85		
ECOG PS (1 vs. 0)	0.63 (0.28–1.4)	0.263		
Cirrhosis (yes vs. no)	0.63 (0.24–1.7)	0.35		
Hepatitis B etiology (yes vs. no)	1.1 (0.34–3.7)	0.865		
Hepatitis C etiology (yes vs. no)	1.5 (0.68–3.1)	0.335		
Alcohol etiology (yes vs. no)	0.61 (0.3–1.2)	0.164		
TACE history (Yes vs. No)	1.2 (0.61–2.5)	0.578		
PVI (yes vs. no)	0.55 (0.27–1.1)	0.099	0.66 (0.29–1.53)	0.337
Child–Pugh score (B vs. A)	1.5 (0.59–4.1)	0.377		
BCLC stage (C vs. B)	0.57 (0.26–1.2)	0.155		
Beyond up-to-7 (yes vs. no)	1.5 (0.44–4.8)	0.536		
Tumor diameter (≥ 65 vs. < 65 mm)	2.2 (1.1–4.4)	**0.021**	1.31 (0.54–3.18)	0.545
AFP (≥ 400 vs < 400 ng/mL)	1.1 (0.54–2.4)	0.745		
Diffuse disease (≥ 10 lesions)	1.3 (0.64–2.7)	0.457		
Extrahepatic disease	1.6 (0.48–5.5)	0.431		
Albumin	0.37 (0.17–0.77)	**0.008**	0.72 (0.29–1.8)	0.483
Bilirubin	0.51 (0.25–1)	0.058	0.83 (0.38–1.84)	0.649

Bold type indicates statistical significance

had a tendency to be higher in patients with worse performance status and larger tumors, IL-8 was associated with tumor diameter and partly correlated with bilirubin values. These findings are consistent with the previous studies (Jang et al. 2012; Sanmamed et al. 2014). In addition to these, when patients were scored according to IL levels (2 being both high, 1 either IL-6 or IL-8 high, and 0 both low), those with higher scores had significantly shorter overall survival. This highlights the importance of recognizing both baseline IL-6 and IL-8 values as interacting prognostic factors to cover both of liver inflammation/injury and tumor-related factors.

Recently, Imbrave150 trial has shown that the combination of atezolizumab with bevacizumab improved both overall and progression-free survival as compared to sorafenib (Finn et al. 2020). Despite the promising results of this and similar studies, not all patients are ideal candidates for atezolizumab and bevacizumab therapy. For example, patients with autoimmune diseases or organ transplant recipients were excluded from the Imbrave150 trial, as well as patients with Child–Pugh class B liver function. The high cost, iv. application need, and toxicity of the immunotherapies might prevent rapid worldwide implementation of these therapies. Additionally, atezolizumab–bevacizumab has shown to be not cost-effective as compared to sorafenib (Wang et al. 2021), which will restrict its use, especially in resource-limited countries. Furthermore, sorafenib will remain an important second-line treatment option in patients who progressed after atezolizumab–bevacizumab (Kudo 2021), and optimized treatment decision-making needs to be supported. For example, the survival rate at 12 months of atezolizumab–bevacizumab-treated patients was 67.2% in the IMbrave150 study (Finn et al. 2020), and patients with IL-6 or IL-8 values lower than cut-off values had similar rates of overall survival (66.6%) at the same time point in our study under sorafenib. For better utilization of limited resources, baseline IL values can potentially serve in risk stratification and patient allocation into therapies once validated. Therefore, further evaluation of the additional benefit of therapies suppressing IL pathways to current therapies in HCC patients with high baseline IL values is warranted. Besides this, baseline measurements of IL-6 and IL-8 should be used to stratify patients between treatment arms in future phase 3 trials for new drugs to improve patient selection for the therapy and avoid confounders.

This study has some limitations. Blood sampling for the translational program was not mandatory in the SORAMIC trial, and 18 of 38 centers participated in the translational study, and samples for IL analyses were available in 23.8% of the patients

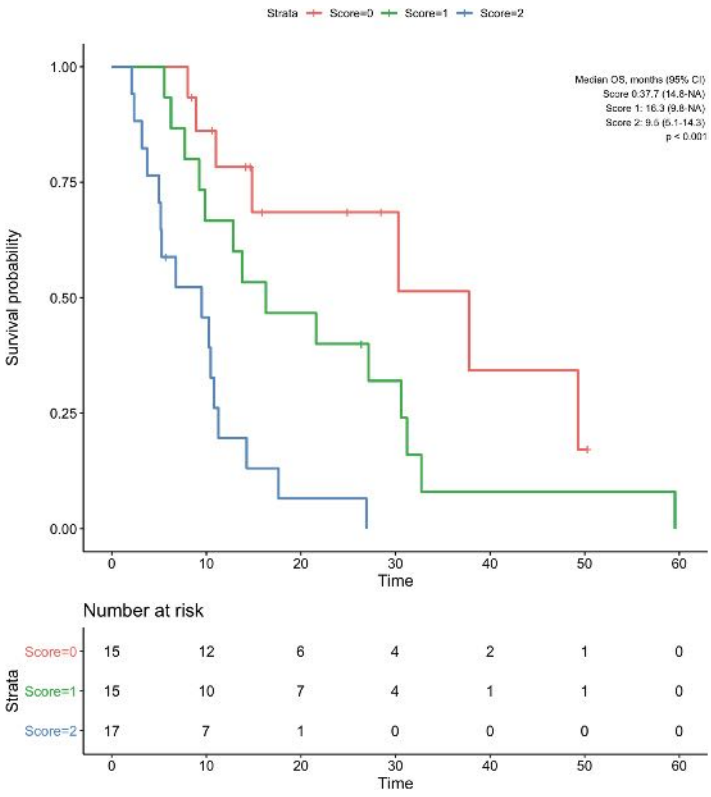

Fig. 5 Kaplan–Meier curve showing overall survival of patients according to interleukin score

received sorafenib monotherapy. However, our study represents the single cohort proving the prognostic role of baseline IL-6 and IL-8 values after sorafenib treatment of HCC in a Western cohort comprising high-quality data collected prospectively within a multicenter randomized trial. Nevertheless, further validation of these cut-off values in a larger cohort of patients receiving sorafenib treatment clearly is needed.

In conclusion, our study identified the prognostic value of baseline IL-6 and IL-8 values in advanced HCC patients receiving sorafenib treatment. The described cut-off values might be useful for individual patient allocation between different therapies in the era of checkpoint inhibitors or aggressive combination treatments. Additionally, baseline cytokine measurements should be included in future trials the assessing benefit of a new therapeutic regimen in advanced HCC. However, further validation of these cut-off values in larger cohorts is warranted.

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Availability of data and materials Data are available through corresponding author upon reasonable request.

Declarations

Conflict of interest Maciej Pech: Grants: Sirtex, Bayer; Personal fees: Sirtex. Peter Malfertheiner: Grants: Bayer, Sirtex. Jens Ricke: Grants: Sirtex, Bayer; Personal fees: Sirtex, Bayer. Max Seidensticker: Personal fees: Bayer, Sirtex.

Ethic approval and patient consent The study protocol was approved by the competent authorities as well as the institutional review board, and all patients gave written informed consent.

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Authors and Affiliations

Osman Öcal[1] · Kerstin Schütte[2] · Juozas Kupčinskas[3] · Egidijus Morkunas[3] · Gabija Jurkeviciute[3] · Enrico N. de Toni[4] · Najib Ben Khaled[4] · Thomas Berg[5] · Peter Malfertheiner[4] · Heinz Josef Klümpen[6] · Christian Sengel[7] · Bristi Basu[8] · Juan W. Valle[9] · Julia Benckert[10] · Antonio Gasbarrini[11] · Daniel Palmer[12] · Ricarda Seidensticker[1] · Moritz Wildgruber[1] · Bruno Sangro[13] · Maciej Pech[14] · Jens Ricke[1] · Max Seidensticker[1]

[1] Department of Radiology, University Hospital, Ludwig Maximilian University of Munich, Marchioninistrasse 15, 81377 Munich, Germany

[2] Department of Internal Medicine and Gastroenterology, Niels-Stensen-Kliniken Marienhospital, Osnabrück, Germany

[3] Institute for Digestive Research and Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania

[4] Department of Medicine II, University Hospital, LMU Munich, Munich, Germany

[5] Klinik Und Poliklinik Für Gastroenterologie, Sektion Hepatologie, Universitätsklinikum Leipzig, Leipzig, Germany

[6] Department of Medical Oncology, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, The Netherlands

[7] Radiology Department, Grenoble University Hospital, La Tronche, France

[8] Department of Oncology, University of Cambridge, Cambridge, UK

[9] Division of Cancer Sciences and Department of Medical Oncology, The Christie NHS Foundation Trust, University of Manchester, Manchester, UK

[10] Department of Hepatology and Gastroenterology, Charité-Universitätsmedizin Berlin, Campus Virchow Klinikum, Berlin, Germany

[11] Fondazione Policlinico Universitario Gemelli IRCCS, Universita' Cattolica del Sacro Cuore, Roma, Italy

[12] Molecular and Clinical Cancer Medicine, University Hospitals and Clatterbridge, University of Liverpool, Liverpool, UK

[13] Liver Unit, Clínica Universidad de Navarra, Pamplona, Spain

[14] Departments of Radiology and Nuclear Medicine, University of Magdeburg, Magdeburg, Germany

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Title: Prognostic value of baseline interleukin 6 levels in liver decompensation and survival in HCC patients undergoing radioembolization.

Authors: Öcal, Osman; Kupčinskas, Juozas; Morkūnas, Egidijus; Amthauer, Holger; Schütte, Kerstin; Malfertheiner, Peter; Klümpen, Heinz Josef; Sengel, Christian; Benckert, Julia; Seidensticker, Ricarda; Sangro, Bruno; Wildgruber, Moritz; Pech, Maciej; Bartenstein, Peter; Ricke, Jens; Seidensticker, Max.

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ORIGINAL RESEARCH **Open Access**

Prognostic value of baseline interleukin 6 levels in liver decompensation and survival in HCC patients undergoing radioembolization

Osman Öcal[1], Juozas Kupčinskas[2], Egidijus Morkunas[2], Holger Amthauer[3], Kerstin Schütte[4], Peter Malfertheiner[5], Heinz Josef Klümpen[6], Christian Sengel[7], Julia Benckert[8], Ricarda Seidensticker[1], Bruno Sangro[9], Moritz Wildgruber[1], Maciej Pech[10], Peter Bartenstein[11], Jens Ricke[1] and Max Seidensticker[1*]

Abstract

Background: To confirm the prognostic value of previously published baseline interleukin 6 (IL6) and IL8 cutoff values in survival and liver dysfunction in patients with advanced HCC undergoing ^{90}Y radioembolization.

Methods: A total of 83 patients (77 male) represented a subset of HCC patients undergoing ^{90}Y radioembolization combined with sorafenib as part of the prospective multicenter phase II trial SORAMIC. IL6 and IL8 levels were determined in serum samples collected at baseline. In this post hoc analysis, we sought to confirm the prognostic value of baseline cutoff values of 6.53 pg/mL and 60.8 pg/mL for IL6 and IL8, respectively, in overall survival (OS) or liver dysfunction (grade 2 bilirubin increase) after treatment.

Results: Median OS was 12.0 months. While low baseline albumin and high bilirubin values were associated with high IL6, liver cirrhosis, alcoholic liver disease, and portal vein infiltration were associated with high IL8.

In univariate analysis, high baseline IL6 and IL8 were associated with significantly shorter overall survival (7.8 vs. 19.0 months for IL6 and 8.4 vs. 16.0 months for IL8). In addition to IL values, liver cirrhosis, Child–Pugh grade, baseline albumin (< 36 g/dL), and total bilirubin (≥ 17 µmol/L), and higher mALBI grade (2b &3) values were associated with OS. At multivariate analysis, high baseline IL6 was the only independent prognostic factor for OS (HR 2.35 [1.35–4.1], p = 0.002).

Risk factors for liver dysfunction were high baseline IL6, albumin, and total bilirubin, and mALBI grade as found in univariate analysis. High baseline IL6 (HR 2.67 [1.21–5.94], p = 0.016) and total bilirubin ≥ 17 µmol/L (HR 3.73 [1.72–8.06], p < 0.001) were independently associated with liver dysfunction.

Conclusion: In advanced HCC patients receiving ^{90}Y radioembolization combined with sorafenib, baseline IL6 values proved to be prognostic, confirming previous findings in patients undergoing ^{90}Yradioembolization. IL6 might be useful for patient selection or stratification in future trials.

Keywords: Hepatocellular carcinoma, Radioembolization, Interleukin, Survival, Liver decompensation

Background

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, and in up to 90% of patients, HCC develops in a cirrhotic liver [1]. The most common etiologies of liver cirrhosis are chronic hepatitis B or C infections, alcoholic liver disease, and non-alcoholic steatohepatitis [2]. Chronic viral infections, alcohol

*Correspondence: max.seidensticker@med.uni-muenchen.de
[1] Department of Radiology, University Hospital, LMU Munich, Marchioninistrasse 15, 81377 Munich, Germany
Full list of author information is available at the end of the article

abuse, and intracellular fat accumulation interrupt the regulation of the hepatic immune system and induce liver inflammation. Chronic inflammation causes epithelial cell death, but the high regenerative capacity of the liver compensates for this damage by inducing cell proliferation. During this process, the accumulation of reactive oxygen species and DNA mutations cause hepatocarcinogenesis. Cytokine signaling, especially pro-inflammatory cytokines (such as IL6 and IL8), plays a key trigger role in inflammation [3]. Previous authors have found increased interleukin 6 levels in patients with chronic liver disease [4].

Reduced release of IL6 from Kupffer cells by inhibition of estrogen in women has been proposed as the cause of a lower incidence of HCC women as compared to men [5]. By blocking the IL6 pathway, an HCC mouse model has demonstrated reduced tumor burden in the liver, presuming as a result of decreased chronic inflammation [6]. A recent meta-analysis has demonstrated that IL6 levels are higher in HCC patients than patients with chronic liver diseases [4]. Similarly, IL-8 has been shown to mirror tumor burden in various tumors including HCC and correlate with tumor stage in HCC patients [7].

Beyond hepatocarcinogenesis, IL6 is also associated with poorer outcomes in HCC patients. The cytokines IL6 and IL8 have been shown to predict treatment response and survival after transarterial chemoembolization (TACE) in patients with primary and metastatic liver tumors [8]. A prospective exploratory study evaluating multiple cytokines has shown that a cutoff value of 6.53 pg/mL for IL6 and 60.8 pg/mL for IL8 was associated with overall survival irrespective of tumor entity after ^{90}Y radioembolization (RE) in patients with HCC or metastatic disease [9].

RE delivers radionuclide embedded microspheres to the liver tumors with much higher concentrations than liver parenchyma via injection into the hepatic artery. The results of SORAfenib in combination with local MICro-therapy guided by gadolinium-EOB-DTPA-enhanced MRI (SORAMIC, EudraCT 2009–012,576-27, NCT01126645), a prospective, phase II, randomized, controlled study in HCC patients with three study arms, has been already published [10]. In the palliative arm of the study, HCC patients were randomized to sorafenib treatment either alone or combined with RE, and the addition of RE treatment failed to show benefit over sorafenib monotherapy [10]. This post hoc analysis of the palliative arm of the SORAMIC trial aimed to validate the prognostic value of previously reported baseline IL cutoff values for overall survival in patients receiving ^{90}Y-radioembolization combined with sorafenib [9].

Methods

Study design and patient population

This study was a post hoc analysis of the palliative arm of the SORAMIC trial. Inclusion and exclusion criteria for the SORAMIC trial have been described previously [10]. In summary, patients aged 18 to 85 years with a diagnosis of HCC in the intermediate stage (BCLC B, not eligible for TACE) or the advanced stage (BCLC-C), preserved liver function (Child–Pugh scores A to B7), an Eastern Cooperative Oncology Group performance status ≤ 2 were eligible. Extrahepatic metastases were permitted if the disease was liver-dominant, and lungs were not involved. In this post hoc analysis of baseline interleukin levels, we included only patients randomized to the combination arm (RE and sorafenib) of the study. Subjects were eligible if baseline blood samples for the evaluation of IL6 and IL8 values were available.

The study protocol was approved by the institutional review boards of each participating center, and all patients gave written informed consent for study participation, including blood sampling and evaluation.

Treatment protocol

Patients underwent RE in a lobar fashion starting from the dominant-diseased liver lobe with semi-empiric BSA method of activity prescription. In patients with bilobar disease, treatments of the contralateral lobes were performed 4–6 weeks later. Sorafenib treatment was initiated 3 days after the last RE session. The starting dose of sorafenib was 200 mg twice daily, and if tolerated, it was escalated to 400 mg twice daily after one week.

Follow-up and laboratory analysis

There was a preplanned participation option for the translational research within the SORAMIC study, and patients were asked to participate in additional blood sampling for cytokine analysis. From patients agreeing to participate, blood samples were obtained before the initiation of the assigned treatment. Serum levels of IL-6 and IL-8 were measured with enzyme-linked immunosorbent assay (ELISA). The following ELISA kits were used in this study: Human IL-6 Quantikine ELISA Kit (R&D Sys; D6050), and Human IL-8/CXCL8 Quantikine ELISA Kit (R&D Sys; D8000C). All analytical procedures were performed according to manufacturers' instructions. After optical density measurements at 450 nm and 570 nm (as the reference) wavelengths using Tecan Sunrise absorbance microplate reader, concentrations were calculated using a four-parameter logistic regression (4-PL) curve fitting model. By using enzyme-linked immunosorbent assays, serum levels of the IL6 and IL8 were measured. Serum

IL6 and IL8 levels were defined as high or low, according to previously published cutoff values of 6.53 and 60.8 pg/mL, respectively [9].

Baseline albumin and total bilirubin values were recorded for each patient, and albumin–bilirubin (ALBI) score was calculated. Modified ALBI (mALBI) grade was used, and grade 1 was grouped together with grade 2a [11]. Within trial, patients were assessed every 2 months for a minimum of 2 years or until death, and at each visit liver function tests, including albumin and bilirubin, were repeated. The presence of any grade ≥ 2 bilirubin increases according to CTCAE (Common Terminology Criteria for Adverse Events) version 5.0 was defined as liver dysfunction. Time to liver dysfunction was recorded for each patient, and for patients with no grade ≥ 2 bilirubin increases, time to liver dysfunction was censored at the last available laboratory follow-up. The presence of RE-induced liver disease (REILD), which was defined as symptomatic ascites and jaundice (total bilirubin > 3 mg/dl) in the absence of tumor progression and biliary obstruction within the 8 weeks after RE [12], was also evaluated. Additionally, progression-free survival (PFS), based on local investigator assessment, was recorded.

Statistical analysis

All statistical analyses were performed using R statistical and computing software, version 3.5.0 (http://www.r-project.org). Categorical variables were reported as counts and percentages, and continuous variables as means and standard deviations. Correlations were evaluated with Chi-square and Fisher's exact tests, and t test was used to compare two groups. The Kaplan–Meier method was used for estimates of overall survival, PFS, and time-to-liver dysfunction. We employed cutoff values of 6.58 for IL6 and 60.8 for IL8 as previously reported by an exploratory analysis [9]. The accrual goal was 26 and 49 patients per IL 6 cutoff value to provide a statistical power of 80% and 90% at a significance level of 0.05. In order to eliminate the effects of disease progression on liver dysfunction, time-to-liver dysfunction analyses for IL6 and IL8 were repeated using the Kaplan–Meier method censoring patients at the time of progression who had disease progression before grade ≥ 2 bilirubin increase.

Cox regression models were used to assess the effects of cofounding factors on overall survival and liver dysfunction. Statistically significant variables in the univariate analyses were analyzed in multivariate Cox regression using two models to explore prognostic factors of overall survival and liver dysfunction. While Model 1 included albumin and total bilirubin separately, mALBI grade was used in Model 2.

