Index

Dear Participants of the 9th DLR,

Welcome to the 9th Dutch Liver Retreat!
Please find the program below:

1. A main program with oral presentations with junior scientists appointed as session chairs.
2. A scientific speed-date session and a competition for the best liver research idea of DLR 2019.
3. A group session. More information will follow.
4. Ample opportunities to meet and discuss with your fellow liver scientists.

The DLR is organized by the section Experimental Hepatology of the Netherlands Association for the Study of the Liver (NASL or NVH). The DLR2019 offers members a strong discount on the registration fee. Furthermore, members are eligible for the NASL/NVH travel grants to go to international scientific meetings and funds for printing your PhD thesis. Moreover, you will receive our quarterly magazine “Lever” with all the news and views on Dutch Hepatology.

We look forward to an exciting DLR2019!

Ronit Sverdlov (UM), Sven van IJzendoorn (UMCG), Stan van de Graaf (AUMC, loc. AMC) and Sandra van de Wiel (AUMC, loc.AMC)

Thursday February 7, 2019

9.45 – 10.50 Registration with coffee/tea

10.50 – 11.00 Welcome by organizing committee

Full presentations (F) are 15 min with 5 min discussion
Short Pitch (P) are 5 min with 5 min discussion

11.00 – 12.30 Session 1
Session chairs: V.E. Gomez-Mellado & J.M. Ramos Pittol

F	J.C. Chang	AUMC, loc. AMC
F	M. Blankestijn	UMCG
P	S.F.J. van de Graaf	AUMC, loc. AMC
F	E.J.C.A. Kamp	Erasmus MC
F	J.A. Hoogerland	UMCG

12.30 – 13.30 Lunch

13.30 – 14.50 Session 2
Session chairs: J.C. Chang & M. Blankestijn

F	N.F. Smith-Cortinez	UMCG
P	G. Sari	Erasmus MC
F	T. Yadati	MUMC
P	J. Yang	UMCG
F	S.M.W. van de Wiel	AUMC, loc. AMC

14.50 – 15.20 Coffee/Tea break

15.20 – 16.30 Session 3:
Session chairs: S.M.W. van de Wiel & T. Yadati

P	Y. Oligschlaeger	MUMC
F	D.W. Kurniawan	U. van Twente
F	J.A.G.M. Langedijk	AUMC, loc. AMC
F	F. van Dijk	UMCG

16.30 – 18.30 **Speed date session**

18.30 – 20.00 **Dinner**

20.00 – 21.00 **Group session**

21.00 – … **Bar, Drinks, Dance**

Friday February 8, 2019

7.00 – 8.30 Breakfast

08.30 – 10.00 Session 4

Session chairs: J.A.G.M. Langedijk & Y. Oligschlaeger

F	T. Damba	UMCG
F	V.E. Gomez Mellado	AUMC, loc. AMC
P	M. Rios	UMCG
F	F.J.M. Roos	Erasmus MC
F	S. Go	AUMC, loc. AMC

10.00 – 10.30 Coffee/Tea break

10.30 – 11.50 Session 5

Session chairs: S. Go & M. Rios

F	D. van der Helm	LUMC
P	Y. Geng	UMCG
F	R . Lieshout	Erasmus MC
P	M.D. Appelman	AUMC, loc. AMC
F	H.D. de Vries	UMCG

11.50 – 13.00 Lunch

13.00 – 14.10 Session 6

Session chairs: M.D. Appelman & R. Lieshout

P	T. Herta	AUMC, loc. AMC
F	M.G.S. Rutten	UMCG
F	J.M. Ramos Pittol	UMCU
F	R.S. Bakker	AUMC, loc. AMC

14.10 – 14.30 Coffee/Tea break

14.30 – 15.45 Presentations Speed date session

15.45 – 16.00 Final Remarks & Closure

Session 1

Soluble adenylyl cyclase regulates glycogen metabolism and complex I activity to maintain energy homeostasis

J.C. Chang[1], E.H. Gilglioni[2], S. Go[1], A.J. Verhoeven[1], R.P.J. Oude Elferink[1]. [1]Tytgat Institute for Liver and Intestinal Research, Amsterdam UMC, Amsterdam, The Netherlands. [2]University of Maringá, Maringá, Brazil.

Background: The evolutionarily conserved soluble adenylyl cyclase (sAC, ADCY10) is the only cytoplasmic cAMP-generating enzyme. As a result of both mRNA splicing and post-translational processing, sAC has been localized to cytosol, mitochondria, and others subcellular compartments. We had previously demonstrated that sAC reciprocally regulates glycolysis and oxidative phosphorylation. Here, we report on the possible mechanism responsible for this reciprocal bioenergetic regulation.

Methods: Human hepatoma cell line HepG2, immortalized human cholangiocyte H69 (known to have high sAC activity), and primary mouse hepatocytes were used as models. The second-generation sAC inhibitor LRE-1 was used. Effect of inhibitors were confirmed with shRNA knockdown. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured by Seahorse XF96 extracellular flux analyzer. ATP production rates were derived from OCR and ECAR. Glycogen and medium metabolites were assayed enzymatically.

Results: In the coupled, physiological state, the inhibition of sAC stably increased ECAR with concomitant reduction of OCR in the presence of glucose. Despite the reciprocal changes in glycolysis and oxidative phosphorylation, sAC inhibition compromised neither the overall ATP production rate nor the adenylate energy charge. When medium glucose was removed or replaced by octanoate (a pure mitochondrial substrate), the effect of LRE-1 on ECAR and OCR in the coupled state became only transient and could be abolished by 2-deoxyglucose and glycogen phosphorylase inhibitor CP-91149, suggesting that sAC inhibition caused glycogen breakdown. In permeabilized HepG2 cells, sAC inhibition suppressed complex I-dependent OCR in both the ADP-coupled respiration and the FCCP-uncoupled respiration. However, the inhibition of sAC significantly mobilized more glycogen than the inhibition of complex I by rotenone, suggesting the extra-mitochondrial targets might regulates glycogen metabolism. Consistently, inhibition of sAC promoted glycogen breakdown in both HepG2 cells and H69 cells.

Conclusions: sAC-dependent cAMP signaling maintains energy homeostasis of cells by balancing the ATP production by glycogenolysis and glycolysis and the ATP production by oxidative phosphorylation. The mechanism involves the regulation of complex I activity in mitochondria and the regulation of glycogen metabolism in the cytosol.

Session 1

Characterization of the role of peroxisomal membrane protein 4 (PXMP4) in cholesterol homeostasis and peroxisomal function in mice

M. Blankestijn, J.F. de Boer, V.W. Bloks, R. Havinga, A.J.A. van der Sluis, F. Kuipers, J.W. Jonker, H.J. Verkade. Department of Pediatrics, University Medical Center Groningen, Groningen, The Netherlands.

Background: Peroxisomes are important in the β-oxidation of very long-chain fatty acids (VLCFA), α-oxidation of phytanic acid and bile acids and cholesterol metabolism. The number and activity of peroxisomes are increased by stimulation of peroxisome proliferator-activated receptors (PPARs). PPARα is a major player in hepatic lipid metabolism and ligands for this nuclear receptor include fatty acids and synthetic compounds such as fenofibrate. Peroxisomal membrane protein 4 (PXMP4) is a peroxisomal membrane protein (Reguenga et al., 1999) with unknown function and a target of PPARα in mice (Rakhshandehroo, Hooiveld, Muller, & Kersten, 2009). Interestingly, by transcriptome analysis we observed an association between PXMP4 expression and stimulation of fecal neutral sterol excretion in different mouse models. In the current study, we investigated whether PXMP4 plays an important role in peroxisomal metabolism of cholesterol, BA synthesis and lipids in the body, including fecal neutral sterol excretion.
Methods: We generated a total-body PXMP4 knockout mouse model (Pxmp4-/-) on a C57Bl/6 background using CRISPR-Cas9 mediated gene editing. In all our studies we used groups of Pxmp4-/- male and female mice and compared them to corresponding wildtype littermates. For PPARα activation studies animals were fed with chow or with chow supplemented with fenofibrate (0.2 w/w%) for a period of 2 weeks.
Results: Pxmp4-/- mice were viable, fertile and displayed no obvious phenotype. No changes were found in peroxisomal function as determined by levels of plasma VLCFA or bile acids concentration or composition. Further, no differences were found in basal biliary cholesterol or total fecal neutral sterol or bile acid output. However, basal levels of pristanic acid are slighty higher in Pxmp4-/- mice compared to wildtype animals. Finally, stimulation with the PPARα agonist fenofibrate also did not reveal any differences between wildtype and Pxmp4-/- mice.
Conclusion: So far, our studies show that PXMP4 does not play a critical role in the normal function of peroxisomes or excretion of fecal neutral sterols, neither under basal conditions nor by stimulation of the PPARα receptor. Because of small changes in pristanic acid levels, a future study challenging mice with phytol could reveal a role in peroxisomal α-oxidation of phytanic acid.

