# 2ND INTERNATIONAL RESEARCH CONFERENCE ON LIVER CANCER

## “NOVEL DIRECTIONS IN LIVER CANCER RESEARCH”

**October 11 – 13, 2023**
**DKFZ HEIDELBERG**


**www.livercancer.de**

LIVER CANCER SFB/TR 209 · DFG Deutsche Forschungsgemeinschaft · Boehringer Ingelheim Stiftung · ZPM Heidelberg Zentrum für Personalisierte Medizin · LCCH Liver Cancer Center Heidelberg

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Dear Colleagues,

It is a great pleasure for us to welcome you at the 2nd International Liver Cancer Meeting in Heidelberg. We hope that you will find our charming old university city and the modern science campus that is especially dedicated to cancer research an adequate venue.

The meeting primarily originated from the Transregional Consortional Research Grant (SFB/TR 209 supported by the German Research Foundation DFG), Liver Cancer - New mechanistic and therapeutic concepts in a solid tumor model' that was located in Heidelberg, Tübingen and Hannover and coordinated by myself. Our SFB/TRR209 existed from 2017 until 2023 and comprised 25 research groups with over 40 PIs and associated programs dedicated to liver cancer research. Therefore, a notably part of the contributions originates from the SFB/TR 209 and we are looking forward to discuss the data during the meeting and also set the basis for further national and international collaborations.

Once decided upon the meeting it rapidly became evident that there are numerous liver cancer meetings worldwide that have a strong emphasis on clinical aspects and clinical research aspects, but that there is a lack of meetings covering the research aspects, despite the strong international interest in liver cancer research, thus, it is truly international and support this concept to all organisations.

In this context, we would be excited, if our meeting may act as a starting point for further research project collaborations. We are also especially happy to welcome our international speakers. In addition, we have provided significant time slots for poster discussions and I want to motivate everybody to take this opportunity and discuss at the posters in detail. The quality of the abstracts is highly promising and will certainly make them a central aspect of the meeting.

Our deepest thanks go to Prof. Michael Baumann and the DKFZ for providing the meeting venue and its facilities and to all those that have contributed to the preparation of the meeting, especially UniKT as the organizing company, Dr. Mandy Skunde, who did most of the preparative work and the colleagues of the organizing committee from Heidelberg, Tübingen and Hannover. Most importantly, we thank all of you for your participation and contributions!

We wish us all a successful research conference with interesting oral and poster presentations, stimulating discussions and many resulting collaborations. We are convinced that Heidelberg and its science campus will provide an adequate forum and we hope that you will enjoy the conference.

So again, in the name of the organizing committee I want to welcome you to Heidelberg.
Peter Schirmacher



## ORGANIZATION

**Conference Chair:**
Prof. P. Schirmacher
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Institute of Pathology
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Prof. S. Roessler, University Hospital Heidelberg
Prof. M. Heikenwälder, German Cancer Research Center and University of Tübingen
PD Dr. A. Saborowski, Hannover Medical School
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**Meeting Venue:**
German Cancer Research Center (DKFZ)
Hörsaal Kommunikationszentrum
Im Neuenheimer Feld 280
69120 Heidelberg
www.dkfz.de

# GENERAL INFORMATION

## CONFERENCE REGISTRATION DESK

Will be opened for registration
Wednesday, October 11th, 11:00 am – 07:30 pm
Thursday, October 12th, 08:30 am – 4:00 pm
Friday, October 13th, 08:00 am – 10:30 am
Direct contact: ++49(0)6221-424242

## MEDIA CHECK

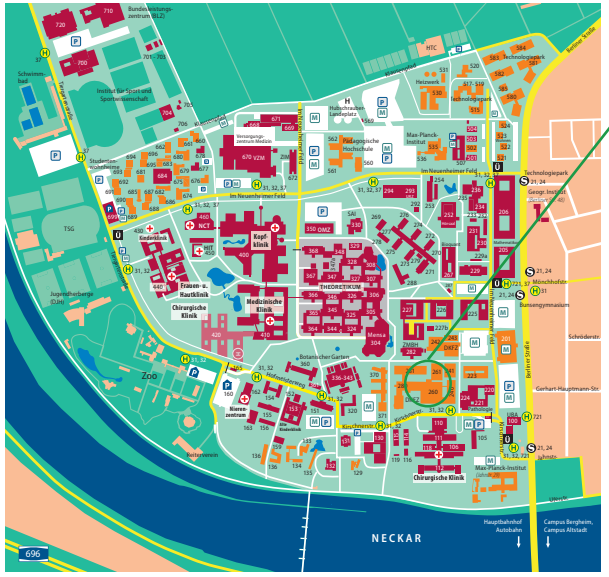
The Media Check is located between the entrances of the lecture hall and opened for talk submission. Open daily one hour before the start of the conference and during break times.
Talks have to be handed in as powerpoint presentation latest one hour before the according session starts at the media check in.

## INTERNET ACCESS

WiFi is accessible.
Username: guest-0089 / Password: kXk6Hk9G (cannot be changed)
Validity period: Tuesday, October 10, 2023 - Saturday, October 14, 2023

You are entitled to connect to the internet via unencrypted WiFi. Make sure you have entered „guest“ in the SSID field of your WiFi client software, otherwise you will not be able to connect to the wireless network.
Please note the Terms of Use in the login process to the guest WiFi.

## HOW TO GET THERE






**By Tramway:**
Take the north exit (Kurfürstenanlage) of the Central Station to the tram stop. Take tram number 24 to „Handschuhsheim Nord“, or tram number 21 to „Hans-Thoma-Platz“. With both lines, leave at stop „Jahnstraße“ (right after the Neckar bridge). Cross the street to the left and go to „Jahnstrasse“. Follow that street until you reach the gate on the left. Pass the gate and walk to „Kirschnerstrasse“. About 100 meters down the road you will see the DKFZ main entrance on your right.

**By Bus:**
The bus stop is located next to the tram stop. Take Bus number 32 to „Neuenheimer Feld - Kopfklinik“ or number 20 to „Neuenheimer Feld – Sportzentrum Nord“ and get off at stop „Kirschnerstrasse“. About 100 meters down the road you will see the DKFZ main entrance on your right.
For the Rhein-Neckar-Verkehr GmbH timetable information go to https://www.rnv-online.de/fahrt-info/.

**Walk:**
As the German Cancer Research Center is located only about 1.5 km from the Central Station, one may even walk (15-20 minutes). Upon exiting Central Station, head north and cross „Kurfürsten-Anlage“ (large 4-lane-street). Walk north along „Mittermaierstrasse“ crossing the Neckar river. Right after the bridge, make a left into „Jahnstrasse“. Follow that street until you reach the gate on the left. Pass the gate and walk to „Kirschnerstrasse“. About 100 meters down the road you will see the DKFZ main entrance to your right.

**Photos:** J. Jung / DKFZ (top), T. Schwerdt / DKFZ (bottom)

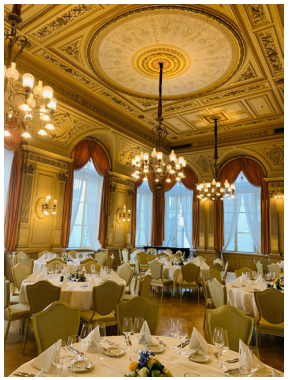
**By car:**
Approaching Heidelberg on Autobahn A5 from Frankfurt or Karlsruhe, change to Autobahn A656 from direction Mannheim at the junction „Heidelberger Kreuz“. Continue on Autobahn A 656 to Heidelberg, which ends at the Heidelberg City limits. As you enter the city of Heidelberg, follow the signs „Eberbach/HD-Wieblingen/Chirurgie“: Turn left at first traffic light, then right at second. Follow the curve at the next light. Underpass the Neckar Bridge, then turn right twice heading for „Chirurgie/DKFZ“. Having crossed the Neckar River, take the left lane at the first intersection and head for „Chirurgie/DKFZ“. Follow this street (Jahnstr.). Follow the signs to the public paid parking lots. The German Cancer Research Center is the tall building in the street Kirschnerstr. (for your orientation, you can find the house numbers on the top of each building; DKFZ has 280). Go straight ahead along the building, until you can see the main entrance on the right.
Because of the limited number of parking areas in the Neuenheimer Feld, it is recommended to arrive by public transport.
The public parking P25 is located about 10 min walk from DKFZ. If you are using a navigation device, please enter „Kirschnerstraße“ as the destination address.
There is no parking in front of the DKFZ Communication Center.

## PARTICIPATION CONFIRMATION

You can download your confirmation of participation in the registration tool Conftool. (https://www.conftool.com/hcc2023/) Please log in with your access data and download the confirmation. Download will be possible after October 13th.



## CONFERENCE DINNER, Thursday, October 12th



The Evening Get Together will take place at the Palais Prinz Carl.
The Palais Prinz Carl is located in the Old Town of Heidelberg with a beautiful view on Heidelberg Castle.

**Address:**
Prinz Carl Palais, Kornmarkt 1, 69117 Heidelberg

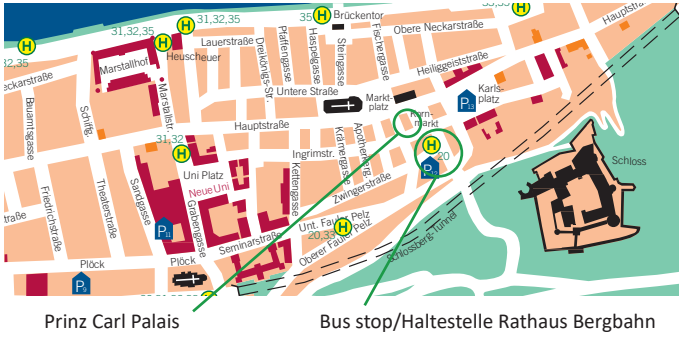
## How to get there from the venue:

**By Tramway/ Bus** (timetable see: www.rnv-online.de/fahrtinfo)
To reach the Conference Dinner by public transport, you can choose bus line 20. Get on the bus at the stop “Neuenheim, Kirschnerstraße” in the direction of “S-Bahnhof Altstadt”. The bus stop is about 100m away from the conference venue.
You take the bus for about 16 minutes to the stop “Rathaus/Bergbahn”.
Get off there and walk approx. 1 minute to the Prinz Carl Palais.

**Walk**
You can reach the Conference Dinner location with a walk along the Neckar river and through the beautiful Old Town within about a 50 minutes walk.
When you leave the Conference Venue, turn right towards the „Marsilius Kolleg“ (several tall buildings). There you leave the “Kirschnerstraße” and walk the footpath towards the Neckar. Along the Neckar river, passing the „Neckarwiese“, you can enjoy the view of the castle. You cross the river with the help of the Theodor-Heuss-Bridge and walk straight ahead until you reach the “Bismarckplatz”. There you turn into the “Hauptstraße”, the main shopping street in Heidelberg, and follow it to the end at the „Kornmarkt“. Prinz Carl Palais is on the right hand side at Kornmarkt.

**By car**
From the conference location you can reach Prinz Carl Palais in about 15 minutes. There are several paid parking garages near the location. To get to the nearest parking garage „Kornmarkt/Schloss P12“, please enter the following address into your navigation device: Zwingerstraße 20, 69117 Heidelberg. You can follow the parking guidance system with the indication „P12“ to the parking garage. From there you can reach Prinz Carl Palais with a few steps .
You will find another parking possibility at „P13 Karlsplatz“. The address is: Hauptstraße 214, 69117 Heidelberg. You can follow the parking guidance system with the indication „P13“ to the parking garage. From here it is only a short walk to the location.
Please note that parking in the Old Town is not possible outside the parking garages.

**By Taxi**
For this purpose, you can order a cab by calling +49 6221 302030.



# CONFERENCE PROGRAM

## WEDNESDAY, October 11th 2023

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| **11:00 – 13:00** | **Registration and poster set-up** |
| **13:00 – 13:15** | **Welcome**<br>Peter Schirmacher and Michael Boutros (Heidelberg) |
| **13:15 – 14:45** | **Session 1: Liver cancer – the impact of etiology**<br>*Chair: Silvia Affo, Niklas Björkström* |
| 13:15 – 13:45 | **HCC etiology with focus on NASH - possible impacts for therapy**<br>Mathias Heikenwälder (Heidelberg/Tübingen, Germany) |
| 13:45 – 14:15 | **Epidemiology and risk factors of cholangiocarcinoma**<br>Shahid A Khan (London, UK) |
| 14:15 – 14:30 | **ABS-117: Loss of Fibroblast growth factor 21 impairs liver regeneration and accelerates hepatocelullar carcinoma development in a murine model of liver injury**<br>Hanna Luisa Redeker (Hannover, Germany) |
| 14:30 – 14:45 | **ABS-155: WISP1 is a downstream target of TGF-beta signaling, highly expressed in cirrhotic HCC microenvironment and linked to better survival**<br>Nadja Meindl-Beinker (Mannheim, Germany) |
| *14:45 – 15:45* | *Coffee break* and **Poster viewing** |
| **15:45 – 18:00** | **Session 2: Tumor cell microenvironment and immune-mediated hepatocarcinogenesis**<br>*Chair: Stephanie Roessler, Satdarshan (Paul) S. Monga* |
| 15:45 – 16:15 | **Heterogeneity of cancer-associated fibroblasts in cholangiocarcinoma**<br>Silvia Affo (Barcelona, Spain) |
| 16:15 – 16:45 | **The role of natural killer cells in the pathogenesis of liver cancer development**<br>Niklas K Björkström (Stockholm, Sweden) |
| 16:45 – 17:00 | **ABS-120: The contribution of autophagy in hepatic stellate cells on tumour initiation and progression**<br>Veronika Büttner (Düsseldorf, Germany) |
| 17:00 – 17:15 | **ABS-130: Targeting mucosal-associated invariant T (MAIT) cells for immunotherapy of HCC**<br>Benjamin Ruf (Tübingen, Germany) |
| 17:15 – 17:30 | **ABS-116: A novel murine organoid-based FGFR2 fusion-driven cholangiocarcinoma model to study the impact of frequently co-altered genes on the tumor microenvironment**<br>Nugzar Lekiashvili (Heidelberg, Germany) |
| 17:30 – 17:45 | **ABS-175: High-dimensional spatial profiling of the hepatocellular carcinoma tumor microenvironment reveals spatial immune types informing immune checkpoint inhibitor therapy response**<br>Henrike Salié (Freiburg, Germany) |
| 17:45 – 18:00 | **ABS-127: Mucosal-associated invariant T cells are rendered dysfunctional within the tumour microenvironment in HCC in a cell-contact dependent manner**<br>Karin Böttcher (Munich, Germany) |
| **18:00 – 19:30** | **Guided Poster session 1 (odd poster numbers)** |
| *19:30* | *Get-together at the venue (DKFZ)* |

## THURSDAY, October 12th 2023

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| **9:00 – 10:45** | **Session 3: Diagnosis, early detection and progression of hepatobiliary carcinoma**<br>*Chair: Anna Saborowski, Michele Vacca* |
| 9:00 – 9:30 | **Molecular alterations of preneoplastic lesions and progression to cholangiocarcinoma**<br>Stephanie Roessler (Heidelberg, Germany) |
| 9:30 – 10:00 | **Liquid biopsy: what is its role in cholangiocarcinoma?**<br>Chiara Braconi (Glasgow, UK) |
| 10:00 – 10:15 | **ABS-133: Integrative Analysis of Signal Transduction and Metabolism to Promote Early Detection of Liver Cancer**<br>Christina Mölders (Heidelberg, Germany) |
| 10:15 – 10:30 | **ABS-172: N-cadherin: a Diagnostic Marker to help Discriminate Primary Liver Carcinomas from Extrahepatic Carcinomas**<br>Tiemo Sven Gerber (Mainz, Germany) |
| 10:30 – 10:45 | **ABS-153: Deep learning-enabled diagnosis of liver adenocarcinoma**<br>Thomas Albrecht (Heidelberg, Germany) |
| *10:45 – 11:30* | *Coffee break* and **Poster viewing** |
| **11:30 – 13:00** | **Session 4: Microbiome and tumor metabolism**<br>*Chair: Mathias Heikenwälder, Fernando Camargo* |
| 11:30 – 12:00 | **Intermittent fasting in NASH and HCC development**<br>Suchira Gallage (Heidelberg, Germany) |
| 12:00 – 12:30 | **Metabolic rewiring (and dysfunction) in liver regeneration and HCC**<br>Michele Vacca (Bari, Italy / London, UK) |
| 12:30 – 12:45 | **ABS-159: Discovery Proteomics: Comparative Serum Profiling of Hepatocellular Carcinoma**<br>Amber Ilyas (Karachi, Pakistan) |
| 12:45 – 13:00 | **ABS-171: Genetically Predicted Circulating Concentrations of Bacterial Metabolites and Hepatobiliary Cancer: A Mendelian Randomization Study**<br>Daniel, Neil (Dublin, Ireland) |
| *13:00 – 15:00* | *Lunch* and **Guided Poster session 2 (even poster numbers)** |
| **15:00 – 17:00** | **Session 5: Tumor cell plasticity**<br>*Chair: Jean-Charles Nault, Kai Breuhahn* |
| 15:00 – 15:30 | **The role of YAP and Hippo signaling in liver growth and cancer**<br>Fernando Camargo (Boston, US) |
| 15:30 – 16:00 | **Harnessing genetic and epigenetic changes for liver cancer therapy**<br>Darjus Tschaharganeh (Heidelberg, Germany) |
| 16:00 – 16:30 | **Beta-catenin mutations in HCC: Implications in tumor biology and therapeutics**<br>Satdarshan (Paul) S. Monga (Pittsburgh, US) |
| 16:30 – 16:45 | **ABS-149: LZTR1 acts as a potent tumor suppressor gene in liver cancer by safeguarding aberrant MAPK activity via posttranslational control of RAS GTPases**<br>Peter Macsek (Heidelberg, Germany) |
| 16:45 – 17:00 | **ABS-109: Prediction of YAP/TAZ activation in liver cancer patients based on gene expression and clinical data**<br>Sofia M.E. Weiler (Heidelberg, Germany) |
| *19:00* | *Conference and networking dinner at restaurant Palais Prinz Carl* |

## FRIDAY, October 13th 2023

| **8:30 – 10:15** | **Session 6: Genetic and epigenetic alterations and targeted therapies**<br>*Chair: Shahid A Khan, Arndt Vogel* |
|---|---|
| 08:30 – 09:00 | **Prognostic and theranostic biomarkers in hepatocellular carcinoma**<br>Jean-Charles Nault (Bobigny, France) |
| 09:00 – 09:30 | **Classification of CCA subtypes based on molecular and genomic features – implications for future trial design**<br>Anna Saborowski (Hannover, Germany) |
| 09:30 – 09:45 | **ABS-160: Myc-dependent replicative response to therapeutic genotoxins in liver cancer cells**<br>Nikita Popov (Tübingen, Germany) |
| 9:45 – 10:00 | **ABS-111: Investigating the Role of YB-1 and DNMTs in Cisplatin-Induced Chemoresistance in Cholangiocarcinoma**<br>Tao Lin (Mannheim, Germany) |
| 10:00 – 10:15 | **ABS-152: PARP-1 inhibition preferentially impairs KRAS mutated intrahepatic cholangiocarcinoma and is mediated by CHK1 kinase**<br>Darko Castven (Lübeck, Germany) |
| *10:15 – 11:00* | *Coffee break and* **Poster viewing** |
| **11:00 – 13:15** | **Session 7: Innovative therapies and novel therapeutic approaches**<br>*Chair: Peter Schirmacher, Chiara Braconi* |
| 11:00 – 11:30 | **Precision medicine in biliary tract cancer**<br>Angela Lamarca (Madrid, Spain) |
| 11:30 – 12:00 | **Systemic therapies in hepatocellular carcinoma – moving the needle on early disease stages**<br>Arndt Vogel (Hannover, Germany; Toronto, Canada) |
| 12:00 – 12:15 | **ABS-176: Multi-center study to evaluate the safety and efficacy of atezolizumab/bevacizumab or lenvatinib in treatment of hepatocellular carcinoma with special focus on bleeding- and thromboembolic-events - Real-world experience from 464 patients**<br>Florian Reiter (Würzburg, Germany) |
| 12:15 – 12:30 | **ABS-139: Exploiting LXRalpha activation for lipotoxic cancer therapies**<br>Pascal Wölffing (Tübingen, Germany) |
| 12:30 – 12:45 | **ABS-173: Plectin-mediated cytoskeletal cross-talk as a target for suppression of hepatocellular carcinoma growth and metastasis**<br>Zuzana Outlá (Prague, Czech Republic) |
| 12:45 – 13:00 | **ABS-162: A tetracycline-inducible model for preclinical testing of immunostimulatory transgenes in solid tumors**<br>Thomas Wirth (Hannover, Germany) |
| 13:00 – 13:15 | **ABS-168: Repurposing passenger amplifications for specific therapeutic targeting of liver and other solid cancers**<br>Sonia Jimenez Vazquez (Heidelberg, Germany) |
| 13:15 – 13:30 | **Closing remarks**<br>Peter Schirmacher (Heidelberg) |



| Poster Number | Abstract ID (ABS) |
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| 1 | 117 |
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| Poster Number | Abstract ID (ABS) |
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# Abstracts





## A long non-coding RNA (lncRNA) signature in blood defines activity of the oncogene yes-associated protein (YAP) in tumor cells of liver cancer patients

Fabian Rose[1], Nada El-Ekiaby[2], Lilija Wehling[1], Sofia M.E. Weiler[1], Marcell Tóth[1], Carsten Sticht[5], Rossella Pellegrino[1], Injie Omar Fawzy[2], Merna Hatem Mohamed Hamad[2], Mohamed Negm[2], Dina Omar[7], Hossam Eldeen Soliman[8], Gamal Esmat[9], Thomas Longerich[1,10], Thomas Illig[4], Bruno C. Köhler[6,10], Anna Saborowski[3], Heike Bantel[3], Arndt Vogel[3], Peter Schirmacher[1,10], Ahmed Ihab Abdelaziz[2,] Kai Breuhahn[1]

1 - Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany
2 - School of Medicine, NewGiza University (NGU), Giza, Egypt
3 - Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School (MHH), Hanover, Germany
4 - Hanover Unified Biobank (HUB), Hannover Medical School (MHH), Hannover, Germany
5 - Core Facility Next Generation Sequencing, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany
6 - Department of Medical Oncology, National Center for Tumor Diseases (NCT) Heidelberg, University Hospital Heidelberg, Heidelberg, Germany
7 - Department of Pathology, Kasr Alainy Faculty of Medicine, Cairo University, Cairo, Egypt
8 - Department of Hepatobiliary and Pancreatic Surgery, National Liver Institute, Menoufiya University, Shebin Elkom, Egypt
9 - Department of Endemic Medicine and Hepatology, Cairo University, Cairo, Egypt
10 - Liver Cancer Center Heidelberg, Heidelberg University Hospital, Heidelberg, Germany

**Question(s):** Body fluids from cancer patients are informative regarding alterations in tumor tissues; however, robust blood biomarkers for the detection of oncogene activity in tumor cells do not exist. Here, we aim to define a set of long non-coding RNAs (lncRNAs) that are transcriptionally regulated by the Hippo pathway-controlled oncogenes yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ).

**Methods used**: NGS data of liver cancer cells after YAP/TAZ silencing, ChIP-seq results as well as patient expression data derived from different tumor types (TCGA) were used to identify differentially regulated lncRNAs. Bioinformatic random forest-based Boruta ranking was used to define predictive lncRNAs. YAP-dependency of lncRNAs and their functional impact were investigated in vitro (e.g., proliferation assay, ChIP analysis) and in vivo (inducible and hepatocyte-specific expression of active $YAP^{S127A}$). YAP expression (immunohistochemistry) and lncRNA signature abundance (in situ hybridization) in human HCCs were investigated. The lncRNA signature in serum of liver cancer patients was correlated with nuclear YAP activity in corresponding tumor tissue (n=24). Receiver operating characteristics (ROC) was used to calculate the predictive power the lncRNAs.

**Result(s):** YAP but not TAZ controls the expression of four lncRNAs in hepatocellular carcinoma (HCC) cells and other cancer cell types (e.g., non-small cell lung cancer). The lncRNA signature consists of CYTOR, MIR4435-2HG, SNHG1, and SNHG17 and defines patients with poor overall survival and the presence of YAP target genes. All tested lncRNAs are transcriptionally regulated by YAP and TEA domain family member (TEAD) transcription factor family members. Equally, lncRNAs are elevated in $YAP^{S127A}$-induced murine tumors and human HCCs with nuclear YAP enrichment. In vitro, CYTOR, MIR4435-2HG, and SNHG17 support HCC cell viability and proliferation. Nuclear YAP positivity if HCC tissues significantly correlates with the presence of the lncRNA signature in corresponding serum samples. The signature in serum predicts YAP activity in HCC tissues with high specificity and sensitivity (AUC: 98%). Lastly, the lncRNA signature characterize HCC cells that respond to YAP-directed pharmacological inhibition.

**Conclusion(s):** We demonstrate that lncRNA signatures in patient serum represent a robust proxy for the activity of druggable oncogenic transcriptional regulators in tumor cells. As the YAP-specific lncRNA signature is detectable in several tumor entities, it may serve as pan-cancer biomarkers for diagnostics and in therapy.

**Support & Funding:** The project was supported by the Deutsche Forschungsgemeinschaft (DFG) and by the SFB/TR 209. In addition, financial support was received from the Alexander von Humboldt-Stiftung.

## α-catenin interaction with YAP/FoxM1/TEAD-induced CEP55 supports liver cancer cell migration

Yingyue Tang[1], Lena Thiess[1], Sofia M.E. Weiler[1], Marcell Tóth[1], Fabian Rose[1], Sabine Merker[2], Thomas Ruppert[2], Peter Schirmacher[1], Kai Breuhahn[1]

1 - Institute of Pathology, University Hospital Heidelberg, Germany
2 - CFMP, Core Facility for Mass Spectrometry & Proteomics at the Center for Molecular Biology (ZMBH), Heidelberg University, Heidelberg, Germany

**Question(s):** Adherens junctions (AJs) facilitate cell-cell contact and contribute to cellular communication; however, the role of α-catenin in hepatocarcinogenesis is controversially discussed. In this study, we aim to investigate how α-catenin contributes to liver cancer formation.

**Methods**: TCGA data was used to identify expression changes in 23 human tumor types. For protein detection, liver cancer tissue microarrays were analyzed by immunohistochemistry. After gene silencing, liver cancer cell lines were used for viability, proliferation, and migration analyses. Mass spectrometry was performed to identify α-catenin binding partners. Results were confirmed by proximity ligation and co-immunoprecipitation assays. Physical binding of transcriptional regulators at gene promoters was investigated using chromatin-immunoprecipitation.

**Result(s):** α-catenin mRNA was significantly reduced in some malignancies (e.g., colon adenocarcinoma). In contrast, elevated α-catenin expression in other cancer entities was associated with poor clinical outcomes (e.g., for hepatocellular carcinoma; HCC). In HCC cells, α-catenin was detectable at the membrane and cytoplasm, supporting tumor cell proliferation and migration. Cytokinesis regulator centrosomal protein 55 (CEP55) was identified as a novel α-catenin-binding protein in the cytoplasm of HCC cells. The physical interaction between α-catenin and CEP55 was associated with CEP55 stabilization. CEP55 was highly expressed in human HCC tissues and its overexpression correlated with poor overall survival and cancer recurrence. Next to the α-catenin-dependent protein stabilization, CEP55 was transcriptionally induced by a complex consisting of TEA domain transcription factors (TEADs), forkhead box M1 (FoxM1), and yes-associated protein (YAP). Surprisingly, CEP55 did not affect HCC cell proliferation but significantly supported migration in conjunction with α-catenin.