Results

Baseline characteristics

Out of 424 patients included in the palliative arm of the SORAMIC trial, 216 patients were randomized to RE and sorafenib treatment. Thirty-three patients did not receive RE. Out of the remaining 183 patients, 83 (45.3%) who underwent baseline blood sampling accessible for IL assessment were the study population (Fig. 1). All of these 83 patients received sorafenib following RE. At the end of the study, 73 (87.9%) patients had died; the median OS of the post hoc study population was 12.0 (95% CI 9.7–16.0) months. Analysis according to predefined cutoffs revealed 48 (57.8%) patients with high IL6 value and 38 (45.7%) patients with high IL8.

Baseline characteristics are displayed in Table 1. There were significantly more patients with high IL6 in patients with total bilirubin ≥ 17 µmol/L (39.5% vs. 17.1%, p = 0.027), albumin < 36 g/L (35.4% vs. 8.5%, p = 0.004), and mALBI grade 2b and 3 (37.5% vs. 8.5%, p = 0.002). High IL8 was associated with liver cirrhosis (94.7% vs. 75.5%, p = 0.030), alcoholic liver disease (68.4% vs. 40.0%, p = 0.009), and portal vein invasion (52.6% vs. 24.4%, p = 0.008). Besides these, there was a trend for high IL6 in patients with cirrhosis (p = 0.065), portal vein infiltration (p = 0.061), and higher BCLC classification (p = 0.053); and a trend for high IL8 in patients with diffuse disease (≥ 10 lesions, p = 0.072), higher Child–Pugh grade (p = 0.088), and lower albumin (p = 0.054).

Overall survival and treatment response

Median OS was 19.0 months (95% CI 15.0–24.7) in patients with low IL6 and 7.8 months (95% CI 6.6–11.8) in patients with high IL6 (HR 2.4 [95% CI 1.5–3.8]; p < 0.001, Fig. 2a). In addition, patients with high IL8

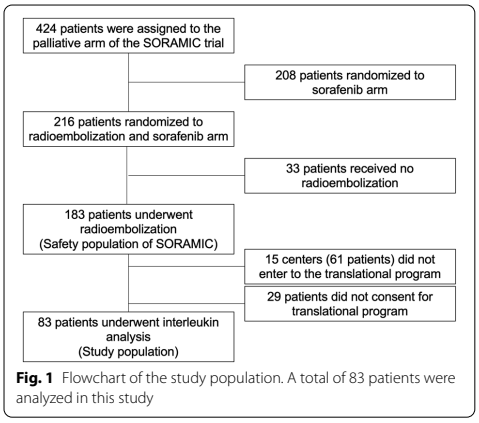

Fig. 1 Flowchart of the study population. A total of 83 patients were analyzed in this study

Table 1 Patient demographics and comparison of baseline characteristics of patients

	All cohort (n = 83)	IL6 low (n = 35)	IL6 high (n = 48)	p	IL8 low (n = 45)	IL8 high (n = 38)	p
Gender (male)	77 (92.7)	34 (97.1)	43 (89.5)	0.393	42 (93.3)	35 (92.1)	> 0.999
Age (≥ 65 years)	42 (50.6)	18 (51.4)	24 (50.0)	> 0.999	24 (53.3)	18 (47.3)	0.588
Race (White)	74 (89.1)	32 (91.4)	42 (87.5)	0.727	41 (91.1)	33 (86.8)	0.725
ECOG							
0	58 (69.8)	28 (80.0)	30 (62.5)	0.111	35 (77.8)	23 (60.5)	0.122
1	24 (28.9)	7 (20.0)	17 (35.4)		10 (22.2)	14 (36.8)	
Missing	1 (1.2)		1 (2.1)			1 (2.6)	
Liver cirrhosis (yes)	70 (84.3)	26 (74.2)	44 (91.6)	0.065	34 (75.5)	36 (94.7)	**0.03**
HCC etiology							
Hepatitis B	4 (4.8)	2 (5.7)	2 (4.1)	> 0.999	3 (6.6)	1 (2.6)	0.621
Hepatitis C	19 (22.8)	6 (17.1)	13 (27.0)	0.287	10 (22.2)	9 (23.6)	0.874
Alcohol	44 (53)	15 (42.8)	29 (60.4)	0.113	18 (40)	26 (68.4)	**0.009**
Previous TACE	18 (15.6)	9 (25.7)	9 (18.7)	0.447	11 (24.4)	7 (18.4)	0.507
Diffuse disease (≥ 10 lesion)	48 (49.3)	20 (57.1)	28 (58.3)	0.913	22 (48.8)	26 (68.4)	0.072
Median (mean) target lesion size, mm	68 (72.4)	66 (74.2)	70 (69.8)	0.65	56 (65.8)	73.5 (80.1)	0.13
Portal vein infiltration	31 (37.3)	9 (25.7)	22 (45.8)	0.061	11 (24.4)	20 (52.6)	**0.008**
Extrahepatic spread	21 (25.3)	6 (17.1)	15 (31.2)	0.144	11 (24.4)	10 (26.3)	0.845
Child–Pugh							
A	77 (92.7)	34 (97.1)	43 (89.5)	0.393	44 (97.7)	33 (86.8)	0.088
B	6 (7.2)	1 (2.8)	5 (10.4)		1 (2.2)	5 (13.1)	
BCLC							
B	26 (31.3)	15 (42.8)	11 (22.9)	0.053	16 (35.6)	10 (26.3)	0.365
C	57 (68.6)	20 (57.1)	37 (77.0)		29 (64.4)	28 (73.6)	
Up-to-7 criteria (outside)	71 (85.5)	29 (82.8)	42 (87.5)	0.552	37 (82.2)	34 (89.4)	0.532
Total bilirubin ≥ 17 µmol/L	25 (30.1)	6 (17.1)	19 (39.5)	**0.027**	11 (24.4)	14 (36.8)	0.22
Albumin < 36 g/L	20 (24.1)	3 (8.5)	17 (35.4)	**0.004**	7 (15.5)	13 (34.2)	0.054
AFP ≥ 400	29 (34.9)	11 (31.4)	18 (37.5)	0.519	13 (28.8)	16 (42.1)	0.235
mALBI grade (2b and 3)	21 (25.3)	3 (8.5)	18 (37.5)	**0.002**	8 (17.7)	13 (34.2)	0.129

Bold type indicates statistical significance;

IL, interleukin; ECOG, Eastern Cooperative Oncology Group; HCC, hepatocellular carcinoma; mALBI, modified albumin–bilirubin; TACE, transarterial chemoembolization; BCLC, Barcelona Clinic Liver Cancer; AFP, alfa fetoprotein

had significantly shorter median OS than patients with low IL8 (8.4 vs. 16.0 months, HR 1.8 [95% CI 1.1–2.9]; p = 0.009, Fig. 2b). Table 2 shows the prognostic factors associated with OS in univariate analysis. Besides high IL6 and high IL8, liver cirrhosis (p = 0.032), Child–Pugh class B (p = 0.014), albumin < 36 g/L (p = 0.024), total bilirubin ≥ 17 µmol/L (p = 0.009), and higher (2b and 3) mALBI grade (p = 0.007) were associated with worse outcome. Multivariate Cox regression analysis using Model 1 (excluding ALBI grade in order to avoid interactions) revealed high IL6 (HR 2.35, [95% CI 1.35–4.1] p = 0.002) as the only independent prognostic factor for shorter overall survival (Table 2). There was a tendency for shorter survival in patients with cirrhosis (HR 2.33, [95% CI 0.94–5.81] p = 0.069), Child–Pugh B (HR 2.91, [95% CI 0.98–8.64] p = 0.055), high total bilirubin (HR 0.58, [95% CI 0.32–1.05] p = 0.073). Similarly, high IL6 (HR 2.2, [95% CI 1.28–3.8] p = 0.005) was the only significant variable in Model 2 (including mALBI grade), and although the difference was not statistically significant, cirrhosis (HR 2.45, [95% CI 0.99–6.1] p = 0.053) and Child–Pugh B (HR 2.69, [95% CI 0.98–7.34] p = 0.054) were associated with shorter overall survival. Additionally, in separate models using each of IL6, IL8, albumin, bilirubin, and ALBI score as continuous variables, IL6 maintained the significant association with overall survival (Additional file 1: Figures S1a–e).

PFS information was missing in one patient. Patients with high IL-6 had significantly shorter progression-free survival than patients with low IL6 (Additional

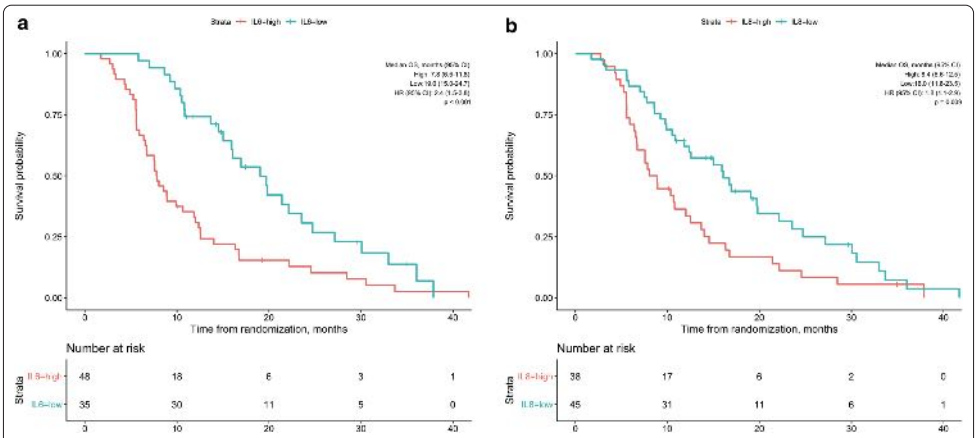

Fig. 2 Kaplan–Meier curves showing overall survival of patients grouped by high and low baseline IL6 (**a**) and IL8 (**b**) values. P values were calculated using the log rank test

Table 2 Univariate and multivariate analysis of factors associated with overall survival

Parameter	Univariate analysis		Multivariate analysis			
			Model 1[a]		Model 2[b]	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
IL6 (> 6.53 pg/mL)	2.4 (1.5–3.88)	**< 0.001**	2.35 (1.35–4.1)	**0.002**	**2.2 (1.28–3.8)**	**0.005**
IL8 (> 60.8 pg/mL)	1.8 (1.1–2.9)	**0.011**	1.32 (0.77–2.3)	0.305	1.2(0.71–2.0)	0.481
Sex (Male vs. Female)	0.59 (0.23–1.5)	0.26				
Age (≥ 65 vs. < 65 years)	0.8 (0.5–1.3)	0.36				
ECOG (1 vs. 0)	0.96 (0.57–1.6)	0.87				
Cirrhosis (Yes vs. No)	2.4 (1.1–5.2)	**0.032**	2.33 (0.94–5.81)	0.069	2.4 (0.99–6.1)	0.053
Hepatitis B Etiology (Yes vs. No)	1.5 (0.53–4)	0.47				
Hepatitis C Etiology (Yes vs. No)	1.3 (0.73–2.2)	0.39				
Alcohol Etiology (Yes vs. No)	1.2 (0.78–2)	0.36				
Previous TACE (Yes vs. No)	0.87 (0.5–1.5)	0.63				
PVI (Yes vs. No)	1.3 (0.81–2.1)	0.28				
Child–Pugh (B vs. A)	3.3 (1.3–8.5)	**0.014**	2.91 (0.98–8.64)	0.055	2.7 (0.98–7.3)	0.054
BCLC (C vs. B)	1.1 (0.53–2.3)	0.78				
Albumin (< 36 g/L)	1.9 (1.1–3.3)	**0.024**	0.68 (0.33–1.43)	0.31	–	–
Total bilirubin (≥ 17 µmol/L)	2 (1.2–3.2)	**0.009**	1.71 (0.95–3.09)	0.073	–	–
AFP (≥ 400 vs < 400 ng/mL)	0.86 (0.53–1.4)	0.53				
Diffuse disease (≥ 10 lesions)	0.77 (0.48–1.2)	0.28				
Extrahepatic disease	0.96 (0.57–1.6)	0.89				
mALBI grade (2b and 3 vs. 1 and 2a)	2.1 (1.2–3.6)	**0.007**	–	–	1.13 (0.62–2.07)	0.694

Bold type indicates statistical significance;

IL, interleukin; ECOG, Eastern Cooperative Oncology Group; TACE, transarterial chemoembolization; PVI, Portal vein invasion; BCLC, Barcelona Clinic Liver Cancer; AFP, alfa fetoprotein; mALBI, modified albumin–bilirubin

[a] Model 1 was identified using Cox regression with albumin and total bilirubin, excluding mALBI grade

[b] Model 2 was identified using Cox regression with mALBI grade as a composite factor, excluding albumin and total bilirubin

file 1: Figure S2a; 5.5 vs. 17.9, $p < 0.001$). Similarly, patients with high IL8 had shorter progression-free survival (Additional file 2: Figure S2b; 7.8 vs. 15, $p = 0.017$) than patients with low IL6.

Liver dysfunction

Follow-up bilirubin values were available in 78 patients. There was no case of REILD. Liver dysfunction (grade ≥ 2 bilirubin increase) was seen in 33 (42.3%) patients. Patients with high IL6 had significantly shorter time to liver dysfunction than patients with low IL6 (Fig. 3a; 9.7 vs. 32.6 months, HR 3.1 [95% CI 1.4–6.6]; $p = 0.003$). Although there was a tendency for a shorter time-to-liver dysfunction in patients with high IL8 (9.7 vs. 30.4 months), the result was not significant (Fig. 3b; $p = 0.25$). In addition to IL6, low albumin (HR 3.0, [95% CI 1.4–6.3]; $p = 0.003$), high total bilirubin values (HR 4.4, [95% CI 2.2–9.0]; $p < 0.001$), and higher mALBI grade (HR 3.9, [95% CI 1.9–8.1]; $p < 0.001$) were associated with shorter time-to-liver dysfunction. In Model 1, multivariate analysis revealed that high IL6 (HR 2.67, [95% CI 1.21–5.94], $p = 0.016$) and high total bilirubin values (HR 3.73, [95% CI 1.72–8.06], $p < 0.001$) were independent prognostic factors of liver dysfunction (Table 3). In Model 2, both of high IL6 (HR 2.5, [95% CI 1.1–5.4], $p = 0.024$) and higher (2b and 3) mALBI grade (HR 3.1, [95% CI 1.5–6.5], $p = 0.003$) were associated with liver dysfunction.

Out of 33 patients who had liver dysfunction, only four patients were diagnosed with disease progression at the time of liver dysfunction. Time-to-liver dysfunction analysis was repeated, censoring these four patients at the time of disease progression in order to eliminate effects of tumor progression in deterioration of liver function. Similar to the first analysis, high IL6 was significantly associated with shorter median time-to-liver dysfunction (Additional file 3: Figure S3a; 9.7 [5.87-NA] vs. NA [32.6-NA] months, $p = 0.01$). Also, although the patients with high IL8 had shorter median time-to-liver dysfunction (Additional file 3: Figure S3b; 9.7 [5.87-NA] vs. 32.6 [11.74-NA] months, $p = 0.22$), the difference was not significant.

Discussion

Our results have shown that baseline IL6 values are independent prognostic factor for overall survival and liver dysfunction in advanced HCC patients who received RE combined with sorafenib. IL8 was associated with overall survival, although the statistical significance was lost in multivariate analysis with other prognostic factors. Also, IL6 and IL8 were significantly associated with markers of advanced disease and worse liver functions.

IL6 plays a crucial role in the acute inflammatory reactions and stimulates the production of acute-phase reactants in the liver. Long-term increased IL6 levels lead to increased proliferation, resistance to apoptosis, chemoresistance, and metastatic potential in HCC [14]. IL8 is a macrophage-derived angiogenesis mediator and pro-inflammatory chemotactic factor for neutrophils that enhances tumor cell growth and promotes angiogenesis

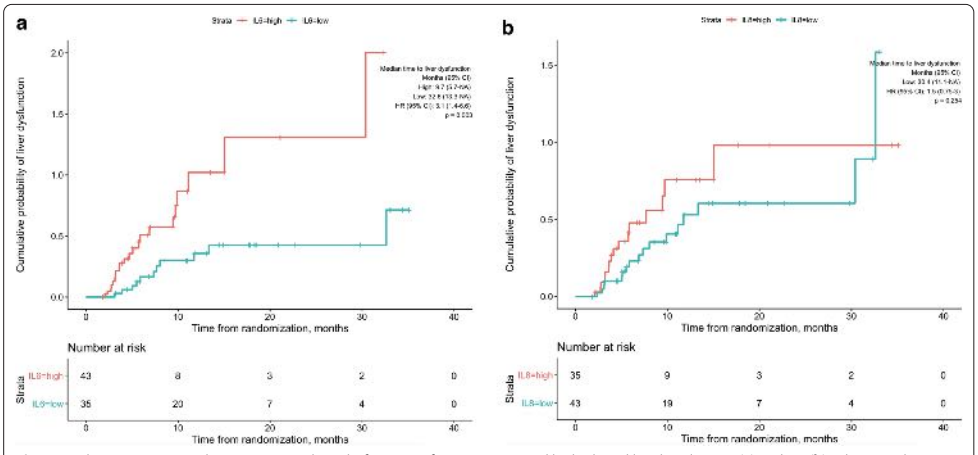

Fig. 3 Kaplan–Meier curves showing time-to-liver dysfunction of patients grouped by high and low baseline IL6 (**a**) and IL8 (**b**) values. P values were calculated using the log rank test

Table 3 Univariate and multivariate analysis of factors associated with liver dysfunction

Parameter	Univariate analysis		Multivariate analysis			
			Model 1[a]		Model 2[b]	
	HR (95% CI)	p value	HR (95% CI)	p value	HR(95% CI)	p value
IL6 (> 6.53 pg/mL)	3.1 (1.4–6.6)	**0.003**	2.67 (1.21–5.94)	**0.016**	**2.5 (1.1–5.4)**	**0.024**
IL8 (> 60.8 pg/mL)	1.5 (0.75–3)	0.25				
Sex (Male vs. Female)	0.66 (0.2–2.2)	0.49				
Age (≥ 65 vs. < 65 years)	0.99 (0.5–2)	0.97				
ECOG (1 vs. 0)	0.84 (0.38–1.9)	0.68				
Cirrhosis (Yes vs. No)	2 (0.62–6.6)	0.25				
Hepatitis B Etiology (Yes vs. No)	1.3 (0.17–9.3)	0.82				
Hepatitis C Etiology (Yes vs. No)	1.9 (0.88–3.9)	0.1				
Alcohol Etiology (Yes vs. No)	1.1 (0.54–2.2)	0.82				
Previous TACE (Yes vs. No)	1.6 (0.73–3.3)	0.25				
PVI (Yes vs. No)	1.4 (0.68–2.9)	0.37				
Child–Pugh (B vs. A)	3.3 (0.98–11)	0.053				
BCLC (C vs. B)	1.1 (0.53–2.3)	0.78				
Albumin (< 36 g/L)	3.0 (1.4–6.3)	**0.003**	1.41 (0.61–3.23)	0.421	–	–
Total bilirubin (≥ 17 µmol/L)	4.4 (2.2–9)	**< 0.001**	3.73 (1.72–8.06)	**< 0.001**	**–**	**–**
AFP (≥ 400 vs < 400 ng/mL)	1.6 (0.73–3.4)	0.25				
Diffuse disease (≥ 10 lesions)	0.68 (0.34–1.3)	0.27				
Extrahepatic disease	1.1 (0.53–2.5)	0.74				
mALBI grade (2b and 3 vs. 1 and 2a)	3.9 (1.9–8.1)	< 0.001	**–**	**–**	**3.1 (1.5–6.5)**	**0.003**

Bold type indicates statistical significance;

IL, interleukin; ECOG, Eastern Cooperative Oncology Group; TACE, transarterial chemoembolization; PVI, Portal vein invasion; BCLC, Barcelona Clinic Liver Cancer; AFP, alfa fetoprotein; mALBI, modified albumin–bilirubin

[a] Model 1 was identified using Cox regression with albumin and total bilirubin, excluding mALBI grade

[b] Model 2 was identified using Cox regression with mALBI grade as a composite factor, excluding albumin and total bilirubin

[7, 15]. Similar to previous reports, high IL6 and IL8 values were associated with advanced tumor stage and impaired liver functions in our study [7, 16, 17]. In our study, high IL6 values were associated with high total bilirubin, low albumin, and high mALBI grade; and high IL8 values were associated with liver cirrhosis, alcoholic liver disease, and portal vein invasion.