Session 1

Modified U1 snRNA to rescue splice defects in liver diseases

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Inherited diseases are individually rare, but together ~35 million individuals suffer from such diseases. Effective medical therapy options are not available for many of these diseases. In our search for a solution for ATP8B1 deficiency (a hereditary liver disease) we recently encountered a possible broadly applicable way to treat hereditary splicing defects in very different diseases. Expression of a sequence modified U1 snRNA, an essential component of the spliceosome, can completely restore splicing *in vitro*. We aim to investigate whether this strategy is effective and safe *in vivo*. However, animal models for cholestatic liver diseases resulting from splice defects are not available. Therefore, we used mice models for OTC deficiency and for Type I tyrosinemia (FAH deficiency) and treated these mice with adeno-associated virus encoding either wild-type U1 snRNA as a control or a modified U1 snRNA specially designed to (partially) restore splicing of the FAH or OTC transcripts. Restoration of splicing was very modest for FAH, both *in vitro* and *in vivo*. However, the correct OTC transcript was abundantly restored using a modified U1 snRNA, leading to prolonged survival of mice with an OTC splice site mutation even when challenged with a high-protein diet.

Session 1

Primary Sclerosing Cholangitis -associated biliary neoplasia demonstrate a high inter- and intratumour heterogeneity of p53 and p16 protein expression

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Tumour heterogeneity in primary sclerosing cholangitis (PSC)-associated cholangiocarcinoma (CCA) may attribute to the diagnostic limitations of cytology and FISH analysis of brushes obtained during ERCP. In addition, these tumours are remarkably resistant to widely different chemotherapeutic drugs. A possible explanation may lie in that PSC-CCA is made up of divergent clones, each with their own genetic defense mechanisms to counteract therapeutic agents. In this study, tumour heterogeneity was assessed through p53 and p16 protein expression analysis in PSC-associated biliary neoplasia.
Formalin-fixed paraffin-embedded tissue samples from resection material of PSC-CCA patients or PSC-patients with biliary dysplasia were selected. Sections with CCA and foci with dysplastic epithelium were identified by two independent GI-pathologists. Immunohistochemistry with p53 and p16 monoclonal antibodies was performed. Two investigators independently scored protein expression, p53 null *mutation/ wildtype/ overexpression* and p16 *negative/ heterogeneous/ positive*.
A total of 18 resection specimens of PSC-CCA and 1 PSC-explant with dysplasia were included, and *37 tumour and 13 dysplasia sections* were selected. P53 protein expression was classified as null mutation, wildtype and overexpression in 3/22/11 in CCA and 2/7/3 in dysplasia. In 5 patients, 7 CCA and 2 samples with dysplasia showed null mutation or overexpression surrounded by neoplastic cells with wildtype expression. In one patient, 1 CCA and 1 sample with dysplasia showed p53 overexpression with an abrupt transition to null mutation. Next generation sequencing in this patient showed different patterns of genomic instability in overexpression compared to null mutation. P16 protein expression was classified as negative, heterogeneous and positive in 13/18/6 in CCA and 6/5/2 in dysplasia. In several patients different p16 protein expression patterns were observed within the tumour.
PSC-associated biliary neoplasia are characterized by a high inter- and intratumour heterogeneity of p53 and p16 protein expression, indicating that such cancers consist of multiple clones with substantially different genetic makeup. By using next generation sequencing genetic intratumour heterogeneity of *TP53* have been detected. These observations may explain the difficulty encountered to reliably diagnosis of PSC-CCA using brush cytology and FISH diagnostic strategies and provides a rational explanation for the poor response to a large spectrum of chemotherapy.

Session 1

Pharmacological FXR activation reduces non-alcoholic fatty liver disease and hypercholesterolemia in a mouse model of Glycogen storage disease type 1a

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Introduction: Glycogen storage disease type 1a (GSD Ia), caused by a defect in glucose-6-phosphatase (G6PC) activity, is characterized by hypertriglyceridemia, hypercholesterolemia and non-alcoholic fatty liver disease (NAFLD). FXR is a key transcription factor that controls bile acid, lipid and glucose metabolism. Pharmacological FXR activation has been shown to improve hyperlipidemia and NAFLD, by regulating VLDL metabolism, cholesterol balance and hepatic fatty acid synthesis. Therefore, we aimed to characterize the consequence of FXR activation on hypertriglyceridemia, hypercholesterolemia and fatty liver disease in L-*G6pc-/-* mice, a liver-specific model for GSD Ia.
Methods: FXR agonist was administered to L-*G6pc-/-* mice and their wildtype (L-*G6pc+/+*) littermates via the food for 10 days. We collected bile and quantified hepatic fatty acid synthesis, hepatic glucose-6-phosphate, glycogen and triglyceride contents, and plasma lipid levels.
Results: L-*G6pc-/-* mice were characterized by accumulation of hepatic glucose-6-phosphatase (G6P) and glycogen, and showed a 5-fold increase in hepatic triglyceride levels compared to L-*G6pc+/+* mice. FXR activation further increased hepatic G6P and glycogen contents in L-*G6pc-/-* mice by 35% and 70%, respectively, while hepatic triglyceride content was decreased by 22%. This was associated with reduced glycolysis and *de novo* lipogenesis. FXR activation did not reduce plasma triglyceride levels, yet, plasma cholesterol levels were partially normalized: treatment with FXR agonist reduced plasma cholesterol levels in L-*G6pc-/-* mice by 22%. In parallel, fecal neutral sterol loss was increased in treated L-*G6pc+/+* and L-*G6pc-/-* mice, associated with a more hydrophilic bile composition in both strains.

Conclusions: Pharmacological FXR activation reduces NAFLD in our mouse model of GSD Ia. Further studies are needed the assess the consequences of prolonged FXR activation on advanced liver disease in GSD Ia, such as liver tumor formation.

Session 2

Vitamin C is required for effective collagen release by human hepatic stellate cells; should vitamin C supplementation be re-evaluated in chronic liver disease?

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Background: Vitamin C (e.g. L-ascorbic acid) is an essential nutrient for humans and is required as cofactor in enzymatic processes in wound healing, neurotransmitter synthesis and acts as a potent anti-oxidant. In contrast to many animals, including laboratory animals, humans lack the enzyme gulonolactone oxidase to produce vitamin C themselves. Cellular uptake of vitamin C is facilitated by selective sodium-dependent transporters (SLC23A1/SVCT1 and SLC23A2/SVCT2) and non-selectively by glucose transporters (GLUT1, GLUT3, GLUT4). Vitamin C is a cofactor of prolyl hydroxylases that promote collagen maturation and secretion, a process studied in detail in wound healing. Remarkably, the putative role of vitamin C in liver fibrosis, especially related to human, is less well studied. Here, we analyzed expression of vitamin C transporters in human hepatic stellate cells (HSC), the main cell type producing collagenous scar tissue in chronic liver disease, and the effect of vitamin C on collagen production and secretion.

Methods: Primary human HSC (p-hHSC) and hepatocytes were isolated from healthy parts of surgical resections. p-hHSC and LX-2 cells were (co-)treated *in vitro* with or without ascorbic acid (0.2 – 2 mM) and TGF-β (10 ng/mL) for 1, 2 and 6 days. Expression of vitamin C transporters, collagen 1A1, and pro-collagen were analyzed by q-PCR, Western blotting and immunofluorescence microscopy.