**Conclusion(s):** Migration-supporting CEP55 is induced by two independent mechanisms in HCC cells: stabilization through interaction with the AJ protein α-catenin and transcriptional activation via the FoxM1/TEAD/YAP complex.

**Support & Funding:** KB was supported by the Deutsche Forschungsgemeinschaft SFB/TR 209 “Liver Cancer” (31490504) and SPP1782 (414059188). SMEW was supported by the Physician Scientist Program of the Medical Faculty Heidelberg (University Hospital of Heidelberg, Germany). YT was supported by the Chinese Scholarship Council (CSC; 201906230334).





## Chromosome 8p-engineering reveals increased metastatic potential targetable by patient-specific synthetic lethality in liver

Huth, Thorben[1], Dreher, Emely C[1], Lemke, Steffen[2], Fritzsche, Sarah[1], Sugiyanto, Raisatun N[1], Ibberson, David[4], Jauch, Anna[2], Nahnsen, Sven[2], Schirmacher, Peter[1], Roessler, Stephanie[1]

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**Question(s):** Large-scale chromosomal aberrations are prevalent in human cancer but their functional effects remain poorly understood. In hepatocellular carcinoma (HCC), chromosome 8p (chr8p) loss of heterozygosity (chr8pLOH) is observed predominantly and correlates with poor overall patient survival. However, inhibition of no single chr8p tumor suppressor gene revealed strong pro-tumorigenic effects accounting for the increased mortality. Given the extent and complexity of chr8pLOH, this suggested that co-suppression of multiple genes can concomitantly promote tumor growth.

**Methods and Result(s):** To investigate the concurrent loss of over 250 chr8p genes simultaneously, we established chromosome-engineered HCC cell lines using a dual-guided CRISPR-Cas9 genome editing strategy. A 33 mega base-sized region mimicking the frequently observed genomic loss on chr8p was heterozygously deleted and single cell clones were generated. Comparison of chr8pLOH cells with isogenic wildtype clones delineated the functional consequences of chr8p loss. Chr8pLOH cells exhibited a strongly enhanced metastasizing potential and led to the identification of multiple metastasis suppressor genes explaining increased patient mortality.

Subsequently, chromosome-engineered cell clones were used to unravel vulnerabilities caused by the loss of potential bystander genes. Genome-wide CRISPR-Cas9 viability screenings in the isogenic chr8p-deleted liver cancer cells served as a powerful tool for the discovery of novel synthetic lethal targets accompanying this patient-specific chromosomal alteration. Using this target identification strategy, we found that loss of chr8p sensitized tumor cells to targeting of the reactive oxygen sanitizing enzyme Nudix hydrolase 17 (NUDT17). This dependency could be explained by the loss of the paralog NUDT18 located on chr8p. Combinatory loss of both genes resulted in increased oxidized damage and cell cycle arrest confirming a novel synthetic lethal relationship of NUDT17 and NUDT18. In the future, this dependency might be selectively targetable in patients with chr8pLOH.

**Conclusion(s):** Taken together, we describe the engineering of chromosome-scale deletions by CRISPR-Cas9 and its further use as a platform to identify functionally relevant genes and novel vulnerabilities acquired by the arm-level loss of chr8p.

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## Investigating the SLIT-ROBO signaling pathway in cholangiocarcinoma

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**Question(s):** Cholangiocarcinoma (CCA) is a rare but highly aggressive malignancy of the bile duct with inadequate therapy options and poor prognosis. A recent study identified genetic alterations in SLIT/ROBO pathway exclusively in the invasive form of CCA (Goeppert et al. 2021). The SLIT/ROBO pathway acts as axon-guidance-cue during neuronal development and, it additionally orchestrates multiple processes in organ development, like cellular growth, attachment, and motility (Blockus and Chédotal 2016). The aim of this project is to functionally characterize the SLIT/ROBO signaling pathway in CCA.

**Methods used:** ROBO1 and ROBO2 receptors were introduced into CCA cells with a doxycycline-inducible expression system by lentiviral transduction. Using small-interference RNA SLIT1,2,3 were knocked down. Functional analyses were applied to assess the role of ROBO receptors and SLIT ligands in viability, migration, wound healing and colony formation. RNA-seq analysis was performed to detect downstream effects of ROBO signaling. The proximity-based labelling method was applied to identify interacting partners of ROBO2.

**Result(s):** Upon doxycycline-induced overexpression of ROBO1 and ROBO2 receptor, migration ability of CCA cell line was decreased in addition to cell viability. Colony formation assays resulted in decreased colony size and area upon ROBO1 or ROBO2 expression. Furthermore, knockdown of SLIT2 resulted in increased migration and colony area which is consistent with the observed inhibitory role of ROBO receptors. RNA-seq analysis revealed multiple pathways involved in migration and cancer to be regulated by ROBO receptors. Proximity-based biotin ligation assay identified interacting partners of ROBO2 receptor.

**Conclusion(s):** SLIT/ROBO signaling suppressed migration, clonogenicity and cell viability of cholangiocarcinoma cells suggesting a tumor suppressive role in CCA. Further experiments will reveal the role of ROBO interaction partners and crosstalk between signaling pathways in SLIT/ROBO mediated migration suppression.

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## Characterization of CEACAM6 molecular function and mechanism in gallbladder cancer aggressiveness

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**Question(s):** Gallbladder cancer (GBC) is an aggressive malignancy and represents the most common biliary tract cancer (BTC)[1]. There is growing evidence that molecular profiles of GBC are different from other BTC diseases, but the molecular drivers for GBC aggressiveness are poorly identified. Carcinoembryonic Antigen-related Cell Adhesion Molecule 6 (CEACAM6) has been reported to be oncogenic in various tumor entities[2-4], yet CEACAM6’s function in GBC is unclear. This study combines proteomic analysis of GBC patient samples, functional characterization of CEACAM6, and molecular investigation of CEACAM6 in supporting GBC aggressiveness.

**Methods used:** Mass-spectrometric analysis of 5 GBC and 5 healthy gallbladder FFPE tissues was performed. Differentially expressed proteins were analyzed to elucidate oncogenic proteins in GBC. Transient siRNA knockdown and inducible cell lines were established to understand the CEACAM6 molecular function in vitro. RNA sequencing was conducted to reveal significant pathways perturbed by CEACAM6 expression. The in-vivo function of CEACAM6 was accomplished by lateral tail-vein of GBC cells into ATYM-Foxn1nu/nu nude mice and tumor cell growth in the lungs was observed for 4 weeks using the in vivo imaging system (IVIS). BirA-BioID followed by mass-spectrometry was conducted to identify CEACAM6 interaction partners. Co-immunoprecipitation (CoIP), proximity ligation assay (PLA), and siRNA knockdown experiment were performed to validate the interaction partners and their molecular function.

**Result(s):** CEACAM6 presented as one of the strongest upregulated proteins in GBC proteomics (fold-change=5.54, adjusted pval≤0.01). CEACAM6 overexpression promoted migration and invasion of GBC cells, but reduced cell adhesion and colony formation. Less cell adhesion and tumor growth of cells with higher CEACAM6 expression were observed in in vivo upon lateral tail-vein injection. Conversely, the knockdown of CEACAM6 reduced cell proliferation, colony formation, and migration, whereas cell adhesion increased. BirA-BioID followed by CoIP and PLA revealed that CEACAM6 directly interacted with integrin alpha-2 (ITGA2), integrin beta-1 (ITGB1) and protein kinase c delta (PRKCD). Furthermore, CEACAM6 signaling depended on ITGB1. Correspondingly, integrin, AKT, and ERK signaling pathways were among the most significantly enriched pathways after CEACAM6 overexpression. Inhibition of AKT and ERK with inhibitors abrogated their downstream signaling and counter-attacked the CEACAM6-induced migration.

**Conclusion(s):** CEACAM6 supports GBC aggressiveness by promoting cell migration and invasion, but reducing cell adhesion, through the interaction with ITGB1 and PRKCD suggesting a role of CEACAM6 in malignant cell dissemination. Inhibition of CEACAM6 and/or administration of AKT and ERK inhibitors might be potential strategies to abrogate GBC aggressiveness.

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# Analysis of metabolism change in murine hepatocellular carcinoma (mHCC) to identify therapeutic vulnerabilities

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**Question:** Despite the continued success of advanced cancer therapies, hepatocellular carcinoma (HCC) represents a substantial challenge to current treatment modalities that are not sufficiently effective for management of the disease, particularly in more advanced stages. Metabolic reprogramming is a hallmark of cancer as many oncogenic signalling pathways impact on cellular metabolism. In this study, we investigated changes in metabolism in oncogene-driven HCC mouse model to identify metabolic vulnerabilities associated with HCC that could be implicated in tumor initiation and progression

**Methods used:** Transposon-based vectors coding for oncogenes, Myc and Akt1$^{Myr}$ (CamiA) or Myc and Nras$^{G12V}$ (CamiN) were injected by hydrodynamic tail vein injection into C57BL/6 mice. The resulting liver tumours and control livers were subjected to a comprehensive profiling of changes in gene expression (transcriptomics), polar metabolites (metabolomics) and lipids (lipidomics and total fatty acid profiling).

**Results:** We found substantial lipidome remodelling in liver tumours, indicating high levels of lysophospholipids (LPC) in CamiN tumors, while CamiA tumors display higher levels of ether phospholipids compared to normal liver. Moreover, the proportion of polyunsaturated fatty acids (PUFAs) was enhanced in lipids from CamiN tumors, while monounsaturated fatty acid (MUFAs) levels and proportion were enriched in CamiA. Furthermore, tumors from both genotypes produced higher levels of oxylipins, a class of lipid mediators involved in signalling and inflammation, compared to normal liver. Transcriptome analysis confirmed these metabolic alterations and revealed evidence for enhanced inflammation. In addition, analysis of tumor microenvironment composition disclosed specific differences in stromal components.

**Conclusion:** These analyses form the basis for further investigations to identify metabolic vulnerabilities in liver cancer, particularly changes in specific lipid species, that are involved in the production of lipid mediators or function as substrates for lipid peroxidation. The impact of lipid metabolism on immune modulation as well as changing tumor mircoenviornment will be also investigated.





# Prediction of YAP/TAZ activation in liver cancer patients based on gene expression and clinical data

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**Question(s):** The Hippo pathway with its two effector proteins YAP and TAZ is a crucial regulator of cell proliferation and tissue homeostasis [1]. YAP and TAZ are transcriptional regulators and are potent oncogenes in many different tumor entities, e.g. breast and lung cancer. In Hepatocellular carcinoma (HCC), YAP and TAZ are dysregulated in about 60% of HCC patients, correlating with poor survival and early cancer recurrence [2].Currently, several pharmaceutical companies develop specific YAP/TAZ inhibitors that could be used in the future to target cancers with YAP/TAZ activation. However, so far there is no possibility to predict whether a patient is suitable for YAP/TAZ targeted therapy.

**Methods used:** Gene expression and clinical data of 370 HCC patients was obtained from the cancer genome atlas (TCGA) consortium [3]. Two published YAP/TAZ target gene signatures were used for clustering of patient expression data using partioning around medoids algorithm. Differences in survival were assessed by Kaplan-Meier estimates and log-rank test. Correlation with clinical features was done using cross-validated logistic regression. Selection of genes was performed with elastic net regression.

**Result(s):** HCC patients were stratified into different groups according to YAP/TAZ signature expression. Interestingly, subgroups of patients with mutual exclusive activation of one YAP/TAZ target gene signature as well as patients with both signatures exist. Patients with high signature expression showed the worst overall survival and correlated with high AFP serum level, p53 mutation and poor tumor grading. Based on clinical characteristics a predicitve model for YAP/TAZ activation was build. Moreover, a new panel of the most important genes for patient stratification were extracted.

**Conclusion(s):** The results from our analysis could help to identify patients with YAP/TAZ activation based on clinical parameters and a minimal panel of genes. Thus, the combination of clinical data and biomarker expression could be used to robustly select HCC patients eligible for YAP/TAZ-directed therapies.

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## SEPTIN10-mediated crosstalk between cytoskeletal networks controls mechanotransduction and oncogene activity in hepatocarcinogenesis

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**Question(s):** The transcriptional co-activators of the Hippo pathway, YAP and TAZ (*yes-associated protein, transcriptional co-activator with PDZ-binding domain*), are key mechanotrandsducers. Information about cell shape and extracellular matrix composition is relayed to YAP and TAZ mainly through changes in the actin cytoskeleton by promoting their nuclear accumulation and target gene induction[1] However, if and how YAP and TAZ influence structural dynamics of different cytoskeletal elements and oncogenic signaling in hepatocarcinogenesis is unknown.

**Methods used:** Target gene expression in human hepatocellular carcinoma (HCC) cells was analyzed after siRNA inhibition of YAP/TAZ followed by expression profiling. Real-time PCR, Western Blotting and Chromatin-Immunoprecipitation were used to verify target genes. Cell viability, colony formation, migration and spheroid-based invasion assays were used to asses the cellular function of genes. Immunofluorescence, Co-Immunoprecipitation and proximity-ligation assays confirmed interaction partners. Mass-spectrometry identified new interacting partners of the target gene. Analysis of HCC patient data assesed the clincal relevance.

**Result(s):** We show that in liver cancer cells the oncogenes YAP and TAZ transcriptionally control the expression of SEPTIN10, which belongs to the filament-forming cytoskeletal protein family of septins. SEPTIN10 overexpression correlates with poor survival and vascular invasion in *hepatocellular carcinoma* (HCC) patients and promotes YAP/TAZ-dependent cancer cell viability, migration and invasion. Interestingly, SEPTIN10 interacts with actin and microtubule filaments supporting actin stress fibre formation and intracellular tension while inhibiting microtubule polymerization. Mechanistically, SEPTIN10 binds to the actin capping protein CAPZA and prevents its interaction with actin thus promoting actin stress fibre formation. Vice versa, SEPTIN10 inhibits microtubule polymerization by binding to the microtubule stabilizing protein MAP4. This functional antagonism is important for cytoskeleton-dependent feedback activation of YAP/TAZ, as microtubule depolymerization induces actin stress fibre formation and subsequently supports YAP/TAZ activity. Importantly, the crosstalk between microfilament and microtubule networks is mediated by SEPTIN10 as its loss abrogates actin stress fibre formation after microtubule disruption followed by reduced transcriptional YAP/TAZ activity.

**Conclusion(s):** In liver cancer cells, YAP/TAZ-induced SEPTIN10 acts as novel regulator of mechanotransduction by controling the interplay between actin-generated tension and tubulin polymerization. Moreover, our study shows that YAP and TAZ promote their own activation by upregulating SEPTIN10 expression and thus shifting the intracellular tension balance towards active actin stress fibres which subsequently foster YAP/TAZ activation.

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## Investigating the role of YB-1 and DNMTs in Cisplatin-induced chemoresistance in Cholangiocarcinoma

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**Question(s):** Cholangiocarcinoma in advanced stage is mainly treated by cisplatin and gemcitabine. However, efficiency of these drugs is limited due to chemoresistance. To date, the precise mechanisms underlying chemoresistance in cholangiocarcinoma remain largely unknown. This study aims to investigate the role of Y-box binding protein-1 (YB-1) and DNA methyltransferases (DNMTs) in cisplatin-induced cholangiocarcinoma chemoresistance.

**Methods used:**The expression of YB-1 was examined by immunohistochemistry in 28 cholangiocarcinoma patients. Among them, 10 patients received chemotherapy post-operation. The effects and the underlying mechanisms of YB-1 and DNMTs were investigated in vitro.

**Result(s):** YB-1 expression in cancer cells, particularly in the nuclei, was closely associated with clinical outcome of chemotherapy. We followed up 10 patients receiving chemotherapy more than five years. At the end of follow-up, 2 patients without YB-1 expression survived while only 3 out of 8 YB-1 positive patients were alive. In vitro, cisplatin, but not gemcitabine, induced YB-1 nuclear translocation in cholangiocarcinoma cells. Knockdown of YB-1 decreased the cell viability inhibited by cisplatin. The development of chemoresistance to cisplatin was dependent on upregulated MDR1 expression. Cisplatin treatment suppressed the expression of DNMTs, leading to a significant CpG methylation reduction on the MDR1 promoter, which resulted in local chromatin open. YB-1 thus bound to the promoter and increased MDR1 transcription.

**Conclusion(s):** YB-1 plays a critical role in cisplatin-induced chemoresistance in cholangiocarcinoma. YB-1 might be a valuable biomarker to predict chemotherapy efficacy in cholangiocarcinoma. In addition, interfering with YB-1 to improve chemotherapy should be investigated in the future.

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# Recovering the inhibitory impact of miR-105 on IGF1R via knocking down YAP/TAZ-MIR4435-2HG lncRNA axis in HCC

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**Question:** MicroRNAs (miRs) are powerful modulators of gene expression, contributing to the pathogenesis of several diseases [1]. They are sponged by another class of non-coding RNAs, the long non-coding RNAs (lncRNAs), which hinder them from affecting their target genes [2]. We have previously shown the role of some miRs in hepatocellular carcinoma (HCC) pathogenesis via targeting various members of the oncogenic IGF axis[3], [4]. We have characterized 3 oncogenic lncRNAs to be regulated by IGF2BP1 in HCC [5]. Moreover, we have shown YAP and TAZ as upstream regulators of a set of lncRNAs in HCC (see poster F. Rose et al.). Our preliminary bioinformatic analyses predicted one of these lncRNAs (MIR4435-2HG) to sponge miR-105 which may in turn target IGF1R. Therefore, the aim of this work is to experimentally validate the interplay between YAP/TAZ and IGF1R via non-coding RNAs in HCC.

**Methods Used:** miR-105 was screened in serum samples of 8 HCC patients and 10 healthy controls. YAP/TAZ and MIR4435-2HG were knocked down, while miR-105 was overexpressed in Huh-7 cells. We carried out RNA quantification as well as functional analysis experiments, where cell proliferation and clonogenicity were assessed.

**Results:** Knockdown of YAP/TAZ in Huh-7 cells caused a decrease in MIR4435-2HG, an increase in the sponged miR-105, and consequently a decrease in its predicted target gene, IGF1R compared to cells transfected with negative control oligonucleotides. Moreover, upon knocking down MIR4435-2HG, the expression of miR-105 was induced, while IGF1R was suppressed, indicating that MIR4435-2HG mediates its effect on IGF1R via sponging miR-105. Our results also showed that mimicking of miR-105 led to a decrease in IGF1R levels and reduced tumor cell proliferation and colony formation. The biomarker potential of miR-105 was explored, where it was found to be under-expressed in the serum of HCC patients compared to healthy controls.

**Conclusion:** Our data shows that YAP and TAZ act as regulators for IGF1R. They may potentially mediate their oncogenic impact via enhancing the expression of lncRNA MIR4435-2HG, which in turn sponges miR-105, thus reversing its inhibitory effect on IGF1R. miR-105 shows a pattern of downregulation in the serum of HCC patients, which goes along with our previous finding that demonstrated a significant upregulation in the lncRNA MIR4435-2HG. These findings highlight the importance of YAP/TAZ and their downstream ncRNAs as potential therapeutic targets and miR-105 as a possible future biomarker in HCC.

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# Profile of Innate Lymphoid Cells in a real-world cohort of HCC patients treated with Atezolizumab/Bevacizumab

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**Questions:** Hepatocellular carcinoma (HCC) is a very heterogeneous type of cancer. Standard therapy for patients with advanced HCC, atezolizumab (anti-PD-L1) / bevacizumab (anti-VEGF) (Atezo/Bev) intervenes in HCC microenvironment and modifies immune responses. Therefore, we hypothesize that a unique pattern of Innate Lymphoid Cell (ILC) – frequency and –function correlates with clinical characteristics of HCC.

**Methods:** We performed flow-cytometry of PBMCs derived from 75 HCC patients with viral and metabolic etiology of liver disease with and without cirrhosis. We compared ILCs, NK and T cell frequencies between four study groups: healthy subjects, patients with HCC before and after therapy with Atezo/Bev (HCC baseline – first follow-up (3 weeks) – three months follow-up) and tumor-free patients after ablative or surgical therapy (recurrence free) at the time of analysis. Clinical parameters were correlated with flow-cytometry results and therapy response. Future experiments will include single-cell RNA-sequencing analysis including AbSeq surface protein analysis for detailed characterization of the immune cell populations.

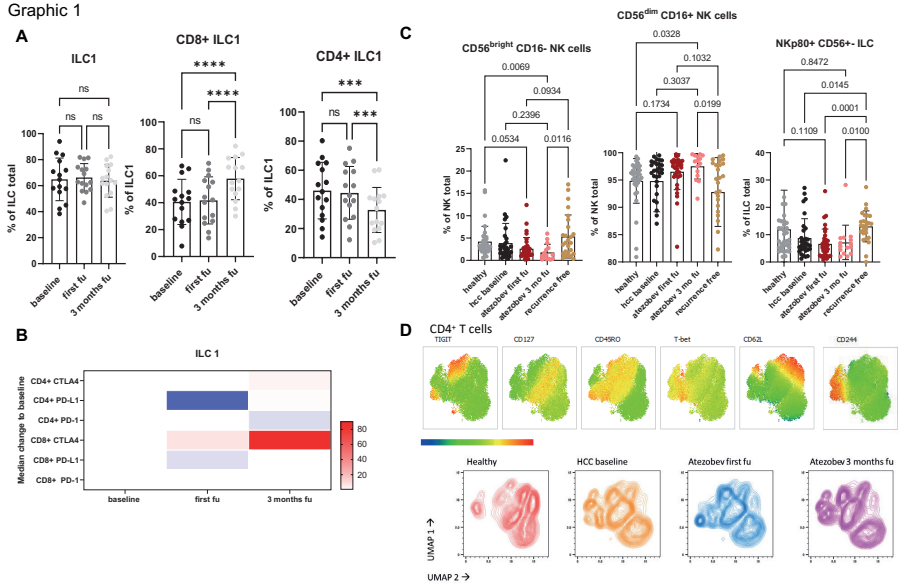
**Results:** Subgroup analysis of ILCs revealed a significant increase of $CD8^+$ ILC1s and decrease of $CD4^+$ ILC1s in the 3 months followup group compared to first followup and baseline HCC patients **(Graphic 1A)**. For further phenotypical analysis, we stained ILC1s for PD-1, CTLA-4 and PD-L1 showing the highest median fluorescence intensity of CTLA4 in three months followup compared to baseline HCC and first follow-up patient group. PD-1 and PD-L1 were very low expressed in all groups **(Graphic 1B)**.

CD56bright NK cells showed a decrease in frequency after systemic therapy in parallel with an increase of CD56dim NK cells. Interestingly, NK cells frequencies were similar in healthy controls and recurrence-free patients, including a high frequency of a previously described cytotoxic $NKp80^+$ NK cell subgroup **(Graphic 1C)**.

Moreover, flow cytometry identified increased exhausted effector memory Th1 phenotype (CD45RO+ CD244+ T-bet+ TIGIT+) in HCC patients compared to healthy controls, independent of treatment with Atezo/Bev **(Graphic 1D)**.

**Conclusions:** Our results suggest that treatment with Atezo/Bev alters ILCs, NK and T cell composition in PBMCs of patients with HCC. Composition of ILCs indicates a more mature but exhausted phenotype after therapy. Further analyses may reveal connections between ILCs and the underlying liver diseases and the response to treatment.

Graphic 1



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Graphic 1: A) Frequencies of ILC1s in PBMCs of patients with HCC before and after therapy with Atezo/Bev. Baseline=before therapy; first fu=first follow up (3 weeks); 3 months fu=follow up. B) Relative expression of checkpoint inhibitors PD1, PD-L1 and CTLA-4 on ILC1s measured by flow cytometry. D) UMAP showing expression of activation and exhaustion marker on CD4+ T cells. Same UMAP showing density of cells within different patient groups.

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**Association of miRNAs expression with patient outcome in non-viral hepatocellular carcinoma**

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**Question:** Are patient outcomes associated with miRNA expression in non-viral HCC [1]. Can miRNA be considered as potential biomarker in non-viral HCC?

**Methods:** Tissue samples were acquired from 51 HCC of non-viral etiology patients, who had undergone resection but had not received neo-adjuvant therapy prior operations. miRNA profiling with Agilent SurePrint G3 arrays was performed on 45 pairs of tumor and adjacent non-tumor tissue and 6 additional tumor samples. Microarray results were analyzed using the GeneSpring v14. Raw data were normalized by quantile normalisation. Significant differences were determined by paired *t*-tests with Benjamini-Hochberg correction. miRNA was considered differentially expressed when FC ≥ 1.5 and corrected p-value ≤ 0.05

**Results:** We found 39 differentially expressed miRNA. Among those miR-381-3p was associated with time to recurrence (TTR) in Univariable cox model and with TTR (p = 0.0004) and disease-free survival (DFS) (p=0.007) in Kaplan–Meier (K-M) analysis shown in (figure 1). miR-21-5p, which was upregulated in tumor tissues, showed significantly longer overall survival (OS) (p = 0.002) in subset of patients with diagnosed NASH. Moreover, correlation between clinic-pathological variables such as, tumor grade, AFP concentration, TNM rank and presence of multiple nodules were also observed.

**Conclusion:** Our results suggest that certain miRNAs may affect patient survival and might be used as a diagnostic biomarker non-viral liver cancer.

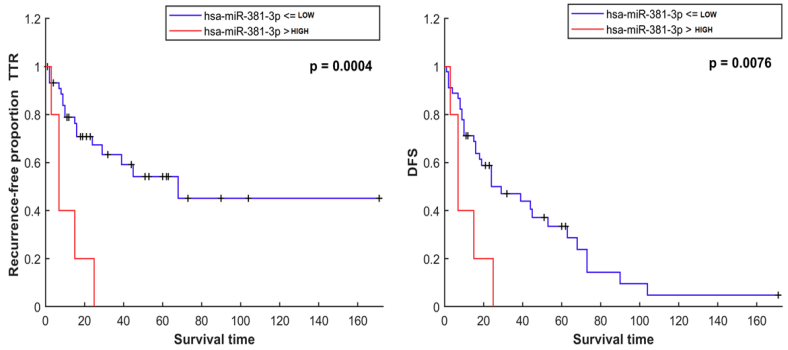


Figure 1 shown K-M analysis miR-381-3p TTR and DFS





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Funding

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## A novel murine organoid-based FGFR2 fusion-driven cholangiocarcinoma model to study the impact of frequently co-altered genes on the tumor microenvironment

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**Question(s):** Fibroblast growth factor receptor 2 (FGFR2) gene rearrangements are among the most common oncogenic alterations found in human intrahepatic cholangiocarcinoma (iCCA) at a frequency of around 12-15%.[1] Several FGFR inhibitors are in clinical development or already used in clinical routine but with limited efficacy both in terms of objective response rates (ORR) and duration of response.[2] Co-alterations in tumor suppressor genes such as *BAP1*, *CDKN2A/B*, *PBRM1*, and *PTEN*, commonly found in FGFR2 fusion iCCAs, might influence the tumor biology and therefore therapeutic response.[3] Due to the overall low frequency of these co-occurring alterations, clinical data is so far not suitable enough to assess the impact of these co-mutations in the context of FGFR inhibition. Current FGFR2 fusion iCCA mouse models rely on overexpression of a human fusion transcript and used additional oncogenes, such as $Kras^{G12D}$, that seem to affect growth and anti-tumor immune response patterns.[4,5]
We therefore aimed to generate a preclinical model developing iCCAs with an endogenous FGFR2 fusion in an immune-competent system to investigate these iCCA subtypes.