Our study confirms previous results of an exploratory study investigating the correlation between multiple cytokines and treatment outcomes in patients receiving RE, which showed with cutoff values of 6.53 and 60.8 pg/mL, IL6 and IL8 values could predict survival [9]. Up to date, no other study to validate these cut-off values have been reported.

RE has been proposed as an alternative treatment option for HCC patients with liver dominant disease who are not candidates for potentially curative treatments or cannot tolerate systemic therapies [2]. Although three randomized trials have failed to show superiority or additional benefit of RE over sorafenib [10, 18, 19], a recent meta-analysis of these trials has suggested non-inferiority to sorafenib, and also higher tolerability of RE [20].

High baseline IL6 values predict recurrence after resection in early-stage HCC patients [21]. However, there are contradictory reports on prognostic value of IL after locoregional therapies. In a report of 22 patients (seven had HCC) received RE, patients with more than six months of survival had significantly lower baseline IL8 values, but there was no significant difference in IL6 values [22]. Another study evaluated patients who underwent TACE, and while post-intervention (day 1) IL6 values were significantly associated with survival, baseline values were not [23]. The largest reported cohort (110 patients) evaluating the association between IL6 values and survival in HCC patients after TACE showed baseline IL6 values > 10 pg/mL is significantly associated with poor overall survival [16]. A study explored IL6 in an Asian cohort of patients with HCC (55 and 73 patients in exploration and validation cohorts) receiving sorafenib showed a cutoff value of 4.28 pg/mL is correlated with survival [24]. These differences in the outcome might be a result of low sample size or retrospective nature of the studies.

Our study confirmed the association between survival and previously reported cutoff values for IL6 (6.53 pg/mL) and IL8 (60.8 pg/mL) in patients who underwent RE followed by sorafenib. The same cutoff value for IL6 was also correlated with liver dysfunction. Although most of the evaluated patients had liver functions precluding inclusion to HCC trials, a recent study has shown a cutoff value of 7.0 pg/mL for IL6 is correlated with clinical decompensation in patients with advanced chronic liver disease [25]. The same study also showed IL6 values are independent predictors of a need for liver transplantation or death. Another study that retrospectively evaluated patients with end-stage liver disease showed IL6 values have a similar predictive value of 90-day and 1-year mortality with MELD score [26]. There were no cases with REILD in our cohort, and liver dysfunction was defined as grade 2 bilirubin increase to detect more subtle changes in liver function seen in patients after RE [12]. IL6 was significantly associated with liver dysfunction. While in patients with high IL6, median time-to-liver dysfunction was 9.7 months; in patients with low IL6, it was 32.6 months. Furthermore, IL6 was independently associated with liver dysfunction in multivariate analysis. Only four of 33 patients with liver dysfunction were already diagnosed with tumor progression at that time. To eliminate the role of tumor progression in liver dysfunction, time-to-liver dysfunction analysis was repeated, censoring these four patients at the time of tumor progression. It showed that the association between IL6 and deterioration in liver function is independent of HCC progression. A previous study has shown that RE induces a sustained increase in circulating IL6 and IL8 and activates inflammation and coagulation cascade, which has been suggested as pathogenic steps of REILD [27], and higher baseline values might be amplified after RE. Considering this study with our findings together suggests that high baseline IL6 values might be related to higher toxicity after RE. Besides, high baseline IL6 and IL8 were associated with disease progression. PFS was significantly shorter in patients with high IL6, as well as in patients with high IL8. In summary, our results show that IL6 is associated with deterioration in liver function independently from tumor progression, as well as progression-free survival. The prognosis of patients with HCC is heavily linked to liver function and tumor burden, and our findings show that IL6 could serve as a marker of the synthesis of both.

Since IL6 plays an essential role in immunity, cell proliferation, and differentiation, several therapeutics have been evaluated to suppress IL6 production or signaling pathways. Initial phase I and II studies have been promising results in Castleman's disease or renal cell carcinoma [28, 29]. Combining anti-IL6 agents with current therapies or suppressing these pathways before initiation of treatment in HCC patients with high baseline IL6 should be evaluated, and currently tocilizumab (anti-IL6) is under investigation in patients with HCC (MORPHEUS-liver trial, EudraCT 2020-001743-10).

Our results confirmed baseline IL6 as an independent prognosticator of survival and liver dysfunction in patients with advanced HCC. Biomarkers play a key role in the decision-making process in many tumor types, but the evaluation of many biomarkers have failed to predict treatment benefit in HCC patients [30]. Baseline IL6 values might be used to predict treatment benefit and can be integrated into prognostic calculators [31, 32], and patients can be allocated to more aggressive treatments or not according to high or low IL6 values. Besides this, baseline measurements of IL6 should be used to stratify patients between treatment arms in future phase 3 trials for new drugs to improve patient selection for the therapy and avoid confounders. An additional advantage of IL6 analysis is the reasonable price which is around 10–15 EUR and hence significantly less than other recently proposed markers [33, 34].

This study has some limitations. IL sampling was not mandatory in the SORAMIC trial, and samples were not available in all recruited patients. Also, the initial study [9], which reported the cutoff values, included patients with different tumor types, and only patients with good liver functions (Child–Pugh A). Moreover, the study population had a narrow margin of tumor burden, a limitation for the prognostic value of IL8, which has been shown to correlate with tumor burden. Additionally, although HCC patients also received additional therapies in that study, all patients evaluated in this post hoc analysis received ^{90}Y radioembolization combined with sorafenib. Also, further research is needed to clarify if IL6 and IL8 could serve as predictors of therapy benefit, however, this is beyond the scope of this analysis. Despite all these limitations, our study provides validation to previously published cutoff value for IL6 in a multinational prospective cohort.

Conclusions

Baseline IL6 value is an independent prognostic factor for overall survival in HCC patients treated with ^{90}Y radioembolization and sorafenib. IL6 could therefore be used as a stratification factor in future clinical trials of radioembolization. Furthermore, IL6 could be studied as part of prognostic tools to improve patient selection.

Abbreviations

BCLC: Barcelona Clinic Liver Cancer; CTCAE: Common Terminology Criteria for Adverse Events; ELISA: Enzyme-linked immunosorbent assay; HCC:

Hepatocellular carcinoma; IL: Interleukin; OS: Overall survival; PFS: Progression-free survival; RE: ^{90}Y radioembolization; TACE: Transarterial chemoembolization.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13550-021-00791-w.

Additional file 1. Supplementary figure 1. Kaplan-Meier curves showing progression-free survival of patients grouped by high and low baseline IL6 (a) and IL8 (b) values. P values were calculated using the log rank test.

Additional file 2. Supplementary figure 2. Results of seperate multivariable analysis models using following parameters as continous variables: (a) IL6, (b) IL8, (c) Albumin, (d) Bilirubin, (e) ALBI score.

Additional file 3. Supplementary figure 3. Kaplan-Meier curves showing cumulative probability of liver dysfunction without disease progression according to baseline IL6 (a) and IL8 (b) values. P values were calculated using the log rank test.

Authors' contributions

Osman Öcal, Peter Malfertheiner, Heinz Josef Klümpen, Bruno Sangro, Jens Ricke, Max Seidensticker: Conception and design of the study; Generation, collection, assembly, analysis and/or interpretation of data; Drafting or revision of the manuscript; Approval of the final version of the manuscript. Juozas Kupčinskas, Egidijus Morkunas, Holger Amthauer, Kerstin Schütte, Christian Sengel, Julia Benckert, Ricarda Seidensticker, Moritz Wildgruber, Maciej Pech, Peter Bartenstein: Generation, collection, assembly, analysis and/or interpretation of data; Drafting or revision of the manuscript; Approval of the final version of the manuscript.

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Availability of data and materials

Data are available from the corresponding author on reasonable request.

Declarations

Ethics approval

The study protocol was approved by the institutional review boards of each participating center, and competing authorities.

Consent to participate

All patients gave written informed consent for study participation.

Competing interests

Holger Amthauer: Grants: Sirtex, Bayer. Personal fees: Sirtex, GE Healthcare, Novartis, outside the submitted work. Kerstin Schütte: Personal fees: Bayer. Peter Malfertheiner: Grants: Bayer, Sirtex. Heinz Josef Klümpen: Grants: Bayer, Personal fees: Ipsen, outside the submitted work. Bruno Sangro: Personal fees: Sirtex, BTG, Bayer, BMS, Astra Zeneca, Eli Lilly, Merck, Novartis, Terumo, Adaptimmune; Non-financial support: Sirtex, BMS, outside the submitted work. Maciej Pech: Grants: Sirtex, Bayer; Personal fees: Sirtex. Peter Bartenstein: Grants: Sirtex. Jens Ricke: Grants: Sirtex, Bayer; Personal fees: Sirtex, Bayer. Max Seidensticker: Personal fees: Bayer, Sirtex.

Author details

[1]Department of Radiology, University Hospital, LMU Munich, Marchioninistrasse 15, 81377 Munich, Germany. [2]Institute for Digestive Research and Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania. [3]Department of Nuclear Medicine, Charité-Universitätsmedizin Berlin, Berlin, Germany. [4]Department of Internal Medicine and Gastroenterology, Niels-Stensen-Kliniken Marienhospital, Osnabrück, Germany. [5]Department of Internal Medicine II, University Hospital, LMU Munich, Munich, Germany. [6]Department of Medical Oncology, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, the Netherlands. [7]Radiology Department, Grenoble University Hospital, La Tronche, France. [8]Department of Hepatology and Gastroenterology, Charité – Universitätsmedizin Berlin, Campus Virchow Klinikum, Berlin, Germany. [9]Liver Unit, Clínica Universidad de Navarra, Pamplona, Spain. [10]Departments of Radiology and Nuclear Medicine, University of Magdeburg, Magdeburg, Germany. [11]Department of Nuclear Medicine, University Hospital, LMU Munich, Munich, Germany.

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Dynamics in Circulating Proinflammatory Biomarkers for Prognostic Assessment of Patients With Advanced HCC – A Substudy From the SORAMIC Trial

*Kerstin Schütte[1,2,3], Juozas Kupčinskas[4], Egidijus Morkunas[4], Osman Öcal[5], Regina Schinner[5], Max Seidensticker[5], Enrico N. De Toni[6], Najib Ben Khaled[6], Maciej Pech[7], Daniel Palmer[8], Thomas Berg[9], Christian Sengel[10], Bristi Basu[11], Juan W. Valle[12], Julia Benckert[13], Antonio Gasbarrini[14], Bruno Sangro[15], Peter Malfertheiner[6] and Jens Ricke[5]**

[1] Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg, Germany, [2] Department of Internal Medicine and Gastroenterology, Niels-Stensen-Kliniken Marienhospital, Osnabrück, Germany, [3] Department of Gastroenterology Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany, [4] Institute for Digestive Research and Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania, [5] Department of Radiology, University Hospital, Ludwig-Maximilians-University (LMU) Munich, Munich, Germany, [6] Department of Medicine II, University Hospital, LMU Munich, Munich, Germany, [7] Department of Radiology and Nuclear Medicine, Otto-von-Guericke University, Magdeburg, Germany, [8] Molecular and Clinical Cancer Medicine, University Hospitals & Clatterbridge, University of Liverpool, Liverpool, United Kingdom, [9] Division of Hepatology, Department of Medicine II, Leipzig University Medical Center, Leipzig, Germany, [10] Radiology Department, Grenoble University Hospital, La Tronche, France, [11] Department of Oncology, University of Cambridge, Cambridge, United Kingdom, [12] Division of Cancer Sciences and Department of Medical Oncology, The Christie National Health Service (NHS) Foundation Trust, University of Manchester, Manchester, United Kingdom, [13] Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Hepatology and Gastroenterology, Berlin, Germany, [14] Fondazione Policlinico Universitario Gemelli IRCCS, Universita Cattolica del Sacro Cuore, Roma, Italy, [15] Liver Unit, Clinica Universidad de Navarra and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Pamplona, Spain*

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Ankur Sharma, Harry Perkins Institute of Medical Research, Australia

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***Correspondence:**
Jens Ricke
jens.ricke@med.uni-muenchen.de

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Introduction: Prediction of response to treatment in patients with advanced hepatocellular carcinoma (HCC) may assist in the selection of personalized management.

Objective: This exploratory analysis of the palliative arm of the SORAMIC trial (ClinicalTrials.gov NCT01126645) evaluated the prognostic potential of basal and dynamic changes in systemic levels of interleukin 6 (IL-6), interleukin 8 (IL-8), systemic vascular endothelial growth factor (VEGF), and lipopolysaccharide (LPS).

Methods: We evaluated the correlations between overall survival (OS) and concentrations of IL-6, IL-8, VEGF, and LPS at follow-up approximately 7-9 weeks after treatment initialization (FU) compared to baseline (BL) in 90 patients treated either with ^{90}Yttrium (^{90}Y) microspheres combined with sorafenib (n = 44) or with sorafenib (n = 46) alone.

Results: Changes in IL-6 concentration during treatment showed correlations with the outcome. An increase in IL-6 concentration of less than 16.8 pg/mL over baseline

readings was associated with better survival [median OS 16.3 months compared with 8.9 months (p = 0.0354)]. Correlations with survival were not observed for VEGF or LPS concentrations at baseline, at FU, or changes between these time points.

Conclusions: Changes in IL 6 serum levels at 7-9 weeks after treatment initialization but not in IL 8, VEGF, or LPS add important information on the outcome of advanced HCC patients treated palliatively within the SORAMIC trial.

Keywords: IL-8, IL-6, biomarker, prognosis, HCC, LPS, VEGF

INTRODUCTION

The predominant causes of hepatocellular carcinoma (HCC) in Europe are alcoholic liver disease, non-alcoholic steatohepatitis (NASH), and chronic Hepatitis B or C virus infection (1). Development of HCC is regarded as closely linked to a state of chronic inflammation, independent of its cause, and end-stage inflammatory liver disease is commonly associated with liver cirrhosis. Intrahepatic and systemic inflammatory events interact in the development of HCC. The tumor microenvironment (TME) with its local pro-inflammatory and pro-fibrotic elements promotes hepatic carcinogenesis. Several circulating inflammatory cytokines, including interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α), participate in chronic hepatic inflammation and contribute to the neoplastic transformation of hepatocytes (2, 3).

Within the prospective randomized multicenter SORAMIC clinical study patients with advanced HCC were randomized to receive treatment with sorafenib with or without ^{90}Yttrium (^{90}Y) radioembolization. In previous substudies of the SORAMIC trial we confirmed that baseline IL-6 and IL-8 levels could correlate with survival outcomes of sorafenib-treated patients with HCC (4, 5). In addition, baseline IL-6 showed predictive value for overall survival in patients with advanced HCC undergoing radioembolization (4, 5). This exploratory analysis evaluated whether dynamic changes in systemic levels of the cytokines IL-6 and IL-8 could add prognostic value over basal concentrations. The relevance of VEGF and LPS levels was also assessed in this cohort.

METHODS

The cohort evaluated in this exploratory sub-analysis comprised patients within the palliative treatment arm of the randomized, controlled, multicenter phase II SORAMIC study, which evaluated the impact of ^{90}Y selective internal radiation therapy (SIRT) combined with sorafenib compared to sorafenib alone on survival in patients with advanced HCC (6).

Patients were included in this analysis if they received study treatment within the palliative arm of SORAMIC and took part in the translational program of the study with blood sample analysis at baseline and at first follow-up at approximately 7-9 weeks of treatment (FU). Of the 424 patients randomized after assignment to the palliative arm, 90 fulfilled these criteria within the intention to treat (ITT) population, comprising 46 patients treated with sorafenib alone and 44 patients treated with the combination of SIRT and sorafenib (**Figure 1**). No statistically significant differences were present between the two treatment cohorts with respect to age, gender distribution, presence of liver cirrhosis, liver function, or tumor stage according to Barcelona Clinic Liver Cancer (BCLC) stage (patient characteristics in **Table 1**).

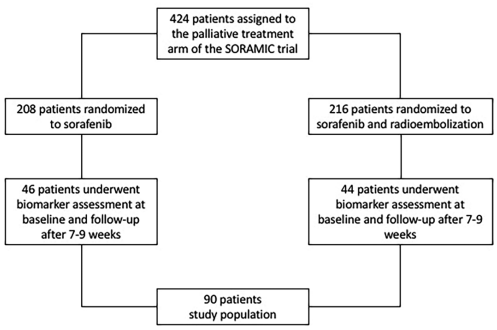

FIGURE 1 | Consort diagram of the study population.

TABLE 1 | Baseline characteristics of cohort.

	Total (N=90)	SIRT/Sorafenib (N=44)	Sorafenib (N=46)	P-value
Gender				0.7396
Female	7 (7.8)	3 (6.8)	4 (8.7)	
Male	83 (92.2)	41 (93.2)	42 (91.3)	
Age				0.2743
Mean (SD)	65.1 (8.3)	64.1 (8.6)	66.1 (8.1)	
Median (IQR)	64.5 (14.0)	63.5 (13.5)	66.0 (13.0)	
HCC Etiology/Liver Cirrhosis				
Alcohol etiology	47 (52.2)	24 (54.5)	23 (50.0)	0.6661
Hepatitis B etiology	4 (4.4)	0	4 (8.7)	0.0454
Hepatitis C etiology	19 (21.1)	10 (22.7)	9 (19.6)	0.7133
Liver Cirrhosis	76 (84.4)	36 (81.8)	40 (87.0)	0.5014
Child Pugh				0.4489
5	56 (62.2)	30 (68.2)	26 (56.5)	
6	25 (27.8)	11 (25.0)	14 (30.4)	
7-8	9 (10.0)	3 (6.8)	6 (13.0)	
BCLC				0.7149
BCLC A/B	23 (25.6)	12 (27.3)	11 (23.9)	
BCLC C	67 (74.4)	32 (72.7)	35 (76.1)	
Number of Lesions				0.3671
1	6 (6.7)	2 (4.5)	4 (8.7)	
2	9 (10.0)	3 (6.8)	6 (13.0)	
3-30	17 (18.9)	11 (25.0)	6 (13.0)	
diffuse	58 (64.4)	28 (63.6)	30 (65.2)	
Max. Diameter of Largest Lesion				0.0518
Nmiss (%)	3 (3.3)	2 (4.5)	1 (2.2)	
Mean (SD)	66.3 (40.7)	75.2 (46.1)	58.0 (33.4)	
Median (IQR)	55.0 (53.0)	69.0 (59.0)	51.0 (29.0)	
Further disease classification				
Liver Dominant Disease	86 (95.6)	41 (93.2)	45 (97.8)	0.2852
Extrahepatic Metastases	16 (17.8)	11 (25.0)	5 (10.9)	0.0797
Portal Vein Invasion	46 (51.1)	19 (43.2)	27 (58.7)	0.1411
Bilirubin at Baseline [µmol/L]				0.3396
Mean (SD)	15.0 (8.2)	14.2 (9.3)	15.8 (6.9)	
Median (IQR)	13.6 (10.3)	11.2 (11.1)	15.2 (9.5)	
Albumin at Baseline [g/L]				0.0514
Mean (SD)	38.8 (6.5)	40.1 (4.6)	37.5 (7.7)	
Median (IQR)	39.1 (6.8)	40.0 (7.4)	37.7 (7.7)	
Albi Value at Baseline				0.0202
Mean (SD)	-2.6 (0.6)	-2.7 (0.5)	-2.4 (0.7)	
Median (IQR)	-2.5 (0.7)	-2.8 (0.7)	-2.4 (0.7)	
Albi Score at Baseline				0.1222
Grade 1 (Median survival 18.5-85.6 months)	42 (46.7)	25 (56.8)	17 (37.0)	
Grade 2 (Median survival 5.3-46.5 months)	47 (52.2)	19 (43.2)	28 (60.9)	
Sorafenib: Days Treated				0.4034
Mean (SD)	338.8 (289.0)	312.8 (224.2)	363.7 (340.4)	
Median (IQR)	279.0 (299.0)	293.0 (280.5)	266.0 (316.0)	
Min-Max	10.0-1534.0	10.0-1050.0	56.0-1534.0	
Sorafenib: Daily Dose				0.0576
Mean (SD)	552.1 (204.6)	510.3 (202.0)	592.1 (201.2)	
Median (IQR)	561.1 (344.6)	464.5 (358.4)	706.1 (314.6)	
SIRT: Total Activity				
Mean (SD)		1.8 (0.5)		
Median (IQR)		1.9 (0.5)		
SIRT: Lobes Treated				
bilobar treatment		29 (65.9)		
lobar treatment		14 (31.8)		
unspecified		1 (2.3)		

AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; BMI, body mass index; ECOG, Eastern Cooperative Oncology Group; SIRT, selective internal radiation treatment: Three patients had discontinued sorafenib intake before the follow-up visit (finished 1 day, 35 days, and 50 days before FU).