Results: p-hHSC and LX-2 cells express *SLC23A2*, but not *SLC23A1*, while human hepatocytes express high levels of both. TGF-β strongly enhanced collagen production by LX-2 cells, which remained almost completely intracellular in the absence of vitamin C. Co-treatment with vitamin C rapidly (already at day 2) stimulated the secretion and deposition of collagen with only minor intracellular amounts detected at day 6 (Figure 1).

Conclusions: Human hepatic stellate cells strongly depend on vitamin C to mature and secrete collagen. This implies that vitamin C supplementation may promote liver fibrosis, although this may be counter balanced by its potent anti-oxidant and co-factor functions. Moreover, inhibition of SLC23A2 is a potential target to suppress liver fibrosis.

Session 2

The Lymphocytic Choriomeningitis Virus (LCMV) Mouse Model for Checkpoint Modulation Studies

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Immunotherapy applies induction, suppression, or enhancement of an immune response in order to obtain a therapeutic effect and clinical benefit. Based on the better understanding of T cell exhaustion and activation during persistent antigen stimulation, immune checkpoint modulators are widely studied as immunotherapeutics and several of them are able to recover an exhausted CD8 T cell phenotype. Several immunomodulators are currently FDA approved in the field of immune-oncology and many more are in clinical or preclinical development. Good preclinical models are needed in order to rapidly evaluate the tolerance and clinical benefit of these candidate compounds.
Lymphocytic Choriomeningitis Virus (LCMV) is a natural mouse pathogen, of which a specific isolate (clone 13) results in persistent infections, characterized by ongoing viral replication in several body compartments and resulting virus-specific T cell exhaustion. We apply the chronic LCMV clone 13 mouse model to assess the efficacy of candidate immune checkpoint inhibitors as future therapeutics in cancer and infectious diseases. We have thoroughly assessed the tolerance, status of T cell exhaustion (both phenotypically as functionally) and the antiviral efficacy of the prototype immune modulators anti-PD-1, anti-Tim-3 and their combination. This allowed us to define the optimal conditions for future studies on candidate checkpoint inhibitors in collaboration with pharmaceutical companies. As a first readout parameter, we test the tolerability of the compounds. Then, the decrease in viral load in serum, recovery of the exhausted phenotype of LCMV specific and total CD8 T cells, change in IFNɣ production capacity of CD8 T cells are tested to analyze the effects of compounds. Also, proliferation of both LCMV-specific and total CD8 T cells are tested by Ki67 staining following *ex vivo* stimulations.
We believe that the clone13 LCMV mouse model is an important and key method to explore a wide range of questions regarding T cell activation and immunomodulatory therapies and will illustrate the different critical factors in establishing this model.

Session 2

Inhibiting extracellular Cathepsin D reduces hepatic steatosis and fibrosis in rodent models of fatty liver disease

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Background: Dietary and lifestyle changes are leading to the increased incidence of Non-alcoholic fatty liver disease (NAFLD). The complexity of NAFLD pathogenesis is hampering the development of new pharmacotherapies. Previous studies from our group highlighted the role of the lysosomal protease, cathepsin D (CTSD) as plasma marker for different stages of NAFLD in both adults and children. Strikingly, we also demonstrated that inhibition of CTSD leads to dramatic improvement in lipid metabolism and inflammation. As inhibition of *cellular* CTSD is highly toxic, our aim in the current study was to determine whether the same beneficial effects could be achieved in different stages of NAFLD by specifically targeting *plasma* CTSD.
Methods: Small molecular inhibitor against plasma CTSD was developed and tested in rodent models of hepatic steatosis and fibrosis. For diet-induced steatosis, Sprague-Dawley rats were fed either low fat diet (LFD) or high fat diet (HFD) for 3 weeks with bi-weekly injections of plasma CTSD inhibitor. Liver fibrosis was induced in C57BL/6 mice by intraperitoneal administration of carbon tetrachloride (CCl4; 5ul/g body weight), twice a week for 6 weeks. Separately, mice were injected with plasma CTSD inhibitor(50mg/kg), 30 minutes post CCl4 dosing.
Results: Rats that received the inhibitor showed reduced steatosis and inflammation. In addition, plasma insulin and transaminases levels were significantly reduced relative to the HFD-fed rats. On the other hand, plasma CTSD inhibition in mice demonstrated protection against hepatic injury. CCl4 mice that received inhibitor showed reduced liver fibrosis, collagen deposition, transaminases levels when compared to CCl4 control mice.

Conclusion: Our collective findings demonstrate for the first time the specific role of plasma CTSD in the NAFLD progression and its potential as a novel therapeutic target for intervention.

Session 2

Farnesoid X Receptor overexpression alters adipose tissue architecture and limits its storage capacity leading to ectopic fat deposition and insulin resistance

J. Yang, T. van Zutphen, J. Stroeve, F. Kuipers. UMCG, Groningen, The Netherlands.

Background: The bile acid-activated nuclear receptor Farnesoid X receptor (FXR, NR1H4) is mostly known for its role in regulating bile acid homeostasis in liver and intestine, but has also been implicated in control of lipid and energy metabolism. Its role in fat tissue is not understood, where unlike the high expression of FXR in the liver, expression is moderate. In view of the recent development of FXR-targeting therapeutics for treatment of human metabolic diseases, understanding of tissue-specifications of FXR is essential.
Methods: We generated transgenic mice that overexpress FXR (NR1H4) under control of the aP2 (Fabp4) promoter. Adipocyte size, liver and plasma parameters were determined. Affymetrix microarray analysis and quantitative profiling of epididymal WAT secretome were performed. We also examined anti-Lamin A/C and anti-Col-1 level using Western blot analysis.
Results: AP2-hFXR mice have markedly enlarged adipocytes and show extensive extracellular matrix remodelling. Unexpectedly these mice also showed increased plasma bile acids with altered composition. Ageing and exposure to obesogenic conditions revealed a strongly limited capacity for adipose expansion and development of adipose fibrosis. This was associated with impaired lipid storage capacity, accumulation of triglycerides and cholesterol esters in the liver as well as insulin resistance.
Conclusion: These studies establish that adipose FXR is a determinant of adipose tissue architecture and contributes to whole-body lipid homeostasis.

Session 2

The role of PI3Kgamma in OSTa-OSTβ mediated bile acid transport

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Background: The organic solute transporter alpha-beta (OSTα-OSTβ) facilitates bile acid efflux mainly in ileal enterocytes, liver and kidney, which is expected to protect cells from an overload of bile acids. Deficiency of OSTα-OSTβ *in vitro* and *in vivo* leads to bile acid accumulation in the enterocyte accompanied by prolonged activation of FXR. In addition, the recent discovery of OSTβ deficient patients, that suffer from chronic diarrhea and mild cholestasis, highlights the role of OSTα-OSTβ in hepatocytes as well. The aim of this study is to identify kinases that play a role in the post-translational modification of OSTα-OSTβ.
Methods: A screening library containing 155 kinase inhibitors was used to find out which kinases modulate bile acid transport by OSTα-OSTβ in HepG2 cells. Hits were further validated using a transcellular transport assay in MDCKII cells overexpressing ASBT and OSTα-OSTβ, that were plated on transwell filters and resembles the *in vivo* situation where OSTα-OSTβ mainly functions as an efflux transporter. The ratio total to plasma membrane protein expression of OSTα and OSTβ was examined by biotinylation and immunofluorescence.
Results: The kinases CaMKII, MEK, ERK and PI3Kγ were identified as positive hits that modulated OSTα-OSTβ mediated transport in the primary screen. Both CaMKII and PI3Kγ inhibition decreased transcellular transport in MDCKII cells. In line with this result, a significant increase of intracellular bile acids was observed, suggesting that bile acids accumulate in cells. ERK and MEK inhibition did not influence transcellular transport in MDCKII cells. Furthermore, in contrast to CaMKII, PI3Kγ did not influence bile acid uptake by ASBT. No differences in OSTα-OSTβ protein levels were observed.
Conclusion: PI3Kγ modulates bile acid transport by OSTα-OSTβ and may play a role in the protection against bile acid overload in cells. We hypothesize that it could act as an alternative but more direct protective mechanism by post-translational modification of OSTαβ, initiated by activation of PI3Kγ by hydrophobic and hepatotoxic bile acids.