**Methods used:** We used CRISPR/Cas9 genetic engineering and functional selection to introduce an endogenous *Fgfr2* fusion with *Ahcyl1*, one of the most common fusion partner genes, in murine cholangiocyte organoids, and afterwards developed various FGFR2 fusion-driven iCCA subtypes by separately deleting the frequently co-altered genes *Bap1*, *Cdkn2a/b*, *Pbrm1,* and *Pten*. The iCCA lines were then orthotopically injected into the mouse liver in a syngeneic system. The harvested tumors are being evaluated for differences in growth patterns, morphological and immunological variabilities by image and molecular analyses based on H&E staining and specific immunohistochemical markers. The spatial distribution of immune cell populations such as CD8+ and CD4+ T cells, Treg-s, M1 and M2 TAMs are being analysed in each subtype.

**Result(s):** We successfully generated endogenous *Fgfr2-Ahcyl1* fusion in *Trp53*-deleted murine cholangiocyte organoid clones, using CRISPR/Cas9 genetic engineering and functional selection. The expression of the fusion protein and its ligand-independent activity have been confirmed by Western Blot. We then successfully knocked-out the selected co-altered genes to create a library of various FGFR2 fusion-driven iCCA subtypes. Upon intrahepatic implantation, *Cdkn2a/b* and *Pten* co-deleted FGFR2 fusion-driven organoid iCCA lines so far have successfully developed into iCCA. The orthotopically grown tumors show morphological and immunohistochemical characteristics of iCCA and attract host immune and mesenchymal cells to generate a complex tumor microenvironment.

**Conclusion(s):** This novel cholangiocyte organoid-based FGFR2 fusion-driven iCCA implantation model is a promising tool to systematically investigate oncogenic effects on tumor biology and TME formation in a syngeneic system.

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## Loss of Fibroblast growth factor 21 impairs liver regeneration and accelerates hepatocelullar carcinoma development in a murine model of liver injury

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**Question(s):** Hepatocellular carcinoma (HCC) is the most common primary liver cancer and a leading cause of cancer-related deaths worldwide. In order to identify potential biomarkers for cancer therapies, a better molecular understanding of the development of HCC will be critical. Recent data provide evidence that fibroblast growth factor 21 (FGF21), mainly expressed in the liver, is a novel therapeutic target for non-alcoholic fatty-liver disease (NASH), in which loss of FGF21 promotes tumorigenesis. Here, we aimed to investigate the role of FGF21 in liver damage, regeneration and hepatocarcinogenesis in the *Fah* model, a non-metabolic liver injury model.

**Methods used:** $Fah^{-/-}$ mice, which were used, develop liver injury and HCC due to the accumulation of the toxic metabolite fumarylacetoacetate, which can be prevented by treatment with nitisinone (NTBC). The $Fah^{-/-}$ mice were crossbred to $FGF21^{-/-}$ mice. Liver tissues of $Fah/FGF21^{-/-}$ and the respective $Fah^{-/-}$ controls that received either a cyclic treatment or reduced dose of NTBC were examined macroscopically and histologically and compared to healthy livers (100 % NTBC). To determine tumor penetrance and the degree of liver damage, H&E, Sirius Red and Oil Red stainings were performed. These results were validated by liver transaminases as biochemical marker of increased liver injury. Liver weight and Ki67 expression were used as surrogates for the regenerative capacity either after acute liver injury or partial hepatectomy (PH).

**Result(s):** After FGF21 loss, tumor onset were accelerated and tumor penetrance was higher in the setting of moderate liver injury (reduced NTBC dose), although liver injury was not increased. Whereas in mice with severe liver injury (cyclic NTBC treatment) loss of FGF21 did not accelerate carcinogenesis, but excaberated liver fibrosis. In the acute liver injury, basal regeneration rate of $Fah/FGF21^{-/-}$ livers was significantly impaired compared to $Fah^{-/-}$ control mice, despite increased liver injury in these livers. However, the regeneration capacity of $FGF21^{-/-}$ livers was not impaired compared with $FGF21^{wt/wt}$ livers. RNA Sequencing just performed will show first results on downstream signaling pathways regulated by FGF21. The distribution of immune cells in the injured liver will also be investigated.

**Conclusion(s):** Overall, FGF21 was found to play a critical role in the regenerative capacity of livers after acute liver injury but not in healthy livers after PH, indicating a context-dependent role of FGF21 for liver regeneration. Further, our data revealed that loss of FGF21 significantly accelerates hepatocarcinogenesis in the setting of moderate liver injury in the *Fah* model. Recently published phase-II clinical trial showed that an FGF21 analogue significantly improved a number of metabolic and inflammatory parameters in patients with NASH. Our data suggest that FGF21 may also be an interesting chemopreventive agent for patients suffering from non-metabolic liver diseases.

# Assessing the role of microbial metabolites in hepatocellular cancer development through targeted mining of high-resolution untargeted metabolomics data

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**Question:** Dysbiosis of the human gut microbiome is implicated in the development of hepatocellular carcinoma (HCC). Some gut-derived microbial metabolites may be pro-inflammatory and growth-promoting, while others may be cancer protective. Currently most known information about metabolic changes occurring in HCC derives from case-control studies, while information from prospective, observational cohort studies remains limited. We explored associations between circulating metabolites related to gut microbial metabolism and HCC risk in a case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

**Methods used:** A detailed literature search of peer-reviewed articles and on-line metabolite databases was conducted to compile a diverse target list of microbial metabolism-related compounds to be examined in association with HCC development. We employed untargeted, high-resolution liquid chromatography-mass spectrometry (LC-MS), using reversed phase and hydrophilic interaction chromatography both in positive and negative polarities, to detect thousands of metabolic features in pre-diagnostically collected serum samples from n=110 HCC cases matched 1:1 with healthy control participants from the EPIC cohort. In EPIC detailed lifestyle data and biospecimens were collected at baseline from >520,000 apparently healthy participants who were then followed up over time for cancer diagnoses. Gut microbiome-related metabolites were annotated from LC-MS features detected in the serum samples. Multivariable adjusted conditional logistic regression models were applied to assess the relationship between each matched metabolite and HCC risk.

**Results:** A large number of LC-MS features were successfully matched with the 473 microbiome-related metabolites identified in the literature search based on chemical formula and isotopic pattern. After Benjamini-Hochberg correction for multiple testing, HCC risk associations were observed for 14 annotated metabolites from diverse chemical classes. In general, bile acids, amino acids and their metabolites, tocopherols, amines and cholines were associated with risk of HCC.

**Conclusions:** Our findings contribute to the evidence base on microbial metabolism-related compounds and HCC risk. This project provides preliminary insight into metabolic perturbations linked to gut microbiome dysbiosis and their potential involvement in HCC development. They also highlight a unique approach of targeted analysis of specific metabolites from within untargeted metabolomic features.





# Establishment and characterization of patient derived xenografts and patient cell lines as preclinical model of intrahepatic cholangiocarcinoma

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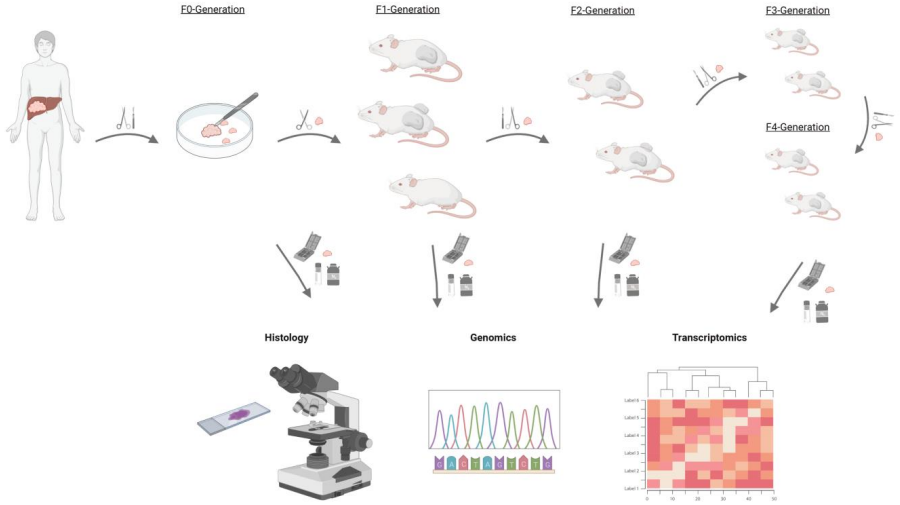
**Question(s):** Cholangiocarcinoma (CCA) is the second common type of liver cancer. 5-year-survival remains below 10 %, mainly caused by the aggressive biology, late diagnosis and lack of curative therapies. In order to develop more effective therapies, there is an urgent need for a better molecular understanding of CCA as well as for appropriate preclinical models for drug testing. Primary tumor cells allow for *in vitro* studies and subsequent *in vivo* experiments, whereas patient derived xenografts (PDX) serve as valuable tools to mimic physiologic conditions *in vivo*. Here, we generated and characterized PDX and tumor cell models of intrahepatic cholangiocarcinoma (iCCA).

**Methods used:** Primary tumor material from resected patients (n = 12) was subcutaneously implanted into immunodeficient mice, and monitored for tumor growth. Successfully engrafted tumors were serially re-transplanted for up to four generations. In addition, primary and PDX tumors were enzymatically digested in order to generate two-dimensional cell lines. Established cell lines were subsequently injected into immunodeficient mice. PDX and cell line-derived tumors were subjected to comparative histologic evaluation and to molecular characterization by DNA as well as RNA sequencing.

**Result(s):** PDXs were established with a success rate of 50% (n = 6/12) with a latency between 39 and 280 days. Upon re-transplantation of PDX tumor tissue, latency was significantly accelerated and penetrance was nearly complete across up to three subsequent transplantations. Histologically, PDX tumors overall resembled the parental tumors, but stroma content was reduced. The morphology of replicate PDXs upon re-transplantation was highly comparable. Succes rate of tumor cell line generation was 17 % (n = 2/12), and for both established cell lines, matching PDX models are available. Based on transcriptome analysis, parental tumors and the respective PDXs clustered closely together, and stromal signatures were longitudinally downregulated upon *in vivo* passaging. Further characterization was performed on a PDX/matched primary tumor-derived cell line pair with ERBB2 overexpression.

**Conclusion(s):** We established and serially passaged six PDXs from iCCA patients undergoing surgical resections. PDXs retained key morphological and molecular characteristics of the original patient tumor. Additionally, we were able to generate primary tumor cell lines from two patients. We expect, that thoroughly characterized preclinical models will serve as valuable tool for drug testing and to understand primary and secondary resistance mechanisms to targeted therapies.



## The contribution of autophagy in hepatic stellate cells on tumour initiation and progression.

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**Question(s):** The tumour microenvironment (TME) interacts with cancer cells, which leads to the development and metastasis of tumours. A major component of the TME are cancer-associated fibroblasts (CAFs), which play a crucial role in proliferation, metastasis, and angiogenesis of cancer. One hallmark of CAFs is the induction of autophagy, which supports the cancer cells to overcome metabolic stress, maintain homeostasis, and surviving. In hepatocellular carcinoma those CAFs derive from activated hepatic stellate cells (HSC). It has been already shown that HSCs are involved in HCC initiation due to a crosstalk with hepatocytes and immune cells. However, the contribution of autophagy in HSCs during HCC development remains still elusive. In this study we investigated the impact of disturbed autophagy in HSCs during liver cancer development.

**Methods used:** To investigate the impact of impaired autophagy of HSCs we crossed mice with transgenic Cre-expression under the control of HSC specific lecithinretinoal acetyltransferease promoter (lrat-Cre) with $mTOR^{flox}$ mice ($mTOR^{HSC\text{-}KO}$) resulting in hyperactivated autophagy, as well as $AGT5^{flox}$ mice ($ATG5^{HSC\text{-}KO}$) resulting in inhibited autophagy in HSCs and used the well-established DEN/$CCl_4$-HCC model in these mice. In this model, mice received a single *i.p.* injection of the genotoxic agent diethylnitrosamine (DEN), followed by repetitive injections of the toxin carbon tetraclorid ($CCl_4$) leading to chronic liver injury, activation of HSCs, liver fibrosis, and HCC development. After sacrifice, serum and liver tissue samples were collected and analysed by western blot, qPCR, and immunohistochemistry.

**Result(s):** The deletion of *mTOR* in HSCs almost completely prevented cancer development after DEN/$CCl_4$ treatment whereas no difference in HCC development in $ATG5^{HSC\text{-}KO}$ mice compared to WT controls was detectable. Furthermore, we found that ablation of *mTOR* in HSCs was also associated with highly significant decrease in liver weight of untreated 19-month-old animals. In contrast, $ATG5^{HSC\text{-}KO}$ animals did not develop a spontaneous phenotype.

**Conclusion(s):** Contrary to our initial assumption, our data suggest that impairment of autophagy in HSCs does not contribute significantly to HCC development in our selected DEN/$CCl_4$ cancer model. Despite this, we hypothesise that mTOR signalling in HSCs is a critical regulator of liver growth as well as communication between HSCs and tumour cells during hepatocarcinogenesis. In addition, mTOR has been shown to be involved in the regulation of several fundamental cellular processes such as metabolism, proliferation and cell death. Therefore, we believe that the mTOR signalling pathway in HSCs could be an interesting target for anti-tumour therapy research.

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## Dissecting cell death and NF-κB in TAK1-deficient livers.

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**Question:** Hepatocellular carcinoma (HCC), the common end stage of chronic liver diseases, arises almost exclusively in the context of chronic hepatic inflammation. In chronic liver disease, hepatocyte cell death is a prominent feature driving inflammation and progression to hepatic fibrosis and finally HCC. The transcription factor NF-κB is one of the key regulators of inflammatory processes, but its function in hepatocarcinogenesis has remained controversial. Mice with conditional deletion of *Tak1* (TGF-β-Activated-Kinase-1) in liver parenchymal cells (LPC; $TAK1^{LPC\text{-}KO}$) display severe hepatic inflammation at young age characterized by inhibition of the NF-κB signaling pathway and LPC apoptosis and necroptosis, proceeding over time to liver fibrosis and liver cancer, but also to severe lethal cholestasis. While either apoptosis or necroptosis has a fundamentally different impact on inflammation, cholestasis, and hepatocarcinogenesis in $TAK1^{LPC\text{-}KO}$ mice, the function of NF-κB remains elusive.

**Methods used:** To examine the impact of reactivation of the NF-κB signaling in $TAK1^{LPC\text{-}KO}$ mice, we crossed mice expressing a Cre-dependent, dominant active form of the NF-κB-inducing kinase IKK2 ($IKK2^{ca}$) with $TAK1^{LPC\text{-}KO}$ mice ($TAK1^{LPC\text{-}KO}$/$IKK2^{LPC\text{-}ca}$). The spontaneous phenotype of these mice was characterized and the molecular mechanisms underlying this phenotype were analysed by genetic, histological, and biochemical methods.

**Results:** We demonstrated that reactivation of NF-κB abrogated hepatocarcinogenesis in young $TAK1^{LPC\text{-}KO}$ mice due to the inhibition of LPC apoptosis, but exacerbated lethal cholestasis due to enhanced ductopenia.

**Conclusion:** While reactivation of the NF-κB signaling pathway in $TAK1^{LPC\text{-}KO}$ mice prevented hepatocarcinogenesis, the active NF-κB signaling exacerbated lethal cholestasis due to a previously unknown function of NF-κB in cholangiocytes associated with the loss of bile ducts.

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**Support & Funding:** This work was funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program through the ERC Consolidator Grant ''PhaseControl'' (grant agreement 771083) and the German Research Foundation (DFG – LU 1360/3-2 (279874820), LU 1360/4- (146170493) and the German Cancer Aid (Deutsche Krebshilfe —70114893).

## Spatial analysis and clinical correlation of the immune tumor microenvironment in extrahepatic cholangiocarcinoma using multiplex immunohistochemistry.

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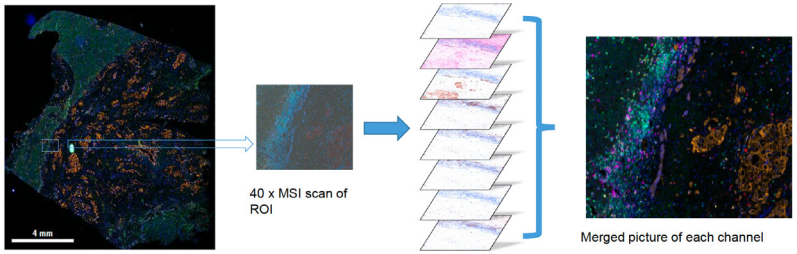
**Questions:** Immune checkpoint inhibitors (ICIs) that specifically modify programmed cell death protein-1/-ligand 1 (PD-1/PD-L1) or cytotoxic T-lymphocyte antigen-4 (CTLA-4) have been proposed for therapeutic approaches against extrahepatic cholangiocarcinoma (eCCA). Alternative ICIs are currently also under preclinical and clinical investigations. A deeper understanding of the immune tumor microenvironment (iTME) of eCAA will be critical to identify potential biomarkers and to develop a more stratified strategy of using immunomodulatory cancer therapies for patients with eCCAs. Here, we set out to spatially resolve and clinically correlate the immune infiltrates as well as immune checkpoint expressions in eCCAs using multiplex immunohistochemistry (mIHC).

**Methods used:** FFPE tissues from 97 resected and clinically annotated eCCAs were selected by pathologist based on H&E stains. Using a mIHC platform (Phenoptics system, Akoya Bioscience), the tissue sections derived from primary eCCAs will be stained for the following markers: CD3, CD4, CD8, FoxP3, CD20, CD68, CD15, CD34, PD-1, PD-L1, CTLA-4, TIGIT, LAG-3 and pan-Cytokeratin mix AE1/3. Regions of interests (ROIs) for subsequent multispectral high-resolution images will go through machine learning process for quantitative analysis.

**Results:** Intertumoral heterogeneity will be studied by separating tissue sections into distinct regions, such as tumor center, margin, stroma, etc. The spatial compositions and densities of different immune cells will be detected by marker panels across all regions, while quantitative assessments of immune checkpoint expression levels will be performed in the same setting. Clinical correlations of both immune cell infiltrations and immune checkpoint expressions will be investigated respectively.

**Conclusions:** Our study on the iTME not only will reveal spatial structures of primary eCCAs, but also could show potential for a more precise estimation of prognosis and response to immunomodulatory cancer therapies in patients with eCCAs. In addition, quantitative analysis and better understanding of the spatially resolved and clinically annotated iTME might be necessary for identifying translationally relevant biomarkers, which are critical for developing novel therapeutic approaches and conducting clinical trials of immunotherapies.




Graphic 1: Regions of interests will be chosen and subsequent multispectral high-resolution images will be taken in differernt tissue regions. Each channel represents one distinct marker in the tumor microenvironment and all channels for the same tissue sections will be merged together for quantitative assessments.

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Support & Funding: AstraZeneca PLC.





## Hypofractionated Carbon Ion Radiation vs. Photon based SBRT in primary liver cancer

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**Question(s):**
Treatment resistance and delayed symptom onset contribute to liver cancer being the third most common cause of cancer-related fatalities. The utilization of external beam radiotherapy (EBRT) for the management of inoperable liver cancer remains undefined.
In recent years, safety and efficacy of EBRT have improved dramatically by implementing stereotactic body radiation (SBRT) and the use of particle therapies such as carbon ion radiation therapy (CIRT). Photon based SBRT is included within the European guidelines for treatment of HCC but European data on CIRT for liver cancer is lacking. The present study analyses efficacy and safety of hypofractionated CIRT for liver cancer compared to photon-based SBRT in a single-center retrospective trial.

**Methods used:** Thirty-six (n = 36) patients with primary malignant liver tumors (Hepatocellular Carcinoma n=32, Cholangiocarcinoma n=3, Mixed n=1) were treated with hypofractionated CIRT between 2011 and 2022 and retrospectively compared to Twenty-two (n = 22) patients with primary liver cancer (17 patients with HCC, 5 patients with iCCA) who were treated with photon based SBRT concerning survival, local control and toxicity. For a matched pair analysis, 18 patients of each cohort could be included and matched for tumor size, tumor type, sex and prior tumor treatment.

**Results:** Comparison of oncological outcomes in the two cohorts revealed a longer distant Progression Free Survival (D-PFS) for CIRT vs. photon based SBRT (2-year D-PFS 63% vs. 25%; p = 0.0182). In a matched pair analysis, CIRT showed a significantly better Overall Survival compared to SBRT (2-year OS 100% vs. 63%; p = 0.0369). The median total applied dose of CIRT was 38 Gy (RBE, Range: 35.2 – 42 Gy), divided into 4 single doses every other day. The median biological equivalent dose (BED, $\alpha/\beta$=10) of the photon based SBRT cohort was 83 Gy (Range: 48 -150 Gy, 3-10 fractions). Median size of irradiated lesions was 3.5 cm (Range: 1.3 – 9.6 cm) for CIRT and 2.9 cm (Range: 1.3 – 6.8 cm) for photon based SBRT. No dose limiting toxicities were reported in both cohorts.

**Conclusion(s):** In this retrospective comparison to photon based SBRT, hypofractionated CIRT seems to score with both better Overall and Progression Free Survival. By minimizing the radiation dose to the

surrounding liver tissue, CIRT has the potential to expand the therapeutic range of liver irradiation for individuals with significantly compromised liver function.

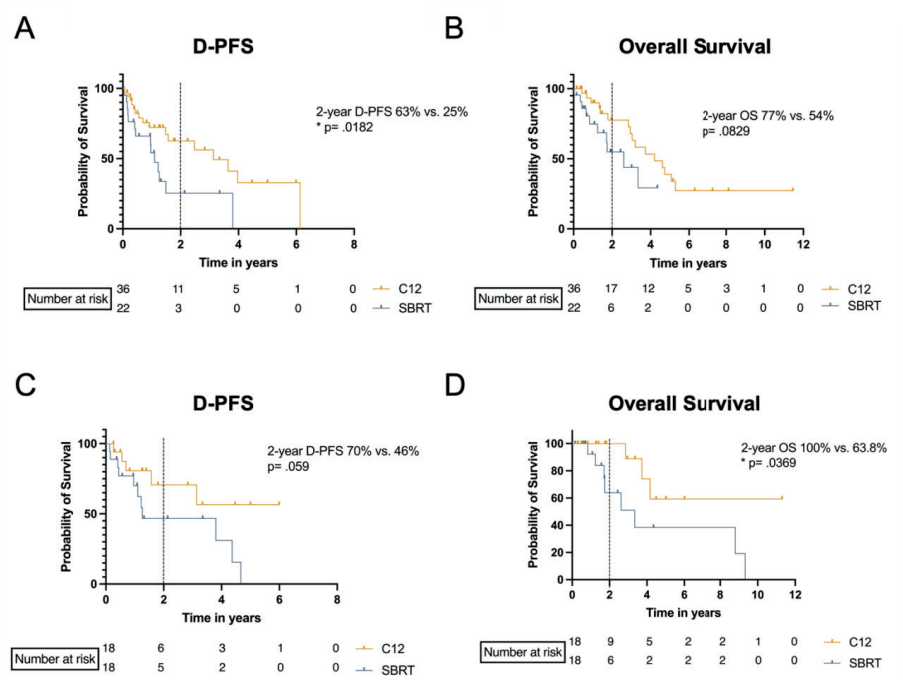


Fig. 1: Hypofractionated CIRT (C12) shows higher distant Progression Free Survival (D-PFS) and Overall Survival (OS) than Photon based SBRT within the whole cohort (A-B) as well as in a matched pair analysis (C-D).





## Prognostic role of innate and adaptive immune cells in the microenvironment of hepatocellular carcinoma after resection

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Question: Infiltration by innate and adaptive immune cells at the microenvironment of resected hepatocellular carcinoma (HCC) was associated significantly with patients' prognosis (1). However, the prognostic significance of immune cells in different intra- and peritumoral regions is still unclear (2). Are the spatial distribution of specific immune cells in resected tissues of HCC and the combination of their different subtypes could impact patients 'survival?

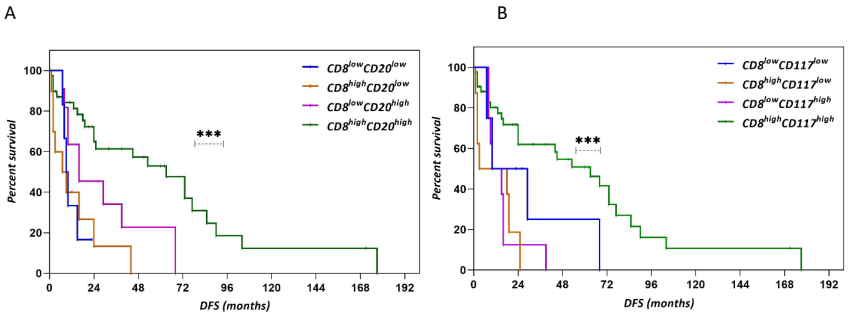
Methods used: The clinicopathological and follow-up data of 67 patients, who underwent curative resection of HCC, were collected. Lymphocytes (CD8+ T cells and CD20+ B cells) and innate immune cells (CD68+, CD163+ macrophages and CD117+ mast cells (MCs)), were evaluated by immunohistochemistry on representative resected specimens of HCC and adjacent tissues. In the tumor center (TC), inner margin (IM), outer margin (OM), and peritumor (PT) liver, the density of lymphocytes was estimated stereologically whereas the area fraction of innate immune cells was defined automatically using QuPath image analysis software. The raw data of density and area fraction were converted into percentilles and categorized into low (<25th) and high (>25th). To highlight possible interactions between two different types of cells, we assigned patients into low/low, high/low, low/high and high/high groups. We used the Kaplan– Meier analysis to test the association of detected immune cells individually and in pairs with disease-free survival (DFS).

Results: Taken alone, $CD20^{high}$, $CD8^{high}$, $CD68^{high}$ and $CD117^{high}$ in the IM was associated with longer DFS. $CD163^{high}$ in PT liver correlated with shorter DFS.

Among combinations of two cell types, which individually predicted significant DFS, patients with $CD8^{high}CD20^{high}$ in IM showed significantly longer DFS compared to groups of high/low and low/high. (Graphic 1). Similarly, $CD8^{high}CD117^{high}$ in IM had longer DFS compared to $CD8^{high}$ or $CD117^{high}$ only groups (Graphic 1).

Conclusion: groups of high lymphocytes, MCs and CD68+ macrophages in the IM were associated with a longer DFS, indicating the antitumor effect of both adaptive and innate immune cells and the specific role of IM region. CD163+ cells in PT liver had a protumor effect. Synergism between CD8+ T cells on one side and CD20+ B cells and CD117+ MCS on the other side may suggest their pair cooperation for the creation of a long/lasting memory immune response against HCC.