Serum levels of VEGF, IL-6, IL-8, and LPS were measured with enzyme-linked immunosorbent assay (ELISA) from patients' serum which were obtained at BL and FU. Human VEGF Quantikine ELISA Kit (DVE00; R&D systems, Minneapolis, MN, USA), Human IL-6 Quantikine ELISA KIT (D6050; R&D systems, MN, USA), Human IL-8/CXCL8 Quantikine ELISA Kit (D8000C, R&D systems, MN, USA), and Human Lipopolysaccharides (LPS) ELISA Kit (CSB-E09945h;

Cusabio, China) were used in the study. Optical density evaluations were performed at 450 nm and 570 nm (as the reference) using Tecan Sunrise absorbance microplate reader, concentrations were calculated using four parameter logistic regression (4-PL) curve fitting model.

Statistical Analyses

All statistical analyses were performed using SAS (SAS version 9.4 for Windows; Copyright SAS Institute Inc., Cary, NC, USA). Numerical data are presented as means with standard deviations. For categorical data, results are given as absolute numbers with percentages. For comparison of categorical data, chi-square tests were applied. T-tests or Mann-Whitney U tests were used for testing homogeneity of independent samples in continuous data. We used receiver operating characteristic (ROC) curves to determine the cut-off value for differences in concentrations of IL-6, IL-8, and VEGF that could produce the highest sensitivity and specificity to predict individual survival shorter than the median overall survival. Univariate and multivariate Cox regression analyses were performed to identify risk factors for mortality. The Kaplan-Meier method was used for estimates of overall survival, and the log-rank test was used to compare survival groups. All tests were carried out two-sided. The level of significance was set to 0.05 without adjusting for multiplicity.

RESULTS

At data closure for this substudy 72 patients (80%) had died. The median overall survival (OS) of the cohort analyzed within this sub-analysis was 14.5 months: OS was 14.3 months in the sorafenib only arm and 15.0 months in the combined treatment arm (p=0.5964).

IL-6 concentrations at BL (rho = -0.3024, p = 0.0038) and FU (rho = -0.3192, p = 0.0022) negatively correlated with OS.

ROC curve analysis identified a FU concentration of 24.18 pg/mL best distinguished patients with a median survival longer than 12 months (sensitivity 42.9%, specificity 87.5%). Median OS was 17.0 months if FU IL-6 was lower than 24.18 pg/mL and was 7.7 months in the 23 patients (28% of patients) with higher FU IL-6 levels (p < 0.0001) (**Table 2** and **Figure 2A**). Patients with lower IL-6 at FU also had a significantly longer progression-free survival (median PFS: 9.6 months vs. 5.0 months; p=0.0278) (**Figure 2C**).

ROC curve analysis identified that absolute increases in IL-6 from BL to FU with a cut-off of 16.8 pg/mL optimally distinguished patients with OS shorter than 12 months from those with an OS exceeding 12 months (sensitivity 38.1%, specificity 85.4%). Patients showing absolute increase in IL-6 concentration less than 16.8 pg/mL had a median OS of 16.3

TABLE 2 | Statistical analyses of prognostic value for overall survival.

Parameter		Descriptive statistics(N=90)				Correlation of IL-6/IL-8/ VEGF/LPS with overall survival		Best cut-off from ROC-analysis (Youden Index), distinguishing between OS </>12 months				Kaplan Meier for OS in patients with high vs. low parameter values		
		Mean	SD	Min.	Max.	Spearman Correlation Coefficients	P-Value H0: Rho=0	Cut-Off	Sensitivity	Specificity	Youden Index	Median OS lower than ROC cut-off	Median OS higher than ROC cut-off	P-Value LogRank
IL-6	BL	14.88	25.16	1.39	205.71	-0.3024	0.0038	9.70	59.5%	70.8%	0.304	21.41	10.26	<0.0001
[pg/	FU	26.95	43.61	2.25	298.60	-0.3192	0.0022	24.18	42.9%	87.5%	0.304	16.95	7.74	<0.0001
ml]	abs. diff.	12.07	31.45	-60.95	138.37	-0.1774	0.0943	16.83	38.1%	85.4%	0.235	16.30	8.89	0.0354
	pct. diff.	183.14	303.49	-76.99	1754.95	-0.0633	0.5536	154.52	45.2%	70.8%	0.161	15.02	10.85	0.2170
IL-8	BL	150.96	384.75	2.91	3414.00	-0.3913	0.0001	80.35	64.3%	79.2%	0.435	21.41	9.51	<0.0001
[pg/	FU	205.76	409.82	0.15	3328.55	-0.4370	<0.0001	75.25	70.7%	63.6%	0.344	28.46	10.26	<0.0001
ml]	abs. diff.	48.82	538.50	-3358.83	3251.94	-0.0991	0.3667	-29.45	31.7%	95.5%	0.272	10.82	15.02	0.6516
	pct. diff.	179.11	538.59	-99.52	4244.91	-0.0202	0.8544	-23.60	31.7%	81.8%	0.135	26.92	14.52	0.1295
VEGF	BL	673.86	683.54	5.45	3806.36	-0.1319	0.2180	857.04	39.0%	77.1%	0.161	16.75	12.00	0.2120
[pg/	FU	409.12	276.52	73.46	1178.69	-0.1991	0.0600	263.02	71.4%	52.1%	0.235	19.80	12.52	0.0935
ml]	abs. diff.	-261.71	640.29	-3475.61	645.03	0.0115	0.9146	-456.70	31.7%	81.3%	0.130	12.52	16.30	0.5028
	pct. diff.	65.02	283.12	-95.00	1924.31	-0.0059	0.9562	42.31	34.1%	77.1%	0.112	14.82	12.85	0.8798
LPS	BL	175.00	141.06	7.40	896.08	0.0341	0.7495	58.76	88.1%	22.9%	0.110	17.64	14.26	0.2054
[pg/	FU	148.82	110.76	3.00	841.00	-0.1045	0.3298	273.78	100.0%	8.5%	0.085	14.26	18.84	0.6043
ml]	abs. diff.	-27.77	148.91	-666.94	703.65	-0.0991	0.3556	100.30	95.2%	14.9%	0.101	13.80	16.07	0.7488
	pct. diff.	17.74	112.56	-86.73	512.27	-0.1278	0.2326	156.76	100.0%	12.8%	0.128	13.80	31.21	0.0874

IL-6 interleukin 6; IL-8 interleukin-8; VEGF systemic vascular endothelial growth factor; LPS lipopolysaccharide; ROC receiver operating curve; BL baseline; FU follow-up at 7-9 weeks; abs. diff. absolute difference; pct. diff: percentage difference.

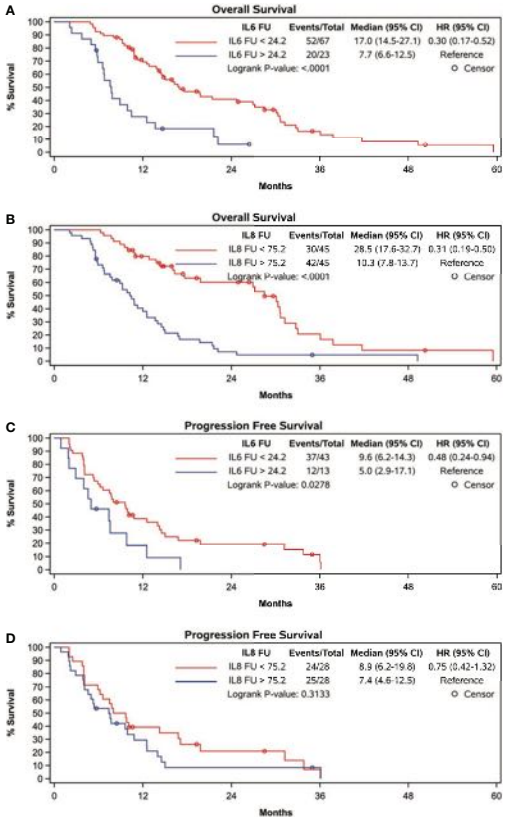

FIGURE 2 | Kaplan-Meier curves grouped by follow-up levels according to cut-off in ROC-curve-analyses **(A)** OS and IL-6, cut-off at 24.2 pg/ml, **(B)** OS and IL-8, cut-off at 75.2 pg/ml, **(C)** PFS and IL-6, cut-off at 24.2 pg/ml, and **(D)** PFS and IL-8, cut-off at 75.2 pg/ml.

months versus 8.9 months for those with greater IL-6 increase (p = 0.0354) (**Figure 3A**).

A Cox regression with BL IL-6 as well as absolute IL-6 change from BL to FU in the model showed significant p-values for both effects (BL: p<0.0001; abs. change BL to FU: p=0.0260).

In patients with a BL IL-6 concentration < 9.7 pg/mL the additional FU IL-6 did not add prognostic value. However, in patients with a BL IL-6 concentration exceeding 9.7 pg/ml (n=39), the additional FU value identified a subgroup with worse expectation of survival. An increase of more than 16.8 pg/mL at FU was associated with a median OS of only 6.8 months compared with median OS of 11.3 months in those showing a less pronounced increase within this subgroup (p = 0.0337) (**Figure 4**).

The percentage increase in IL-6 concentration did not correlate with OS nor did the absolute or percentage increase in IL-6 concentration correlate with PFS (**Figure 3C**).

Both baseline (rho = -0.3913, p = 0.0001) and FU concentrations (rho = -0.4370, p = <.0001) of IL-8 significantly correlated with OS (**Table 2** zand **Figure 2B**). At follow-up, patients with IL-8 concentrations below the threshold of 75.25 pg/ml had a significantly longer OS than those above threshold (28.5 months compared to 10.3 months, p < 0.0001). However, there was no correlation with PFS at this cut-off (**Figure 2D**).

Furthermore, the absolute or percentage difference in IL-8 concentrations between both time points did not correlate with OS (rho = -0.0991, p = 0.3667) (**Figure 3B**) nor with PFS (**Figure 3D**).

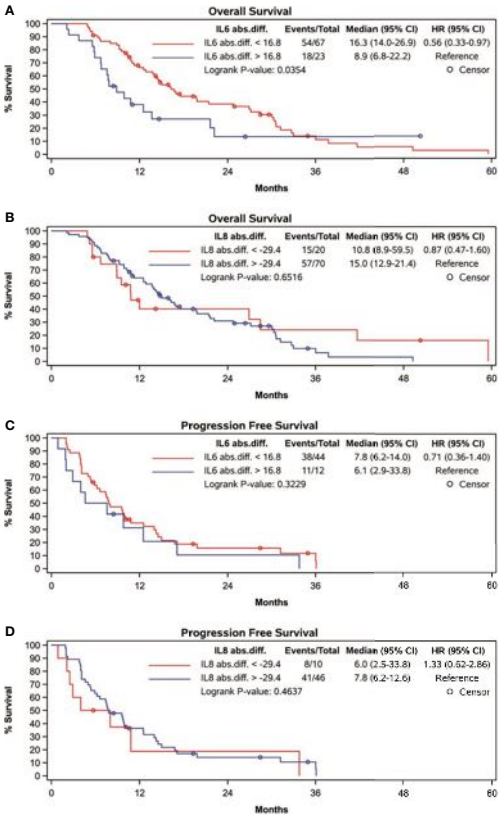

FIGURE 3 | Kaplan-Meier curves grouped by absolute difference between baseline and follow-up levels according to cut-off in ROC-curve-analyses. **(A)** OS and abs. difference in IL-6, cut-off at 16.8 pg/ml, **(B)** OS and abs. difference in IL-8, cut-off at -29.4 pg/ml, **(C)** PFS and abs. difference in IL-6, cut-off at 16.8 pg/ml and **(D)** PFS and abs. difference in IL-8, cut-off at -29.4 pg/ml.

Multivariate Cox regression analyses integrating also treatment modality, liver function, portal vein thrombosis, and tumor distribution revealed that baseline concentrations of IL-6 and IL-8 as well as liver function and the presence of portal vein thrombosis are independent factors impacting on OS, while absolute differences in IL-6 and IL-8 concentrations are not (**Tables 3A, B** and **Supplementary Table S1**).

Serum VEGF and LPS concentrations did not show any correlation with survival in this cohort of patients, either at baseline, follow-up level, or the changes between these time points (**Table 2**).

While baseline IL-6 levels did not correlate with liver decompensation indicated by an increase in ALBI grade at FU ($p = 0.2413$), baseline IL-8 concentration ($p = 0.0275$) as well as IL-6 and IL-8 concentration at FU did ($p = 0.0267$ and $p = 0.0109$) (**Figures 5A–D**).

DISCUSSION

We have previously reported on the prognostic value of baseline IL-6 concentrations in patients treated within the palliative arm of the SORAMIC study (4, 5). This current analysis suggests that repeated measurement of IL-6 7-9 weeks after treatment initialization is of prognostic value in these patients and that dynamic changes in IL-6 concentrations further helps to identify

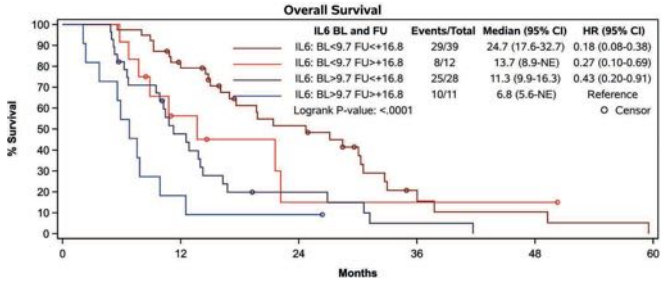

FIGURE 4 | Survival of patients according to their prognostic groups identified by IL-6 levels. Patients with a baseline IL-6 concentration exceeding 9.7 pg/ml and an increase of 16.8 pg/mL or more at FU presented with a median OS of 6.8 months while patients with less pronounced increase showed a median OS of 11.3 months (p = 0.0337). Patients with low IL-6 levels at baseline and increase of less than 16.8 pg/ml at FU had the most favorable prognosis.

TABLE 3A | Impact follow-up assessment of IL-6 concentration: univariate and multivariate analysis.

		Parameter estimate	Standard error	P-value	Hazard ratio	95%	Confidence limits for HR
Univariate analyses	IL6 FU < 24,2	-1.21	0.28	<0.0001	0.30	0.17	0.52
	IL6 abs. diff. < 16,8	-0.58	0.28	0.038	0.56	0.33	0.97
Multivariate analysis	IL6 BL < 9,7	-0.99	0.27	0.0002	0.37	0.22	0.63
	IL6 abs. diff. < 16,8	-0.53	0.30	0.0747	0.59	0.33	1.05
	SIRT/Sorafenib treatment (vs. Sorafenib only)	0.19	0.25	0.4395	1.21	0.74	1.98
	Child-Pugh A (vs. B)	-2.05	0.44	<0.0001	0.13	0.06	0.31
	Portal Vein Infiltration YES (vs. NO)	-0.64	0.28	0.0207	0.53	0.31	0.91
	Liver-Dominant Disease YES (vs. NO)	-0.72	0.53	0.1754	0.48	0.17	1.38

TABLE 3B | Impact of follow-up assessment of IL-8 concentration: univariate and multivariate analysis.

		Parameter estimate	Standard error	P-value	Hazard ratio	95%	Confidence limits for HR
Univariate analyses	IL8 FU < 75,2	-1.18	0.25	<0.0001	0.31	0.19	0.50
	IL8 abs. diff. < -29,4	-0.14	0.31	0.6549	0.87	0.47	1.60
Multivariate analysis	IL8 BL < 80,3	-1.12	0.29	0.0001	0.33	0.19	0.58
	IL8 abs. diff. < -29,4	-0.64	0.35	0.0641	0.53	0.27	1.04
	SIRT/Sorafenib treatment (vs. Sorafenib only)	-0.17	0.26	0.529	0.85	0.50	1.42
	Child-Pugh A (vs. B)	-2.05	0.44	<0.0001	0.13	0.05	0.30
	Portal Vein Infiltration YES (vs. NO)	-0.57	0.28	0.0417	0.57	0.33	0.98
	Liver-Dominant Disease YES (vs. NO)	-0.30	0.55	0.5874	0.74	0.25	2.18

patients with dismal prognosis. An increase of IL-6 levels above 16.8 pg/ml from baseline is associated with poor prognosis. The subgroup of patients with baseline IL-6 readings of 9.7 pg/ml or over and an increase of IL-6 level of more than 16.8 ppg/ml have particularly poor outcomes with median OS of only 6.8 months. Favorable prognosis (median OS of 24.7 months) was associated with low IL-6 levels at baseline and increases of less than 16.8 pg/ml at follow-up. Contrary to initial expectation, no additional value in the prognostic assessment of these palliative HCC patients was conferred by the analysis of the dynamics of IL-8, VEGF, or LPS.

Clinical factors influencing survival of HCC patients in numerous studies including the SORAMIC trial, relate to tumor burden (number and size of HCC nodules within the liver, invasion of portal vein, existence of extrahepatic metastases), clinical performance status, and liver function (1,

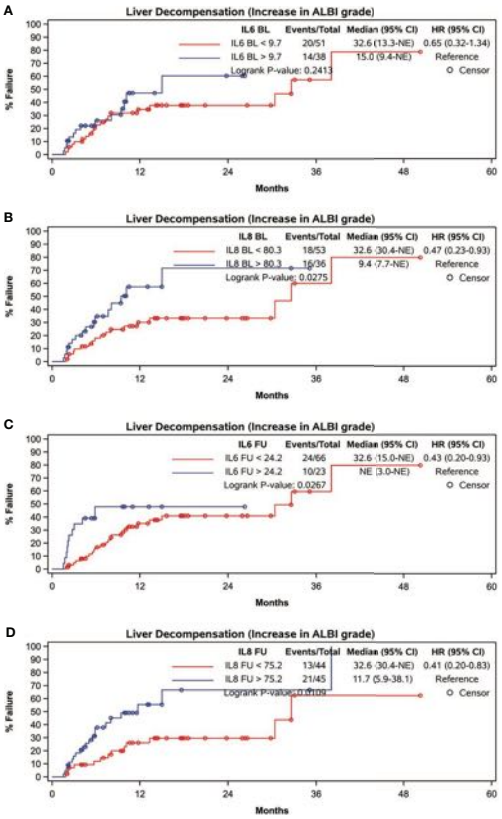

FIGURE 5 | Time to liver function deterioration assessed by ALBI-grade. **(A)** baseline IL-6, cut-off at 9.7 pg/ml, **(B)** baseline IL-8, cut-off at 80.3 pg/ml, **(C)** FU IL-6, cut-off at 24.2 pg/ml, **(D)** FU IL-8, cut-off at 75.2 pg/ml.