Session 3

Plasma CTSD activity is negatively associated with hepatic insulin sensitivity in overweight and obese patients

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Background: Obesity-associated insulin resistance (in skeletal muscle and liver), is considered the primary defect underlying the development of type 2 diabetes (T2DM). The hyperinsulinemic-euglycemic clamp is considered the gold standard to assess peripheral and hepatic insulin sensitivity, yet it is a costly and labor-intensive procedure. Therefore, there is a need for easier, cost-effective and organ-specific approaches to determine insulin sensitivity and, consequently, enable organ-specific interventions. Current studies have indicated that a lysosomal enzyme, plasma cathepsin D (CTSD), is associated with insulin resistance and liver inflammation in T2DM and non-alcoholic fatty liver disease (NAFLD) patients, respectively. The aim of the present study was to investigate the association between plasma CTSD and hepatic insulin sensitivity.
Methods: 94 overweight/obese adults were included in this study (BMI: 25-35 kg/m2). A two-step hyperinsulinemic-euglycemic clamp with a [6,6-2H2]-glucose tracer was performed to assess peripheral and hepatic insulin sensitivity (Rate of glucose disappearance at the highest step of the clamp (Rd40) and % suppression of endogenous glucose output at the lowest step (%EGP), respectively). Moreover, plasma CTSD levels and activity as well as the levels of plasma inflammatory cytokines were measured.
Results: Plasma CTSD activity was negatively associated with hepatic insulin sensitivity (%EGP suppression; standardized β=-0.206, p=0.043), independent of age, sex, BMI and waist, but not with peripheral insulin resistance. Furthermore, plasma CTSD levels were positively related with the pro-inflammatory cytokine: IL-8 and TNF-α (IL-8: standardized β=0.492, p=0.000; TNF-α: standardized β=0.273, p=0.011), even after adjustment for age, sex, BMI and waist (IL-8: standardized β=0.333, p=0.001; TNF-α: standardized β=0.276, p=0.008).
Conclusions: These data demonstrate that plasma CTSD activity is independently associated with hepatic insulin sensitivity, suggesting that plasma CTSD activity may be used as a novel biomarker to non-invasively estimate hepatic insulin sensitivity.

Session 3

Inhibition of Src kinase signaling pathway attenuates Nonalcoholic Steatohepatitis

D.W. Kurniawan[1], A. Jajoriya[2], G. Dhawan[2], D. Mishra[2], D. Oosterhuis[3], P. Olinga[3], G. Storm[1], D.P. Mishra[2], J. Prakash[1], R. Bansal[1]. [1]Biomaterials Science and Technology, University of Twente, Enschede, The Netherlands. [2]CSIR-Central Drug Research Institute, Lucknow, India. [3]University of Groningen, Groningen, The Netherlands.

Background: The prevalence of non-alcoholic steatohepatitis (NASH) is growing rapidly due to the western lifestyle and is becoming a leading and growing cause of mortality worldwide. Currently, there is an unmet need for effective and safe therapies for the treatment of this disease. Inflammatory macrophages play a crucial role in the pathogenesis of NASH. Therefore, molecular therapies inhibiting macrophage activation would be a highly promising therapy for the treatment of NASH.
Aim: In this study, we investigated the implication of Src signaling pathway inhibition in inflammatory macrophages and NASH.
Methods: We used KX2-391, a small-molecule Src kinase inhibitor, to achieve the goals of this project. We tested the therapeutic efficacy and toxicity of KX2-391 in the differentiated inflammatory RAW macrophages, bone marrow-derived macrophages, and murine precision-cut liver slices (PCLS). Eventually, we evaluated the therapeutic effects of KX2-391 *in vivo* in methionine choline deficient (MCD)-diet NASH model.

Results / Conclusion: Expression of Src kinase and M1-specific genes (iNOS, CCL2, FcɣR1) was found to be significantly upregulated in inflammatory macrophages. Src kinase pathway activation (Src phosphorylation) was confirmed in inflammatory macrophages. KX2-391 concentration-dependently inhibited the phosphorylation of Src. Furthermore, KX2-391 significantly attenuated M1-induced nitric oxide (NO) release (an indicator of M1 activation) and expression of M1 markers in RAW macrophages, BMDMs, and PCLS. KX2-391 showed significant inhibition of M1 macrophage markers in PCLS without inducing toxicity. Finally, we performed an *in vivo* study on MCD diet-induced murine model of NASH and showed that KX2-391 ameliorated inflammation, fibrosis, and steatosis in mice. KX2-391 significantly decreased ALT, AST, total cholesterol, and total triglycerides serum levels. These results suggest that Src inhibitor KX2-391 can be a potential therapeutic approach to treat Non-alcoholic Steatohepatitis.

Session 3

Cholephilic compounds in bile of cholestatic patients can activate Transient Potential Channels and thereby influence itch perception.

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Background: Cholestatic diseases often lead to chronic itch. Cholephilic compounds, normally secreted into bile, are increased in serum during cholestasis. Therapeutic interruption of the enterohepatic circulation (EHC), e.g. by nasobiliary drainage markedly diminishes itch intensity, suggesting that cholephilic compounds (in)directly cause pruritus.
Here, we studied the effect of cholephilic compounds on their capacity to activate channels involved in pain and itch signaling. Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) are normally located on sensory neurons but in this experiment they are overexpressed on Human Embyonic Kidney cells (HEK) and neuroblastoma cells (SH-SY5Y), respectively.
Methods: Bile was obtained from different cholestatic patients undergoing biliary diversion by nasobiliary drainage. This bile was deproteinized and desalted by acetonitrile-treatment, and was separated by reverse phase HPLC into different fractions. These fractions were tested for bile salt levels and effect on transient potential channels TRPA1 and TRPV1, measured by calcium-fluorescence.
Results: Several fractions containing bile salts showed activation of the TRPA1 channel. High concentrations of bile salts are able to weakly activate this channel. Interestingly, also multiple fractions that did not contain bile salts showed activation of the TRPA1 channel and others showed activation of the TRPV1 channel. This indicates that cholestatic bile contains several compounds that are able to activate transient potential channels.

Conclusion: We show here that cholephilic compounds present in bile directly activate the TRPA1 and TRPV1 channels, which will influence itch perception in cholestatic patients.

Session 3

En route to treat liver fibrosis: the sustained release of a HSC-selective rho-kinase inhibitor

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Background. Liver fibrosis is characterized by progressive production of extracellular matrix proteins by myofibroblasts, highly expressing the platelet-derived growth factor β receptor (PDGFβR). As liver fibrosis is a growing health problem, there is a need for effective therapies. Our aim was to develop a patient-friendly formulation providing sustained and controlled release of antifibrotic protein therapeutics, by preparing polymeric microspheres that ensure prolonged release *in vivo*. We produced microspheres containing either human- (HSA) or mouse serum albumin (MSA) enriched with PDGFβR-recognizing peptide moieties (pPB) targeting the PDGFβR. The antifibrotic effects of the encapsulated rho-kinase inhibitor Y27632 coupled to the albumin carrier were tested in Mdr2-/- mice. Moreover, we determined the immune response against the drug carrier.
Methods. Biodegradable microspheres, based on poly-ε-caprolactone, polyethylene glycol and poly-l-lactic acid, containing HSA, pPB-HSA, pPB-MSA or pPB-MSA-Y27632 were characterized by electron microscopy, laser diffraction and *in vitro* protein release. Mdr2-/- mice received microspheres containing 5 wt% albumin conjugate and were sacrificed at day 7. Plasma and livers were assessed for albumin conjugates (ELISA), livers for antifibrotic effects (PCR, IH staining), and plasma for antibodies against albumin (ELISA).
Results. All microspheres showed similar morphology and particle size (median ~23 μm), and displayed sustained protein release *in vitro* for up to 14 days. *In vivo*, the release of pPB-HSA from the subcutaneously residing microspheres reached steady state concentrations in both plasma and fibrotic liver within 1 day after injection. This declined after 7 days, paralleled by an antibody response against HSA in these mice, which was not observed with MSA-based constructs. We proceeded with pPB-MSA as carrier and showed a significant reduction in hepatic fibrotic parameters on the gene (collagen1a1 (34%) and fibronectin (19%)) and protein level (collagen I/III) only when microspheres contained Y27632 coupled to pPB-MSA.