Graphic1:




Graph1. DFS according to combined low vs. high CD8/CD20 (A) and CD8/CD117 (B) in the inner margin.

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Support & Funding:

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# Deciphering the role of VPS72 in hepatocellular carcinoma

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**Question(s):** Liver cancer is the sixth most prevalent cancer in terms of incident cases and the second leading cause of cancer related mortality worldwide[1]. Unlike other cancer types, mortality of liver cancer is drastically increasing and underlying molecular mechanisms are still not deciphered completely. Hepatocellular carcinoma (HCC) is a major liver cancer subtype, having an incidence/mortality ratio close to 1.0, presented with limited treatment options[2]. Hence, it is of utmost importance to hunt for novel molecular targets to treat HCC. The etiologies of liver cancer drive unique genetic alterations and epigenetic modifications. Past research in liver cancer was majorly focused on studying genetic alterations, leaving large scope of research in elucidating the role of and targeting epigenetic modifications. Chromatin remodeling is an important epigenetic event which alter nucleosome architecture that governs transcriptional control of genome. The chromatin remodeling complexes SRCAP and TRRAP have been reported to be derugulated in different cancer types; however, their exact role in liver cancer still remains elusive. VPS72 is the H2A.Z incorporation chaperon and the common subunit of SRCAP and TRRAP complex. Recently, it was shown that both H2A.Z. and VPS72 are oncogenic in HCC[3,4]. However, the exact underlying detailed mechanism how VPS72 regulate HCC is still elusive.

**Methods used:** In this study, we performed TCGA bioinformatic analysis to analyze the expression levels of SRCAP and TRRAP components in liver cancer. *In vitro* cell culture assays were used to investigate the effect of the gain or loss of function of VPS72 on cancer hallmarks like colony formation, migration, and cell cycle. Differential gene expression analysis was performed by using bulk RNA-sequencing to elucidate the underlying mechanism of VPS72 that drives HCC. Hydrodynamic tail vein injection (HDTVi) of VPS72 alone, and with other drivers of HCC was also performed to check effect of VPS72 on cancer development or progression.

**Result(s):** VPS72 was most upregulated and amplified among all subunits of SRCAP and TRRAP complex. High VPS72 expression correlated with overall and progression free survival in HCC. High expression of VPS72 in HCC was grade dependent but etiology independent. VPS72 gain of function increased anchorage dependant colony formation ability, while VPS72 loss of function reduced anchorage dependant colony formation and migration ability of HCC cells. Loss of function of VPS72 arrested cells in G0/G1 and G2/M phase. HDTVi of VPS72 alone did not induce tumors, but HDTVi of VPS72 in combination with other cancer drivers like Nicd1 and cMyc induced liver tumors.

**Conclusion(s):** VPS72 is a novel molecular target in HCC and inhibiting it could be effective treatment option for HCC.

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# Hepatic stellate cell-derived Rspo3 sensitizes Hepatocytes to peri-central liver damage

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**Question(s):** In the mouse liver, both liver sinusoidal endothelial cell (LSEC) and hepatic stellate cell (HSC) express Rspondin 3 (Rspo3). Working as a Wnt signaling regulator, LSEC-derived (angiocrine) Wnt signals control liver growth and metabolic maturation1. However, the role of HSC-derived Wnt signals remains elusive, regardless of its higher expression than LSEC in the liver. Recently, it has been shown that HSC-secreted Wnt is dispensable for zonation and the development of liver fibrosis2. Therefore, elucidating the function of HSC-derived Wnt signaling, especially Rspo3, is firmly required for a comprehensive understanding about the Wnt signaling in the liver. This study aims at unveiling the ambiguous functions of HSC-expressed Rspo3 in the liver, in regulating physiological and pathological liver.

**Methods used:** Hepatic stellate cell-specific Rspo3 knockout mouse is generated. With different kind of liver injury models, including CCl4 and APAP administration, bile duct ligation, and partial hepatectomy, the functional roles of HSC-derived Rspo3 in regulating physiological and pathological liver is studied. HSC is isolated and RNA-seq is performed for unveiling the mechanism of HSC-expressed Rspo3 during fibrosis and HCC progression.

**Result(s):** In physiological condition, HSC-specific Rspo3 knockout mice show similar liver function and well established zonation as in wild type littermates, as well as no effects in hepatocyte proliferation after partial hepatectomy. However, in pathological conditions, with peri-central liver injury models, HSC-Rspo3 knockout mice show mitigated liver damage than the control animals (Fig. A-D). In per-portal liver injury model, both group show comparable liver damage (Fig. E-G). LSEC-specific Rspo3 knockout animals, as well as HSC-specific Wnt signal knockout mice with same challenges did not show any similar phenotypes (Fig. H-K).

**Conclusion(s):** Our results indicate that HSC-derived Rspo3 shows limited effects in establishing the homeostasis of the liver. However, it plays critical roles during fibrogensis in the peri-central liver injury models, but not peri-portal liver injury models. This may serve as a potential target for anti-fibrosis therapy.

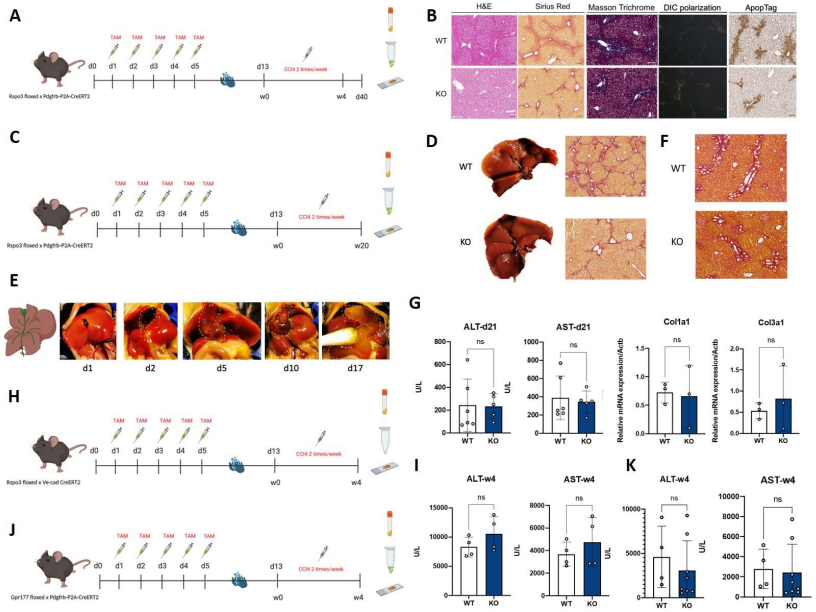






Figure 1: Key data for the presented project. (A) Shematic overview of CCl4-induced liver injury model. (B) Representative images of H&E, Sirius red, Masson trichrome, Differential Interference Contrast, and ApopTag staining images from the CCl4-treated mouse liver. (C) Shematic overview of CCl4-induced HCC model. (D) Representative photos of the lesion-bearing liver and Sirius red staining images. (E) Overview of the BDL model and photos of the liver and gall bladder at different time points. (F) Representative Sirius Red Staining images from the d21 bile duct ligated mouse. (G) Plasma biochemisty and qPCR analyses of the bile duct ligated mouse. (H) Shematic overview of CCl4-induced liver injury model with LSEC-specific Rspo3 KO mice. (I) Plasma biochemisty analysis of the LSEC-specific Rspo3 KO mice. (J) Shematic overview of CCl4-induced liver injury model with HSC-specific Gpr177 KO mice. (K) Plasma biochemisty analysis of the HSC-specific Gpr177 KO mice.

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# Mucosal-associated invariant T cells are rendered dysfunctional within the tumour microenvironment in HCC in a cell-contact dependent manner

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**Question:** Hepatocellular carcinoma (HCC) is one of the major causes of cancer death worldwide[1]. Immunotherapy has recently become the first-line treatment for advanced HCC, although response rates remain low[2]. MAIT cells, innate-like T cells particularly enriched in the liver, express pro-inflammatory cytokines as well as cytolytic molecules[3-5] and have therefore been attributed anti-tumour properties. In this study, we aim to decipher direct interactions between MAIT cells and HCC cells in order to unravel mechanisms of immune cell exhaustion within the tumour microenvironment in HCC.

**Methods:** MAIT cells were isolated from tumour tissue, adjacent liver tissue and peripheral blood of human patients with HCC or other liver tumours and healthy controls. Primary MAIT cells were co-cultured with various HCC cell lines *in vitro* and MAIT cell phenotype and function was analysed by multi-colour flow cytometry.

**Results:** We show that MAIT cells frequency is significantly reduced in peripheral blood of HCC patients compared to healthy controls, as well as in HCC tumour tissue compared to the adjacent liver tissue. Such MAIT cells loss was specific, since frequency of conventional T cell subsets was unaffected in HCC and MAIT cell frequency was unchanged in other liver tumours. Whereas MAIT cells from peripheral blood of HCC patients remained functional, tumour-educated liver-derived MAIT cells showed increased exhaustion marker expression and significantly impaired effector function, suggesting MAIT cell exhaustion within the tumour microenvironment in HCC. Interestingly, such MAIT cell dysfunction could be induced by co-culture of MAIT cells with different HCC cell lines, while the hepatocyte-like cell line HepG2 was unable to induce MAIT cell exhaustion. Mechanistically, induction of MAIT cell exhaustion by HCC cells was dependent on direct cell-cell contact.

**Conclusions:** Taken together, we show that MAIT cells, innate-like T cells with anti-cancer potential, are rendered dysfunctional within the HCC microenvironment, suggesting MAIT cells as a potential target for novel anti-cancer therapies in HCC. Understanding mechanisms of local MAIT cell exhaustion in HCC may facilitate the development of novel immunotherapeutic strategies against HCC.

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ABS-127, Poster Number 12

**Mining the "dark proteome" to identify novel biomarkers and target molecules in hepatocellular carcinoma**

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**Questions:** A key remaining frontier in our understanding of biological systems is the "dark proteome"—that is, proteins encoded by long noncoding RNAs (lncRNAs) where the molecular function is largely unknown. The key aspect of this work is that it combines big data mining and pathology to explore the "dark proteome" in hepatocellular carcinoma (HCC), a highly aggressive cancer with limited therapeutic options. Considering that there are very few accurate molecular biomarkers for HCC detection, understanding function for the entities involved and their potential role in diagnosis and patient stratification will bring substantial impact in HCC therapy.

**Method used:** We performed DNase I hypersensitive sites sequencing and RNA-seq for HepG2 cells and control liver to map open chromatin regions that associate with transcription of HCC-specific lncRNAs. Polysome profiling and ribosome sequencing were applied to identify lncRNAs that are translated. Peptide specific antibodies were generated for C20orf204-189AA and Linc013026-68AA, two of HCC-specific lncRNA-encoded small proteins. Immunohistochemical staining and biochemical assays were performed to examine the expression of these novel proteins in a HCC cohort and the underlying molecular functions.

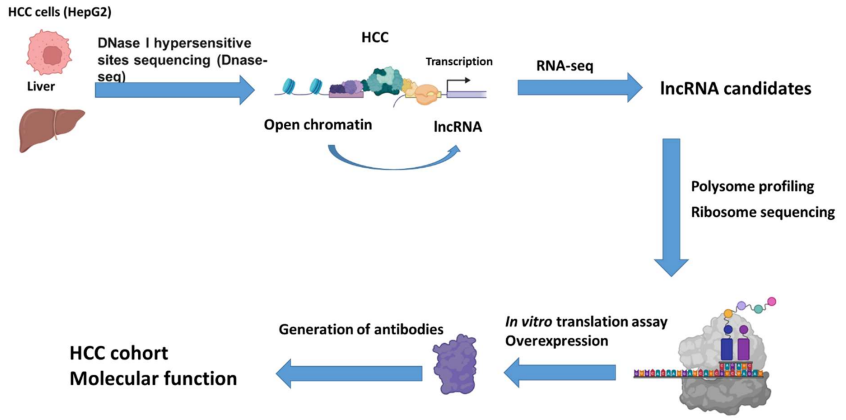
**Results:** We successfully generated specific antibodies for C20orf204-189AA and Linc013026-68AA, two of HCC-specific lncRNA-encoded small proteins. Both proteins promote cancer cell proliferation. At the molecular level, we show that C20orf204-189AA participates in ribosomal RNA transcription, while Linc013026-68AA may be phosphorylated by Epidermal Growth Factor Receptor (EGFR) and extracellular signal-regulated kinase (ERK). Remarkably, C20orf204-189AA protein was detected in 70% of primary HCCs but not in but not in control livers, suggesting that HCC-specific lncRNA-encoded proteins may represent a novel class of biomarkers and HCC targets.

**Conclusion:** Our finding provides important insights into molecular functions of small proteins originating from "dark proteome" and their potential value as biomarkers or drug targets in HCC.

**Figure:** Strategy to identify HCC-specific lncRNA encoded small proteins (created with BioRender.com)



ABS-128, Poster Number 23




**Support & Funding:** German Research Foundation (DFG), die Gesellschaft der Freunde der MHH (GdF)

## The context-dependent role of *c-myc* in liver regeneration and hepatocarcinogenesis in chronic liver injury

MARHENKE, SILKE[1], DE CASTRO, TIAGO[1], TRAN, DOAN DUY HAI[1], KLETT, STEPHANIE[1], GUENTHER, GEORGIA[2], HEIKENWAELDER, MATHIAS[3], VARTAK, NACHIKET[2], HENGSTLER, JAN[2], SABOROWSKI, ANNA[1], VOGEL, ARNDT[1]

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**Question(s):** The highly pleiotropic transcription factor C-MYC is known to control a variety of cellular processes, like cell cycle progression, proliferation, growth, adhesion, differentiation, apoptosis, and metabolism. The expression of C-MYC is frequently dysregulated in human hepatocellular carcinoma (HCC), and considered to be a driver of malignant transformation. Despite its impact on HCC, the physiological role of *c-myc* during acute and chronic liver injury remains enigmatic. We used different murine models to explore loss of *c-myc* on liver injury, regeneration and carcinogenesis.

**Methods used:** To investigate the effect of *c-myc* in different liver disease settings, we crossed conditional *c-myc*$^{fl/fl}$ to *Mdr2*$^{-/-}$ and *Fah*$^{-/-}$ mice, and excise *c-myc* expression by liver-specific Cre's. The mice were analyzed for biochemical and metabolic parameters and tumor development. Additionally, partial hepatectomies (PH) were performed to assess regenerative response, and bile duct ligations (BDL) were performed to describe the effects of acute biliary injury. Furthermore, the influence of *c-myc* on the bile flow were investigated.

**Result(s):** Consistent with its role as a driver of hepatocarcinogenesis, loss of *c-myc* delayed tumor development in *Fah*$^{-/-}$ mice. In contrast, tumor development was significantly accelerated in *Mdr2*$^{-/-}$ mice after loss of *c-myc*. Consequently, the biochemical parameters of liver injury were elevated in *Mdr2/c-myc*$^{\Delta/\Delta}$ mice, accompanied by more fibrosis. The increased liver injury was concomitant with an impaired regenerative response, which is likely a direct consequence of the loss of *c-myc*, as indicated by decreased number of Ki67-positive cells in *c-myc*$^{\Delta/\Delta}$ mice after PH. In contrast, liver regeneration was not affected in *Fah/c-myc*$^{\Delta/\Delta}$ mice, suggesting a context specific role of *c-myc* in liver regeneration. *Mdr2/c-myc*$^{\Delta/\Delta}$ exhibited altered energy metabolism, reflected by decreased baseline ATP levels, and an imbalanced metabolite profile of the TCA cycle. In addition, bile acid composition was severely altered in *Mdr2/c-myc*$^{\Delta/\Delta}$ mice, and transcriptome analysis revealed downregulation of a number of bile acid transporters in the liver, such as Slc10a1 and Slco1a1. Previously, increased susceptibility to cholestatic damage was reported in *Slco1a1* knockout mice.[1] Of note, liver injury was markedly increased in *c-myc*$^{\Delta/\Delta}$ mice following BDL, suggesting *c-myc* regulated Slco1a1 protects mice from bile acid-induced liver injury.

**Conclusion(s):** Our results reveal a context dependent role of *c-myc* during liver injury and injury-induced tumorigenesis. Of note, dysregulation of bile duct transporters after loss of c-myc sensitizes mice to cholestatic damage, leading to massively increased liver injury and accelerated tumorigenesis

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## Targeting mucosal-associated invariant T (MAIT) cells for immunotherapy of HCC

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**Question(s):** The cellular composition of the Hepatocellular Carcinoma (HCC) tumor microenvironment (TME) has a major impact on tumor initiation, progress, and therapy response [1]. Mucosal-associated invariant T (MAIT) are an abundant T cell subtype in the human liver and play a crucial role in regulating immunity and inflammation [2]. Yet, their role in HCC and their potential for cancer immunotherapy remains elusive [3]. Here, we studied MAIT cell phenotype and function in HCC using primary patient samples and murine models.

**Methods used:** High-dimensional flow cytometry (n=37) and single-cell RNA sequencing (n=8) was used to analyze MAIT cell phenotypic changes in tumor tissue from HCC patients. We used highly multiplexed immunofluorescence microscopy (CODEX[4], n=15) to simultaneously profile in situ expression of 37 proteins. A novel machine learning (ML) algorithm (S3-CIMA[5]) was developed to quantify the MAIT cell interaction network at the HCC invasive front. Murine models of orthotopic, syngeneic HCC were used for in vivo validation. In vitro co-culture systems using MAITs from primary human liver tissue were established (**Figure 1**).

**Result(s):** MAIT-deficient *Mr1*$^{-/-}$ mice showed a lower tumor burden ($p<0.01$) of orthotopic HCC compared to WT mice, indicating a protective role of MAIT cells. scRNA-seq uncovered MAIT cell heterogeneity in HCC as MAIT cells in patient samples were characterized by impaired infiltration ($p<0.001$) into tumors, increasing dysfunction and loss-of-cytotoxicity within the TME. Spatial CODEX imaging revealed the cellular interaction network underlying MAIT cell dysfunction. S3-CIMA analysis identified interactions of CSF1R$^{+}$PD-L1$^{+}$ tumor-associated macrophages (TAMs) and MAIT cells localized in the non-tumor liver as key regulatory elements of MAIT cell dysfunction. Ex vivo co-culture of MAITs show suppressive activity of autologous TAMs. Perturbation of this cell-cell interaction in vitro or in vivo through conditional knockout of PD-L1 on murine TAMs reinvigorated the cytotoxic MAIT cell phenotype ($p<0.01$). Lastly, targeting MAIT cells through aPD-L1 or MAIT-ligand 5-OP-RU demonstrated that these cells can be potent orchestrators of anti-tumor immunity in vivo with pronounced anti-tumor activity against various models of liver cancer.

**Conclusion(s):** We show that MAIT anti-tumor immunity and response to aPD-L1 relies on organized, spatially nuanced interactions between MAITs and TAMs within the TME. These studies also identify MAITs as a novel target for immunotherapy in HCC.

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Figure 1: Study design

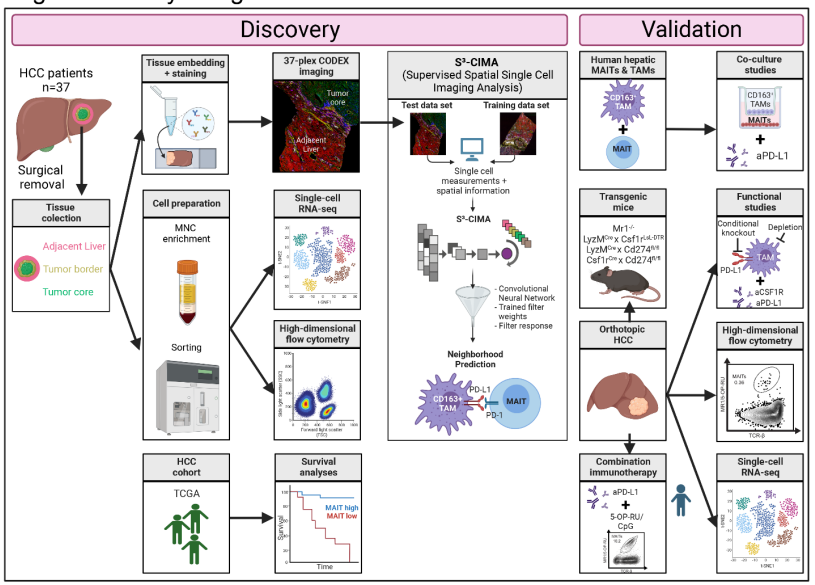


***Figure 1: Study design***

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# Building a data science ecosystem for the SFB/TR 209 liver cancer research data

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**Question(s):**
Projects within the collaborative research areas of the Liver Cancer SFB/TR 209 produced complex data including high-throughput raw datasets and its bioinformatics data analysis results. A professional data management plan was required that allowed not only to store this data alongside its associated metadata, but also to share and re-use the data across many groups and sites.

**Methods used:**
In order to tackle these challenges, the core facility infrastructure of the Quantitative Biology Center (QBiC) of the University of Tübingen was used, to provide efficient and scalable solutions for project- and data management and bioinformatics workflow systems primarily based on nf-core (https://nf-co.re/).
Experimental metadata, raw data files and bioinformatics results corresponding to different -omics datasets (e.g. RNA, DNA or proteins) were collected and stored in the database of QBiC from 2017 to 2022, which is accessible for collaborators via its web interface qPortal using a dedicated LDAP server system.
QBiC assisted the end users throughout the whole life cycle of a typical research project starting from experimental design towards final data analysis. As QBiC is also a central part of nf-core, analysis was done using highly automated best-practice nf-core pipelines that allowed for standardization and reproducibility of the analysis. In addition, QBiC's data secure concept assures that data privacy is assured but re-use of existing data can be requested by collaborators and granted by the data owner.

**Result(s):**
QBiC supported 38 projects from different research areas, which could be distributed into 6 main categories of analysis: RNA-Seq (47%), DNA-Seq (16%), Proteomics (16%), Single Cell (5%), Microarrays (5%), Data Management-"only" (projects without Bioinformatic support) (5%), Amplicons (3%) and Multi-Omics (3%). 1653 samples were registered corresponding to a total of ~25 TB distributed mostly into DNA-seq (15.1 TB), followed by Proteomics (4.4 TB), RNA-Seq (2.8 TB), and Project Management (1.3 TB). Overall, the collaboration within the SFB/TR 209 led to 15 publications in different research areas of Hepatocellular Carcinoma: Molecular Biology, signalling and treatment (n=5), Metabolism (n=2), Data Science Methods for Cancer Research (n=5) and Genetic Factors (n=3).

**Conclusion(s):**
QBiC was able to provide a sustainable data science ecosystem with geo-redundant and long-term data storage, in which raw data is embedded in an existing data model that allows for intuitive association with biological samples, its metadata, derivatives (intermediate and final results) as well as the underlying experimental design. Bringing raw data and its metadata close together with a nf-core workflow-based repository, along with existing computation resources allowed also for efficient data analysis using state-of-the-art bioinformatics pipelines.

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## Transcriptional profiling of tumor-specific CD8 T cells shows contribution of TIGIT to T cell exhaustion in liver cancer

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**Question(s):** Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death and the fifth most common kind of cancer worldwide. This cancer has a 5-year survival rate of 10% and its increasing incidence requires the development of efficacious treatments against HCC. Recent advances in immunotherapy demonstrated its capability in treatment of cancer, but there is still potential for further development of immunotherapies against liver cancer. Immune responses against cancer are often hampered by upregulation of co-inhibitory receptors on the surface of CD8 T cells. This inhibition leads to emergence of T cell exhaustion, where tumor-infiltrating lymphocytes (TILs) show a reduced proliferative capacity and low production of effector cytokines IFNγ and TNFα, a mechanism that impedes tumor rejection by CD8 T cells. We wanted to analyse molecular changes in exhausted tumor-specific CD8 T cells in liver cancer.

**Methods used:** For the generation of an autochtonous liver cancer mouse model we utilized the *Sleeping beauty*" (SB) and transposon system. Adoptive transfer of specific CD8 T cells, followed by T cell vaccination were used for induction of a measurable tumor-specific T cell response that was characterized by flow cytometry. Whole transcriptome microarray was performed for elucidation of changes in exhausted tumor-specific CD8 T cells.

**Result(s):** By using the *Sleeping beauty*" (SB) and transposon system we developed an autochtonous HCC mouse model. Using adoptive T cell transfer allowed us in-depth phenotyping of tumor-specific CD8 T cells and we could demonstrate pronounced upregulation of co-inhibitory receptors PD-1, TIM-3, CD160, LAG-3, 2B4 on T cell surface. The tumor-specific CD8 T cells also showed a reduced cytokine production and degranulation capacity, indicating the emergence of T cell exhaustion. In order to elucidate the molecular cause of tumor-induced T cell exhaustion we have performed the first whole transcriptome microarray analysis of tumor-specific CD8 T cells in a murine autochthonous liver cancer model, that allowed us to compare the mRNA profiles of naive, functional effector and exhausted tumor-specific CD8 T cells. The comprehensive transcriptomic data represents a means for the identification of candidate genes and pathways that play a role in T cell exhaustion. Particularly, the substantial upregulation of TIGIT suggested the involvement of this inhibitory T cell receptor in T cell exhaustion in liver cancer. Utilization of immune checkpoint-blockade against TIGIT in combination with PD-1 inhibition prolonged survival of tumor-bearing mice, compared to single inhibition of PD-1. We could further verify the expression of TIGIT on tumor-infiltrating CD8 T cells in patients with liver cancer.

**Conclusion(s):** Our results suggest that TIGIT is involved in the appearance of T cell exhaustion in human liver cancer and presents a potential target for combination treatment by immune checkpoint blockade.

**Support & Funding:** Deutsche Forschungsgemeinschaft (DFG)

# Integrative Analysis of Signal Transduction and Metabolism to Promote Early Detection of Liver Cancer

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## Abstract

Liver cancer is becoming one of the most prevalent cancers in Western countries with hepatocellular carcinoma (HCC) being the most common form. Chronic obesity frequently leads to non-alcoholic fatty liver disease (NAFLD) that can progress to HCC even in absence of cirrhosis. As the prevalence of NAFLD is massively increasing, case numbers of NAFLD-associated non-cirrhotic HCC are on the rise as well.