6). As previously published, our analysis confirms liver function and portal vein infiltration as prognostic factors in patients with advanced HCC included in the SORAMIC trial (7). A pooled analysis of the two phase III studies that led to approval of sorafenib for treatment of patients with advanced HCC revealed that presence of macrovascular invasion, high alpha-fetoprotein (AFP) levels, and high neutrophil-to-lymphocyte ratio (NLR) were prognostic factors of poorer OS. Patients had a benefit of the treatment with sorafenib irrespective of prognostic factors, although lack of extrahepatic metastases, lower NLR, and chronic hepatitis C virus infection were predictive for greater survival benefit (8, 9). Since the approval of sorafenib, the evaluation of additional systemic biomarkers applicable either in a prognostic or in a predictive manner has been the focus of many studies.

The chronically inflamed microenvironment in the liver induces macrophages to adopt the M2 phenotype and transform into tumor associated macrophages (TAMs) which are responsible for continuous local proinflammatory signaling leading to parenchymal cell transformation (10). TAMs secrete IL-8 which activates the PI3K/AKT/HIF-1a signaling pathway involved in invasiveness and metastatic progress of HCC (10–12). They also secrete further proinflammatory cytokines including IL-6 with antiapoptotic activity in HCC cell lines (12). TAMs stimulate tumor growth also by suppression of the adaptive immune system through expression of high levels of

programmed cell death-ligand 1 (PD-L1), and by this mechanism suppress the antitumor cytotoxic T-cell responses (13). Tumor cells also secrete a variety of inflammatory factors and chemokines to recruit TAMs including IL-6, IL-8, and IL-34 (14). Increased levels of proinflammatory cytokines in patients with chronic liver disease are associated with increased risk of HCC and correlate with survival in patients with advanced HCC (15, 16). IL-6 activates signal transducer and activator of transcription 3 (STAT3) which is implicated in induction of sorafenib resistance in patients with HCC (2, 17), but IL-6 levels have also been shown to be a predictor of survival even in patients with liver cirrhosis without HCC (18).

IL-8 in addition to its pro-tumorigenic activity is a potent angiogenic factor and produced by most HCC cell lines (19, 20). The inhibition of IL-8 signaling increases the sensitivity of liver cancer cells to sorafenib (21). We and others have previously reported that baseline IL-8 values predict outcome in patients with advanced HCC treated with sorafenib (5). However, we did not find an add-on value by follow-up measurement or the assessment of dynamics of IL-8 concentration with respect to survival.

The analysis of larger panels focusing on factors relevant to the proinflammatory tumor microenvironment and giving a more holistic view on systemic consequences of chronic tumor associated inflammation might result in more accurate results with respect to individual prognosis in patients with advanced HCC. In recent years, a clinical breakthrough in systemic therapy of HCC was reached with the introduction of checkpoint-inhibitors targeting programmed death-1, programmed death-ligand 1, and cytotoxic T lymphocyte antigen-4. These molecules resolve T-cell activation to maintain inflammatory homeostasis, protect tissue integrity, and prevent unwanted autoimmunity under physiological condition (22). In patients with tumors, the administration of checkpoint inhibitors unleashes tumor-directed cytotoxic T-cells specific against an unknown spectrum of tumor-associated antigens (22, 23). The immune contexture of HCC before treatment is the most promising predictive marker for response to immunotherapies (24). The identification of a subset of patients with high grade of tumor associated inflammation might therefore guide treatment decisions toward early systemic immune-checkpoint inhibition.

Concentrations of IL-6 and IL-8 at FU both were of prognostic value with respect to future deterioration of liver function assessed by the ALBI-Grade (25). This is of special clinical interest, as preserved liver function is the key factor in decisions on applicability of sequential treatments, and the repeated measurement of IL-6 and IL-8 potentially help to identify patients at risk to become untreatable because of impairment of liver function rather than because of tumor progression. In a previous analysis on patients treated within SORAMIC in palliative intent with the combination of radioembolization and sorafenib high IL-6 concentrations at baseline were associated with a significant shorter time to liver dysfunction. Although there was a tendency for a shorter time-to-liver dysfunction in patients with high IL-8 concentrations in that analysis, the result was not significant (4). As in that paper, liver function deterioration was defined by a significant increase in bilirubin levels, cholestasis, and secretory function of the liver are assumed to be the dominant factors in this setting. The current analysis also takes liver synthesis mirrored by albumin concentration into account which might explain differences in the results.

Vascular endothelial growth factor (VEGF), a protein that promotes angiogenesis, supports tumor cell survival, proliferation, and vessel formation has been shown to have prognostic value in HCC patients (26, 27). Sorafenib, a multi-tyrosine kinase inhibitor with inhibitory activity against vascular endothelial growth factor receptor (VEGFR) 2 (28) was the standard treatment in patients with advanced HCC for more than a decade. Baseline concentrations of IL-6 and IL-8 have been demonstrated to be predictive for response to sorafenib monotherapy in patients with advanced HCC (5). Recently, the combination of atezolizumab, a humanized anti-PDL1 antibody to restore antitumor T-cell activity, with bevacizumab, binding to VEGF, has been defined as the new first line treatment standard (29, 30).

Previously, it has been reported that circulating concentrations of VEGF are of prognostic value in patients with HCC undergoing liver resection, liver transplantation, or locoregional therapy (26). A decrease in VEGF levels at week 8 of sorafenib treatment was prognostic for better survival in advanced HCC patients (mostly on the background of chronic viral hepatitis) (31). We however did not observe any prognostic potential of systemic VEGF levels in our cohort. It is unclear whether this discrepancy is related to the patient characteristics within this cohort, with low numbers of patients with viral etiology of HCC.

Alterations in gut microbiota composition are associated with hepatic inflammatory diseases and may play a contributory role in hepatic carcinogenesis (32). Dysbiotic gut microbiota composition leads to increased hepatic exposure with gut-derived microbiota-associated molecular patterns (MAMPs) that include lipopolysaccharides (LPS), a cell wall component of Gram-negative bacteria.

Bacterial compounds reach the liver *via* the portal blood flow and are eliminated by Kupffer cells under normal conditions (33). In a mouse model, LPS levels increase during the course of chronic liver disease fostering proinflammatory responses in the periphery and in the liver (32, 34). Plasma LPS concentrations correlate with the degree of liver dysfunction (35, 36).

An increased exposure to LPS contributes to hepatocarcinogenesis *via* activation of Toll-like receptor-4 signaling in mouse models (37). By this, LPS promote hepatic inflammation, fibrosis, proliferation, and the activation of anti-apoptotic signaling (37). In an analysis of fecal samples of patients with HCC and liver cirrhosis LPS-producing genera were increased compared to patients with liver cirrhosis (38).

The proinflammatory tumor microenvironment in part induced by pathogen-associated molecular patterns (PAMPs) including LPS also promotes tumor progression. High concentrations of infiltrating TAMs are associated with poor prognosis of HCC (12, 39). However, our study does not support the idea of measuring systemic concentrations of LPS for prognostic purposes in patients receiving treatment with sorafenib with or without radioembolization in patients with advanced HCC. It is likely that concentrations of LPS in the

portal blood flow and in the liver are of higher significance for progression of HCC than systemic concentrations.

Our analysis is limited by the rather small number of patients that could be included into the analysis compared to the whole study population of SORAMIC.

CONCLUSION

As availability of effective systemic treatment options for advanced HCC patients increases, sequential treatments are now a realistic strategy for management. Methods to expeditiously identify patient groups who do well on particular treatments, or select patients with more dismal prognosis, may help to guide optimal management. Although clinical factors have previously been utilized in this aim, the baseline assessment of proinflammatory cytokines IL-6 and IL-8 in addition to inclusion of the dynamic changes of IL-6 values at 7-9 weeks after treatment initiation (sorafenib with or without radioembolization used in the SORAMIC trial) appears of value in the prediction of patients who do better on palliative treatments within the SORAMIC study. Early re-assessment of IL-6 and IL-8 may also help to identify patients at risk for deterioration of liver function which potentially precludes further lines of treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical committee Otto-von-Guericke University Magdeburg, Germany and the local ethics committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KS, PM, and JR: Conception and design of the study; Generation, collection, assembly, analysis, and/or interpretation of data; Drafting or revision of the manuscript; Approval of the final version of the manuscript. JK, EM, OÖ, RS, MS, ED, NBK, MP, DP, TB, CS, BB, JV, JB, AG, and BS: Generation, collection, assembly, analysis, and/or interpretation of data; Drafting or revision of the manuscript; Approval of the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgstr.2022.939192/full#supplementary-material

Supplementary Figure 1 | Boxplots indicating concentrations of IL-6, IL-8, VEGF and LPS, each comparing absolute levels at baseline and follow-up stratified by treatment, overall survival and a combination of both.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors: Morkūnas, Egidijus; Vaitkevičiūtė, Evelina; Varkalaitė, Greta; Pilvinis, Vidas; Skiecevičienė, Jurgita; Kupčinskas, Juozas.

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Diagnostic and Prognostic Value of IL-10, FABP2 and LPS Levels in HCC Patients

Egidijus Morkunas [1,*], Evelina Vaitkeviciute [2], Greta Varkalaite [2], Vidas Pilvinis [3], Jurgita Skieceviciene [2] and Juozas Kupcinskas [1,2]

[1] Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, 50161 Kaunas, Lithuania; juozas.kupcinskas@lsmuni.lt
[2] Institute for Digestive Research, Medical Academy, Lithuanian University of Health Sciences, 50161 Kaunas, Lithuania; evelina.vaitkeviciute1@gmail.com (E.V.)
[3] Department of Anesthesiology, Medical Academy, Lithuanian University of Health Sciences, 50161 Kaunas, Lithuania
* Correspondence: egidijus.morkunas@lsmu.lt

Abstract: Hepatocellular carcinoma (HCC) still lacks valuable diagnostic and prognostic tools. This study aimed to investigate the potential diagnostic and prognostic value of baseline interleukin (IL)-10, fatty acid-binding protein 2 (FABP2) and lipopolysaccharide (LPS) levels in patients with HCC. Serum levels of IL-10, FABP2 and LPS in 47 newly diagnosed HCC patients and 50 healthy individuals were estimated and compared. The best cut-off points for baseline IL-10, FABP2 and LPS levels predicting overall survival (OS) were evaluated. Both levels of FABP2 and IL-10 were significantly higher in HCC patients vs. control group (median 2095 vs. 1772 pg/mL, $p = 0.026$; 9.94 vs. 4.89 pg/mL, $p < 0.001$) and may serve as potential biomarkers in complex HCC diagnostic tools. The cut-off value of 2479 pg/mL for FABP2 was determined to have the highest sensitivity (66.7%) and specificity (55.6%) to distinguish patients with a median OS longer than 17 months. However, the median OS of patients with high and low levels of FABP2 were not significantly different ($p = 0.896$). The prognostic value of LPS as well as FABP2 and IL-10 for HCC patients appears to be limited.

Keywords: hepatocellular carcinoma; HCC; prognosis; biomarker; FABP2; IL-10; LPS

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer which is the third leading cause of cancer-associated mortality [1]. In up to 90% of patients HCC develops in a cirrhotic liver and is associated with chronic liver inflammation mostly due to alcoholic liver disease, chronic hepatitis B or C infections and non-alcoholic steatohepatitis [2,3]. Hepatic carcinogenesis is multifactorial process. It is promoted by tumor microenvironment with its local proinflammatory and profibrotic elements as well as systemic inflammatory factors. Several circulating proinflammatory cytokines and chemokines, including interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α), participate in mechanisms of chronic hepatic inflammation and neoplastic transformation of hepatocytes [4,5]. Endotoxins such as lipopolysaccharide (LPS) are not directly involved in chronic inflammation by inducing Kupffer cells to release radical oxygen species (ROS) and proinflammatory cytokines and chemokines [6]. Not only proinflammatory but also anti-inflammatory cytokines, such as IL-10, take part in liver cirrhosis and possibly in HCC development and progression [7]. Microbial translocation from the gut is as well involved in the pathogenesis of liver damage. One of possible markers for increased gut permeability is fatty acid-binding protein 2 (FABP2) [8].

Recent findings show that inflammatory cytokines IL-6 and IL-8 as well as IL-10 are associated with poorer outcomes in HCC patients [7,9]. Loosen and colleagues demonstrated that IL-6 and IL-8 can be valuable in predicting treatment response and survival after

transarterial chemoembolization in patients with primary and metastatic liver tumors [9]. A study by Seidensticker et al. evaluated multiple cytokines and delivered cut-off values for IL-6 and IL-8 which were associated with overall survival after 90Y radioembolization in patients with HCC or metastatic disease [10]. The post hoc analysis of the palliative arm of the SORAMIC trial demonstrated that high baseline IL-6 and IL-8 were associated with significantly shorter overall survival in HCC patients undergoing radioembolization or treated with sorafenib monotherapy. Baseline IL-6 and IL-8 with respective cut-off values predicted objective response rates for sorafenib-treated patients [11,12].

As there is evidence that inflammatory molecules such as IL-6 and IL-8 could have diagnostic, prognostic and predictive value for HCC patients, it is important to identify other molecules related to chronic liver inflammation and potentially carcinogenesis which could be involved in future complex tools for better approach for HCC patients.

This exploratory analysis aimed to explore the potential diagnostic and prognostic value of baseline IL-10, LPS and FABP2 in patients with HCC, and provide additional information for possible future complex tools for better HCC diagnosis and prognosis.

2. Materials and Methods

The study included 47 patients with newly diagnosed HCC and 50 healthy individuals as controls. Demographic and clinical characteristics of the patients with HCC are provided in Table 1. All participants were enrolled retrospectively at the Department of Gastroenterology of Lithuanian University of Health Sciences from June 2010 to May 2021. Demographic data and clinical parameters were collected at the time of inclusion to the study. Healthy control individuals (volunteers) were free of any chronic diseases and had not received any medications during the previous 3 months prior to inclusion to the study. A schematic of the workflow of the study is shown in Figure 1.

Table 1. Demographic and clinical characteristics of subject groups.

	Controls (n = 50)	**HCC Patients (n = 47)**	***p* Value**
Age, mean ± SD	58.8 ± 4.9	61.3 ± 9.3	0.101
Gender (male)	25 (50%)	39 (83.0%)	<0.001
Liver cirrhosis (yes)		45 (95.7%)	
HCC etiology			
Hepatitis B		7 (14.9%)	
Hepatitis C		21 (44.7%)	
Other (alcohol)		19 (40.4%)	
BCLC stage			
0		2 (4.3%)	
A		15 (31.9%)	
B		17 (36.2%)	
C		7 (14.9%)	
D		6 (12.8%)	
Cytokine levels, median (min-max) (pg/mL)			
FABP2	1327 (518.5–8388)	2345 (326.3–4587)	0.026
IL-10	4.89 (0.036–53) *	9.94 (0.04–564.1) *	<0.001
LPS	56.38 (4.74–436.4)	51.95 (12.37–148.8)	0.263

* Due to insufficient amount of blood for analysis of IL-10, samples of 45 HCC patients and 45 controls were used. SD—standard deviation; HCC—hepatocellular carcinoma; BCLC—the Barcelona Clinic Liver Cancer staging system; min—minimum value; max—maximum value; FABP2—fatty acid-binding protein 2; IL—interleukin; LPS—lipopolysaccharides.

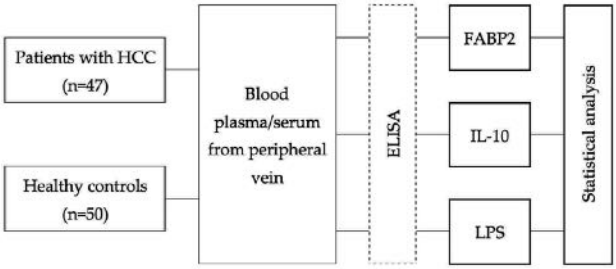

Figure 1. Workflow of the study. HCC; ELISA—enzyme-linked immunosorbent assay; FABP2; IL; LPS.

Peripheral blood samples were drawn from all subjects at the time of enrollment in the study. Within 1 h after drawing, the serum samples were placed at −80 °C and stored until further processing. A Human FABP2/I-FABP Quantikine ELISA Kit (DFBP20; R&D Systems, Minneapolis, MN, USA), Human IL-10 Quantikine ELISA Kit (D1000B; R&D Systems, Minneapolis, MN, USA), and Human LPS ELISA Kit (CSB-E09945h; Cusabio, Houston, TX, USA) were used to quantify serum levels of FABP2, IL-10, and LPS in HCC patients and the healthy control subjects.

All statistical analyses were performed using IBM SPSS Statistics 27.0 (Armonk, NY, USA). Numerical data are presented as mean with standard deviation for age and as medians with minimum and maximum values for cytokine levels due to abnormal distribution. For categorical data, results are given as absolute numbers with percentages. For comparison of categorical data, chi-square tests were applied. *t*-tests and Mann–Whitney U tests were used for testing homogeneity and comparing of independent samples in continuous data. ROC curves were used to determine the cut-off value for differences in concentrations of FABP2, IL-10, and LPS that could produce the highest sensitivity and specificity to predict individual survival shorter than the median overall survival. The Kaplan–Meier method was used for estimates of overall survival, and the log-rank test was used to compare survival groups. All tests were carried out two-sided. The level of significance was set to <0.05 without adjusting for multiplicity.

3. Results

Blood levels of three possible biomarkers—FABP2, IL-10, and LPS—were measured in both HCC and control groups. Levels of FABP2 and IL-10 were significantly higher in HCC patients vs. control group (median 2345 vs. 1327 pg/mL, $p = 0.026$; 9.94 vs. 4.89 pg/mL, $p < 0.001$). Levels of LPS did not reach significant difference and were higher in healthy patients than in HCC patients (51.95 pg/mL in HCC patients vs. 56.38 pg/mL in control group, $p = 0.263$) (Table 1).

At data lock point for this study, 33 HCC patients (70.2%) had died. The median OS of these patients was 17 months.

Using receiver operating characteristic (ROC) curve analysis, a cut-off value of 2479 pg/mL for FABP2 was determined to have the highest sensitivity (66.7%) and specificity (55.6%) to distinguish patients with a median OS longer than 17 months with the area under the curve (AUC) of 0.622 (Figure 2a). However, the median OS of patients with high and low levels of FABP2 were not significantly different ($p = 0.893$) (Figure 3).