Conclusion. The carrier pPB-MSA was steadily released from microspheres into the plasma and detected in fibrotic livers with increased PDGFβR-expression up to 7 days after injection. We demonstrated the antifibrotic potential of this formulation for a PDGFβR-targeted rho-kinase inhibitor. Our studies show that sustained controlled release formulations can be applied for large therapeutic proteins in treating chronic diseases like fibrosis.

Session 4

Hydrogen sulfide dose-dependently stimulates hepatic stellate cell proliferation

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Hepatic fibrosis is caused by the accumulation of an excessive amount of extracellular matrix (ECM) that is produced by activated hepatic stellate cells (HSCs) during oxidative stress or inflammation. Hydrogen sulfide (H_2S) has been demonstrated in the mammalian cell as a gasotransmitter that regulates many different physiological processes, including inflammation,proliferation and cell death. Recent *in vivo* studies showed that H_2S ameliorates hepatic fibrosis. On the other hand, H_2S increases cell proliferation due to its pro-inflammatory characteristics and increasing mitochondrial bioenergetics. The AIM of this study was to elucidate the dynamics of endogenously produced and/or exogenously administered H_2S in primary rat HSCs *in vitro*. In the experiment, primary rat HSCs were cultured 7 days for activation and then treated with different types of H_2S releasing donors (slow releasing donor GYY4137, fast releasing donor NaHS) or inhibitors (PAG, AOAA) that decrease endogenous production of H_2S releasing enzymes (CSE, CBS). The results show that exogenously administered H_2S dose-dependently modified HSC proliferation. A relatively low concentration of H_2S increased HSC proliferation and fibrotic markers, while a relatively high concentration of H_2S suppressed activated HSC proliferation. Moreover, inhibition of endogenous production of hydrogen sulfide decreased HSC proliferation. The H_2S donors' and inhibitors' toxicity was determined on activated HSC by Sytox Green (necrosis) and caspase-3 activity (apoptosis). No toxicity of donors and inhibitors was observed in the concentration range used. In summary, we demonstrated that HSC proliferation is dose-dependently regulated by hydrogen sulfide. In addition, endogenous production of hydrogen sulfide promoted HSC activation during hepatic fibrosis.

Session 4

ATP8B1 Deficiency Affects Lipid Metabolism in Human Macrophages

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Background: ATP8B1 deficiency causes cholestatic liver disease characterized by a continuous disease spectrum, from intermittent in Benign Recurrent Intrahepatic Cholestasis type 1 (BRIC1) to progressive in Progressive Familial Intrahepatic Cholestasis type 1 (PFIC1). PFIC1 usually is associated with dyslipidemia, manifested by low serum HDL, high oxidized LDL (oxLDL) and hypertriglyceridemia, while serum total cholesterol levels are low to normal. The etiology of dyslipidemia is not understood. Here we studied the role of ATP8B1 and CDC50A (the obligate heterodimer of ATP8B1) in cholesterol metabolism in human macrophages.
Methods: Experiments were performed in human CDC50A- and ATP8B1-depleted THP-1 macrophages. Fluorescently-labeled native low-density lipoprotein (LDL) uptake and accumulation was measured by FACS analysis. Cholesterol levels were quantified enzymatically. Distribution of cholesterol and neutral lipids were studied by filipin and Nile Red staining, respectively. Protein levels were quantified after administration of Liver X Receptor (LXR) agonist T0901317 by surface biotinylation and Western analysis, and mRNA expression by quantitative RT-PCR.
Results: CDC50A and ATP8B1 knockdown macrophages showed increased accumulation of fluorescence after incubation with fluorescently-labeled LDL. Pulse labeling with LDL followed by a 24-hour chase resulted in a higher free cholesterol content in both knockdown cells. Interestingly, filipin- and Nile Red staining showed affected distribution of cholesterol and neutral lipids, respectively. Quantitative PCR showed downregulation of transcription factors and targets involved in cholesterol and fatty acid metabolism, including *LXRα*, *SREBP1*, *HMG-CoA reductase, FAS*, *SCD1 and ABCA1*. Importantly, ABCA1 protein was strongly decreased under both lipoprotein-supplemented and -deficient conditions, however recovered completely upon incubation with T0901317 as shown by surface biotinylation experiments.

Conclusion: ATP8B1-CDC50A heterodimer plays an important role in cholesterol and fatty acid metabolism in human macrophages. ATP8B1 deficiency in macrophages may contribute to dyslipidemia in PFIC1, and may predispose to atherosclerosis, an hypothesis that is presently under study.

Session 4

Analytical methods to measure kinetics of fermentation of fibers inside the human gut, for a computational model on gut-liver axis

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Background:Gut microbiota use fibers as substrates to produce short chain fatty acids (SCFAs). Consumption of fibers has been linked to many health benefits in humans, and SCFAs influx into the host have been associated with improvements of metabolic markers, as hepatic triglycerides in mice. However, detailed knowledge of the exact fate and impact of fibers in the human intestinal tract, and their potential effect in the liver is lacking. Access to this inner world of gut microbiota in humans is now possible using novel capsules that allow sampling of the lumen content in a non-invasive way. The IntelliCap system will be applied in a nutritional intervention to study kinetics of fiber fermentation and SCFA uptake. All data collected will be integrated in a computational model that will connect gut microbiota with liver metabolism and improves in metabolic markers.
Methods and results:A specific challenge of the IntelliCaps is the need for a stabilizing reagent used to stop further fermentation in the sample until it is excreted. We developed a quencher reagent to be pre-loaded in the IntelliCaps and tested its effect on the oligosaccharides (HPAEC) and SCFA (GCMS) analysis. The quencher interfered with the analysis and this was overcome by a pre-treatment of the sample with KCl. To test the effectiveness of the quencher, in vitro batch fermentations with human ileum and feces inocula with dietary fibers (inulin, fructo- and galacto oligosaccharides) were performed. The quencher reagent was shown to block microbial fermentation up to 48 h: it stabilized oligosaccharides degradation and did not result in SCFA formation. Considering the small volume of recovered sample, a mixed protocol was developed to measure oligosaccharides, SCFAs and succinate from a small sample, without splitting it and using only one organic solvent extraction.
Conclusion:This above-described part of the project give us a set of analytical tools to use with the new IntelliCap system to obtain as many data using a small representative sample of the human colon. A computational approach will be used to model how this fiber fermentation and SCFAs uptake can affect lipids metabolism in the liver in humans.

Session 4

Identification of new drug targets to prevent ischemia-induced bile toxicity using a human biliary organoid model.

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Introduction. The study of bile duct disease has been hampered by the lack of good cholangiocyte models. The establishment of human liver-derived organoids, enabled long-term expansion of cholangiocyte-like cells with which we aim to investigate the role of cystic fibrosis transmembrane conductance regulator (CFTR) in ischemia-induced bile duct injury. Liver ischema (transplantation-related) might accelerate bile toxicity in cholangiocytes, due to insufficient protection by CFTR-related bicarbonate (NH_3) secretion. We investigated if liver-derived organoids have functional cholangiocyte-transport channels and can be used for drug-discovery.