We employ a systems biological approach connecting alterations of metabolic function as a consequence of NAFLD to changes in signal transduction. These changes may promote NAFLD progression towards non-cirrhotic HCC. High heterogeneity and non-linearity of such alterations represent a major obstacle in understanding molecular mechanisms of dynamic disease progression. Mathematical models are a powerful tool to handle complexity and to overcome such difficulties. To this end, we are developing an integrated mathematical model based on ordinary differential equations which considers both the metabolic state of the cell as well as hepatocyte growth factor (HGF)-induced signal transduction. We examined the time-resolved dynamics of HGF-induced signal transduction in the hepatoma cell line Huh7 in the presence or upon deprivation of glucose or glutamine using quantitative immunoblotting. Global proteome analysis indicated changes in the PI3K/AKT signal transduction pathway when glucose or glutamine was depleted. Upon glucose deprivation, we observed a HGF-dependent decrease in AMP-activated protein kinase (AMPK) phosphorylation and similarities in the dynamics of AMPK and AKT phosphorylation. Furthermore, we showed that AKT phosphorylates the key enzyme executing the rate limiting step in glycolysis, PFKFB2. These observations point to a close interlink between AKT signaling and sensing of the metabolic state. To validate the observations obtained in the cell line Huh7, we are investigating the HGF-induced signal transduction in isolated primary human hepatocytes (PHH) from NAFLD patients at different disease stages. This data indicated a decreased HGF-induced phosphorylation of the MET receptor in steatotic hepatocytes compared to non-steatotic hepatocytes. We are currently investigating the global proteome of the same PHHs employing an in depth global proteomics approach based on data independent acquisition (DIA). Our mathematical model is applied to identify characteristic proteomic alterations characteristic for NAFLD progression. The data emphasizes the deep interconnection between metabolism and signal transduction and provides evidence on how alterations in either part of the system affect the other. With our mathematical model we aim to understand the molecular circuits in a more holistic way. These insights may offer new perspectives for cancer prevention in the context of NAFLD.





Abstract

# Disentangling synergies: Unraveling TGFβ and GAS6/AXL signal transduction in cirrhosis and HCC

***Elisa Holstein*, Severin Bang, Viktor Makarenko, Daniel Lill, Seddik Hammad, Steven Dooley, Lorenza A. D'Alessandro, Barbara Helm, Marcel Schilling, Jens Timmer and Ursula Klingmüller**

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Chronic liver damage comprising fibrosis and development of cirrhosis is the main risk factor for HCC development. Fibrosis and cirrhosis are characterized by hepatic stellate cell activation, which in this stage drive fundamental changes in the liver architecture due to alteration of the extracellular matrix (ECM) composition and its associated proteins, i.e. the matrisome. One of the known drivers for hepatic stellate cell activation is transforming growth factor (TGFβ), but also the growth arrest specific (GAS) 6/AXL pathway plays an important role in fibrosis and cirrhosis. To understand the complex non-linear reactions induced by TGFβ and GAS6/AXL signal transduction contributing to hepatic stellate cell activation and fibrogenesis, we employed a systems biology approach utilizing ordinary differential equations (ODE) for mechanistic modeling. We calibrated the mechanistic model for TGFβ signal transduction, which was developed in hepatoma cells (Lucarelli et al. Cell Syst 2018), to the hepatic stellate cell line LX2 based on time and dose resolved immunoblotting data. The differences in Smad protein abundance were key to explain the cell type specific TGFβ induced target gene expression by the model. Next, we developed a mathematical model for GAS6/AXL signal transduction, which revealed that the differences in phosphorylated S6 kinase (pS6K) was able to explain distinct phosphorylation dynamics of GAS6/AXL pathway components in hepatoma and hepatic stellate cells. Proteomic changes in the hepatic stellate cell line LX2 in response to TGFβ and GAS6 were characterized by employing data-independent-acquisition mass spectrometry (DIA-MS). We found that co-stimulation with TGFβ and GAS6 leads to higher collagen protein production in LX2 compared to stimulation with TGFβ or GAS6 alone, which implies a synergistic effect. Currently, the TGFβ and GAS6/AXL models are being connected to investigate how both pathways influence each other to describe and predict their impact on ECM protein abundance and cell proliferation. Since collagen production was recently identified as critical matrisomal parameter in liver carcinogenesis, we hypothesize that these synergies are able to accelerate crossing the tipping point towards HCC by preparing the pre-tumor microenvironment.

## Deciphering the role of RAGE in cholangiocytes in diet induced mouse model of liver injury and HCC progression.

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**Question(s):** To detemine the role of RAGE (receptor for advanced glycation end products) signaling in liver injury induced by choline deficient ethionine diet (CDE).
Is RAGE in cholangiocytes a key regulator of DR and fibrosis upon chronic liver injury?

**Methods used:** We generated tamoxifen-inducible cholangiocyte-specific RAGE KO mice using HNF1beta promoter- driven Cre recombination. These mice also express Cre- dependent tdTomato thereby exclusively labelling bile duct- lining cells. RAGE KO mice and WT controls were subjected to choline-deficient ethionine (CDE) diet or normal diet for 3 weeks. Intravital imaging was performed at weeks 0 and 3 onmouse livers to determine bile transport function *in vivo* using the bile salt analog CLF and directly imaging its uptake from blood and subsequent secretion into the biliary system. Additionally, we performed serum chemistry for biomarkers of liver damage, inflammation and cholestasis. Mouse livers from Week 0 and Week 3 were harvested for immunohistochemical analysis of KRT19 and sirius red in order to visalize the extent of ductular reaction (DR) and liver fibrosis, respectively.

**Result(s):** WT mice on CDE diet showed an extensive DR, that almost completely replaced the bile canalicular network in the liver parenchyma. In contrast, RAGE KO mice on CDE diet did neither display a massive DR, nor branching and/or invasion of cholangiocytes. WT mice on CDE diet also had severely compromised bile transport function and elevated ALT/AST levels indicating CDE mediated liver injury. Serum bile acids in these mice were increased upto 5-fold corroborating their severe cholestatic state due compromised bile transport. On the other hand, RAGE KO mice on CDE diet also displayed elevated ALT/AST as markers of liver injury, but had bile transport kinetics indistinguishable from WT or RAGE KO mice that were on nomal diet.

**Conclusion(s):** In sum, these results indicate that RAGE receptor activation and signaling during liver injury is essential to trigger DR. Despite its function to clear bile during acute liver injury, under chronic conditions the inflammatory component of the ductular reaction appears to be the primary cause of loss of bile transport function in hepatocytes resulting in cholestasis. These findings suggest that during chronic liver disease, functionality of the liver may be more efficiently maintained by inhibiting RAGE signaling in cholangiocytes in order to prevent an invasive ductular reaction and, thus, attenuate fibrosis and progression to HCC.

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DKFZ microscope core Facility: Dr. Felix Bestvater





## Identification of Basal MET Phosphorylation as Indicator of Hepatocyte Dysregulation in Liver Disease

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Chronic liver diseases are worldwide on the rise. Due to the rapidly increasing incidence, in particular in Western countries, Non-alcoholic fatty liver disease (NAFLD) is gaining importance as the disease considerably increases the risk of hepatocellular carcinoma. Lipid accumulation in hepatocytes has been identified as the characteristic structural change in NAFLD development, but the molecular mechanisms responsible for disease progression remained unresolved. Here, we uncover in primary hepatocytes from a preclinical model fed with a Western diet (WD) a strong downregulation of the PI3K-AKT pathway and an upregulation of the MAPK pathway. Furthermore, we employ dynamic pathway modeling of hepatocyte growth factor (HGF) signal transduction combined with global proteomics, and we identify that an elevated basal MET phosphorylation rate is the main driver of altered signaling in WD-hepatocytes. Importantly, the elevated basal phosphorylation of MET leads to increased proliferation of primary hepatocytes, which may imply that this dysregulation could favor cancer formation. Lastly, by adapting our model to patient-derived hepatocytes we reveal that patient-specific variability in basal MET phosphorylation correlates with patient outcome after liver surgery. Thus, dysregulated basal MET phosphorylation could be an indicator for the health status of the liver and thereby inform on the risk of a patient to suffer from liver failure after surgery.

# Dissecting the tumor heterogeneity and tumor microenvironment in intrahepatic cholangiocarcinoma using spatial transcriptomics.

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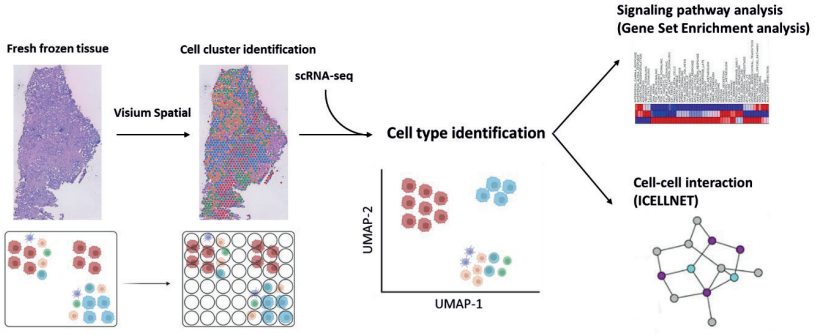
**Question(s):** Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary liver malignancy after hepatocellular carcinoma (HCC) with limited therapeutic options. Immunotherapy (IO) has revolutionized cancer therapy during the last decade, offering durable responses with an acceptable safety profile. In biliary malignancies, addition of the immune checkpoint inhibitor to Gemcitabine/Cisplatin has recently become the standard of care in first line treatment, however, the overall survival benefit is moderate in an all-comer population. To facilitate patient stratification, and to explore new strategies, it will be critical to characterize and better understand the interplay between tumor cells and the immune microenvironment.

**Methods used:** We combined spatial transcriptomics (ST) with published single-cell RNA sequencing (scRNA-seq) of iCCA patients to identify the subpopulations of tumor cells and spatial structures in the tumor microenvironment. We also inferred cell-to-cell relationships from high throughput ligand-receptor interaction measurements within tissue sections.

**Result(s):** Spatial transcriptomic analysis at high resolution uncovers distinct subpopulations of tumor cells exhibiting unique patterns of signaling pathway signatures such as KRAS, Myc, TGFß, and Wnt/beta-catenin. Intriguingly, we have identified specific tumor subpopulations that express a set of genes either minimally or not expressed in other tumor subpopulations or non-tumor cells. We further validated these novel gene sets in an iCCA cohort (n= 134). Notably, one of these gene sets exhibited a significant association with FGFR2-fusion and NRAS mutation, implying that the utilization of spatial transcriptomics for profiling tumor subpopulations could aid in the identification of novel iCCA subgroups. Moreover, our observations revealed a higher prevalence of a tumor immune barrier structure in stroma-rich tumors, while stroma-poor tumor cells exhibited a greater infiltration of macrophages. Analysis of cell-to-cell communication unveiled that interactions between ligand-receptor pairs, namely CCL2/CCR2, CCL18/PITPNM3, and CX3CL1/CX3CR1, were more frequent in tumor cells infiltrated with macrophages. Notably, most of macrophage populations are associated with myeloid-derived suppressor cell (MDSC) gene signature. In both, stroma rich and poor tumor, tumor cells express vascular endothelial growth factor VEGFA, which can stimulate endothelial cells through VEGF receptor suggesting the pro-angiogenic shift in the state of the tumor-associated endothelial subpopulation.

**Conclusion(s):** Our data suggest that an immune suppressive tumor microenvironment associates with suppressive myeloid populations, in addition to high stromal angiogenic activity. Our work thus provides a highly detailed and comprehensive analysis of the iCCA tumor microenvironment and an exploratory analysis of tumor-stromal cell interactions.




Graphic 1: Workflow description (created with BioRender.com)



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## Loss of tumor suppressive function of PRSS23 fosters hepatocellular carcinoma development in the context of activated c-Myc signaling

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**Question:** Human hepatocarcinogenesis is a step-wise process, in which premalignant dysplastic nodules (DN) progress to malignant hepatocellular carcinoma (HCC). During this process, somatic mutations accumulate and are clonally selected, in case they exert a driver gene function. In this study, we aimed to identify and validate tumor suppressive loss-of-function mutations clonally expanded during the malignant transformation of liver cancer cells.

**Methods used:** Whole exome sequencing was performed on the different tissue compartments of an HCC case with nodule-in-nodule appearance to identify clonally expanded variants. These were validated by Sanger sequencing. Loss of function mutations were functionally validated in p53 heterozygous mice using hydro-dynamic tail vein co-injection of a Myc-myrAKT1 transposon vector with a targeted shRNA library. Further *in vivo* validation was performed by single variant injection. Murine tumor-derived isogenic cell lines either overexpressing the wild type (WT) or the mutated (Mut) candidate gene were generated for functional characterization, which included expression profiling, ultrastructural analysis, live cell imaging with Mitotracker staining, biochemical and functional assays.

**Results:** Exome sequencing identified 17 clonally expanded variants. An *in vivo* RNAi screening targeting each of these candidates revealed the enrichment of three independent shRNAs targeting PRSS23. Expression of the PRSS23 variant (p.P230A) in MYC overexpressing *Tp53* heterozygous mice led to higher tumor incidence compared to $PRSS23^{WT}$. Expression profiling of $PRSS23^{Mut}$ and $PRSS23^{WT}$ cells revealed activation of Sirtuin- and PI3K/AKT- and downregulation of p53-, AMPK-, and mTOR signaling pathways in $PRSS23^{Mut}$ expressing cells. In addition, the cell viability and the ATP production rate were increased compared to $PRSS23^{WT}$ expressing cells. Surprisingly, variant-independent PRSS23 showed a distinct mitochondrial localization in liver cancer cells. More interestingly, $PRSS23^{Mut}$ improved the mitochondrial function as morphologically indicated by longer mitochondrial branches, a feature of mitochondrial fusion.

**Conclusions:** Integration of human whole exome sequencing data and murine *in vivo* RNAi screening identified PRSS23 as a new tumor suppressor gene during hepatocarcinogenesis in context of activated c-Myc signaling. Our data assign a new mitochondrial function to PRSS23, which is able to leverage the mitochondrial fitness of liver cancer cells. Further investigations are going on to uncover the interacting proteins and the detailed molecular mechanisms in this protumorigenic process.

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## Exploiting LXRalpha activation for lipotoxic cancer therapies

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The success of molecular therapies targeting specific metabolic pathways in cancer is often limited by the plasticity and adaptability of metabolic networks. We could recently show that an enhanced lipogenesis, triggered by a pharmacological activation of the Liver X receptor alpha (LXRalpha), represents a new therapeutic strategy for the treatment of liver carcinoma (HCC). We found that a combination of LXRalpha-mediated fatty acid synthesis and concomitant Raf suppression results in oxidative stress, induction of a critical ER stress response and subsequently in apoptosis of HCC cells. Mechanistic analyses revealed, that Raf-1 directly interacts with Stearoyl-CoA desaturase-1 (Scd1), the central enzyme for the conversion of saturated into mono-unsaturated fatty acids and maintains Scd1 protein stability. Conformation changing (DFG-out) Raf inhibitors disrupt this interaction, thereby diminishing Scd1 protein abundance, which results in a toxic accumulation of saturated free fatty acids and metabolic stress in cancer cells under sustained lipogenesis. In follow-up studies, we could detect the protein-protein interaction of Raf-1 and Scd1 also in several other tumor entities such as pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung cancer (NSCLC) and found that these tumor entities are also vulnerable to LXRalpha-mediated lipotoxic therapy.

While suitable conformation changing DFG-out Raf inhibitors are already in use for the treatment of cancer patients (e.g. sorafenib), synthetic LXRalpha agonists are currently not available for the clinic. We therefore aimed to develop novel LXR agonists that are efficient and specific for LXRalpha and that may serve in the future as potential compounds for the treatment of patients. We developed two novel hit series and screened the synthesized compounds in LXRalpha- and LXRbeta-specific co-activator FRET assays, cell-based LXR reporter assays and cell viability assays. The results were used to guide the synthesis of 2nd generation ligands that were again tested for activity and specificity. As a result, we were able to establish frontrunner compounds that were highly efficient in activating LXRalpha and more selective for LXRalpha in comparison to established agonists. Furthermore, our novel agonists efficiently induced metabolic stress and cell death in combination with direct or indirect Scd1 inhibition in cells and organoid cultures of liver cancer and other tumor entities.

Taken together, we propose LXRalpha-mediated lipotoxicity as a new strategy for an efficient metabolic targeting of liver cancer and other therapy refractory solid tumors.

# Tumoral macrophages and VETC interplay in HCC immuno-vascular microenvironment: new morphological evidences

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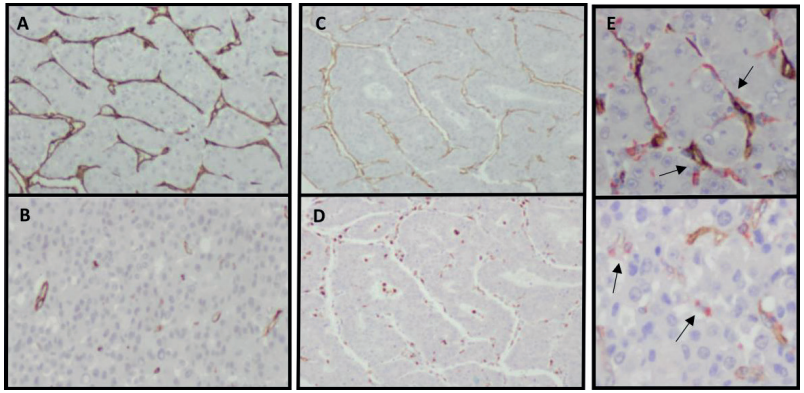
**Question(s):** The genomic and transcriptomic features of HCC have been extensively described in the last decade (1). Despite the improved understanding of HCC biology, results missed to identify therapeutic targets and biomarkers. We previously showed that a peculiar vascular phenotype, namely VETC (vessel that encapsulate tumor cluster), is associated with worst outcome (2). Tumor-associated macrophages (TAMs) play a role in occurrence and development of HCC, including stimulating angiogenesis and promoting metastasis (3). The aim of the study is to investigate at morphological level the relationship between VETC, and TAM features.

**Methods used:** We analysed 48 HCC, evaluating VETC and TAMs. VETC+ was defined as a continuous CD34+ endothelial covering of neoplastic clusters in ≥10% of tumoral cells. Number and size of CD68+/CD163+ TAMs cells were recorded (4).

**Result(s):** TAMs showed different features between VETC+ and VETC- cases. Intratumoral TAMs in VETC+ cases were more numerous (p=0.004), characterized by a peculiar morphology (p=0.003): larger, with irregular border and foamy (4), and spatially related to endothelial cells. In VETC- cases, TAMs were smaller, simpler and far from endothelial cells.

**Conclusion(s):** The presence of an inflammatory milieu of lymphocytes inhibits the effect of angio-genic factor on VETC onset (5). By contrast, VETC+ cases are more frequent in HCC with poor, or even desert, lymphocytic infiltrate (2). We first proved VETC is associated with an increased number of large, foamy TAMs, a peculiar morphology we linked to M2 profile (4), spatially related to endothelium.




Graphic 1: HCC showing VETC (A) and non-VETC (B) phenotype (10x); C, D) VETC (C) and CD68+ TAMs (D) in the same HCC area (10x); E) Double immunostaining [CD34, brown and CD68, red] proving the close relationship between CD68+ TAMs and endothelial cells in VETC+ HCC (20x); F) Double immunostaining in a VETC- HCC case: note the scattered number of TAMs, (40x).



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## Identification of blood based biomarkers for hepatocellular carcinoma screening

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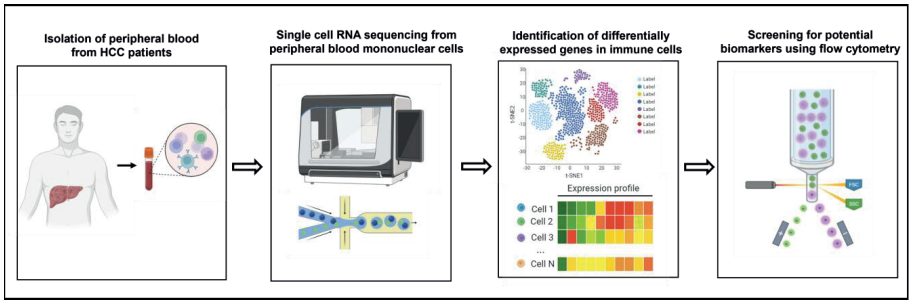
**Question(s):** Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with rising incidence. It is the third leading cause of cancer-related deaths, in part because of inadequate early detection strategies. Current recommendations for screening include biannual ultrasonography with or without alpha-fetoprotein, but has limited sensitivity and accuracy. A blood based non-invasive biomarker could aid in overcoming the deficiencies of the present methods. With the advancements in transcriptomic techniques, the in-depth profiling of the immune cells in the peripheral blood of HCC patients has become possible. From this study, we wish to determine the transcriptional footprint of immune cells in HCC to facilitate the detection of prognostic biomarkers in patients.

**Methods used:** For the study of the immune cell populations, we performed single cell RNA sequencing (10X Genomics platform) on the peripheral blood samples of 9 patients with HCC. Blood sampling was performed prior to and post either surgical resection or ablation of the tumors in patients. The differentially expressed genes from the analysis will be studied for biomarker identification using flow cytometry.

**Result(s):** From the transcriptomics data, the we observed the highest changes in the differentially expressed genes (DEGs) in the monocyte subpopulations in comparison to the other immune cells. Interestingly the DEG profile was contrasting in the patients that relapsed post-surgery in comparison to the non-relapsed patients. Gene set enrichment analysis suggested an upregulation in the type-1 and type-3 interferon response genes in the non-relapsed patients.

**Conclusion(s):** Our results suggest the presence of an immune signature of monocytes in HCC patients which can be used as a potential prognostic biomarker for prediction of recurrence risk.




Graphic 1: Workflow of the project (Figure elements adapted from Biorender)

**Support & Funding: Deutsche Forschungsgemeinschaft (DFG)**





## Where to go for processing and evaluation of animal tissue samples? The Center for Model System and Comparative Pathology (CMCP) – a service platform for research animal pathology - introduces itself

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**Background information:** In 2016, the Center for Model System and Comparative Pathology (CMCP) has been set up at the Institute of Pathology in Heidelberg. It is centrally staffed and lead by a dedicated Veterinarian Pathologist, Dr. T. Poth, and fulfills the demand for high quality animal tissue processing and animal model evaluation / phenotyping and scoring for translational research projects. The CMCP is one of only two facilities in Germany that merge the expertise in human and veterinary pathology, an essential prerequisite for faithful analysis of animal models intended to reflect human disease. In the recent years, the CMCP supported numerous animal projects of two DFG-funded Collaborative Research Centers (CRC): the SFB/TR 209 “Liver Cancer” and the SFB 1118 “Diabetes”. Actually, the CMCP cooperates with many different animal research teams in Heidelberg (incl. DKFZ and HiSTEM) and nationwide and has meanwhile served more than 600 projects in a quality-controlled manner. Regarding tissue-based technologies, it closely collaborates with the NCT tissue bank.

**Summary of the SFB/TR 209 “Liver Cancer” period:** During the running time of the SFB/TR 209, the CMCP was part of the infrastructure project and was technically responsible for the processing of murine tissue samples including histotechnology, immunohistology, and virtual microscopy. The CMCP supported 14 SFB projects working with mouse models, established individual protocols for 77 different antibodies customized for the mouse projects and performed comparative histopathological evaluations including scoring and phenotyping / characterization. Details of the different CMCP services requested by the SFB/TR 209 principle investigators are shown in table 1.

**Conclusion:** Embedded in the network of the infrastructure project, the CMCP supported the SFB/TR 209 mouse projects with customized high-quality services and could therefore contribute to the excellent results of the preclinical research part of the CRC “Liver Cancer” resulting in high-level publications of the CRC community.

**Perspective:** The CMCP realized the high demand for animal pathology in the translational research field and addresses to all researchers working with animal models independently of a DFG funding. The services encompass a broad spectrum of standard and specialized tissue-based technologies including consulting in project planning and histopathological analysis of animal tissue specimens and provide optimal preconditions for a high-quality and sustainable research. Comparative histopathological evaluation, scoring and phenotyping of animal models for human diseases performed by a board-certified expert in veterinary pathology in collaboration with board-certified human pathologists fulfill ideally the requirements for publications in top-class professional journals.

Table 1: Tissue-based services provided by the CMCP during the total running time of the SFB/TR 209.

**Support and funding:** The infrastructure project of the SFB/TR 209 was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - SFB/TRR 209 – 314905040.

## Advancing Personalized Treatment Approaches through Mechanistic Modeling of Type-I Interferon Signal Transduction Pathway

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The global prevalence of chronic hepatitis B virus (HBV) infection is estimated to be around 4% and the number of deaths due to HBV-associated liver failure and cancer has been rising over the last decades. Interferon alpha (IFNα), a central component of the innate immune system, is administered to patients with chronic hepatitis B virus infections. By establishing a mechanistic ordinary differential equation model of IFNα signal transduction, we previously characterized the interplay of feedback components of the system and identified their dose- and time-dependent contributions to pathway sensitization. Following stimulation with IFNα, hundreds of proteins are induced promoting cellular effects, like antiviral effects, inhibition of cell growth and angiogenesis, induction of apoptosis and activation of immune effector cells. These proteins are responsible for the heterogeneity of treatment responses and due to their non-linear dynamics a time-resolved, proteome-wide analysis of the IFNα-induced responses is required.

Therefore, we stimulated the hepatoma cell line HepG2 and with multiple doses of IFNα and acquired time-resolved proteome data by mass spectrometry operated in the data independent acquisition mode. More than 7500 proteins were simultaneously and accurately quantified. A newly developed data analysis pipeline confirmed the induction of a multitude of known interferon-stimulated proteins, but also led to the discovery of several novel candidates. A sequential, wave-like induction of sets of interferon-stimulated proteins could not only be observed in mRNA data but was now also confirmed by proteomics highlighting the importance of longitudinal data acquisition. Furthermore, a group of proteins with decreased abundance after IFNα stimulation compared to the unstimulated condition was identified indicating repressed transcription or enhanced degradation.

We integrated the dynamics of the uncovered interferon-stimulated proteins into our mathematical model IFNα signal transduction in hepatoma cells and *in silico* explored the contributions of the three transcription factors associated to IFNα signal transduction. This linkage provides a valuable tool for fine-tuning the timing (tmax) and maximum concentration (cmax) of sets of interferon-stimulated proteins through personalized IFNα dosing regimens tailored to the specific disease type and patient.

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**Support & Funding:** DFG Transregio 179 - Determinants and dynamics of elimination versus persistence of hepatitis virus infection.





## Expression of CDH6 in Cholangiocarcinogenesis and its Diagnostic Relevance in High-Grade Biliary Intraepithelial Neoplasia

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**Questions:** Cholangiocarcinoma (CCA) is a heterogeneous, highly aggressive malignancy with poor prognosis. Surgical resection is currently the only curative therapy, however, reactive changes caused by inflammation may closely mimic biliary intraepithelial neoplasia (BilIN) precursor lesions hampering detection at a resectable stage. Several cadherins (CDH) have been linked to metastatic progression and downregulation of CDH6 has been reported in biliary carcinogenesis (1). In this study, we focused on the role of CDH6 in early cholangiocarcinogenesis and aimed to assess its use as a diagnostic marker of early malignant transformation in bile ducts.

**Methods used:** Tissue microarrays of CCA samples of a large and clinicopathologically well-characterized patient cohort, containing intrahepatic (iCCA, N=145), perihilar (pCCA, N=145) and distal CCA (dCCA, N=118) as well as high-grade BilIN (N=170) and adjacent non-neoplastic biliary tissue (N=144) were immunohistochemically analyzed for CDH6. The average staining intensity of invasive tumor, high-grade BilIN and non-neoplastic bile duct epithelium was assessed using the immunoreactive score (IRS). Furthermore, full slide specimen of a second control cohort, encompassing resection and brush cytology samples of CCA, high-grade BilIN, non-neoplastic atypic bile ducts, inflamed bile ducts and normal bile ducts were analyzed and assessed by CDH6 immunohistochemistry.