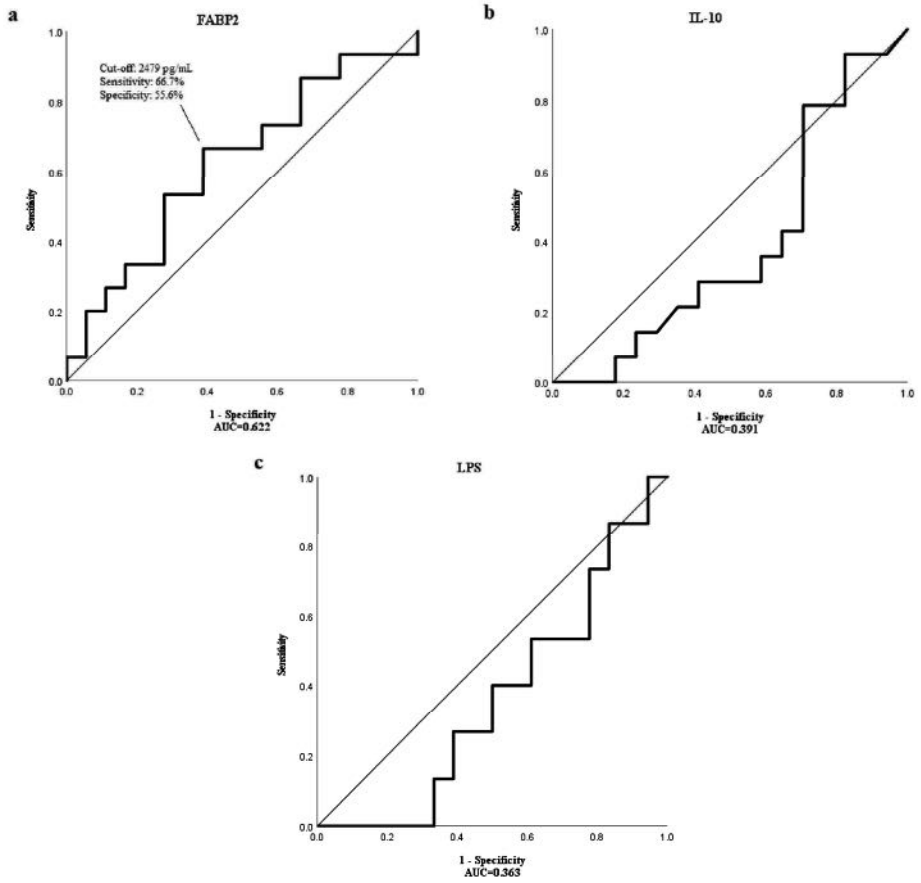

Figure 2. The ROC curve showing the sensitivity and specificity of various cut-off values of baseline (**a**) FABP2, (**b**) IL-10 and (**c**) LPS levels to distinguish patients with the OS longer than a median OS of 17 months.

ROC curve analysis could not identify cut-off values for IL-10 and LPS to distinguish HCC patients with shorter and longer median OS than 17 months as AUC levels were too low (0.391 and 0.363, respectively) (Figure 2b,c).

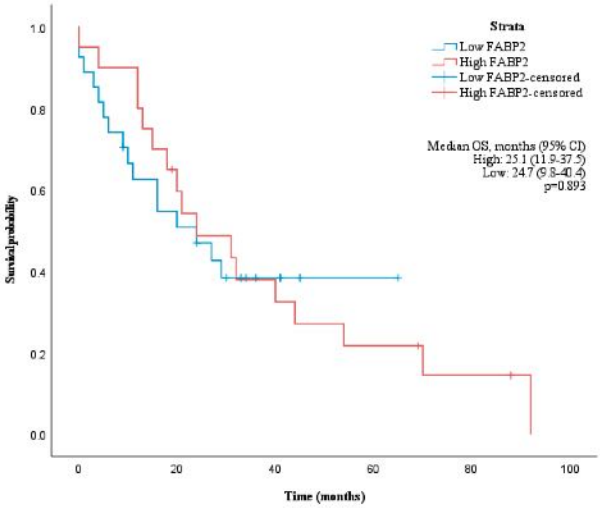

Figure 3. Kaplan–Meier curve showing overall survival of patients grouped by baseline FABP2 values according to cut-off of 2479 pg/mL.

Separate ROC curve analysis was performed discriminating values of FABP2, Il-10 and LPS according to OS of 6 months as well as of 1 and 2 years (Figure 4). The highest AUC (0.622), sensitivity (52.9%) and specificity (84.6%) were obtained for FABP2 to distinguish HCC patients with OS longer than 1 year with the same potential cut-off value of 2479 pg/mL.

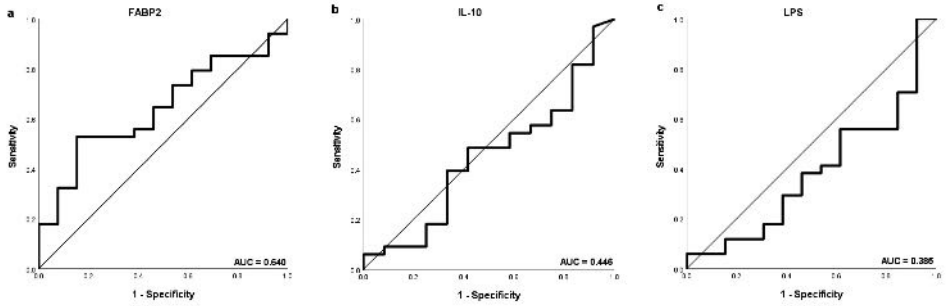

Figure 4. *Cont.*

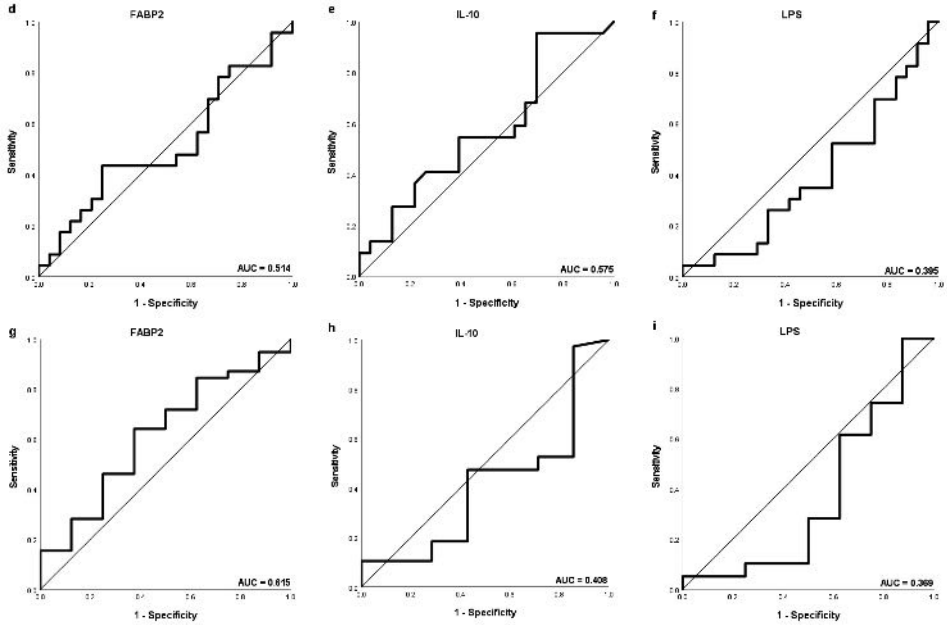

Figure 4. The ROC curve showing the sensitivity and specificity of various cut-off values of baseline (**a**,**d**,**g**) FABP2, (**b**,**e**,**h**) IL-10 and (**c**,**f**,**i**) LPS levels to distinguish patients with the OS longer than 1 year (**a**–**c**), 2 years (**d**–**f**) and 6 months (**g**–**i**).

4. Discussion

In this study, we aimed to investigate LPS, FABP2 and IL-10 as possible diagnostic and prognostic biomarkers in patients with HCC.

HCC development in the chronically inflamed liver tissue is a multifactorial process corresponding to the adaptive immune response and the effector molecules including chemokines, growth factors, metalloproteases, and cytokines. The best studied of them, IL-6 and IL-8 are produced by the tumor-associated macrophages (TAMs) and promote HCC development through different signaling pathways [13,14]. TAMs also suppress the adaptive immune system through expression of high levels of programmed cell death-ligand 1 (PD-L1), causing suppression of the antitumor cytotoxic T-cell responses and stimulation of the tumor growth [15]. Tumor cells themselves produce a variety of inflammatory factors and chemokines to recruit TAMs including IL-6, IL-8, and IL-34 [16]. Recent studies have shown that IL-6 and IL-8 may serve as valuable predictive and prognostic biomarkers in HCC patients. Cut off serum levels distinguishing better and worse survivals than median OS as well as predicting response to various HCC treatment methods were estimated for both IL-6 and IL-8 [9–12].

Altered gut microbiota composition are associated with chronic liver inflammation and may play a contributory role in hepatic carcinogenesis [17]. Dysbiotic gut microbiota composition affect the gut barrier and increase intestinal permeability, promoting the translocation of gut bacteria [18]. This leads to increased hepatic exposure with gut-derived microbiota-associated molecular patterns that include LPS, a cell wall component of Gram-negative bacteria [19]. Ni et al. demonstrated that LPS-producing genera, such as *Bacteroidetes*, *Firmicutes* and *Fusobacteria*, were increased, while butyrate-producing genera

were decreased in early HCC [20]. The accumulation of LPS itself may lead to bacterial translocation and increased gut permeability. Bacterial compounds reach the liver via portal vein and are recognized by pattern recognition receptors, expressed in Kupffer cells, and eliminated under normal conditions [21].

Increasing levels of LPS activate Toll-like receptor 4 signaling pathways, produce proinflammatory cytokines, such as IL-17, TNF-α, IL-6, and IL-1β, and promote liver inflammation. Toll-like receptors also induce tumor proliferation mediated by mitogens such as hepatocyte growth factors, amphiregulin and epiregulin [22]. High concentrations of proinflammatory cytokines producing TAMs are associated with poor HCC prognosis [23]. Plasma LPS concentrations correlate with the degree of liver dysfunction [24].

In this study we found no significant difference in LPS serum levels in healthy individuals vs. HCC patients. On the contrary, the LPS levels were a bit higher in control group. Moreover, this study does not support the measurement of LPS levels for prognostic purposes for HCC patients as the baseline levels do not have impact on the time of survival. It is very likely that LPS concentrations in the liver or the portal system has a higher significance for development and progression of HCC than blood LPS levels.

Increased intestinal permeability may be the primary and one of the most important factors in pathogenesis of chronic liver inflammation by causing bacterial translocation. Enterocytes express FABPs which are thought to be involved in uptake of lipids in the intestine [25]. FABP2, also known as intestinal-type FABP, is rapidly released into the systemic circulation on enterocyte damage and has been shown to be a useful biomarker for diagnosing acute intestinal ischemia [26,27].

Recent studies have demonstrated that fecal FABP2 levels are significantly increased in cases of liver cirrhosis and correlate with disease severity. FABP2 concentration in plasma were found to be different in pattern and absolute levels but as well elevated in liver cirrhosis [28,29]. High FABP2 levels is significantly associated with increased mortality from variceal bleeding in patients with liver cirrhosis [30]. No studies investigating the link between FABP2 levels and HCC were published to date.

This current study demonstrated that serum FABP2 levels were significantly higher in HCC patients compared with healthy controls that might be associated with background of liver cirrhosis in most HCC cases. However, our analysis does not support the idea of measuring FABP2 levels for prognostic purposes in newly diagnosed HCC patients. It is likely that FABP2 is too nonspecific for HCC as it represents only a part of intestinal permeability and is not directly involved in carcinogenesis.

Integrity of the immune system plays the important role in physiologically functioning gut-liver axis. The constant low-level exposure to bacterial components in the liver inhibits the activation of immune cells by specific receptors, such as TLRs, to level, called "endotoxin tolerance", and activates immune suppression via anti-inflammatory cytokines, such as IL-10, transforming growth factor beta (TGFβ), and hepatocyte growth factor [31]. IL-10 is an anti-inflammatory molecule which limits potentially damaging inflammatory response by inhibiting antigen presentation by dendritic cells and inhibiting macrophage activation and infiltration into damaged liver tissue [32]. At the cellular level, IL-10 is thought to act as a posttranscriptional regulatory agent to suppress the messenger RNA (mRNA) promoting the destabilization of inflammatory cytokine mRNA [33]. Furthermore, IL-10 inhibit apoptotic signaling pathways [34].

Being significantly involved in the development of chronic liver inflammation and HCC, IL-10 should be considered as a potential biomarker for liver damage. Recent studies have shown that some IL-10 polymorphisms could act as significant biomarkers of liver cirrhosis or HCC. Higher serum levels of IL-10 were estimated to be associated with higher inflammatory liver disease severity [7,35].

Our analysis confirms the fact that IL-10 serum levels are significantly higher in HCC patients than in healthy individuals. However, according to our study, it appears to be not suitable as prognostic tool for survival of HCC patients. The increase in IL-10 serum levels

may be associated with the progression of the inflammatory process of the liver but not with the carcinogenesis and stage of HCC.

This study has some limitations. It includes rather small number of HCC patients. The HCC group was heterogenous as patients of all BCLC stages were included. It lacks more single patient clinical data such as staging, underlying liver function, results of laboratory tests, radiological imaging, histology availability, performance status, co-morbidities, treatment modalities, cause of death, etc. Therefore, subgroup analysis could not be performed. Larger and more complete studies are needed to better describe the value of these molecules.

In conclusion, our study investigated LPS, FABP2 and IL-10 as possible diagnostic and prognostic biomarkers for newly diagnosed HCC patients. We demonstrated that baseline levels of both FABP2 and IL-10 are elevated in HCC patients and may serve as potential biomarkers in complex HCC diagnostic tools. LPS as well as FABP2 and IL-10 appear not to be suitable for prognosis of survival of HCC patients, however, further investigations are needed.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

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Title: miRNome profiling analysis reveals novel hepatocellular carcinoma diagnostic, prognostic and treatment-related candidate biomarkers: post-hoc analysis of SORAMIC trial.

Authors: Morkūnas, Egidijus; Vaitkevičiūtė, Evelina; Inčiūraitė, Rūta; Kupčinskas, Juozas; Link, Alexander; Skiecevičienė, Jurgita; Alunni-Fabbroni, Marianna; Schütte, Kerstin; Malfertheiner, Peter; Varkalaitė, Greta; Ricke, Jens.

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miRNome Profiling Analysis Reveals Novel Hepatocellular Carcinoma Diagnostic, Prognostic and Treatment-Related Candidate Biomarkers: Post hoc Analysis of SORAMIC Trial

Egidijus Morkunas[a] Evelina Vaitkeviciute[b] Ruta Inciuraite[b] Juozas Kupcinskas[a] Alexander Link[c] Jurgita Skieceviciene[b] Marianna Alunni-Fabbroni[d] Kerstin Schütte[e, f] Peter Malfertheiner[g] Greta Varkalaite[b] Jens Ricke[d]

[a]Institute for Digestive Research and Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania; [b]Institute for Digestive Research, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania; [c]Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Hospital, Magdeburg, Germany; [d]Department of Radiology, University Hospital, LMU Munich, Munich, Germany; [e]Department of Internal Medicine and Gastroenterology, Niels-Stensen-Kliniken Marienhospital, Osnabrück, Germany; [f]Department of Gastroenterology, Hepatology and Endocrinology, Medizinische Hochschule Hannover, Hannover, Germany; [g]Department of Internal Medicine II, University Hospital, LMU Munich, Munich, Germany

Keywords
Hepatocellular carcinoma · Micro RNA · Selective internal radiation therapy · Sorafenib

Abstract
Introduction: Early diagnosis of hepatocellular carcinoma (HCC) as well as evaluation of prognosis and prediction of treatment efficacy remains challenging due to the missing specific non-invasive biomarkers. The aim of this study was to identify disease-specific microRNA (miRNA) patterns for diagnosis, prediction of prognosis, and treatment response in patients with HCC. ***Methods:*** The study population included 42 HCC patients from SORAMIC clinical trial: 22 patients received sorafenib monotherapy, 20 patients underwent ^{90}Y radioembolization in combination with sorafenib. 20 individuals were included in the control group. HCC patients underwent collection of plasma samples before and 7–9 weeks after the beginning of the treatment. Isolation of circulating miRNAs, preparation of small RNA sequencing libraries and next-generation sequencing were performed. Association analysis for novel diagnostic, prognostic, and treatment-related candidate biomarkers was performed. ***Results:*** A total of 42 differentially expressed (16 up-regulated and 26 down-regulated) miRNAs were identified comparing baseline and control group plasma samples. hsa-miR-215-5p and hsa-miR-192-5p were down-regulated, while hsa-miR-483-5p and hsa-miR-23b-3p were up-regulated comparing baseline and 7–9 weeks post-sorafenib monotherapy samples. hsa-miR-215-5p was the sole

Egidijus Morkunas and Evelina Vaitkeviciute contributed equally to this work.
Greta Varkalaite and Jens Ricke contributed equally to this work.

karger@karger.com
www.karger.com/ddi

Correspondence to:
Egidijus Morkunas, egidijus.morkunas@lsmu.lt

down-regulated miRNA in the same combination therapy comparison. hsa-miR-183-5p, hsa-miR-28-3p, and hsa-miR-1246 were found to be significantly up-regulated comparing non-responders versus responders to sorafenib. High hsa-miR-215-5p expression was significantly associated with worse HCC patients' prognosis. ***Conclusions:*** Systematic miRNA profiling of highly characterized samples from SORAMIC study revealed a subset of potential miRNA biomarkers for HCC diagnosis and prognosis of sorafenib-treated patients' survival.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver tumour and the third leading cause of cancer-related death worldwide [1]. The incidence of HCC is increasing and will continue to rise in the future, according to predictions concerning the American population [2]. It remains a crucial demand in HCC management to develop instruments to select the appropriate therapy by including the prediction of treatment efficacy.

Sorafenib is an oral multi-target tyrosine kinase inhibitor, which has been shown to prolong overall survival in patients with HCC in advanced stages [3] and has been the main first-line treatment for more than 10 years. Although novel immunotherapeutic have displaced the sorafenib therapy in first-line or second-line therapies [4], sorafenib will remain an important treatment option, in cases where immunotherapy is contraindicated, unavailable, or ineffective. Although the addition of selective internal radiation therapy (SIRT), such as ^{90}Y radioembolization, has not been shown to significantly improve the efficacy of sorafenib in patients with advanced HCC [5], further analysis has demonstrated positive effect of addition of SIRT to sorafenib on survival rates for selected patients with HCC [6].

As of today, the only prognostic biomarker is alpha-fetoprotein (AFP) used in everyday clinical practice in management and surveillance of HCC as well as in the selection of liver transplant recipients [7]. However, its sensitivity and specificity are rather low as baseline AFP levels are usually normal and may be elevated because of non-malignant diseases (e.g., chronic viral hepatitis) [8, 9]. Several other molecules have been investigated as potential biomarkers to improve the HCC risk assessment, diagnosis, and prognosis. HCC carcinogenesis is associated with chronic liver inflammation, inflammatory cytokines, such as interleukin-6 (IL-6) and IL-8, presented as candidates for improving diagnosis and prognosis in HCC. Their baseline levels are indeed significantly higher in HCC patients compared to patients with chronic liver diseases [10, 11]. Moreover, they were associated with overall survival in HCC patients treated with sorafenib monotherapy or combination with radioembolization [12, 13]. Various additional biochemical and molecular cellular markers (e.g., glypican-3, des-γ-carboxyprothrombin), cancer stem cell markers (e.g., CD44, CD133), non-cellular components (e.g., vascular endothelial growth factor), microRNAs (miRNAs), circular RNAs, somatic genetic, and epigenetic alterations were found to provide useful information on HCC prognosis depending on treatment modalities [14, 15]. Extracellular vesicles-based proteomics also appeared to have predictive characteristics in HCC treatment [16].

miRNAs are small non-coding RNAs regulating gene expression through inhibition of translation or degradation of messenger RNAs (mRNAs). miRNAs act as tumour promoters or suppressors and have a role in the carcinogenesis of HCC [17]. Some studies have directly or indirectly demonstrated the clear link between miRNAs and progression of HCC [18, 19] and even proposed particular miRNAs as a therapeutic target [20]. Analysis of tissue and blood miRNAs has already demonstrated the potential of these molecules as HCC-related biomarkers [21–23]. However, the vast majority of studies report the results of predefined miRNA panels or specific miRNAs analysed by real-time PCR [21–23]. Thus, there is a need for comprehensive high-throughput studies to identify the complete miRNA profile with the best diagnostic and prognostic modalities. This miRNome profiling study as a post hoc analysis of the palliative arm of the SORAMIC trial aimed to identify HCC-associated miRNA diagnostic, prognostic, and predictive biomarkers.