Methods. Analysis on the genetic and protein level of cholangiocyte-specific transporters was done using liver tissue-derived organoids (n=8), cultured from donor livers reserved for liver transplantation. Functionality of these channels was analysed using an Ussing chamber set-up allowing measurement of slow circuit current though 2D-grown organoids (n=42). Forskolin (cAMP activator) added to the basolateral side of the cells, initiated CFTR activation which was inhibited by GIyH. Ischemic conditions were achieved by nitrogen gas (95%N_2/5%CO_2) exposure. To study bile-related toxicity, undiluted bile was added under oxygen and ischemic conditions and cell death was analyzed. Finally, compounds were tested for the ability to abrogate the ischemia-induced inhibition of CFTR**.**

Results. CFTR was expressed in liver organoids on gene (qPCR) and protein (Western blot) level. Moreover, CFTR could be activated by Forskolin and significantly reduced under ischemic conditions compared to oxygenated conditions (1.66±0.45 vs. 4.18±0.48, p=0.005). Furthermore, the activity significantly decreased when switching from oxygenated to ischemic conditions (8.00±1.19 vs. 5.89±1.26, p=0.02). Further experiments showed that bicarbonate is the driving factor for the current, suggesting that bicarbonate excretion is decreased under ischemic conditions. Addition of bile resulted in increasing cell death in ischemic conditions compared to oxygen (31.2%±4.32 vs. 19.18±4.81, p=0.04). Most importantly, addition of compound C (cAMP inhibitor) was able to rescue transporter activity under ischemic conditions.

Conclusion. Liver-derived organoids provide an excellent model to study bile duct transporters. We demonstrate that ischemia inhibits CFTR-related bicarbonate (NH_3) secretion and cAMP inhibitor compound C can restore this. This encourages further clinical studies to test whether cAMP inhibitors can prevent ischemia-related biliary injury during graft preservation and after liver transplantation.

Session 4

Complex post-translational modification of soluble adenylyl cyclase

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Background: The evolutionary conserved soluble adenylyl cyclase (sAC) is an intracellular cAMP generating enzyme. Activity of sAC is regulated by cytosolic bicarbonate, calcium and ATP concentrations but not by G-proteins. sAC-derived cAMP is involved in many general physiological processes, such as oxidative stress-induced apoptosis, cellular metabolism, proliferation and differentiation in multiple cell lines. Hence, studying the biosynthesis of this enzyme will enhance the understanding of a broad range of cellular processes.
Methods: The immortalized H69 cholangiocyte cell line was used to study sAC expression because of its high expression of sAC. Lysates were treated with the deglycosylating enzyme PNGase-F to study N-linked glycosylation. Using the mild detergent digitonin, soluble fractions were extracted to examine the subcellular localization of sAC. A sAC ovexpression construct encoding human full length sAC with N-terminal and C-terminal tags was overexpressed in different human cell lines.
Results: We have observed that post-translational modification of sAC in H69 cholangiocytes is regulated by glucose in a process that involves N-linked glycosylation, where sAC contains multiple high mannose sugar chains. Moreover, digitonin treatment of cells shows that sAC is not a soluble extractable protein, but rather is a membrane-tethered protein. In H69 cholangiocytes and HepG2 cells we observed different C-terminal proteolytic processing of overexpressed full length sAC cDNA, leading to the generation of various truncated sAC forms.

Conclusion: In H69 cholangiocytes, sAC is an N-linked glycosylated and possibly heavily processed protein. Future experiments will determine the role of its N-glycosylation and proteolytic processing in relation to its activity, stability and subcellular localization. In addition, multiple cell lines will be used to investigate how these cellular processes relate to its regulatory functions.

Session 5

VCAM positive, in contrast to VCAM negative, subpopulations of mesenchymal stem cells reverse fibrogenesis in a mouse model for liver fibrosis.

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Background Liver fibrogenesis starts with apoptotic hepatocytes that induce stellate cell proliferation and differentiation into myofibroblasts. Myofibroblasts are the source of extracellular matrix in fibrosis. Mesenchymal stem cells (MSCs) are known to possess pro-regenerative and anti-inflammatory properties, but in relation to the reversal of fibrogenesis opposing findings have been reported. We hypothesised that these differences could be explained by the use of different subpopulations of MSCs. In the present study we compared the pro-regenerative and anti-fibrotic effects of four different subpopulations of MSCs, selected to be double positive, double negative, or single positive for Endoglin or VCAM.
Methods Proliferation, wound healing and trans-well migration experiments were performed to study migratory and pro-regenerative effects of the MSC subpopulations. Basal expression levels of migratory (SDF1, CXCR4) and antifibrotic (TGFβ, VEGF, HGF and IGF) genes were measured by qPCR analysis. Furthermore, the ability of the different subpopulations to reverse fibrogenesis was tested in a mouse model for liver fibrosis. The severity of fibrosis was assessed by collagen deposition visualised by Sirius red staining.
Results Proliferation and migration experiments with damaged HepG2 cells showed that VCAM positive MSC subpopulations have more pro-regenerative capacities compared to the VCAM negative subpopulations. VCAM positive subpopulations of MSCs also have more migratory and anti-fibrotic gene expression profiles. Furthermore, VCAM positive MSC subpopulations, in contrast to VCAM negative MSC popualtions, were able to reverse fibrogenesis in a mouse model for liver fibrosis (85 vs 70% reduction of collagen deposition, $P<0.05$).

Conclusion To conclude, VCAM positive subpopulations of MSCs are superior compared to VCAM negative subpopulations in relation to their anti-fibrotic and pro-regenerative properties. This suggests that the use of different subpopulations of MSCs could be an explanation for conflicting results in the literature.

Session 5

Protective effect of Metformin against palmitate-induced hepatic cell death

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Background and aim: Hepatic cell death represents the major consequence of lipotoxicity and plays an important role in the pathogenesis of Non-alcoholic fatty liver disease (NAFLD). Metformin, a first-line anti-diabetic medicine in clinic, has shown a potential protective effect against NAFLD. However the underlying mechanism is still not clear. In this study, we aim to understand the molecular mechanism of the protective effect of metformin against lipotoxicity.
Methodology: HepG2 cells were exposed to palmitate (PA; 0.5 mM). Metformin was used at a concentration of 1.0 mM. Necrosis was determined by Sytox Green nuclear staining and apoptosis by caspase-3 activity assay. HepG2 cells were treated with Compound c (18μM) a pharmacological AMPK inhibitor or transfected with dominant negative AMPK adenovirus to inhibit AMPK signaling. Generation of reactive oxygen species (ROS) was measured using CellROX assay. The effect of metformin on mitochondrial respiratory chain was compared to that of rotenone (1.0μM and 0.1μM). The function of the mitochondrial respiratory chain was monitored using the Seahorse system. Expression of mRNAs (SOD1, SOD2) was measured by quantitative PCR.
Results: Metformin ameliorated PA-induced necrosis and inhibited PA-induced caspase-3 activity by 53.4%. Both compound c and dnAMPK-Adenovirus did not modulate the protective effect of metformin, indicating that AMPK signaling is not involved in the protective effect of metformin. Metformin decreased ROS production induced by PA, and increased SOD2 mRNA expression. Moderate inhibition of the mitochondrial respiratory chain (specifically complex I), by a low concentration of rotenone (0.1μM) also protected against PA-induced cell death, whereas a high concentration of rotenone (1.0μM) was toxic. Metformin also inhibited mitochondrial complex I.

Conclusions: Metformin protects against PA-induced cell death by partially inhibiting mitochondrial respiratory chain, decreasing intracellular ROS production, and inducing SOD2 expression. Moreover, moderate inhibition of mitochondrial respiratory chain is protective against PA-induced cell death, but complete inhibition aggravates PA-induced cell death. The protective effect of metformin is independent of AMPK-signaling. Our results indicate that metformin may have beneficial actions beyond its glucose-lowering effect and also suggest that mitochondrial complex I may be a therapeutic target in NAFLD/NASH.