**Results:** CDH6 immunoreactivity was absent or reduced significantly in CCA (mean IRS: 2.7) and in high-grade BilIN (mean IRS: 2.5), whereas it was highly expressed in atypic and non-atypic, non-neoplastic bile duct epithelia (mean IRS: 10.4). Statistical analysis confirmed a significant CDH6 protein reduction in all CCA subtypes and in high-grade BilIN, compared to normal bile ducts (each $p<0.001$). Furthermore, the comparison of nonneoplastic bile duct samples with their corresponding intraindividual CCA samples revealed a significant decrease of CDH6-expression in 99% of cases ($p<0.001$). On full slide sections, BilIN and CCA could be clearly differentiated from adjacent non-neoplastic bile ducts due to reduced CDH6 signals (each $p=0.004$), thus validating the tissue microarray data. Brush cytology specimen and bile duct samples with inflammatory changes showed that CDH6 expression significantly differs between inflammatory and neoplastic changes confirming the potential clinical utility.

**Conclusions:** Our results show that reduced expression of CDH6 is indicative for early precursor lesions and invasive CCA and is easily detectable by immunohistochemistry. Brush cytology is a well-established diagnostic tool, however it is known to have low sensitivity, especially in the presence of inflammation. Our results show that loss of CDH6 expression may serve as a diagnostic marker for discrimination of high-grade BilIN from reactive atypia in histologic and cytologic specimens.

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**Support & Funding:** This project was supported by the SFB/TR 209 “Liver cancer”. Fritz Brinkmann received a Gerok stipend funded by the SFB/TR 209.

# Characterization of the effect of chronic immobilization stress on the structural and functional state of the liver of mother rats and their one-month-old offspring as a risk factor for liver cancer

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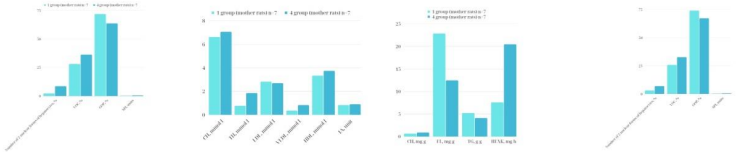
**Question(s):** Gastrointestinal tumors are the second most common cause of disability and death of the able-bodied population both in Ukraine and in other European countries. The influence of various types of stress has been established to be one of the factors resulting in damage to the hepatobiliary system, both in the mother and in the fetus. Thus, it has been proven that conditions unfavorable for the fetus lead to the creation of prerequisites for the development of diseases in adulthood, in particular arterial hypertension and diabetes, liver cancer.

**Methods used:** The study was conducted on 13 WAG female rats, 50% of which were the control group. A complex of morphometric, immunohistochemical and biochemical studies of liver tissue and blood serum was carried out. Seventeen one-month-old offspring were removed from the experiment one month after birth.

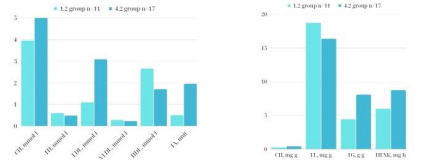
**Result(s):** Morphometric study showed structural and functional changes in the liver in 100% of the mother rats of all groups, namely: moderate discomplexation of the beam-radial structure, fatty dystrophy of hepatocytes, their uneven swelling around the portal tracts, and proliferation of the stroma of the portal tracts and around the central veins with an increase in SPI by 46.2%. In mother rats, the expression of eNOS and iNOS changed, indicating damage to the endothelium of the liver vessels. At the same time, morphological changes in the liver of the offspring were generally similar to those of their mothers, but less pronounced, and there was no dystrophy of hepatocytes and thickening and sclerosis of arterial walls. Lipidography of experimental animals revealed only one regularity: an increase in AI (by 1.2, 3.9 times in females and one-month-old rats), but its causes were somewhat different. In females, it occurred due to a significant increase in VLDL (2.3 times) and a slight increase in HDL (1.1 times) (at a normal level of cholesterol), in one-month-old rats due to an increase in the level of LDL (2.8 times) and a decrease in HDL (1.6 times) (with an increased level of cholesterol by 1.6 times.

**Conclusion(s):** Therefore, according to the findings of this study, it can be concluded that immobilization stress leads to the same type of changes in mother rats and one-month-old offspring, but the extent of changes was greater in mothers. The structural and functional changes found in the organs of the offspring are explained by the inclusion of epigenetic programming mechanisms and may form the basis of the development of tumors in the liver at later stages of ontogenesis.









Graphic 1: Morphometric indicators and structural elements of the liver maternal rats under the influence of chronic stress (Me [25; 75])

Graphic 2: Biochemical indicators of lipid metabolism in the blood serum of maternal rats under influence of chronic stress (Me [25; 75])

Graphic 3: Fraction composition of lipids and glycogen in the liver of maternal rats under the influence of chronic stress (Me [25; 75])

Graphic 4: Morphometric indicators and structural elements of the liver offspring of rat mothers exposed to chronic stress (Me [25; 75])

Graphic 5: Biochemical indicators of lipid in blood serum of the offspring maternal rats exposed to chronic stress (Me [25; 75])

Graphic 6: Fractional composition of lipids in the liver homogenate of rat offspring exposed to chronic stress (Me [25; 75])

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**Support & Funding:** This work is a continuation of cathedral research on this topic.

# Spatial Analysis of α-SMA-Positive Hepatic Stellate Cells and CD68-Positive Macrophages Reveals Altered Phenotypes during the Transition from Cirrhosis to HCC

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## Abstract

Hepatocellular carcinoma is the fourth leading cause of cancer-related mortality worldwide and a main cause of death in cirrhosis. The development and progression of hepatocellular carcinoma are controlled by a complex tumor microenvironment, including hepatic stellate cells (HSC) and macrophages (MØ). In the context of cirrhotic HCC, spatial transcriptomics (GeoMX-DSP) technology has been applied to investigate the gene expression profiles of cellular compartments. Fluorescent imaging using Alpha-Smooth Muscle Actin (α-SMA), Cluster of Differentiation 68 (CD68), and Keratin 8/18 (CK8/18), identified the spatial distribution of activated HSC-, macrophage- and hepatocyte/HCC cell-populations in formalin-fixed paraffin-embedded (FFPE) tissues. The slides were additionally probed with photo-cleavable barcoded DNA oligos (whole transcriptome atlas, 18,000 genes). 57 regions of interest (ROIs) were selected according to the presence of sufficient numbers of the aforementioned cell types in distant cirrhotic regions (C), in cirrhotic tumor boundary (CT), and inside the HCC (T) for analysis. We found that Collagen Type I Alpha 1 (COL1A1), COL1A2, Fibronectin (FN1), and TIMP metallopeptidase inhibitor 1 (TIMP1) are highly upregulated in HSCs located in the T compared with the CT and C compartments, indicating a potential involvement in tumor formation. Furthermore, deconvolution analysis revealed an opposing trend in abundance of cytokine-producing HSC (cyHSC) towards myofibroblastic HSC (myHSC) from the C to T regions, suggesting a phenotypic transition of the microenvironment to facilitate hepatocyte transformation. Ligand-receptor interaction analysis revealed that upregulated COL1a1 and FN1 may signal via parallel induced integrin receptor family members (ITGA5, ITGAV) on hepatocytes/HCC cells, thereby influencing hepatocyte fate. Similarly, MØs in the T regions display significant downregulation of genes belonging to the IgG family, such as IgHG1-4 and IgKC, suggesting a potential implication in the onset of tumorigenesis. Principal component analysis revealed that HSCs and MØs in the CT and T regions cluster together, while hepatocytes do in the CT and C regions, indicating alterations in the tumor microenvironment as drivers of hepatocyte transformation. In conclusion, our spatial profiling investigation provides preliminary evidence that the transition of cyHSCs to myHSCs, accompanied by changes in matrisomal components such as Col1, FN1, etc., change liver stiffness and

viscoelasticity above a critical threshold that is facilitating and induce hepatocyte transformation.





## LZTR1 acts as a potent tumor suppressor gene in liver cancer by safeguarding aberrant MAPK activity via posttranslational control of RAS GTPases

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**Question(s):** Hepatocellular carcinoma (HCC) ranks among the cancers with the highest rate of mortality, yet treating this carcinoma remains challenging due to late diagnosis and poor patient stratification, thereby preventing the utilization of targeted therapeutical approaches. Identifying potential tumorigenic drivers therefore remains a crucial prerequisite for precision treatment of this malignancy.

**Methods used:** Hydrodynamic tail vein injection, primary cell line derivation, CRISPR-Cas9 and -Cas12a mediated genetic perturbation, tetracycline-inducible gene expression induction/suppression, multicolor competition assay, qPCR, immunoblotting analysis.

**Result(s):** Using human genome sequencing data, we identified frequent deleterious alterations of Leucine-zipper-like transcriptional regulator 1 (LZTR1), which plays a crucial role in regulation of RAS-like GTPases (e.g. RIT1) and downstream pathways, such as the mitogen activated protein kinase (MAPK) pathway. Using murine *in vivo* as well as human *in vitro* models, we reveal that loss of function of LZTR1 promotes tumorigenesis *in vivo* as well as cell growth *in vitro*, an effect accompanied by elevated RIT1 expression and subsequent MAPK pathway activation. Moreover, truncated forms of LZTR1 lacking domains crucial for its interaction with RAS molecules phenocopied the effect of LZTR1 loss, further suggesting that this interaction is crucial for tumor suppression. Finally, expression of mutant RIT1 proteins rendering RIT1 non-degradable by LZTR1 in murine livers resulted in liver tumorigenesis comparable to LZTR1 loss.

**Conclusion(s):** Our findings suggest that LZTR1 safeguards MAPK signaling by controlling RAS GTPases in the liver and could therefore potentially be utilized to stratify HCC patients for usage of small molecule inhibitors targeting MAPKs, which are currently only employed in other carcinomas.

**Support & Funding:**

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## Interfering with ß-catenin-induced immunosuppression in HCC by cytokine-armed virotherapy.

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**Question(s):** Mutations of β-catenin belong to the most frequent genetic alterations in hepatocellular carcinoma (HCC) and are supposed to be involved in dysfunctional antigen presentation, immunosuppression, and insensitivity to immunotherapies. Animal models of HCC with defined immunogenicity and activated Wnt/β-catenin pathway are lacking that are suitable for investigations on local immunoactivation by cytokine-armed virotherapy.

**Methods used:** In C57BL/6 mice, we first induced liver tumors in-situ by hydrodynamic injection (HDI) and by local plasmid electroporation to deliver SB13-transposase and transposons encoding c-Myc, shRNAp53, mutated p53-R172H, and with or without ΔN90-β-catenin (ΔN90-β-cat). To introduce immunogenic properties, c-myc expression was genetically linked to immunogenic (neo)epitope arrays. Grown tumors were used to isolate cell lines for the establishment of a s.c. tumor model.

**Result(s):** After HDI, ΔN90-β-cat expression was essentially required for the development of immunogenic HCCs. After electroporation, HCC occurred in both groups but significantly faster and more reliable in the presence of ΔN90-β-cat confirming the impact of β-cat activity in tumor development. Further reduction of EP-induced tissue damage by lowered voltage almost fully prevented tumor development in the group lacking mutant β-cat suggesting that mutated β-cat was in this setting decisive to enable tumor escape from immune surveillance. β-cat-activated tumors showed HCC characteristics as confirmed by a pathologist. To generate a s.c.- β-cat-dependent HCC-model we established the primary cell line HepM-683 from a ΔN90 β cat activated HCC. In this model, we investigated the oncolytic adenovirus hTert-Ad for local virotherapy together with adenoviral vectors expressing immunoactivating cytokines. Compared with virotherapy alone additional expression of Mip1α, Flt3L together with Xcl1, or CCl5 respectively, showed signs of successful TME activation and resulted in significantly reduced growth of β-cat-dysfunctional tumors.

**Conclusion(s):** We have established syngeneic in-situ and s.c. murine HCC models featuring an immunogenic epitope array and β-cat-dependent growth characteristics. These models are well suited to investigate local immunotherapies such as virotherapy to break β-cat-dependent immunosuppression in HCC.

**Support & Funding:** TR209 C6/C7





## Genotype-to-phenotype mapping in mouse models of liver cancer

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Organization(s): 1: Institute of Pathology, UKHD, Germany; 2: Institute of Neuropathology, UKHD, Germany

**Question(s):** Intratumoral genetic heterogeneity and tumor-stroma interactions are emerging topics in cancer research. However, the lack of experimental strategies has so far precluded systematic and unbiased analyses of how heterogenous complex genetic alterations can affect tumor development and the tumor micro-environment in a native context.

**Methods used:** We have devised a novel approach for conducting spatially-resolved in vivo functional genomics and apply this method to an newly developed autochthonous liver tumor heterogeneity mouse model.

**Result(s):** We successfully demonstrate that a single spatial transcriptomics readout platform can be used to study complex genotype-to-phenotype relationships among over 200 cancer clones coexisting within the native tissue environment of a single mouse liver.

**Conclusion(s):** Our proof-of-concept study reveals limitations and opportunities of our current approach.

## PARP-1 inhibition preferentially impairs KRAS mutated intrahepatic cholangiocarcinoma and is mediated by CHK1 kinase

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**Question(s):** Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary liver cancer with an increasing incidence over recent years. Due to the complexity of iCCA pathogenesis and the pronounced genetic heterogeneity treatment options are still limited. Activating KRAS mutations are among the most abundant genetic alterations in iCCA and are associated with early recurrence, poor response to chemotherapy, and reduced overall survival, highlighting the need for novel therapeutic approaches. Poly(ADP-ribose)polymerase-1 (PARP-1) is frequently observed to be upregulated in iCCA. Evidence indicate potential therapeutic relevance for PARP-1 inhibition in iCCA that preferentially affects KRAS-mutated cancers, but exact mechanisms remain unknown.

**Methods used:** PARP-1 depletion was generated by siRNA and CRISPR/Cas9-mediated knockdown/knockout in KRAS-mutated and non-mutated iCCA cell lines. Functional assessment of PARP-1 knockout and inhibition of tumorigenic potential was analyzed by viability assay and colony and sphere formation. RNA sequencing was employed to further decipher PARP-1 regulation. To investigate the impact of PARP-1 deficiency in KRAS-driven tumorigenesis, PARP-1 knockout mice were combined with an inducible KRAS-driven mouse model using hydrodynamic tail vein injection. Molecular analyses including transcriptome profiling were employed to further investigate molecular mechanisms.

**Result(s):** Significant upregulation of PARP-1, as well as enrichment of genes related to PARP-1 activation, was observed in iCCA tissue and KRAS-mutated cell lines. Knockout of PARP-1 in KRASmutated cells led to a reduction in colony and sphere formation. Moreover, KRAS-mutated cell lines showed higher sensitivity to PARP-1 inhibition. In vivo PARP-1 deficiency considerably impaired biliary carcinogenesis and induced a shift from dominant iCCA towards HCC phenotype in a KRAS-dependent manner. Transcriptome analyses of CRISPR/Cas9 PARP-1 knockout clones and in vivo tumors revealed differential expression of DNA damage response pathways (e.g. CHK1) as well as cellular pathways affected by PARP-1, (inflammation, oxidative stress, cell death signaling). The most prominent candidate regulating PARP1 in KRAS cell lines and tumors appeared to be CHK1 kinase, further validated by qRT-PCR, western blot, and drug-screening assays.

**Conclusion(s):** Together, these findings suggest an unrecognized prognostic and therapeutic role of PARP-1 in iCCA patients with oncogenic KRAS signaling and unveil the potential mechanism of PARP-1 regulation by CHK1 kinase.

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## Deep learning-enabled diagnosis of liver adenocarcinoma.

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**Question(s):** Diagnosis of adenocarcinoma in the liver is a frequent scenario in pathology with critical impact on clinical decision-making. However, rendering an accurate diagnosis can be challenging and often requires the integration of diverse ancillary information. We developed a deep learning model (HEPNET) to distinguish intrahepatic cholangiocarcinoma (iCCA) from colorectal liver metastasis (CRM) as two major forms of hepatic adenocarcinoma with clinical-grade accuracy using hematoxylin and eosin-stained whole-slide images.

**Methods used:** HEPNET was trained on 714,589 image tiles from 456 patients who underwent surgical resection or biopsy at Heidelberg University Hospital using an EfficientNet B3 model architecture. The model was evaluated on an internal test set of 115 patients and externally validated on 159 patients from Mainz University Hospital.

**Result(s):** On the internal test set, HEPNET achieved an area under the receiver operating characteristic curve (AUROC) of 0.994 and an accuracy of 96.5% at the patient level. External validation yielded an AUROC of 0.997 and an accuracy of 98.1%. HEPNET surpassed the performance of six pathology experts in a reader study and lifted the performance of resident pathologists to the level of senior pathologists.

**Conclusion(s):** We provide a deep learning-based tool capable of accurately discriminating iCCA and CRM, potentially facilitating routine pathology with respect to definitive diagnosis and guiding ancillary testing. The integration of AI-based tools like HEPNET may ultimately optimize the diagnostic workflow, resulting in potential labor and cost savings. Large-scale multi-institutional studies are needed to further validate the perfomance of HEPNET.

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## Integrated genotype-phenotype analysis of Familial adenomatous polyposis-associated hepatocellular adenomas

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**Question(s):** Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome caused by a germline mutation in the adenomatous polyposis coli (APC) gene, characterized by numerous colorectal adenomas [1]. In addition, extraintestinal manifestations may occur in the patients, such as desmoid tumors or osteomas [1, 2]. Several cases of hepatocellular adenomas (HCA) detected accidentally in FAP-patients have raised the question whether they represent a specific manifestation of FAP or a mere coincidence [3-5].

**Methods used:** Analysis of all resected liver lesions of FAP patients (1991-2021) of the Institute of Pathology of University Hospital Heidelberg resulted in the detection of five hepatocellular adenomas in three patients. Comprehensive morphological, immunohistological and molecular analyses were employed, including targeted next-generation sequencing.

**Result(s):** The analyzed HCAs showed no cytological or histological atypia. The immunohistochemical analysis revealed a diffuse, strong positivity for glutamine synthetase in the absence of a nuclear beta-catenin staining. In two patients, the adenomas showed a moderate immunoreactivity against serum amyloid A. Consistent with the diagnosis of FAP, molecular profiling revealed a pathogenic germline mutation of the APC gene in all analyzed adenomas as well as deleterious somatic second hits. The somatic mutations accumulated between codons 1345 and 1577. No mutations were found in beta-catenin encoding Catenin Beta 1.

**Conclusion(s):** HCA in FAP patients is a specific, although rare neoplastic manifestation of this inborn disease and represents a distinct group of liver cell adenomas. The results highlight that in some rare cases of HCA, strong overexpression of glutamine synthetase alone does not allow to ascribe a HCA to the subtype of beta-catenin-activated HCA and does not necessarily demonstrate an increased risk of malignant transformation. These benign tumors represent an important differential diagnosis to hepatic metastases in FAP patients and require adequate clinical and molecular (diagnostic) assessment for optimal patient guidance.

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## WISP1 is a downstream target of TGF-beta signaling, highly expressed in cirrhotic HCC microenvironment and linked to better survival

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**Question(s):** WNT1-inducible signaling pathway protein 1 (WISP1) has been reported to promote tumor progression in several organs and its higher expression is correlated to poor outcome. WISP1 is also induced in chronic liver diseases (CLD). We aim to investigate its expression and role in the progression from liver fibrosis to cirrhosis to hepatocellular carcinoma (HCC).

**Methods used:** WISP1 mRNA expression was analyzed in publically available microarray datasets of three CLD patient cohorts, Wisp1 protein levels in serum samples by ELISA and by immohistochemical staining of an HCC patient tissue microarray (147 patients). WISP1 expression was correlated to clinical parameters. Spatial distribution of WISP1 expression in patient liver samples was determined by RNAscope (isH) analysis. WISP1 expression regulation was investigated in cytokine stimulated mouse hepatocytes by qRT-PCR and Affymetrix array analysis. By InucyteS3-live cell imaging assays, RT-PCR and immunoblot analysis, outcome of Wisp1 and/or TGF-β1 stimulation was determined in mouse hepatocytes and the human HCC cell line HuH7.

**Result(s):** In a tissue microarray, high WISP1 expression was positively correlated with early HCC stages (TNM I and II) and male sex. Analysis of publically available CLD patient cohorts as well as serum samples of CLD patients demonstrated the highest WISP1 expression in cirrhotic stages as compared to healthy livers, early fibrotic disease or non-cirrhotic HCC. WISP1 RNAScope of cirrhotic HCC patients´ liver tissue displayed significant staining in the cirrhotic surrounding of tumors, but not in the tumor itself. Higher WISP1 expression was found associated with better survival of patients, fitting to its expression in precancerous stages of CLD.

In primary mouse hepatocytes, WISP1 expression was regulated by TGF-β but not HGF, TGFα, TNFα, EGF, Insulin, IL6, amphiregulin or Wnt3a treatment. TGF-β1 dependent induction of WISP1 expression was confirmed in the murine hepatocyte cell line AML12. In line, WISP1 and TGF-β1 expression levels are positively correlated in human HCC patients´ samples ($r>0.55$; $p<0.0001$).

Outcome of TGF-β1 and WISP1 treatment was compared in AML12 cells. Both cytokines led to reduction of AML12 cell numbers within 72 hours of culture with a stronger effect of TGF-β1 than WISP1. This might be attributed to upregulated Bcl2 and Bcl-xL expression and induced AKT phosphorylation in WISP1-treated AML12 cells.

**Conclusion(s):** Our study revealed higher WISP1 expression in cirrhotic liver tissue as compared to earlier or later CLD stages being a marker for better suvival. Although Wisp1 downregulated cell growth of AML12 cell like TGF-β1, its addtional impact on apoptosis regulation and survival signaling might dampen the overall effect compared to TGF-β1. As WISP1 is a target gene of TGF-β signaling itself and correlates with TGF-beta expression in HCC patients, we next aim to understand its regulatory role in CLD in interdependence with TGF-β1.

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## Role of angiocrine Wnt signaling in Hepatocellular Carcinoma.

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**Questions:** Hepatocellular carcinoma (HCC) is the second common cause of cancer-related death. Yet, the therapeutic interventions available are limited. One major bottleneck impeding the drug discovery process is the lack of preclinical tumor models which mirror the complexity of human HCC initiation and progression and the inability to fully recapitualte the tumor microenvironement. This is of particular importance for HCC, since it is a highly vascularized tumor and angiogenesis plays a major role in tumor progression. Recently, it was also described in the IMBrave clinical trial that targeting the endothelium (in combination with anti-PD1) has a better therapeutic potential than the standard care of treatment[1]. However, endothelial cells' contribution to the tumor microenvironment (TME) is not fully understood. Hence, this project aims to first establish different *in vivo* murine models to recapitulate the HCC initiation and progression. Secondly, it investigates how EC-derived Wnt ligands affect, at the angiocrine and autocrine level, HCC development and progression. Specifically, two members of the Wnt signaling pathway were selected based on the contribution for the liver at the physiological level: Rspo3 (Wnt enhancer) and Evi/Wls (Wnt ligand transporter).

**Methods used:** A focal electroporation-based HCC tumor model (EPO) was developed. In this model, different plasmids were electroporated in the left liver lobe targeting some of the most common HCC mutations (Trp53 deletion, KRas or cMyc overexpression). These genes are under the albumin promoter to induce the tumor transformation specifically in hepatocytes. The combination of Trp53 LOF and cMyc GOF was selected for further refinement and optimization. This model was complemented with an inducible HCC GEMM (iAST) model (e.g. Cre delivery induces activation of the oncogenic SV40 large T antigen leading to multinodular tumor formation within 5 to 8 weeks)[2]. Both models were established in the background of inducible EC-specific deletion for either one or both (Rspo3, Evi).

**Results:** Expression of both genes (mostly Rspo3 and to lesser extent Evi/Wls) is decreased in tumor endothelial cells compared to healthy liver endothelial cells. Endothelial abolishment of both enhances tumor initiation and preconditions the microenvironment, resulting in more malignant tumors. Likewise, Rspo3 expression is decreased in human HCC samples in comparison to no tumor sections. Mechanistically, tumor-derived factors decrease the expression of the Wnt signaling enhancer Rspo3. In line with this, treatment with recombinant Rspo3 suggest an anti-tumorigenic effect.

**Conclusions:** Taken together, we have developed novel *in vivo* models to better recapitulate human HCC tumor formation and study the role of endothelial Wnts in HCC initiation and progression. Furthermore, our data suggests angiocrine Wnts (in particular Rspo3) is characteristic of the healthy liver ECs and it may have potential therapeutic role in HCC.



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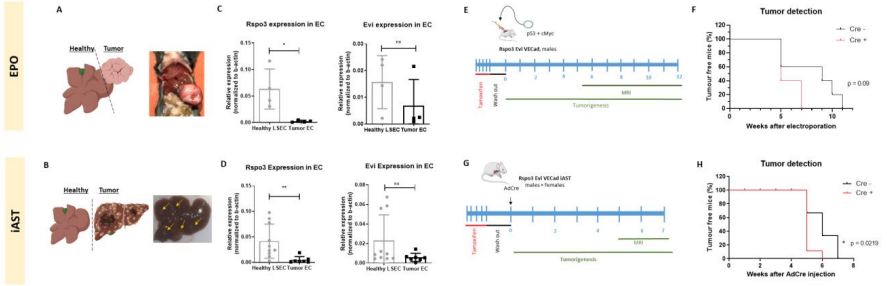


Figure 1: Establishment of in vivo HCC murine models to study angiocrine Wnts, expression of Wnt candidate genes in tumor and healthy liver ECs and effect of their abolishment in HCC tumorigenesis in the established murine HCC models. (A) Schematic representation of healthy and tumor liver from local electroporation model (EPO) and representative image of electroporated liver tumor bearing mice with the selected plasmid combination. (B) Schematic representation of healthy and tumor liver from iAST GEMM mice and representative image of multifocal derived liver tumos. (C) Rspo3 and Evi expression of sorted murine EC (CD45-CD31+CD146+) from healthy liver and tumor from EPO mice. (D) Rspo3 and Evi expression of sorted murine EC (CD45-CD31+CD146+) from healthy liver (AST non AdCre injected mice) and tumor (AST AdCre injected mice). (E) Experimental design of Rspo3-Evi- VECad-creERT2mice where ECKO was induced with five consecutive shots of tamoxifen followed by one week wash out period, before electroporation for tumor induction. (F) Tumor detection graph from MRI in Cre- (Wt) and Cre+ (ECKO) Rspo3-Evi- VECad-creERT2 electroporated mice as described in E. (G) Experimental design of Rspo3-Evi-VECad-creERT2-iASTmice where ECKO was induced with five consecutive shots of tamoxifen followed by one week wash out period, before AdCre injection for tumor induction. (H) Tumor detection graph from MRI in Cre- (Wt) and Cre+ (ECKO) Rspo3-Evi-VECad-creERT2-iAST mice as described in G.

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## STAT1 and STAT3 are Associated with Increased Inflammation in Hepatocellular Carcinoma

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**Questions:** IL6-signaling is important for hepatocyte homeostasis and a strong cell cycle mediator. Constant activation of IL6-signaling, caused for example by chronic inflammation and an increase in infiltrating immune cells, is associated with HCC tumorigenesis. The regulatory function of IL6-signaling is mediated by STAT3, which can be inhibited by SH2D4A by retaining STAT3 dimers in the cytoplasm[1]. In addition, STAT3 and STAT1 show a divergent functionality in HCC tumorigenesis[2]. Our aim was to find a possible association between STAT1 and STAT3 expression and tumoral immune cells.