Materials and Methods

Study Design and Selection of the Patients

We performed a post hoc analysis of plasma samples available from the SORAMIC (SORAfenib in combination with local MICrotherapy guided by gadolinium-EOB-DTPA enhanced MRI) clinical trial (ClinicalTrials.gov No. NCT01126645). Inclusion and exclusion criteria for this trial as well as treatment protocol have been described previously [5]. Twenty-two sorafenib monotherapy-treated patients and 20 patients treated with combinational therapy of radioembolization and sorafenib were included in the study population (see online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000538757). For each patient in this study population plasma, samples were collected at two different time points: before the initiation of the treatment (baseline [BL]) (n = 42) and 7–9 weeks post-therapy (n = 40). Twenty individuals from Hospital of Lithuanian University of Health Sciences Kauno klinikos (Kaunas, Lithuania, ethical approval No. BE-2-31), aged 48–80 years, endoscopically diagnosed with colonic diverticulosis and free of other known chronic or malignant liver diseases (considered as control [CON] individuals for HCC cases) were included in the control group of this study and their plasma samples were collected.

Table 1. Characteristics of study participants

	Total HCC patients (*n* = 42)	Sorafenib (*n* = 22)	Sorafenib + SIRT (*n* = 20)	*p* value**	CON (*n* = 20)	*p* value**
Gender, *n* (%)						
Female	4 (9.5)	2 (9.1)	2 (10.0)	1	3 (15.0)	0.671
Male	38 (90.5)	20 (90.9)	18 (90.0)		17 (85.0)	
Age, years						
Mean±SD	65.8±7.8	65.8±7.9	65.7±8.0	0.962	62.0±6.9	0.062
Race, *n* (%)						
White	38 (90.5)	21 (95.5)	17 (85.0)	0.601	–	–
Asian	1 (2.4)	–	1 (5.0)		–	–
Other	1 (2.4)	–	1 (5.0)		–	–
Unknown	2 (4.8)	1 (4.5)	1 (5.0)		–	–
ECOG, *n* (%)						
0	28 (66.7)	16 (72.7)	12 (60.0)	0.515	–	–
1	14 (33.3)	6 (27.3)	8 (40.0)		–	–
Liver cirrhosis, *n* (%)						
Yes	38 (90.5)	20 (90.9)	18 (90.0)	1	–	–
No	4 (9.5)	2 (9.1)	2 (10.0)		–	–
HCC aetiology, *n* (%)						
Hepatitis B	2 (4.8)	2 (9.1)	–	0.661	–	–
Hepatitis C	13 (31.0)	8 (36.4)	7 (35.0)		–	–
Alcohol	25 (59.5)	13 (59.1)	12 (60.0)		–	–
Child-Pugh score, *n* (%)						
A	35 (83.3)	19 (86.4)	16 (80.0)	0.691	–	–
B	7 (16.7)	3 (13.6)	4 (20.0)		–	–
BCLC stage, *n* (%)						
B	7 (16.7)	5 (22.7)	2 (10.0)	0.414	–	–
C	35 (83.3)	17 (77.3)	18 (90.0)		–	–
Radiological response, *n* (%)*						
Yes	24 (57.1)	14 (63.6)	10 (50.0)	0.727	–	–
No	12 (28.6)	8 (36.4)	4 (20.0)		–	–
Unknown	6 (14.3)	–	6 (30.0)	–	–	–

CON, control; HCC, hepatocellular carcinoma; SIRT, selective internal radiation therapy; SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; BCLC, Barcelona Clinic Liver Cancer. *Radiological response according to mRECIST (modified Response Evaluation Criteria in Solid Tumors) criteria comparing baseline and first control imaging (CT or MRI); complete response, partial response and stable disease were considered as *response* while progressive disease – as non-response. 6 patients treated with combination of sorafenib and SIRT lacked data on control imaging. **Student's *t* test for continuous variables (normally distributed data), non-parametric Wilcoxon rank-sum test (not normally distributed data), and Fisher's exact test for the categorical variables.

Plasma was isolated from whole blood according to SOP and stored at −80°. The study protocol was approved by the Institutional Review Boards of each participating centre and conducted in accordance with the Declaration of Helsinki. All subjects involved in the study gave written informed consent. Characteristics of the study participants are summarized in Table 1.

Isolation of Nucleic Acids, Small RNA-Seq Library Preparation, and Next-Generation Sequencing

Isolation of circulating miRNAs and preparation of sequencing libraries were performed as described previously [24]. Briefly, plasma circulating nucleic acids, including circulating miRNA fraction, were isolated using QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) according to the manufacturer's instructions. Small RNA sequencing (smRNA-seq) libraries were constructed using Illumina TruSeq Small RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's protocol. After the quantification and quality assessment, the small RNA libraries were randomized and sequenced using Illumina HiSeq 2500 (1 × 50 bp single-end reads).

Bioinformatics Analysis of Small RNA-Seq Data

smRNA-seq primary and secondary data analysis were performed as described previously [24]. First, nf-core/smrnaseq pipeline v.1.0.0 was used to perform read quality control, removal

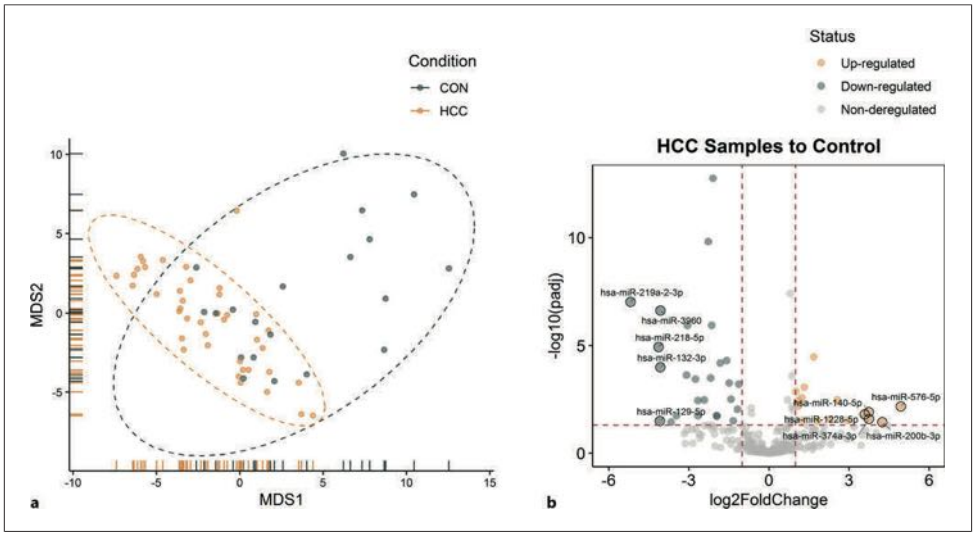


Fig. 1. a Multidimensional scaling plot based on normalised expression values showing the similarity corresponding to control (CON, $n = 20$) and hepatocellular carcinoma (HCC, $n = 42$) plasma samples. Each dot corresponds to a sample, the centroid represents the group mean, and the ellipse presents the mean standard error (SEM) based confidence. The rug plots along the x and y axis show distributions of each dot's value. **b** Volcano plot showing the differentially expressed miRNAs between HCC and controls; the orange and dark grey dots represent the up-regulated and down-regulated miRNAs, the most deregulated miRNAs are highlighted. Dashed lines indicate p value <0.05 and |log2FC| > 1.

of 3′ adapter sequences, mapping to mature and hairpin miRNAs (miRBase v.22.1 [25]), and GRCh37 human reference genome. Further, normalized counts were generated using the isomiRs package and differential expression analysis was carried out using the DESeq2 Bioconductor package v.1.32.0 [26]. The threshold for significant differential expression was Bonferroni adjusted p value <0.05 and absolute value of log2 fold change (FC) | log2FC| > 1.

Statistical Analysis

Statistical analysis was performed using R (v. 4.1.0) and RStudio software (v. 1.4.1106). The Shapiro-Wilk normality test was used to test the distribution of data. For normally distributed data, statistical significance was assessed by Student's t test, and in the case when data did not pass the normality test – a non-parametric Wilcoxon rank-sum test was used. Fisher's exact test was used for categorical variables. The area under the receiver operating characteristic curve (AUC-ROC) analysis was performed using the pROC R package to evaluate the diagnostic value. The Kaplan-Meier method and log-rank test were used to evaluate the prognostic value. p values <0.05 were considered statistically significant.

Results

Characterization of the Participants

Study population was male-predominant (90.5%) with an average of 65.8 ± 7.8 years. The majority (90.5%) of liver cirrhosis cases of HCC patients have been caused by alcoholic hepatic injury (59.5%) and chronic viral hepatitis C (31.0%). All patients have had good or very good performance status (ECOG 0–1). Most of the patients' liver function has been preserved: estimated Child-Pugh score A (35 patients, 83.3%) and B (7 patients, 16.7%). According to the BCLC staging system, 7 HCC patients (16.7%) were at the intermediate stage (BCLC B) and the rest 35 (83.3%) were at the advanced stage (BCLC C). Similarly, the control group included mostly male individuals (85.0%) with an average of 62.0 ± 6.9 years. As described previously, controls had no documented chronic or malignant liver diseases. Statistical analysis showed no gender and age-related differences comparing HCC and CON groups ($p = 0.671$ and $p = 0.062$, respectively) (shown in Table 1).

smRNA-seq Analysis showed Differentially Expressed miRNAs in HCC Baseline Patients Compared to Controls

Plasma smRNA-seq has been performed to evaluate the potential of miRNA transcriptome (miRNome) for clinical applications such as minimally invasive diagnostics. First, expression profiles of miRNA in HCC baseline patients and CONs have been determined. smRNA-seq yielded an average of 140 K raw sequencing reads (from 12 K to 1 M miRNA read counts per sample), and 711 miRNAs (miRBase v22.1) were identified. In total, 301 low-abundant and non-variable miRNAs and 1 outlying sample that did not pass data quality criteria have been removed. After data preprocessing and filtering, 408 miRNAs and 61 samples have been retained for further differential expression analysis (see online suppl. Fig. 2, 3). A total of 42 differentially expressed miRNAs have been identified comparing HCC baseline patients and CON plasma samples (16 up-regulated and 26 down-regulated; full list provided in online suppl. Table 1). Multidimensional scaling analysis of normalized miRNA expression, revealing the similarity of samples by implementing Spearman's correlation distance, has shown the distinct centroids of ellipses, and overlap between 2 analysed clusters of CON and HCC plasma samples (shown in Fig. 1a). Data have also shown that top five deregulated miRNAs were: hsa-miR-576-5p, -200b-3p, -374a-3p, -140-5p, -1228-5p (up-regulated), and hsa-miR-219a-2-3p, -218-5p, -129-5p, -132-3p, -3960 (down-regulated) (shown in Fig. 1b).

miRNA Profile Has Weak Correlation with Clinical Parameters

In order to evaluate the relationship between these deregulated miRNAs and clinical parameters of HCC patients such as plasma AFP levels and BCLC staging, the correlation analysis was performed. However, none of 42 differentially expressed miRNAs was found to have strong correlation with baseline AFP levels as well as absolute and percentage change of AFP levels (see online suppl. Fig. 4–6). Differential expression analysis revealed sole deregulated miRNA (hsa-miR-142-5p) which could distinguish BCLC stages B and C (shown in Fig. 2 and online suppl. Table 2).

Differentially Expressed miRNA Profiles Could Be Implemented for HCC Diagnostics

Normalized miRNA read counts were analysed for discrimination of controls and HCC patients (in BL time point). The analysis involved complete HCC miRNome profiles and was performed based on feature selection and random forest classificator algorithms. The feature classificator showed that 10 miRNAs (hsa-let-7b-5p, hsa-let-7c-5p, hsa-miR-124-3p, hsa-miR-125b-5p, hsa-miR-219a-2-3p, hsa-miR-3960, hsa-miR-432-5p, hsa-miR-6734-5p, hsa-miR-7-5p, hsa-miR-95-3p) were confirmed as an important attribute. Confirmed attributes were used for performance evaluation by using the receiver operating characteristic (ROC) curve (shown in Fig. 3a–y) showing the best performance of hsa-let-7b-5p (AUC = 85.6%, 95% CI = 76.1–95.1%) and hsa-miR-3960 (AUC = 85.9%, 95% CI = 73.3–98.4%). This analysis revealed that combination of all 10 above-mentioned miRNAs resulted in significant AUC – 92.68% when discriminating between control and HCC cases.

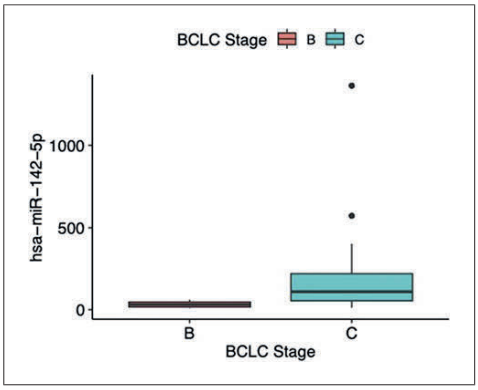
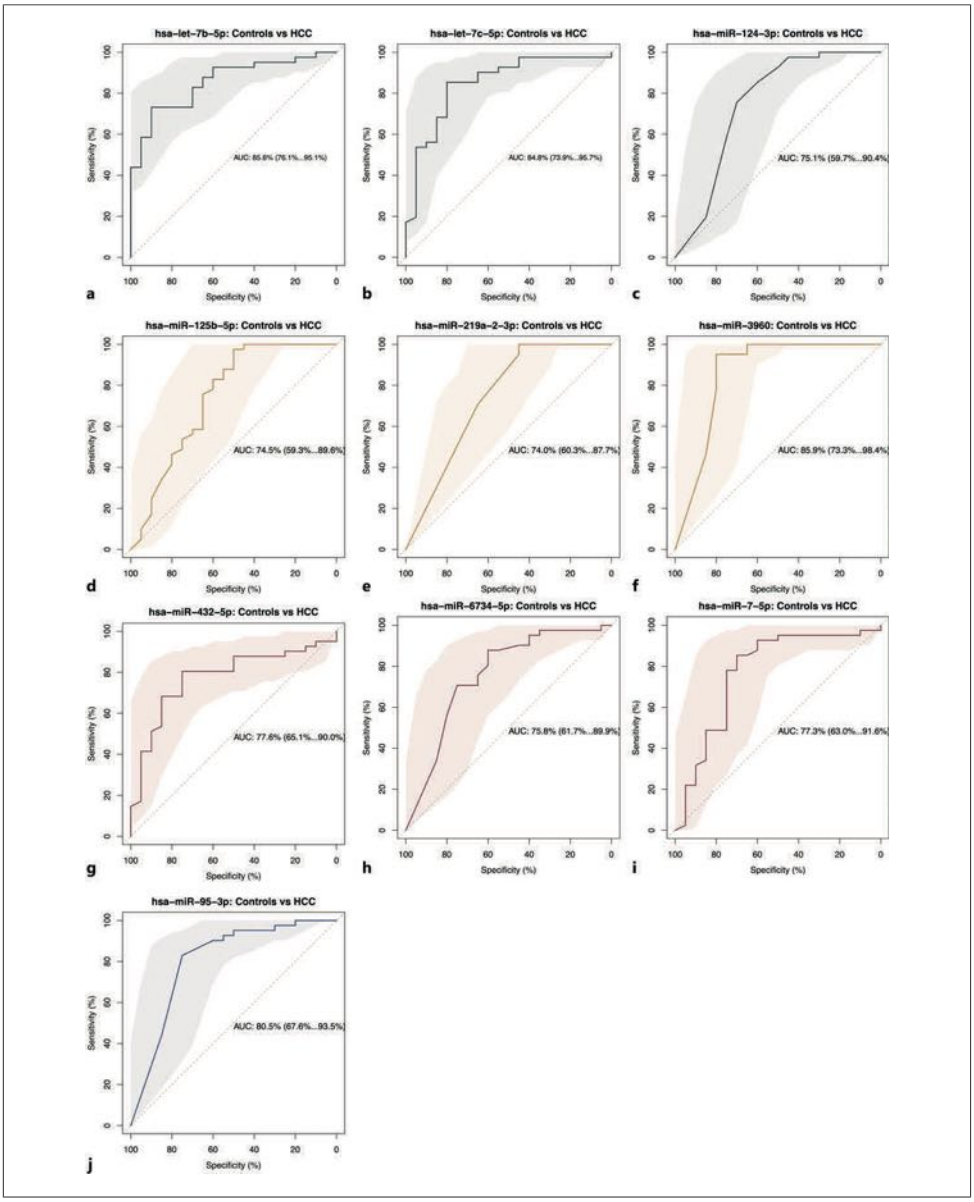


Fig. 2. Graph showing different expression of has-miR-142-5p in patients with HCC of BCLC stage B and C. It was the sole miRNA that could distinguish different HCC stages. The coloured area represents a 95% confidence interval (CI). BCLC, Barcelona Clinic Liver Cancer.

miRNA Expression Patterns Could Be Altered after HCC Therapy

Further the effect of HCC patients' treatment on the expression patterns of plasma miRNAs has been analysed. Two miRNAs, hsa-miR-215-5p (log2FC = −3.65; padj = 7.72×10^{-8}) and hsa-miR-192-5p (log2FC = −2.27; padj = 1.11×10^{-2}), were down-regulated comparing baseline patients versus 7–9 weeks post-sorafenib therapy samples, whereas hsa-miR-483-5p (log2FC = 1.75; padj = 2.30×10^{-2}) and hsa-miR-23b-3p (log2FC = 1.60; padj = 2.96×10^{-2}) were up-regulated in the same comparison



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group (shown in Fig. 4a). Notably, hsa-miR-215-5p (log2FC = −3.88; padj = 4.84×10^{-3}) was the sole down-regulated comparing baseline HCC patients and 7–9 weeks post-treatment groups when analysing combined SIRT/sorafenib therapy cases (shown in Fig. 4b).

Plasma miRNA Expression Could Significantly Discriminate between Responders and Nonresponders Depending on Treatment Strategy

Differential plasma miRNA expression analysis has been performed to identify the association of miRNAs with response to treatment. Comparing non-responders *vs*. responders after 7–9 weeks of sorafenib therapy, three miRNAs were found to be significantly up-regulated (hsa-miR-183-5p [log2FC = 3.17, padj = 3.07×10^{-2}]; hsa-miR-28-3p [log2FC = 4.45, padj = 1.61×10^{-4}]; hsa-miR-1246 ([log2FC = 4.53, padj = 2.26×10^{-3}]). However, no significantly deregulated miRNAs were detected when comparing non-responders versus responders to combined SIRT/sorafenib therapy after 7–9 weeks of treatment. AUC/ROC analysis has shown that hsa-miR-183-5p discrimination performance resulted in an AUC of 75.5% (95% CI = 52.6–98.5%; shown in Fig. 5a), hsa-miR-28-3p resulted in AUC of 83.2% (95% CI = 66.0–100.0%; shown in Fig. 5b), and hsa-miR-1246 - AUC of 78.6% (95% CI = 59.5–97.6%; shown in Fig. 5c).

miRNA Expression Signatures for HCC Patients' Prognosis

Finally, we have performed survival analysis to identify the potential circulating miRNA prognostic markers. We have grouped HCC patients into the following groups: (1) baseline, (2) 7–9 weeks post sorafenib therapy, and (3) 7–9-week post-SIRT/sorafenib therapy. The analysis results revealed that two miRNAs (hsa-miR-215-5p [log2FC = 3.39, padj = 1.06×10^{-2}]; hsa-miR-25-5p [log2FC = −6.05, padj = 1.06×10^{-2}]) were significantly deregulated in group II comparing death and non-death (censored) outcomes of HCC patients. No deregulated miRNAs that could be associated with survival were found in groups I and III. Furthermore, we have grouped HCC patients into two groups relative to the cut-off expression level (above the median – high expression; below the median low expression). Kaplan-Meier survival analysis has shown that high hsa-miR-215-5p expression is significantly associated ($p = 0.031$) with the worse HCC patients' prognosis (median survival time 10.5 and 26.9 months, high and low hsa-miR-215-5p expression, respectively) (shown in Fig. 6).