Session 5

Modeling Cancer Stem Cell and Differentiated Cancer Cell Phenotypes in Liver Cancer Organoids

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Background*:* Cholangiocarcinoma (CCA) is a biliary-type liver tumor with a dismal prognosis, due to late diagnosis, high chemo-resistance and large intra- and inter-tumor heterogeneity. Recently, we established CCA organoid cultures. These long-term 3D cultures retain histological architecture, gene expression profile and genomic landscape of the original tumor and are amenable to drug screening approaches. Due to the stem cell characteristics of organoids, CCA organoids represent a cancer stem cell (CSC)-like phenotype. CSC's are known to be resistant to chemo- and radiation therapy. However, the bulk of cancer cells in a tumor have a differentiated (non-stem cell) phenotype and are more therapy-sensitive. The aim of our study is to establish a model to study both the CSC and differentiated cancer cell phenotypes in CCA organoids and apply this in drug sensitivity screening.
Methods*:* Organoids from CCA, non-tumorous adjacent liver and healthy liver tissue (all n=3-6) were differentiated by blocking cancer stem cell signaling pathways. At day 5, cell viability, proliferation, cell death and differentiation potential was tested on gene expression (qPCR) and protein (immunohistochemistry) level and compared to CSC-like organoids. Drug sensitivity of differentiated and CSC-like CCA organoids was tested with sorafenib.
Results*:* Upon differentiation, all organoid types acquired a more dense appearance containing columnar epithelial cells. Live/dead staining revealed that the level of cell death was similar to CSC-type cultures. Differentiated CCA organoids had a reduced proliferative rate, as demonstrated by reduced EdU incorporation and downregulation of Ki67 gene expression ($p<0.001$). Even though proliferation is inhibited, metabolic activity was stable, indicating a higher metabolic activity per differentiated cell. Gene expression analysis showed that known CSC markers LGR5 ($p<0.001$), CD44 ($p=0.01$) and CD133 ($p<0.01$) were downregulated upon differentiation. Preliminary results from a drug sensitivity assay suggested that differentiation of CCA organoids increased their sensitivity to sorafenib.

Conclusion*:* This study shows that it is feasible to differentiate liver tumor-derived organoids from a CSC-like phenotype towards a differentiated cancer cell phenotype. Differentiated CCA organoids are less proliferative, downregulate CSC markers and are more sensitive to sorafenib. More elaborate drug screenings are ongoing in order to more accurately identify effective compounds to treat CCA.

Session 5

NTCP expression is modulated by ERK1 activation via the FXR-FGF19 gut-liver pathway.

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Background: NTCP mediates the uptake of conjugated bile acid into hepatocytes and thereby regulates the intracellular and systemic bile acid concentration. NTCP expression is known to be regulated at several levels, including posttranslational regulation by kinases. However, the precise mechanisms by which NTCP is post-translationally controlled are not yet fully understood. In the enterocyte, bile acids can activate FXR and thereby inducing FGF19 expression which induces a reduced bile acid synthesis and has an effect on liver metabolism and regeneration. Besides that, FGF19 also induces ERK1 activation in hepatocytes. However whether the FXR-FGF19 gut-liver pathway participates in NTCP regulation via ERK1 activation is unknown.
Hypothesis: We speculate that FXR activation in enterocytes induces FGF19 secretion, which induces ERK activation in hepatocytes lowering NTCP expression at the plasma membrane.
Research Plan: In a screen of a library of kinase inhibitors, ERK was identified as a kinase that influences the functional activity of NTCP by measuring the level of bile acid uptake in HepG2 cells overexpressing NTCP. A phosphorylation prediction screen will be performed to identify amino acid in NTCP which could potentially be the target for a specific kinase. These amino acids will be mutated to either an alanine or an phosphomimetic amino acid (D/E) to verify the target amino acid. Inhibitors in the ERK1 pathway, EGF and FGF19 will be used to verify a possible ERK1 effect on NTCP which will be assessed by TCA uptake and cell-surface biotinylation in combination with western blot.
Anticipated Results: The kinase inhibitor screen identified ERK1 as potential candidate to modulate NTCP bile acid uptake. We expect that FGF19 by inducing ERK1 activation in the liver can phosphorylate NTCP directly. This might lower NTCP expression at the plasma membrane lowering hepatic bile acid accumulation.

Session 5

Effects of A Humanized Bile Acid Pool Induced by Deletion of Cyp2c70 on Pharmacological FXR Activation in Mice

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Background: Bile acids (BAs) facilitate fat absorption but also modulate various metabolic pathways through activation of the BA receptors FXR and TGR5, which have been identified as targets for therapeutic interventions. However, fundamental differences in BA metabolism between humans and mice complicates translation of preclinical data. CYP2C70 was recently proposed to catalyze the formation of rodent-specific muricholic acids (MCAs). We generated mouse models of *Cyp2c70*-deficiency, to clarify its role in BA metabolism *in vivo* and to evaluate whether humanization of the murine bile acid pool modulates the effects of pharmacological FXR activation.
Methods: Two different mouse models of *Cyp2c70*-deficiency were generated, an acute hepatic knock-out model, in which the *Cyp2c70* gene was ablated in adult mouse livers by CRISPR/Cas9-mediated somatic genome editing employing adenovirus-mediated delivery of single-guide RNA to Cas9-transgenic mice (*Cyp2c70* acute knock-out, *Cyp2c70*^{ako}), as well as a full-body *Cyp2c70* knock-out model.
Results: Hepatic CYP2C70 protein levels were reduced by ~95% in *Cyp2c70*^{ako} mice. This translated into strongly increased contributions of chenodeoxycholic (CDCA) and ursodeoxycholic (UDCA) acids and a concomitantly reduced contribution of mouse-specific β-MCA, resulting in a more hydrophobic BA pool ($p<0.001$). Evaluation of *in vivo* CDCA and UDCA metabolism using D4-labeled tracers revealed 6β-hydroxylase as well as C7-epimerase activity of CYP2C70, delineating its importance in generating the characteristic murine BA pool. The reduction of fractional cholesterol absorption in control mice upon FXR activation with PX20606 (54% to 20%, $p<0.001$) was blunted in *Cyp2c70*^{ako} mice (47% to 34%, $p<0.01$). Additionally, augmented fecal cholesterol disposal in response to FXR activation was impaired in *Cyp2c70*^{ako} mice ($p<0.05$), predominantly due to reduced stimulation of transintestinal cholesterol excretion (TICE). Full-body *Cyp2c70*-KO mice were completely devoid of MCAs, indicating that CYP2C70 is the only enzyme responsible for MCA production in mice. In contrast to acute ablation of *Cyp2c70*, lifelong exposure to the more hydrophobic bile acid pool induced moderate cholangiocyte proliferation.

Conclusion: Deletion of *Cyp2c70* in mice translates into a human-like BA pool composition and impacts the response to pharmacological FXR activation, emphasizing the importance to carefully consider the consequences of species-specific (BA) metabolism in pre-clinical studies.

Session 6

Role of the novel IgG4-associated cholangitis autoantigen Annexin A11 in cholangiocyte protection

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Introduction: Annexin A11 was recently identified as the first IgG4/IgG1-autoantigen in IgG4-associated cholangitis (IAC), the hepatobiliary manifestation of a B cell-driven autoimmune disease targeting the biliary epithelium (Gut 2018;67:728). The Ca^{2+}-regulated protein Annexin A11 is known to participate in Ca^{2+}-dependent exoxytosis in pancreatic β-cells. In cholangiocytes, Ca^{2+}-dependent exoxytosis is thought to insert proteins like the Cl^-/HCO_3^- exchanger AE2 into their target membrane, their site of action. AE2 is indispensable for maintenance of human cholangiocellular defense mechanisms against hydrophobic bile salts (referred to as 'biliary HCO_3^- umbrella'; Hepatology 2010;52:1489). Here, we investigate the potential role of Annexin A11 in mediating the transport of AE2 to the cholangiocyte cell membrane.
Methods: Expression pattern of Annexin A11 in human liver tissue sections was visualized by immunohistochemistry. Human SV40-transformed cholangiocytes (H69 cholangiocytes) were transduced with short hairpin RNA (shRNA) against Annexin A11 or scramble shRNA as negative control. AE2 surface expression was examined by surface biotinylation followed by immunoblotting. Intracellular pH was determined using fluorescent probe BCECF.
Results: Immunohistochemistry of healthy liver tissue showed weak immunostaining of Annexin A11 in hepatocytes, but prominent staining in cholangiocytes. After biotinylation Annexin A11 was also detectable on the extracellular site of the plasma membrane. Surface expression of AE2 was reduced by ~50% on Annexin A11 knockdown H69 cholangiocytes compared to sham transduced H69 cholangiocytes. Correspondingly, intracellular pH of Annexin A11 knockdown H69 cells was significantly more alkaline, indicating intracellular HCO_3^- retention.