**Methods used:** A tissue microarray consisting of 127 HCC samples taken from 2006 to 2011 at the Heidelberg University Hospital was constructed. Sections were stained with antibodies against STAT1, STAT3, CD3, CD4, CD8, CD20, CD68, CD117, FOXP3 and PD-L1. STAT1 and STAT3 were scored using the immunoreactivity score combining staining intensity and percentage of positive cells. Immune cells were already counted before[2,3]. PD-L1 was scored in tumor cells using established TP- and CP-Scores. For statistical analysis paired Spearman correlation coefficients were calculated.

**Results:** When stratifying the patient cohort for high and low STAT3 and STAT1 expression, there were no significant differences in clinicopathological parameters. STAT1 expression in tumor cells and STAT1 positive immune cells showed a strong correlation with infiltrating T-cells and an intermediate correlation for B-cells and macrophages. STAT3 expression in tumor cells was moderately correlated with CD4- and FOXP3-positive immune cells. In addition, STAT1 expression in tumor and immune cells showed a strong correlation with PD-L1 TP- and CP-scores in tumor cells and with T- and B-cells. Furthermore, cell counts were compared to verify the results. Here, T- and B-cell counts and macrophages were significantly increased in tumors with high PD-L1 TPS. This effect was also detectable in STAT1 positive immune cells but not in STAT3 positive immune cells. Tumors with high STAT3 expression showed a significantly increased infiltration by FOXP3-positive and CD8-positive T-cells. When analyzing the immune cells, those tumors with a high level of STAT3 expressing immune cells had significantly more infiltrating T- and B-cells. Tumor cells with a high nuclear STAT1 and STAT3 expression showed a high cytoplasmatic STAT1 and STAT3 expression, respectively. In addition, tumor cells with a high nuclear STAT3 expression showed a high nuclear STAT1 expression.

**Conclusions:** We could show a subset of HCC tumor samples with activated STAT1 and STAT3 signaling, associated with increased infiltrating immune cells, mainly consisting of cytotoxic and regulatory T-cells, which are often associated with worse prognosis in HCC. High PD-L1 expression in STAT3 positive tumor cells could indicate a subset of tumors with STAT3-associated immunotolerant behavior, possibly facilitating a new approach for blocking PD1-/PD-L1-axis.

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## Comparative Profiling of ML364 Treated HepG2 Hepatocellular Carcinoma Cells using Bottom-Up Proteomics Approach

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**Question(s):** The current study aims to investigate the involvement of ubiquitination pathway in development of hepatocellulct carcinoma (HCC). The levels of Ubiquitin Specific Proteases (USPs) and its substrates in HCC HepG2 cells are examined using ML364 treatment on the proliferation of HCC HepG2 cells in-vitro. The toxicity generated by ML364 produces anti-proliferative effects on HCC cells. Lable free proteomic studies were conducted in order to quantitate and identify differential protein expression as potential therapeutic targets for the treatment of HCC.
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**Methods used:** ML364 treated and untreated HepG2 HCC cells were lysed using 6M Guanidine HCl in 100mM Tris. All the samples were reduced and alkylated with CAA and TCEP respectively. Samples were digested with Lys-C and trypsin (Mass Spec Grade) followed by desalting using C-18 zip tips and eluted with acetonitrile and formic acid. Eluted peptides were then dried using vacuum concentrator. The peptides were reconstituted in 0.1%TFA and peptide concentration were measured using Nanodrop. All the peptides were appplied on Orbitrap Fusion™ Lumos™ Mass Spectrometer for lable free quantification. Data were analyzed using the DIANN software at mass accuracy of 4.5 ppm and FDR 0.01 for peptide and proteins. Carbamidomethylation and Oxidation (M) were selected as fixed modifications whereas variable modification was acetylation of protein N-termin. Statistical analysis was performed with LIMMA pakage. Statistically signicant proteins were used to generate heatmap. String analysis was performed in order to determine the protein-protein interactions.

**Result(s):** Approximately 3000 proteins were identified in HepG2 cells. Differentially expressed proteins were found to be associated with the ubiquitination pathway. FASN, USP4, and USP7 decreased significantly ($p<0.05$). We have also identified TRIMs which play a significant role in various biological processes such as viral genetic diseases, infections, and carcinogenesis. Significant upregulation of TP53 and P21 in ML364 treated HepG2 HCC cells play a crucial role in tumor growth suppression.

**Conclusion(s):** Differentially expressed proteins might be beneficial biomarkers for HCC. Our study lays a foundation for understanding the role of ML364 induced cell cytotoxicity in HepG2 cells and provides a potential therapeutic target for treating Hepatocellular carcinoma.

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## Discovery Proteomics: Comparative Serum Profiling of Hepatocellular Carcinoma

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**Question(s):** The aim of the study was to investigate the differential protein expression in serum samples from HCC patients and as compared to serum from healthy individuals with normal liver function. The potential role of these identified differentially expressed proteins was investigated in hepatocarcinogenesis. Furthermore, involvement of these differentially expressed proteins in signalling cascades have also been evaluated. The study may provide valuable insight into potential biomarkers or therapeutic targets for HCC. It could help in developing better diagnostic tools and treatment strategies for patients with HCC.

**Methods used:** Serum samples from healthy and HCC patients were used for LFQ LC-MS/MS. Initially serum samples were treated with 6M G-HCl in 100mM Tris added CAA and TCEP. After boiling samples were digsted with LyC and trypsin for over night. Peptides were de salted using C18 column and eluted with 50% ACNin 0.1% TFA. Eluted peptides were vacoufuged and reconstituted with 0.1%TFA. Peptide concentration were measured using nanodrop. Finally extracted peptides were applied on orbitrap Fusion™ Lumos™ mass spectrometry for labelle free quantitation. Data were analyzed using the DIANN software and raw data files were searched against a human database (Uniprot Homo sapiens), using a mass accuracy of 4.5 ppm and 0.01 false discovery rate (FDR) at peptide and protein levels. Carbamidomethylation was specified as fixed modification while methionine oxidation and acetylation of protein N-termini were specified as variable. For statistical analysis the replicates of HCC and normal serum samples were grouped and t-test was employed to find significant changes in protein intensities. . Intensities were then normalized and differential analysis was performed with LIMMA pakage. Protein-protein interaction analysis was analyzed using STRING and involvement of these proteins in various pathways were also investigated using KEGG pathway.

**Result(s):** Approx 600 differentialy expressed proteins were identified in HCC serum. Mostly upregulated proteins are involved in Nitrogen metabolism, Glutathione metabolism, Glycolysis / Gluconeogenesis, Carbon metabolism, HIF−1 signaling pathway. Down regulated protein are mainly responsible for PI3K−Akt signaling pathway, Lipid and atherosclerosis, Protein processing in endoplasmic reticulum, Thyroid hormone synthesis, PPAR signaling pathway, Platelet activation, Glycolysis / Gluconeogenesis, Carbon metabolism ECM−receptor interaction, Glutathione metabolism Complement and coagulation cascades and Cholesterol metabolism.

**Conclusion(s):** These findings confirmed that mass spectrometry-based label-free quantitative proteomics can be used to gain insights into liver carcinogenesis. Identified proteins might be used as theragnostic markers for early stage diagnosis and treatment.

**Reference:**

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**Support & Funding:** Higher Education Commission, Pakistan





## Myc-dependent replicative response to therapeutic genotoxins in liver cancer cells

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**Abstract**

The oncogenic transcription factor Myc is a pleiotropic regulator of transcription, DNA replication and DNA damage response pathways. Myc is overexpressed in many human liver tumors and is essential for tumor development in mouse models of liver cancer. Therefore, analysis of essential Myc-regulatory mechanisms can provide important insights for liver cancer therapy.

Myc is a short-lived protein stringently regulated by the ubiquitin system. The Usp28 deubiquitinase is a key regulator of Myc stability but also plays a critical role in cellular response to DNA damage via interactions with the mediator protein 53bp1. Usp28 forms stable dimers in vitro and in vivo, but the biological role of Usp28 dimerization is unknown.

We show that dimerization limits Usp28-mediated deubiquitination of Myc and that forced expression of monomeric Usp28 leads to ectopic Myc stabilization. Surprisingly, monomeric Usp28 selectively stimulates Myc function in DNA replication with a minimal impact on gene expression. 53bp1 promotes dimerization of Usp28 and thereby limits Myc-dependent DNA replication. Depletion of 53bp1 favors formation of Usp28 monomers, deregulating DNA replication. Genotoxic stress disrupts 53bp1-Usp28 complexes, promotes formation of Usp28 monomers and stabilizes Myc. This triggers ectopic firing of DNA replication origins during early response to genotoxins, amplifying DNA damage. Consistently, inhibition of DNA replication promotes cell survival under genotoxin treatment, whereas acceleration of DNA replication exacerbates genotoxin-induced cell death. Our data thus uncover a molecular stress response to therapeutic genotoxins, which may be exploited for liver cancer therapies.

From genotype to phenotype: how IDH1 mutations alter the landscape of intrahepatic cholangiocarcinoma

Agnieszka Seretny*, Lio Böse, Janine Rückheim, Peter Macsek, Luise Butthof, Kai Volz, Darjus Tschaharganeh

Questions:
We aimed to understand the implications of gain-of-function IDH1 mutations in intrahepatic cholangiocarcinoma (iCCA) progression and how the oncometabolite, 2-hydroxyglutarate (2-HG), influences the disease. We also investigated which elements contribute to the 2-HG-driven phenotype and evaluated whether targeting them in the context of mutated IDH1 could offer a viable therapeutic strategy for iCCA.

Methods:
We utilized a liver-specific mouse model via hydrodynamic tail vein injection to study the impact of IDH1 mutations and 2-HG production in iCCA. A combination of techniques, including RNAseq, methylation array, immunohistochemistry, and a spectrum of biochemical assays, were deployed to elucidate the 2-HG-driven phenotype. Mass spectrometry further enabled molecular profiling of changes in the liver cancer tissue.

Results:
Our results showed that IDH1 mutations can significantly reduce the survival span of tumor-bearing mice. Our data further showed that 2-HG accumulation in tumor tissue leads to increased methylation, tumor differentiation, and altered abundance of stromal and immune cell infiltration.

Conclusions:
Our research highlights the importance of IDH1 mutations in iCCA progression, especially the impact of 2-HG. The findings underscore IDH1's role in shaping the tumor microenvironment and influencing cell differentiation. Our analyses are also shedding light on key elements that contribute to the 2-HG-driven phenotype, providing direction for future therapeutic development in the context IDH1-mutant iCCA.

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Universitätsklinikum Heidelberg, DKFZ, DFG





## A tetracycline-inducible model for preclinical testing of immunostimulatory transgenes in solid tumors.

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**Question(s):** Immune therapy significantly improved treatment of cancer patients and has been established as another standard therapy in just a few years. Treatment success almost exclusively relies on reversion of tumor induced T cell exhaustion by immune checkpoint inhibitors, resulting in an obligatory need for a T cell inflamed tumor bed. However most tumor entities will establish an immune suppressive TME, which is a fundamental problem of any immune therapy. Even today, it is clear that current therapy options will not serve all cancer patients. Furthermore, therapy choice is prone to arbitrariness and follows the try and error principle. Transgenic expression of immunologic key regulators may create an inflammatory TME and improve current immunotherapies. In this study, we demonstrate suitability of a tetracycline-inducible expression system for the preclinical testing of gene candidates.

**Methods used:** Various murine tumor cell lines were stably transduced with immunologic key regulators under the control of a tetracycline responsive promoter (Tet-On). Subcutaneous injection of transgenic tumor cells gave rise to solid tumors and gene expression was induced with a doxycycline-supplemented diet. Progression of tumor growth as well as local and systemic changes in immune cell frequencies were monitored by flow cytometry and multiplex immune histology.

**Result(s):** Functionalitiy of the Tet-On expression system was confirmed by utilization of EGFP transgenic tumor cells. *In vivo* expression kinetics of EGFP in tumor cells were clearly controllable and could be quantified in FACS analysis and Western blots. The testing of gene candidates has shown, that expression of a single immunologic regulator induced obvious changes of intratumoral immune cells, but had very limited effects on tumor growth, even when transgene expression was combined with αPD-1 therapy. Moreover, effects of trangenic gene regulation showed major differences between tumor entities. In contrast, combined expression of multiple immunologic regulators clearly amplified effects on immune cell infiltration into tumors. In most cases these combined effects could not be predicted and were of synergistic - rather than an additive nature. In general, simultaneous targeting of T cells and DCs seemed to be favorable and the triple combination of IL12, CXCL9, FLT3L showed the most convincing therapeutic effects on tumor growth. Interestingly, in these experiments tumor remission correlated with frequency and phenotype changes of local T cells.

**Conclusion(s):** The given date shows a feasable and robust method to test transgenic gene candidates in a preclinical setting. The results demonstrate the need for multifactor approaches to reverse local immune suppression, with a strong focus on simultaneous targeting of T cells and DCs.

**Support & Funding:** Deutsche Forschungsgemeinschaft (DFG)

## Characterization of tumor immune microenvironment revealed enrichment of M2-macrophages, impaired metabolic functions, and the involvement of 14-3-3 protein family in driving poor sorafenib-response of HCC patients.

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**Question(s):** Identification of molecular drivers of poor response to HCC therapy with large focus on novel prognostic markers associated with tumor immune landscapes was our ultimate goal.

**Methods used:** From a cohort of 91 patients treated with sorafenib, we identified 17 HCC patients with particularly good or bad response. Integrative RNA sequencing and whole-exome sequencing analyses were performed to characterize tumor microenvironment and to identify predictive biomarkers of sorafenib resistance. Further, *in vitro* validation of defined targets were performed in a model of sorafenib resistance, followed by subsequent functional and mechanistic validation.

**Result(s):** Patients with worst response to sorafenib (n=7) were characterized by significantly shorter treatment duration and poor overall survival than good responders (n=10). Molecular analyses revealed that acquisition of drug resistance observed in poor responders was associated with impaired immune response reflected by enrichment of M2-immunosuppressive macrophages, upregulation of lipid metabolism and hypoxia-related pathways. From potential biomarkers of poor response, 14-3-3 scaffolding protein family was identified. Specific peptide inhibition of these proteins, in combination with sorafenib, displayed synergistic effects and efficiently reduced cell proliferation and viability. Dual inhibition consequently reversed sorafenib resistance and migratory capacity of tumor cells.

**Conclusion(s):** Defining actionable targets and their subsequent inhibition might greatly help delineate molecular alterations driving drug resistance. Notably, characterization of the immune microenvironment in different subgroups could be of particular importance to depict treatment resistance and warrants further investigations.

**Support & Funding:** N/A





## Non-steroidal analgesics drugs dim the acute phase response but amplify BMP-induced hepcidin expression in liver cancer cells

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**Question(s):** Cancer is frequently associated with chronic inflammation, which can result in cancer related pain. At advanced stages of the disease many patients suffer in addition from anemia characterized by reduced levels of red blood cells, a condition that severly impairs quality of life. Interleukin (IL)-6 is a key inflammatory cytokine and triggers the expression of acute phase proteins in hepatocytes, including hepcidin, a key regulatory hormone of iron availability, which is critical for the production of red blood cells. Among other medication, cancer-pain is commonly treated with prescription-free non-steroidal analgesics like diclofenac (DCF) or acetaminophen (APAP) that are metabolized in hepatocytes. Therefore, we hypothesized that this pain medication might impact IL-6 induced acute phase responses in hepatocytes and thereby aggrevate anemia in cancer patients.

**Methods used:** We used mass spectrometry-based proteomics to characterize the effect of DCF and APAP on IL-6 induced changes in the proteome of the hepatoma cell line HepG2 as well as primary human hepatocytes (PHH). To disentangle regulatory mechanisms and cross-talk, we employed a systems biology approach and developed a dynamic pathway model consisting of ordinary differential equations that was calibrated utilizing time- and dose-resolved data generated by qRT-PCR and quantitative immunoblotting. The mathematical modelling approach was utilized to predict intervention points that were validated by targeted inhibitor studies.

**Result(s):** The global proteome analysis of the hepatoma cell line HepG2 and primary human hepatocytes (PHH) confirmed the IL-6-dependent induction of acute phase proteins and revealed an inhibitory impact of DCF and APAP in both cell types. Dynamic pathway modelling identified that DCF and APAP enhance the induction of the feedback-inhibitor SOCS3 and reduce IL-6 mediated STAT3 phosphorylation in hepatoma cells and PHHs. Surprisingly, at early time points, DCF and APAP co-treatment had a stronger inhibitory effect on the expression of genes coding for acute phase proteins in PHHs. On the contrary, in hepatoma cells we observed an amplified expression of hepcidin upon co-treatment with DCF or APAP. Our mathematical modelling approach revealed that the amplified hepcidin expression is not the result of altered IL-6 signal transduction, but due to an autocrine activation loop of bone morphogenetic protein (BMP) signal transduction in hepatoma cells, which is absent in PHHs.

**Conclusion(s):** Our integrated mathematical model of the IL-6 and BMP signaling pathways did not only identify the processes affected by DCF or APAP regulating hepcidin expression in liver cells, but also predicted that inhibition of BMP signal transduction is the most promising intervention point to reduce excessive hepcidin production.

**Support & Funding:** This work has been funded by the German Federal Ministry of Education and Research (BMBF) within the Liver Systems Medicine network, the e:Bio collaborative research projects “Multi-Scale Modeling of Drug-Induced Liver Injury” (MS_DILI), the EraSysAPP consortium IMOMESIC as well as the Deutsche Forschungsgemeinschaft (DFG) within Germany's Excellence Strategy (CIBSS – EXC-2189), the TRR179 and FerrOs – FOR-5146.

## Analyzing the Association of *ASS1* Gene polymorphism in relation to Arginine Metabolism and the Risk of HCC

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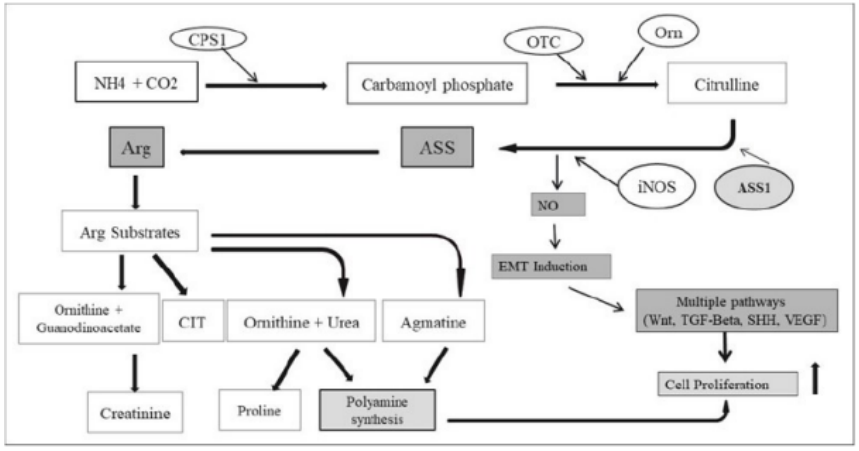
**Question(s):**
The aim of this study is to analyze the ASS1 gene expression, its polymorphic genotype and microsatellite instability among HCC patients from our Pakistani population. In liver, arginosuccinate synthase-1 is a center of arginine metabolism and rate limiting enzyme of urea cycle. It also triggers multiple mechanisms that lead to HCC pathogenesis.[1,2] Variable factors directly impact the disease burden, among them, molecular alterations have been found to play a significant role.[3,4]

**Methods used:** : Blood samples were collected from disease and healthy control individuals. Allele-Specific PCR was performed for SNP analysis. MSI of tri and tetra nucleotide repeats were analyzed by PCR. The differential expression of ASS1 gene was also investigated. Furthermore, the reactome database and STRING software were utilized for finding correlations between ASS1 gene with other associated gene/proteins.

**Result(s):** The GG wild-type genotype was more prevailed in the disease group as compared to the control. Significant downregulation in ASS1 and NOS2 genes was observed. Bioinformatics analysis reveals the correlation between ASS1 polymorphism and HCC development appears to be linked with the EMT pathway and polyamine production. Furthermore, MSI significantly resided in the disease group. Results were analyzed statistically to calculate the significance of obtained results.

**Conclusion(s):** Study concludes that the insight of HCC mechanism through population-specific genetic mutations and altered gene expression of ASS1 might be helpful in early diagnostic and therapeutic purposes.




Graphic 1: *ASS1* regulation with polyamines and *NOS2* in hepatocellular carcinoma

| Genotype | Disease Groups | Control Groups | OR | 95 % CI | P-Value | Chi-Square |
|---|---|---|---|---|---|---|
| **GG** | 22/32 (68.75%) | 8/32 (25%) | **6.600** | 2.2079 - 19.7288 | < 0.001 | $\ast\ast\ast$ $X^2$ =14.492 |
| **GT** | 07/32 (21.87%) | 22/32 (68.75%) | 0.1273 | 0.0414- 0.3913 | | |
| **TT** | 03/32 (9.37%) | 02/32 (6.25%) | 1.5517 | 0.2414- 9.9739 | | |
| **Allele type** | | | | | | |
| **G** | 51/64 (79.68%) | 38/64 (20.31%) | **2.6842** | 1.2216 - 5.8978 | 0.0140* | |
| **T** | 13/64 (20.31) | 26/64 (40.62%) | 0.3725 | 0.1696- 0.8186 | 0.0140* | |

Table 1: Distribution of ASS1 gene SNP; rs_10901080 (G>T) genotype and allele type in HCC patients and control subjects. OR > 1 Represents genotype is associated with disease and vice versa.

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## Repurposing passenger amplifications for specific therapeutic targeting of liver and other solid cancers

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**Question(s):** Current cancer therapies focus on targeting driver alterations responsible for tumorigenesis: either oncogenic point mutations or driver events within large somatic copy number alterations (SCNAs)[1,2]. However, these alterations are often not actionable or are only present in a small subset of patients and, thus, new therapeutic targets are urgently needed. In the case of SCNAs, these regions normally also comprise neutral bystander genes without an active role in neoplastic transformation: we hypothesized that passenger events, specifically in amplified regions, could be therapeutically exploited by providing actionable molecules on the cell surface.

**Methods used:** Using publicly available multi-omics data, we screened cell surface protein-coding genes[3] for their genomic status and expression levels in liver cancer[4]. Subsequently, we performed a thorough validation of our top candidate hit, analyzing its protein levels on human tissues. Lastly, we applied our findings to the development of a chimeric antigen receptor (CAR)-T cell therapy specific against several solid tumor entities.

**Result(s):** We identified the cell-surface protein-coding gene *MPZL1* (Myelin protein zero-like 1), which is amplified in 75% of hepatocellular carcinomas, accompanied high mRNA expression in tumors compared to normal livers. *MPZL1* codes for a glycosylated cell surface receptor[5] and is located on chromosome 1q, one of the most commonly amplified regions across several solid cancer types. We further validated MPZL1 protein expression in a wide range of human cancer entities (n=2038) and normal tissues (n=163) by immunohistochemistry, and found that a high percentage of the tumors in study present scores 2 or 3 (e.g. 48% of HCCs or 89% of TNBCs), whereas healthy tissues are mostly negative or just faintly positive (scores 0 or 1). Next, in order to target MPZL1-expressing cells, we generated a highly specific monoclonal antibody directed to the extracellular domain of human MPZL1 protein and thoroughly characterized its binding capacity. The scFv sequence of this antibody was then used to generate a CAR construct targeting MPZL1. Corresponding CAR-T potently killed various MPZL1-high human cancer cell lines *in vitro* (liver, breast and lung, among others), whereas they failed to kill respective isogenic cell lines with *MPZL1* knockout. Moreover, upon antigen-specific exposure, MPZL1-28ζ CAR-T cells underwent antigen-dependent proliferation and showed increased cytokines production (IFNγ, TNFα, IL-2, GZMB), further confirming their specificity.

**Conclusion(s):** Our findings reveal MPZL1 as a new target for the treatment of 1q-amplified cancers and implement a novel immunotherapeutic strategy based on MPZL1-28ζ CAR-T cells. Furthermore, our work provides a proof of concept that passenger events within large chromosomal amplifications can serve as potent therapeutic targets, opening a new avenue for innovative approaches in the field of anti-cancer drug development.

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## Effectivity and tolerability of chemosaturation of the liver with melphalan for patients with primary or secondary liver tumors

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**Question(s):** Chemosaturation by percutaneous hepatic perfusion (CS-PHP, CHEMOSAT®, Delcath) allows temporary administration of melphalan hydrochloride directly to the liver. This approach was shown to be effective in controlling tumor growth in patients with primary or secondary liver tumors. In those studies, CS-PHP was applied in various frequencies ranging from one to six times. We have retrospectively analysed the effectiveness and tolerability of CS-PHP given on an eight-weekly basis.

**Methods used:** CS-PHP was applied to patients with primary or secondary liver tumors based on board decision in one German center until disease progression, intolerability or complete response. Overall survival (OS) and overall response rates (ORR) were retrospectively assessed according to Response Evaluation Criteria In Solid Tumors (mRECIST). OS was analysed by Kaplan-Meier estimation. ORR included patients achieving complete (CR) or partial response (PR) after each treatment. Toxicity associated with CS-PHP was assessed according to Common Terminology Criteria for adverse events (CTCAEv4.03).

**Result(s):** A total of 31 patients (21 (68%) female) was treated with 90 (median 2, range 1-6) CS-PHP between 2016 and 2022. Included patients had either unresectable intrahepatic metastases of ocular melanoma (OM, n=17), intrahepatic cholangio carcinoma (ICC, n=8), hepatocellular carcinoma (HCC, n=2), ciliary body melanoma (n=1), acinar cell carcinoma (n=1), hepatic spread of pancreatic (PC, n=1) or tonsillar carcinoma (n=1). CS-PHP was performed 6 times in 5, 5 times in 4, 4 times in one, three times in 4, two times in 7 and one time in 10 patients.
The median OS of all patients since decision for CS-PHP was 67 (range, 41.31-92.89) weeks, and for patients with OM 76 (range, 56.5-95.5) weeks and for ICC 37 (range, 32.11-41.88) weeks, respectively. ORR was 66% to all CS-PHP treatments (60/90), including 85% of OM and 31.25% of ICC patients. In 5 patients, CR was achieved after a median of 5 (range, 2-6) CS-PHP. CS-PHP was abandoned due to disease progression in three patients after first CS-PHP, due intolerability in two and due to lost in follow up in 7 patients. Presence of extrahepatic tumor manifestations before CS-PHP was not associated with response or survival. In 12 patients developing extrahepatic tumor progress, one of them with bone marrow infiltration, CS-PHP was continued if liver tumors were still controlled. Hematological AEs included leukopenia (grade 2) or thrombocytopenia (grade 2-3) were transient. Febrile neutropenia occurred in two cases and was treated with G-CSF.

**Conclusion(s):** CS-PHP induced response in the majority of malignant primary or secondary liver tumors. The procedure was safe and had few hematological side effects. The effectivity CS-PHP as long-term treatment needs to be validated in future studies.