Discussion

In the present study, plasma smRNA-seq analysis was performed in HCC and CON groups. Moreover, the HCC patients' group was randomized by treatment (sorafenib and combined SIRT/sorafenib therapy) and followed-up after treatment. The survival rates in both groups were similar [5]. We have identified 16 up-regulated and 26 down-regulated miRNAs comparing HCC baseline and CON plasma samples. At baseline two miRNAs (hsa-miR-215-5p and hsa-miR-192-5p) were found to be down-regulated while two miRNAs (hsa-miR-483-5p and hsa-miR-23b-3p) were up-regulated comparing to 7–9 weeks post-sorafenib monotherapy samples. The only miRNA (hsa-miR-215-5p) was down-regulated at baseline comparing to 7–9 weeks post-sorafenib + SIRT samples. Three miRNAs (hsa-miR-183-5p, hsa-miR-28-3p, and hsa-miR-1246) were found to be significantly up-regulated comparing non-responders versus responders after 7–9 weeks of sorafenib monotherapy. Kaplan-Meier survival analysis has demonstrated significant association between high hsa-miR-215-5p expression and worse prognosis for HCC patients independently of the therapy received.

First, we have shown the distinct circulating plasma miRNome profiles comparing the HCC baseline and CON samples. From the list of the most deregulated miRNAs in HCC patients' plasma, hsa-miR-576-5p has been reported to be up-regulated in non-alcoholic fatty liver disease [27]. However, deregulation of this miRNA in the case of HCC was reported in our study for the first time. Interestingly, expression of hsa-miR-200b-3p has been shown to be down-regulated in HCC tissues [28], while our data suggest significant up-regulation of this miRNA in HCC plasma samples compared to controls. Several functional miRNA studies report the role of hsa-miR-374a-5p and hsa-miR-140-5p in HCC in vitro [29, 30]; however, to our best knowledge there are no studies reporting altered expression of these miRNAs in plasma or tissue in the case of HCC and their potential for HCC

Fig. 3. ROC curves depicting the discrimination performance of (**a**) hsa-let-7b-5p, (**b**) hsa-let-7c-5p, (**c**) hsa-miR-124-3p, (**d**) hsa-miR-125b-5p, (**e**) hsa-miR-219a-2-3p, (**f**) hsa-miR-3960, (**g**) hsa-miR-432-5p, (**h**) hsa-miR-6734-5p, (**i**) hsa-miR-7-5p, (**j**) hsa-miR-95-3p between controls and HCC in the BL group. The shaded area represents a 95% confidence interval (CI). AUC, the area under the receiver operating characteristic curve.

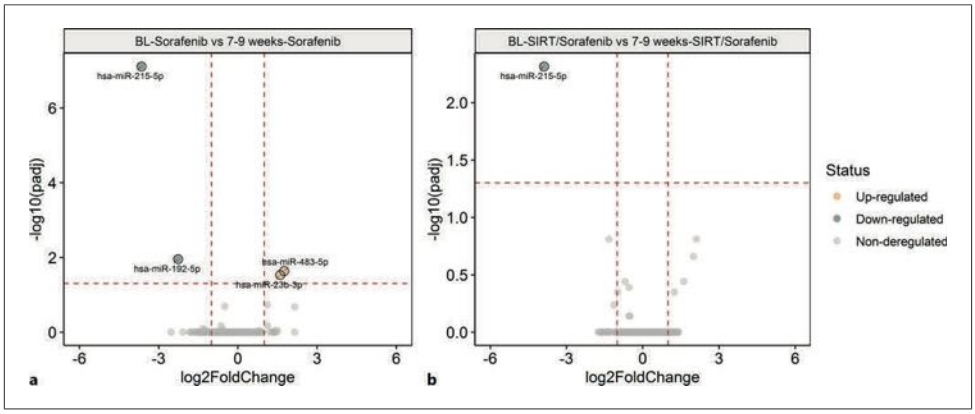

Fig. 4. Volcano plot showing the differentially expressed plasma miRNAs compared to HCC BL and 7–9 weeks post-therapy patients, demonstrating the changes during treatment: (**a**) sorafenib therapy group; (**b**) SIRT/sorafenib therapy group.

diagnosis and prognosis. A reduced level of hsa-miR-1228-5p was reported by Cho et al. [31] in the case of cervical cancer. A study by Sonoda et al. [32] associated hsa-miR-1228-5p with cerebrovascular disorders. However, our study reported up-regulation of this miRNA in HCC patients' plasma for the first time. To our best knowledge, hsa-miR-219-2-3p and hsa-miR-218-5p down-regulation in plasma has not been previously reported in the case of HCC. hsa-miR-219-2-3p has been previously shown to be possibly involved in gastric cancerogenesis, the development of pituitary adenomas, and glioblastomas [33–35]. In addition, functional miRNA studies report the role of hsa-miR-218-5p in the regulation of cell proliferation, metastasis, and invasion in vitro in HCC cell lines [36]. Similar to our results Shaker et al. [37] have shown that hsa-miR-129-5p is down-regulated in the serum samples of HCC patients and showed great performance (AUC = 0.997, sensitivity 100%, specificity 97.2%) in HCC discrimination analysis. Functional studies have supported the important role of hsa-miR-129-5p in HCC. This miRNA may contribute to proliferation and metastasis, affect progression, and regulate HCC progression [38]. We have determined reduced plasma levels of hsa-miR-132-3p. In contrast, Wen et al. [39] reported significantly overexpressed hsa-miR-132-3p in the plasma of HBV-positive HCC patients, and Yuan et al. [40] reported increased expression of hsa-miR-132-3p in HCC tissues. Although a few studies have reported that expression of exosomal or circulating serum, hsa-miR-3960 could be a molecular signature of diffuse large B-cell lymphoma or bladder cancer, respectively [37, 41] and that hsa-let-7b-5p has a link with acute myeloid leukaemia, skin melanoma, and head and neck squamous cell carcinoma [42–44], there is no previously published data showing the potential link of both hsa-miR-3960 and hsa-let-7b-5p with HCC and their association with this disease is reported for the first time in our study.

We compared newly identified differentially expressed miRNA profile with AFP as the only approved biomarker for HCC. However, none of deregulated miRNAs had strong correlation with baseline AFP levels or changes (both absolute and percentage) in AFP levels. Previous studies have shown that combining AFP levels with certain miRNAs levels could improve diagnostic abilities [45]. Further investigations are needed.

Differential expression analysis demonstrated that there is only one miRNA (hsa-miR-142-5p) which deregulation is different depending on the HCC stage and could distinguish BCLC stages B and C. Previously only one study has identified the link between this miRNA and HCC [46]. However, its relationship with differently progressed disease has not been analysed before.

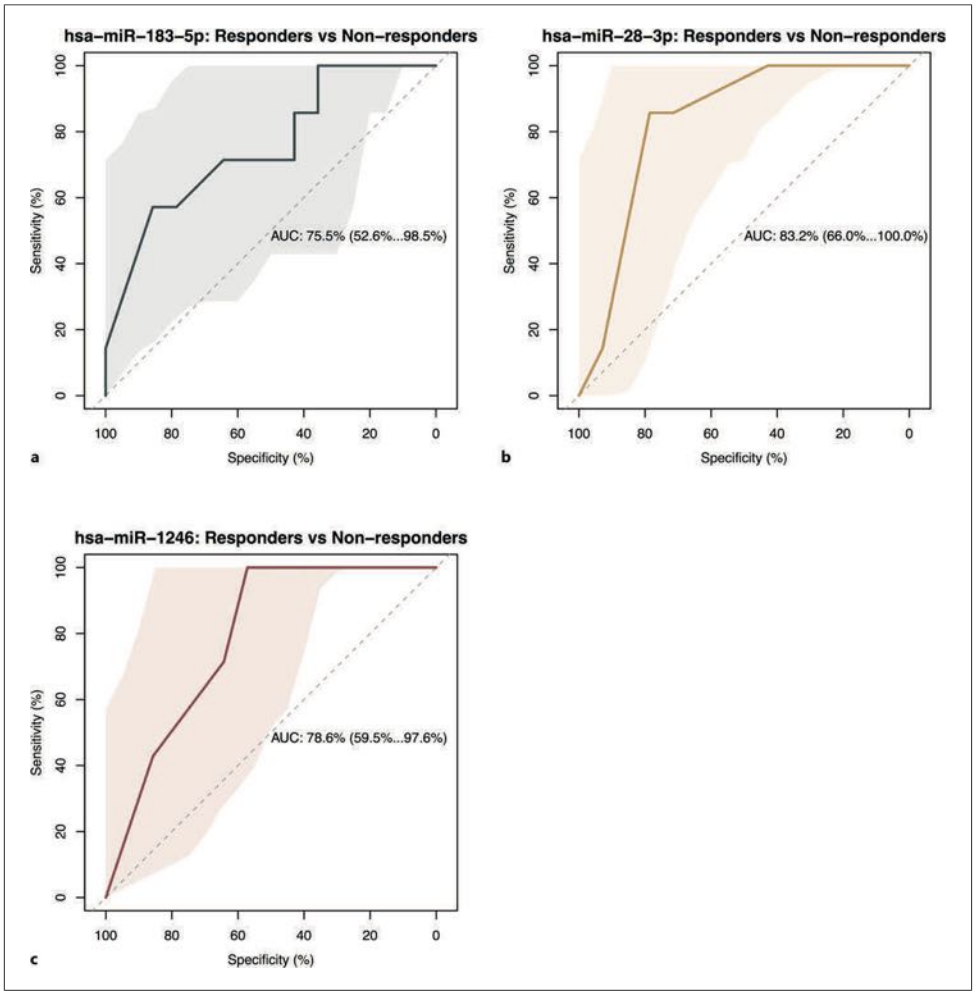

Fig. 5. ROC curves depicting the discrimination performance of (**a**) hsa-miR-183-5p, (**b**) hsa-miR-28-3p, (**c**) hsa-miR-1246 between responders and non-responders in the sorafenib treatment group. The shaded area represents a 95% confidence interval (CI). AUC, the area under the receiver operating characteristic curve.

Next, we have identified several miRNAs such as hsa-miR-215-5p, -192-5p, -483-5p, -23b-3p the expression pattern of which alters depending on the different treatment strategies but irrespective of response to treatment. miRNA hsa-miR-215-5p expression in plasma decreased after 7–9 weeks post-sorafenib and combined

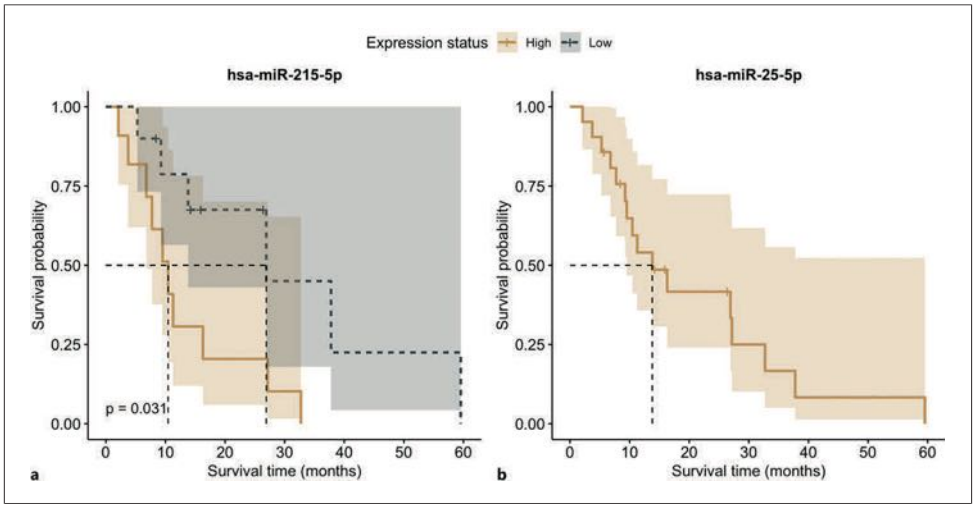

Fig. 6. Kaplan-Meier survival analysis of all study patients with HCC with low (dark grey line) and high (orange line) expression of miRNA in plasma: (**a**) hsa-miR-215-5p, (**b**) hsa-miR-25-5p. A 95% CI (estimated from a log hazard) is presented in the shadowed area. The tick marks indicate censored events, and the dashed line indicates the median survival time.

SIRT/sorafenib treatment; while sorafenib therapy has affected hsa-miR-192-5p, -483-5p, -23b-3p levels in plasma. The listed miRNAs have been previously associated with HCC. Zhang et al. [47] have suggested that increased hsa-miR-215-5p expression in serum could be implemented for HCC diagnostics. A study by Zhou et al. [48] associated this miRNA with hepatitis B virus-related HCC. Lin et al. [49] reported differentially expressed exosomal hsa-miR-483-5p in HCC. However, only one study by Manganelli et al. [50] in concordance with our results revealed that the average amount of miR-23b-3p significantly increased 1 month after starting sorafenib treatment.

Plasma smRNA-seq data analysis revealed that hsa-miR-183-5p, -28-3p, and -1246 could be associated with HCC patients' response to sorafenib treatment. In vitro studies showed that the latter-mentioned miRNAs could be highly important in HCC carcinogenesis [51, 52]. However, their expression relationship with the response to sorafenib treatment in HCC has not been previously described. Pehserl et al. [53] reported that several miRNAs including hsa-miR-1246 were identified to be differentially regulated post-sorafenib treatment in colorectal cancer cell culture Caco-2. Although hsa-miR-28-3p have not been previously related to HCC, we have shown that it reveals quite good AUC of 83.2% when discriminating the patients that received sorafenib therapy.

Finally, survival analysis showed that hsa-miR-215-5p has been also associated with HCC patients' survival and what is important; its expression level could indicate a worse prognosis for HCC patients. It has been previously reported that hsa-miR-215-5p could be applied for HCC diagnostics, but it has not been associated with patients' survival before. Taken together, our data and results from previous studies suggest that in the case of HCC hsa-miR-215-5p acts as an oncomir, its levels in the patient's blood plasma respond to sorafenib treatment, and increased levels could be significantly associated with a worse prognosis (after sorafenib therapy). hsa-miR-215-5p putative gene target set analysis has shown that genes such as *EREG*, *NIPAL1*, *MFAP3*, *CCNT2*, *NKAIN2*, *ZEB2*, etc. could be targeted by this miRNA (see online suppl. Tables 3 and 4 for the full lists of potential targets, TargetScan 8.0 and miRDB [54]). Consequently, this miRNA could be

involved in EGF receptor, angiogenesis, apoptosis, cadherin, Wnt, integrin signalling, and TGF-beta signalling pathways (PANTHER 17.0 [55]) which have been previously shown to be deregulated in cancer, including HCC [56].

Despite the important and novel findings of this study, some limitations should be declared. Our study does not include HCC tumour tissue collection. Therefore, we have retained the open access data sets and performed tissue miRNome analysis comparing normal and HCC samples (TCGA and YM500v3 [57] data bases, see online suppl. Tables 5 and 6). However, the overlap of the tissue and our plasma miRNomes was insignificant. These results could be explained by the origin of tissue (whether it is a healthy subject or adjacent tissue) or even ethnicity of study participants. Next, miRNA analysis was performed from samples of total plasma circulating nucleic acids, as the isolation kit does not include technical steps for specific enrichment of small RNA molecules. However, this provides important data revealing the possible application of multi-layer molecular profiling from a single sample. As the subgroups of HCC patients corresponding different aetiology of chronic liver disease were too small, the detailed analysis was not performed. Despite the limitations mentioned, we believe that this study provides highly relevant and promising data for more precise and minimally invasive HCC diagnostics and prognostics.

Conclusion

We have shown different plasma miRNome profiles comparing HCC and control cases. Our results have suggested possible minimally invasive liquid biopsy-based plasma miRNA biomarkers for HCC. We have revealed potential therapy-related targets showing that hsa-miR-215-5p expression levels in plasma could be related to both sorafenib and SIRT/sorafenib treatment and also survival rates when patients are treated with sorafenib. We have also identified several plasma miRNA-based targets that showed response-dependent expression and that could potentially be applied in a minimally invasive manner for the evaluation of survival prognosis for patients treated with sorafenib therapy.

Statement of Ethics

The Institutional Review Boards of all participating centres approved the study prior to initiation of the SORAMIC trial. Trial registration: EudraCT, 2009-012576-27, registered April 9, 2010, https://www.clinicaltrialsregister.eu/ctr-search/search?query=2009-012576-27; ClinicalTrials.gov, NCT01126645, registered May 20, 2010, https://classic.clinicaltrials.gov/ct2/show/NCT01126645. The inclusion of the control group was approved by Kaunas regional biomedical research Ethics Committee (Kaunas, Lithuania, No. BE-2-31). Written informed consent was obtained from all patients. Study procedures were performed in accordance with the protocol and ethical principles that have their origin in the Declaration of Helsinki and the International Council for Harmonisation-Good Clinical Practice.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

E.M., E.V., G.V., and R.I. analysed, interpreted the data, and were the main contributors in writing the manuscript. J.K., A.L., J.S., M.A.F., K.S., P.M., and J.R. made substantial contributions to the conception and design of the study as well as revision of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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CURRICULUM VITAE

Name, Surname: Egidijus Morkūnas
Mobile: +370 614 72145
E-mail: egidijus.morkunas@lsmu.lt

Education:

2010–2016	Lithuanian University of Health Sciences, Medical Academy, Faculty of Medicine *Master's degree in Medicine*
2016–2020	Lithuanian University of Health Sciences, Medical Academy, Department of Gastroenterology *Residency in Gastroenterology*
2020–2023	Lithuanian University of Health Sciences, Medical Academy, Department of Oncology and Hematology *Residency in Medical Oncology*
2020–2024	Lithuanian University of Health Sciences, Medical Academy, Department of Gastroenterology *PhD studies*

Work experience:

2016–2020	*Resident doctor* at the Department of Gastroenterology, Hospital of Lithuanian University of Health Sciences Kauno klinikos
2016–2022	*Clinical assessor*, Lithuanian State Medicines Agency
2020–2023	*Resident doctor* at the Department of Oncology and Hematology, Hospital of Lithuanian University of Health Sciences Kauno klinikos
2020–present	*Gastroenterologist and endoscopist*, Hospital of Lithuanian University of Health Sciences Kauno klinikos
2023–present	*Medical oncologist*, Hospital of Lithuanian University of Health Sciences Kauno klinikos
2020 –present	*Assistant*, Lithuanian University of Health Sciences

Professional affiliation:

Lithuanian Society of Gastroenterology
Kaunas region's Club of Endoscopists
Lithuanian Society of Medical Oncology
Kaunas region's Society of Oncologists, Hematologists and Transfusiologists
European Society of Digestive Oncology, Young Group member
United European Gastroenterology
European Society of Medical Oncology

International experience:

09/2022	University Hospital of Nantes, Digestive Oncology Center, France
2023–present	"Horizon Europe" project "A European "shield" against colorectal cancer based on novel, more precise and affordable risk-based screening methods and viable policy pathways" (ONCOSCREEN), GA No. 101097036 *Junior researcher*
2023–present	"Horizon Europe" project "Evidence-based Participatory Decision Making for Cancer Prevention through Implementation Research" (ONCODIR), GA No. 101104777 *Junior researcher*

Clinical trial experience:

2021–2023	CANFOUR (CAN04CLIN001) clinical trial, closed *Investigator*
2021–2024	Amgen 20210096 clinical trial, ongoing *Investigator*

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