Conclusion: Annexin A11 may be crucial for robust AE2 membrane expression and function in human cholangiocytes, possibly by mediating Ca^{2+}-dependent fusion of AE2-carrying vesicles with the cell membrane. We speculate that an IgG4/IgG1 antibody response in IAC targeting Annexin A11 may interfere with AE2 membrane insertion and function (see Hepatology 2016;63:524). Thus IAC, next to PBC and PSC, might represent another cholangiopathy characterized by a defective biliary bicarbonate umbrella leading to subtle cholangiocyte injury and bile duct destruction over time.

Session 6

Hepatic ChREBP attenuates the progression of liver disease in a mouse model for Glycogen Storage Disease type 1a

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Background: Glycogen storage disease type 1a (GSD Ia) is an inborn error of metabolism caused by a defect in glucose-6-phoshatase (G6PC) activity. GSD Ia patients exhibit hepatomegaly due to massive accumulation of glycogen and triglycerides in the liver from childhood on, and frequently develop hepatocellular tumors in adulthood. Previously, we have reported that the activity of the glucose-sensitive transcription factor Carbohydrate Response Element Binding Protein (ChREBP) is increased in GSD Ia liver. Moreover, unpublished data from our lab show that short-term (*i.e.* 10-day) normalization of hepatic ChREBP activity aggravates hepatomegaly in GSD Ia due to further accumulation of glycogen and triglycerides. These findings suggest that enhanced activity of ChREBP protects the liver against advanced liver disease in GSD Ia. Therefore, we assessed the impact of prolonged hepatic ChREBP knockdown on the progression of GSD Ia liver disease in mice.
Methods: Liver-specific *G6pc* knockout (L-*G6pc-/-*) and wildtype (L-*G6pc+/+*) mice were treated with either AAV8-shChREBP or AAV8-shScramble. Animals were sacrificed 3 weeks after combined shRNA treatment / *G6pc* knockout.
Results: Hepatic ChREBP knockdown in L-*G6pc-/-* mice led to a further increase in liver weight and hepatomegaly, as well as a progressive increase in plasma ALT levels. Moreover, the liver of these mice showed an increase in fibrosis marker genes (*Col1a1*, *TIMP1*, α*SMA*, *TGFβ*, *MMP9*) and slight increases in the expression of inflammatory genes (*TNFα*, *IL1β*, *IL6*). Histological analysis showed that *G6pc-/-* / shChREBP-treated livers showed the presence of inflammatory foci and Mallory Denk bodies, while Sirius Red staining was comparable among the different experimental groups. Hepatocytes from L-*G6pc-/-* mice treated with shChREBP also showed an increase in γH2A.x staining, mitotic figures, and BrdU-positivity.

Conclusions: Prolonged hepatic ChREBP knockdown in GSD Ia mice aggravates hepatomegaly and sensitizes to liver inflammation and fibrosis, in parallel, to increased chromosomal instability and DNA synthesis and/or repair. Altogether, our data suggest that enhanced ChREBP activity in GSD Ia serves as a protective mechanism against advanced liver disease, and may slow down liver tumor formation.

Session 6

FXR isoforms a2/4 regulate triglyceride levels through a novel mode of activation independent of RXRa

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In the last two decades, the Farnesoid X Receptor (FXR) has drawn great attention because of its role as a bile acid-activated transcription factor with important physiological effects. Importantly, activation of FXR reduced triglyceride (TG) levels in mice and cultured liver organoids. In this context, misregulation of the hepatic de novo lipogenesis is considered central in the development of Non-alcoholic fatty liver disease.

In this study we investigated the mechanisms of activation by FXR isoforms in mouse liver organoids, and their effects in TG regulation; as this system recapitulates many aspects of liver physiology. We reconstituted mouse FXR-/- liver organoids with the different mouse FXR isoforms (α1 to α4).
We found that a previously identified FXR-bound regulatory region in the SHP locus is bound predominantly by FXRα2/4, correlating with the observed FXRα2/4- dependent increase in SHP expression upon FXR-agonist treatment. The upregulation of SHP has been shown to decrease both bile acid and triglyceride synthesis via the attenuation of CYP7a1 and SREBP-1c transcription, respectively. In liver organoids, isoform specific Shp activation correlated with the reduction in TG.
Subsequent ChIP-sequencing analysis revealed that the majority of the detected FXR peaks correspond to FXRα2/4-selective DNA binding locations, enriched for a novel discriminant binding motif. Interestingly, activation by FXR from this DNA element is antagonised by RXR heterodimerization, in contrast to the canonical FXR binding to IR-1 hexamer repeats.
Our data support that SHP and many more genes of metabolic relevance are transcriptionally regulated by FXR α2/4 selectively via this novel mechanism. As the relative expression and availability of the FXR isoforms and RXR has a strong impact on the transcriptional output, the study of regulators of FXR isoform expression may prove instrumental in optimizing the therapeutic efficacy of FXR full agonists against metabolic diseases.

Session 6

Adeno-associated viral gene therapy corrects the phenotype of a mouse model for progressive familial intrahepatic cholestasis type 3

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Background: Progressive familial intrahepatic cholestasis type 3 (PFIC3) leads to severe liver injury at young age with limited therapeutic options. PFIC3 results from mutations in the ATP-Binding Cassette subfamily B member 4 (*ABCB4*) gene, leading to dysfunction of ABCB4-mediated phospholipid secretion into bile. In the *Abcb4* knockout mouse model, absence of phospholipids in bile leads to bile salt–induced cytotoxicity, which is aggravated by a cholate-supplemented diet, resembling the PFIC type 3 phenotype. We hypothesized that adeno-associated virus serotype 8 (AAV8) mediated expression of human *ABCB4* in the *Abcb4-/-* -mouse liver could correct the cholestatic phenotype by restoring the phospholipid content in bile.

Methods: Ten weeks old *Abcb4*$^{-/-}$ mice received a single dose of AAV8-h*ABCB4* (n=8) or -*GFP* (n=8) under control of a liver specific promotor at 5x10^{13} vector genome copies per kg via tail vein injection. All animals were challenged by a 0.1% cholate diet for 2 weeks, either at 8 or 24 weeks after vector administration. Bile duct cannulation at termination enabled analysis of biliary phospholipids. Liver fibrosis was scored as absent (0), periportal (<50% - 1, >50% - 2) or bridging (<50% - 3, >50% - 4).

Results: All animals that received AAV8-h*ABCB4* showed expression of hepatocanalicular ABCB4, resulting in complete normalization of plasma cholestatic markers 2 weeks after administration. Treated animals were resistant to a 0.1% cholate diet, both at 8 or 24 weeks, while the AAV8-*GFP* treated controls developed severe cholestasis (ALP wk10 62±8 vs. 919±332; wk26 73±6 vs. 1152±118U/L; Bilirubin wk10 and wk26 <1 vs. 208±84 µmol/L). The phospholipid content in bile of treated animals was restored to levels comparable to wild-type in contrast to near absence in controls (wild-type 42.2±17.6; wk10 38.5±8.6 vs. 1.5±0.7; wk26 12.3±2.9 vs. 3.3±1.0 nmol/min/100gr). Liver fibrosis scores were nearly normalized in all treated animals in contrast to the significant bridging fibrosis found in controls (wk10 1.0±0.0 vs. 3.75±0.5; wk26 0.6±0.6 vs. 3.7±0.6).

Conclusion: AAV8-mediated expression of h*ABCB4* in the liver of *Abcb4-/-* mice restores phospholipid content in bile, provides long-lasting correction of the cholestatic phenotype and prevents fibrosis. This study supports the feasibility of gene therapy for PFIC3.

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Notes

Notes

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