# Inhibition of the non-canonical NF-κB pathway attenuates lymphotoxin-induced proliferation in HCC cells

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**Question(s):** Non-canonical nuclear factor kappa B (NF-κB) signaling is involved in onset and progression of chronic liver diseases [1]. In various solid tumor types, a protumorigenic role was demonstrated [2-4]. NF-κB activating cytokines Lymphotoxine α (LTA) and β (LTB) are present in virus infected livers and hepatocellular carcinomas (HCCs) [5]. This study aimed to assess how non-canonical NF-κB pathway stimulation through LTB and inhibition through the small molecule NF-κB inducing kinase (NIK) inhibitor B022 affects HCC cell survival and proliferation.

**Methods used:** Liver cancer cell lines Huh7, Hep3B, and HepG2 were exposed to LTB axis activation (LT-α1/β2), NIK inhibition (small molecule NIK inhibitor B022) or a combination of the two. First, Western Blot analyses were performed to compare expression levels and nuclear translocation of non-canonical NF-κB transcription factors RelB and p52. Next, the effect of downstream inhibition of non-canonical NF-κB by NIK inhibition via B022 application on cell proliferation was investigated by continuous impedance measurements. Cell death measurements were conducted by FACS analyses. Finally, qPCR was performed to determine mRNA levels of RELB and NIK.

**Result(s):** In vitro, basal levels of NF-κB proteins were low. Upon LT-α1/β2 treatment, RelB and p52 were upregulated and nuclear translocation was increased. This effect was attenuated through combination of LT-α1/β2 and B022. Proliferation assessment revealed a significant attenuation of proliferation in all three cell lines in the combination treatment group compared to prominent proliferation induction following LT-α1/β2 treatment ($p<0.0001$). B022 treatment led to a slight increase in cell death compared to untreated controls (Hep3B: $p=0.1608$; HepG2: $p=0.0033$; Huh7: $p=0.0217$). qPCR analyses of Hep3B cells showed an increase in mRNA abundance for NIK ($p=0.3955$) and RelB ($p=0.0247$) upon LT-α1/β2 treatment compared to controls and less induction in the LT-α1/β2/B022 combination condition (NIK: $p=0.6091$; RelB: $p=0.6448$) compared to LT-α1/β2 treatment.

**Conclusion(s):** B022 suppresses the LT-α1/β2 induced proliferation phenotype in HCC cells through inhibition of non-canonical NF-κB pathway activity. Hence, NIK could be a potential therapeutic target in HCC. Further analyses using B022 in vivo should be performed to investigate the therapeutic potential of this effect.

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## Genetically Predicted Circulating Concentrations of Bacterial Metabolites and Hepatobiliary Cancer: A Mendelian Randomization Study

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**Question:** Hepatobiliary cancers (HBC) and cancers of the biliary tract including intrahepatic cholangiocarcinoma, share high mortality and rising incidence rates. Recent data suggest a role for the gut microbiome in the development of HBC as the gut microbiome and the liver interact bidirectionally via the gut-liver axis. To complement and inform observational studies, we investigated associations of genetically predicted concentrations of circulating bacterial metabolites with HBC using Mendelian randomization (MR).

**Methods used:** We searched Pubmed, exposome explorer and Human Metabolome Database (HMBD) to identify circulating bacterial metabolites. This yielded 473 metabolites. We then searched Pubmed and the Genome Wide Association Study (GWAS) catalogue for Single Nucleotide Polymorphisms (SNPs) associated with circulating concentrations of these metabolites. This provided 11,796 SNPs with genome wide significance ($p < 5 \times 10\text{-}8$) for 62 metabolites and suggestive significance ($p < 1 \times 10\text{-}5$) for an additional 71 metabolites from published GWASs conducted among individuals of European decent. Two-sample MR was conducted using the malignant neoplasm of liver and intrahepatic bile ducts dataset from UK biobank (cases n=539, controls=419,992). Inverse variance-weighted MR analyses were performed with sensitivity analyses to assess the impact of potential violations of MR assumptions.

**Result(s):** Nominally significant associations for inverse HBC risk associations were observed for higher genetically predicted phenylacetate (Odds ratio per standard deviation [ORSD]: 0.226; p-value = 0.049), histidine (ORSD: 0.423; p-value = 0.024) and acetoacetate (ORSD: 0.174; p-value = 0.024).

**Conclusions**: These results, though based only on genetically predicted circulating metabolite concentrations, provide evidence for possible associations of bacterial metabolites with hepatobiliary carcinogenesis.

**Acknowledgement**: Thanks to the UK Biobank investigators and study participants.





## N-cadherin: a Diagnostic Marker to help Discriminate Primary Liver Carcinomas from Extrahepatic Carcinomas

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**Question(s):** Distinguishing primary liver cancer (PLC), namely hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), from liver metastases is of crucial clinical importance. Histopathology remains the gold-standard, but differential diagnosis may be challenging. While absent in most epithelial, the expression of the adherens junction glycoprotein N-cadherin is commonly restricted to neural and mesenchymal cells, or carcinoma cells that undergo the phenomenon of epithelial-to-mesenchymal transition (EMT). However, we recently established N- and E-cadherin expression as hallmarks of normal hepatocytes and cholangiocytes, which are also preserved in HCC and iCCA. Therefore, we hypothesized that E- and/or N-cadherin may distinguish between carcinoma derived from the liver versus carcinoma of other origins.

**Methods used:** We comprehensively evaluated E- and N-cadherin in 2,489 different tumors in a multicenter study using immunohistochemistry and compared our results with previously published 882 cases of PLC, including 570 HCC and 312 iCCA.

**Result(s):** Most carcinomas showed strong positivity for E-cadherin. Strong N-cadherin positivity was present in HCC and iCCA. However, except for clear cell renal cell carcinoma (23.6% of cases), N-cadherin was rarely and only faintly expressed in adenocarcinomas of the gastrointestinal tract (0-0.5%), lung (7.1%), pancreas (3.9%), gynecological organs (0-7.4%) and breast (2.2%) as well as in urothelial (9.4%) or squamous cell carcinoma (0-5.6%). As expected, N-cadherin was detected in tumors of neuroendocrine origins, such as thyroid cancer (29.2%), neuroendocrine tumors (25-75%), as well as in malignant melanoma (46.2%), and malignant mesothelioma (41%).

**Conclusion(s):** In conclusion, N-cadherin is a useful marker for the distinction of PLC versus liver metastases of extrahepatic carcinomas ($p<.01$).

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## Plectin-mediated cytoskeletal cross-talk as a target for suppression of hepatocellular carcinoma growth and metastasis.

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**Question(s):** Cytoskeleton-based mechanical homeostasis plays a critical role in numerous processes, including carcinogenesis. Plectin, a cytolinker protein that crosslinks and shapes cytoskeletal networks, was found to be upregulated in various tumor types including hepatocellular carcinoma (HCC). Here, we investigate the role of plectin-mediated cytoskeletal crosstalk in the invasion and tumorigenic potential of HCC cells. We also aim to validate plectin as a potential target for HCC treatment.

**Methods used:** First, the immunohistochemistry of HCC patients liver samples was performed to compare plectin levels in tumor and adjacent non-tumor tissue. The effect of plectin ablation on HCC development was inspected by chemical induction of hepatocarcinogenesis using diethylnitrosamine (DEN) in $Ple^{fl/fl}$ and $Ple^{\Delta alb}$ mice. Next, plectin-deficient human HCC cell lines were generated using CRISPR/Cas9 approach, together with plectin functional mutants lacking the intermediate filament-binding domain. Also, the effect of plectin pharmacological targeting was addressed using plecstatin, a high-affinity plectin ligand. The effect of plectin targeting on tumorigenic potential and motility of HCC cells *in vivo* was verified in immunodeficient mice using xenograft tumor formation assay and lung colonization assay. To test the therapeutic potential of plectin targeting in HCC, hepatocarcinogenesis was induced in $Ple^{fl/fl}$ and $Ple^{\Delta alb}$ mice by hydrodynamic gene delivery of transposon vector expressing *c-myc* (Myc) in conjuction with CRISPR/Cas9 construct targeting *Tp53* (sgTp53) via tail vein injection. The tumor development over time was then monitored using magnetic resonance imaging.

**Result(s):** Plectin fluorescence intensity levels were significantly elevated in liver tumor tissue when compared to adjacent non-tumor tissue. A similar effect on plectin levels was observed in DEN-treated $Ple^{fl/fl}$ mice liver tumor and non-tumor tissue using immunohistochemistry and immunoblot analysis. The plectin ablation in $Ple^{\Delta alb}$ mice accounted for a reduced number of tumors formed upon DEN treatment. Both genetic and pharmacological plectin targeting in HCC cells resulted in compromised xenograft formation and growth, together with a lower number and volume of HCC cell loci in the lungs. The plectin depletion in $Ple^{\Delta alb}$ mice reduced tumor development and increased the survival of Myc;sgTp53 injected mice. A similar effect on tumor burden was observed in Myc;sgTp53 injected $Ple^{fl/fl}$ mice orally treated with plecstatin.

**Conclusion(s):** Our findings show that plectin elevation in liver tumor tissue appears to associate with HCC progression, which can be prevented by genetic or pharmacological plectin targeting. Plectin ablation attenuates the oncogenic and invasion potentials of HCC cells, suggesting plectin´s contribution to HCC metastatic capacity. Together, our results show plectin to be a potential therapeutic target in HCC.

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## Poorly Differentiated Hepatocellular Carcinoma with Osteoclast-like Giant Cell Component – A Case Report

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**Background:** Hepatocellular carcinoma with osteoclast-like giant cell component is a rare liver malignancy with a poor prognosis. As to our knowledge only 19 cases have been previously reported. The hepatocellular carcinoma with osteoclast-like giant cell component has a slightly higher incidence in men than in women, as concluded from previous reports, and has a tendency to affect people in their 6th decade of life [1].

**Case Presentation:** We report the case of a poorly differentiated hepatocellular carcinoma with osteoclast-like giant cell component found in a 42-year-old woman. The initial clinical findings included pain in the right upper abdominal quadrant and a few episodes of nausea and emesis. The abdominal ultrasound showed the presence of a tumoral mass in the right hepatic lobe, with multiple hepatic, subhepatic and periportal nodular lesions, and small volume ascites. The MRI examination revealed hepatomegaly and irregular capsule contour with a 14/16/14 cm tumour with a stromal and cystic structure, located in the IV, V and VIII hepatic segments. A central atypical resection of involved hepatic segments was performed. The resected segment was sent for histopathologic examination. The patient died 16 hours post- surgery and an autopsy was performed. Macroscopically, the tumour had large areas of haemorrhage and necrosis with stromal and cystic appearance. Histologically, the tumour was composed of spindle and epithelioid cells with marked pleomorphism and moderate to abundant cytoplasm. Giant osteoclast-like cells were seen. No lymphatic, vascular, or perineural invasion was observed. Immunohistochemistry was positive for CD68 and Glipican3 and negative for CK7, HepPar1, SMA, and DesminA. The autopsy showed multiple organ failure.

**Conclusion:** Hepatocellular carcinoma with osteoclast-like giant cells is rare among patients and has a reserved prognosis with a high mortality rate. Late detection and aggressiveness increase the mortality rate but also increases the risk of surgical complications due to anatomical constraints [2].

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# High-dimensional spatial profiling of the hepatocellular carcinoma tumor microenvironment reveals spatial immune types informing immune checkpoint inhibitor therapy response

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**Question(s):** Hepatocellular carcinomas (HCC) can be grouped into distinct subtypes according to their molecular profiles, including immune subclasses. However, clinical translation of this classification is lacking which may be due to the negligence of spatial context. We hypothesized that the spatial organization of the immune response in the tumor microenvironment (TME) is likely to influence response and survival of HCC patients under immune checkpoint inhibitor (ICI) therapy. Thus, we set out to characterize the HCC TME on a spatially resolved and high-dimensional level using highly multiplexed imaging mass cytometry (IMC).

**Methods used:** We first performed IMC analysis using a 41-marker panel on FFPE sections from a discovery cohort of 54 HCC patients with regions of interested selected in the tumor, interface and adjacent liver. We mapped tumor and non-tumor as well as stromal and parenchymal regions for each image. After channel normalization and cell segmentation, we clustered single-cells using PhenoGraph and identified immune neighborhoods based on spatial immune cell interactions. We reapplied the same workflow to a validaton cohort of 47 HCC patients that received ICI based therapies after tumor biopsy or resection.

**Result(s):** We identified several immune, stroma and tumor/hepatocyte cell clusters that reflect major cell types of the HCC and liver microenvironment. Unsupervised neighborhood detection based on spatial interaction of immune cells identified three immune neighborhoods: A CD8 T cell dominant, a B/CD4 T cell dominant and a macrophage/granulocyte dominant immune neighborhood. Based on key features such as infiltrating CD8 T cells between different tumor regions we identified distinct spatial immune types: an immune-depleted, immune-intermediate and immune-enriched tumor microenviroment. This classification correlated with the unsupervised microanatomic neighborhoods. We observed a differential organization of the TME between the spatial immune types with a reduction of antigen presenting cells in depleted patients, increased tumor stroma in intermediate patients and elevated amounts of PD-1+ and exhausted CD8 T cells in enriched patients. Immune types were not clearly connected to known HCC etiologies but correlated with progression-free survival under ICI based therapies.

**Conclusion(s):** In sum, our in-depth spatial analysis successfully captured the immune heterogeneity of HCC patients. Spatial immune types may be reflective of different immune evasive strategies of the corresponding tumor and represent a potential novel biomarker for ICI based therapies.

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# Multi-center study to evaluate the safety and efficacy of atezolizumab/bevacizumab or lenvatinib in treatment of hepatocellular carcinoma with special focus on bleeding- and thromboembolic-events - Real-world experience from 464 patients

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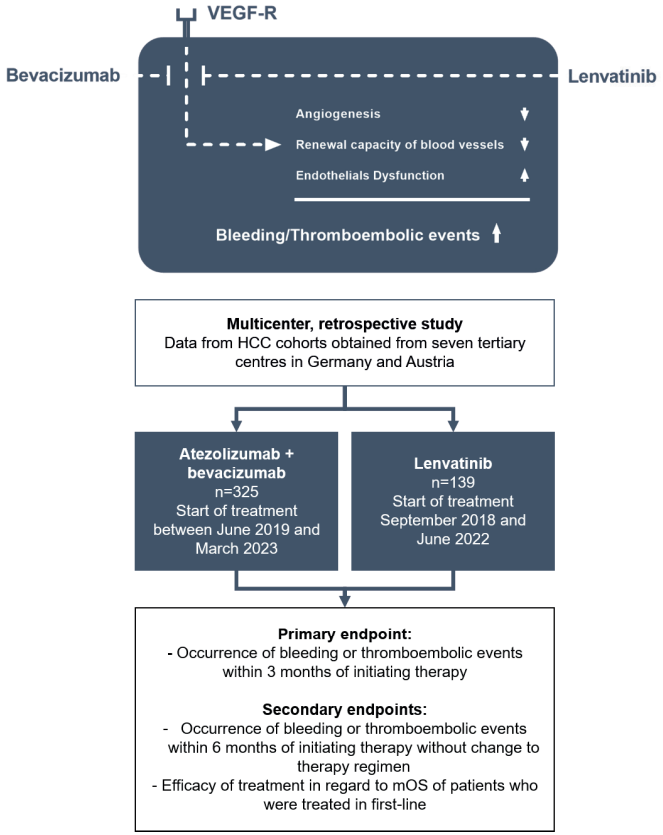
**Question(s):** The combination of atezolizumab and bevacizumab (atezo/bev) or lenvatinib demonstrated efficacy in systemic first-line therapy for hepatocellular carcinoma (HCC). However, a VEGF-inhibition with either bevacizumab or tyrosine-kinase inhibitors is associated with the risk of bleeding and thromboembolic events. In this regard, the IMbrave150 trial reported a numerically higher rate of bleeding and arterial thromboembolic events with atezo/bev compared to sorafenib. A first-line alternative to treatment with atezo/bev is currently lenvatinib, which has demonstrated longer progression-free survival and higher objective response rates compared to sorafenib. Data comparing the safety of atezo/bev and lenvatinib are widely lacking.
In this study, we evaluated the risk of bleeding and thromboembolic events with atezo/bev versus lenvatinib and their efficacy in a large, multi-centric real-world population.

**Methods used:** Data from HCC cohorts of seven tertiary centers in Germany and Austria were used for analysis. In total data from n=464 patients, who were treated between September 2018 and March 2023 with either atezo/bev (n=325) or lenvatinib (n=139) were evaluated. The incidence of bleeding or thromboembolic events within 3 months after initiation of therapy and of patients who received each therapy for 6 months was assessed. Efficacy of both treatments was compared in patients who received therapy in first-line. For statistical analysis Student's t test or Mann Whitney test was used. Fisher's exact test was applied for contingency tables. Survival analysis was performed by Log-rank test

**Result(s):** Both groups were balanced with respect to demographics, presence of liver cirrhosis and variceal status. Days on therapy did not differ. Surrogate markers of portal hypertension such as platelet count or spleen size showed no differences between groups. Bleeding episodes within 3 months of therapy were described in n=57 (18%) patients receiving atezo/bev compared to n=15 (11%) patients receiving lenvatinib (OR 1.76 95 % CI 0.97-3.2; not significant). Variceal hemorrhage occurred in n=11 (3%) patients treated with atezo/bev versus n=4 (3%) patients treated with lenvatinib (OR 1.18 95 % CI 0.40-3.44; not significant). Thromboembolic events were reported in n=19 (6%) of patients in the atezo/bev cohort compared to n=5 (4%) patients in the lenvatinib cohort (OR 1.66 95 % CI 0.63-4.13; not significant). Incidence of overall bleeding, variceal hemorrhage and

thromboembolic events did also not differ significantly in patients, who received either atezo/bev or lenvantinib for 6 months. Median overall survival (mOS) was 12.2 months in the atezo/bev group and 9.9 months in the lenvatinib group (not significant).

**Conclusion(s):** Rates of bleeding and thromboembolic events did not differ significantly between atezo/bev and lenvatinib and may not be helpful in choosing between the two treatments. Future prospective studies are needed to confirm our results.




Graphic 1: Both bevacizumab and lenvatinib are potent VEGF inhibitors and can result in higher rates of bleeding and/or thrombembolic events. The objective of our study was to investigate saftey in a large real-world cohort with 464 patients from seven tertiary centers.

**Support & Funding:** This study was initiated by the IMMUreal study group and was supported by the Bavarian Cancer Research Center (BZKF)





## PROX1 inhibits liver tumorigenesis by maintaining hepatocellular identity and preventing cellular plasticity.

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**Question(s):** Unlocking cellular plasticity and the subsequent loss of cell identity, leading to the acquisition of a malignant phenotype, has emerged as a new hallmark of cancer cells. Primary liver cancer can originate from hepatocytes when they undergo processes such as transdifferentiation, causing them to lose their original identity. However, the specific transcriptomic and epigenomic alterations that drive this transformation are still not well understood. Transcriptional repressors, which play a crucial role in defining and maintaining cell identity, achieve this by repressing genetic programs associated with other lineages, thus preventing cellular plasticity. This characteristic makes them interesting candidates for exploration in the context of liver cancer and this newly recognized hallmark. By defining and characterizing these transcription factors (TFs) within the dual contexts of cell identity and cancer, we can gain valuable insights into disease mechanisms and potentially uncover novel therapeutic approaches.

**Methods used:** We designed and conducted a screening to identify trancriptional repressors using scRNA-seq data from the Tabula Muris dataset. This screening was based on specific criteria, including cell-type-specific and lifelong TF expression, as well as the enrichment of TF DNA motifs at the promoters of repressed genes within each cell type. Transcriptional repressors identified in hepatocytes were then tested in the context of cell identity and cellular plasticity in different forms of liver cancer.

**Result(s):** We identified a total of 33 cell-type-specific and lifelong transcriptional repressors across 18 different cell types. Among the liver candidates tested, PROX1 emerged as the most potent hepatocyte-specific transcriptional repressor, enhancing reprogramming efficiency *in vitro* by suppressing other lineage fates. Furthermore, we observed that PROX1 expression was downregulated in human patients with hepatocellular carcinoma (HCC), and its expression correlated with patient survival. PROX1 overexpression was found to promote apoptosis and downregulate MYC-target genes in cancer cells, resulting in a reduction in the number of tumors and an increase in survival in a mouse model of HCC. Finally, we observed that the further downregulation of PROX1 could drive transdifferentiation from HCC to cholagiocarcinoma (CCA).

**Conclusion(s):** This study demonstrates the critical role of PROX1 in defining and maintaining hepatocellular identity through its function as a transcriptional repressor. Additionally, we have shown that PROX1 plays a role in preventing tumorigenesis by inhibiting cellular plasticity. Identifying and mechanistically characterizing similar factors in other cell types, guided by computational tools such as the one presented here, could facilitate the generation of cells for biomedical applications and reveal potential targets for preventing cell fate plasticity and diseases.

## Upcoming expression of ALK1 in HCC determines the switch from tumor-suppressive to tumor-promoting BMP-9 signaling

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**Introduction**: BMP-9, a hepatic cytokine belonging to the TGF-β superfamily, has garnered attention in recent studies due to its controversial role in hepatocellular carcinoma (HCC). Some investigations indicate BMP-9's promotion of cell proliferation and epithelial-to-mesenchymal transition (EMT), while others suggest anti-proliferative effects and a reduction in mesenchymal markers in HCC cells. With HCC ranking as the third leading cause of cancer-related deaths worldwide, there is an urgent need for new therapeutic targets to enhance treatment strategies. The aim of this study was therefore to better understand BMP-9 signaling in HCC.

**Methods**: We conducted an extensive analysis of available expression data for BMP-9 signaling pathway components using public databases, including The Cancer Genome Atlas (TCGA) and The Cancer Proteome Atlas (TCPA). Expression of ALK1, ALK2, Activin A Receptor Type 2A (ACVR2A), Activin A Receptor Type 2B (ACVR2B), BMPRII (BMPR2), endoglin (ENG), Smad1 (SMAD1) and others were assessed. Further, we determined Smad-1 phosphorylation, cell proliferation, and migration in response to BMP-9 stimulation in two human HCC cell lines (HLE and Hep3B) with Alk1 KO or over-expression using Western blot, real-time PCR, proliferation assays, and wound closure assays.

**Results** *In silico* results show that Alk1 is upregulated in HCC patient samples and the presence of Alk1 is associated with attributes of cancer progression. In line with this, BMP-9 induced proliferation, migration and EMT in HLE cells, which display high levels of Alk1, but not in epithelial Hep3B cells with low Alk1 expression. Over-expression of Alk1 in Hep3B could partially induce tumorigenic effects. Finally, we show that Alk1 expression leads to decreased Smad-1 phosphorylation and an enhanced tumor-promoting expression pattern, whereas non-Alk1 BMP-9 signaling (e.g., via Alk2) uses the canonical Smad-1-pathway for tumor-suppressive signaling.

**Conclusions** These findings suggest that in tumor cells presence of Alk1 promotes tumor progression whereas non-malignant hepatocytes (and some HCC cell lines) do not express Alk1 and respond to BMP-9 via the Smad-1 pathway, thereby stabilizing the differentiated (non-proliferative) parenchymal phenotype and acting tumor-suppressive. Thus, individualized determination of Alk1 expression in HCC patients should help to predict potential responsiveness to BMP-9 targeted therapies.



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## The patient registry of the Liver Cancer Center Heidelberg (LCCH-Register).

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**Question:** At the Liver Cancer Center Heidelberg (LCCH), patients with primary tumors of the hepatobiliary system are diagnosed and treated based on interdisciplinary tumor board decisions. This involves diagnostics that lie beyond the minimum requirement defined in clinical guidelines and offers a wide range of treatment options that go far beyond the repertoire of a certified liver tumor center. Through this comprehensive concept, patients with hepatobiliary tumors can be treated in an interdisciplinary, patient-oriented and individualized manner over the entire course of their disease, which is documented in a structured manner.
The collection and processing of health data is an important prerequisite for medical care and research. Initially, the required data was to a large extent electronically recorded in a wide range of application systems in predominantly different formats. An exchange and comparison between these systems was not systematically established, resulting in redundancies and inconsistencies, which affected data quality. This kind of unstructured data was difficult to use for interdisciplinary evaluations and research.

**Methods used:** Since the LCCH-Register was introduced in 2020, all patients with tumors of the hepatobiliary system who present at the Liver Cancer Center Heidelberg have been systematically recorded to optimize care and therapy in compliance with the current treatment recommendations. Clinically relevant data on the respective diagnosis, the therapy measures carried out and the course of the disease during and after treatment are documented in a standardized manner.

**Results:** Through close collaboration with the Register of the Center for Personalized Medicine (ZPM-Register) and the NCT Cancer Registry, we demonstrate how to leverage synergies in several ways: The registries use established data formats and work together to develop and implement new formats for oncological precision medicine [1-3]. One example is the integration of molecular diagnostic data into our tumor documentation for all registers together to link them with personalized therapy recommendations from molecular tumor boards and follow-up data from internal systems (Hospital Information System, laboratory) and external sources (residents' registration office, external doctors, state cancer registries). Since the already documented data of all registries are available in the same tumor documentation system (ONKOSTAR) in a shared format, duplication of documentation is avoided, all registries are interoperable and data exchange with external researchers is possible via exports in various standard formats. To date, we have provided more than 1400 pseudonymized patient records for 6 different research projects, some of which have already been published [4].

**Conclusion:** The LCCH-Register has established the necessary data base for the continuous improvement of patient care and research at the Liver Cancer Center Heidelberg.

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## Combined methyl-selenocysteine antitumor therapy in hepatocellular carcinoma cell lines

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**Question(s):** Liver cancer related death is the fourth common one worldwide. The cytotoxic effect of selenium compounds on tumor cells provides the opportunity to treat cells highly resistant to cytostatics. Methyl-selenocysteine (MSC) is a natural selenium compound primarily metabolized by KYAT 1. We aimed to answer the question whether the antitumor effect of MSC is different on separate HCC cell lines. Further, our goal was to investigate its effects with different KYAT-1 activators in combination with traditional chemotherapeutic agents and analyze main molecular mechanisms.

**Methods used:** We used Huh7 and HepG2 hepatoma cell lines for our study. We applied two keto acid analogs to stimulate the metabolism of MSC. The antiproliferative effect of MSC, α-keto acids, and sorafenib in monotherapy and in combination treatment was determined using the Alamar Blue Assay technique. Wes Simple was used to analyze protein levels of main signaling pathways of PI3K/AKT/mTOR; Ras/Raf/ERK1-2, which might influence proliferation and EMT.

**Result(s):** After 72 hours of treatment in both cell lines, the addition of α-keto acid increased the cytotoxic efficiency of MSC. In HepG2, after triple-combination treatment, markedly increased inhibition of proliferation was detected compared to sorafenib monotherapy. However, we could not prove this with Huh7. Triple combination treatment reduced the activity of PI3K/AKT/mTOR and MAPK/ERK pathways at the protein level. It may explain the success of combination therapy by simultaneously inhibiting several signaling pathways.

**Conclusion(s):** Our results highlighted the possibility of combined use of selenium compounds and traditional chemotherapeutic agents, especially in late-diagnosed, therapy-resistant tumors. In tumors of different aggressiveness, it is necessary to identify molecular mechanisms to enable personalized, successful therapy in HCC patients.

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