Falk Workshop

Targeted Therapies in Hepatology

January 24 – 25, 2013
Medizinische Hochschule
Hannover
Germany

Abstracts
Poster Abstracts

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Falk Workshop

TARGETED THERAPIES IN HEPATOLOGY

Hannover (Germany)
January 24 – 25, 2013

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Session I

Principles of targeted therapies

The role of tissue analyses for targeted cancer therapies – What can we learn for liver cancer?

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Precision oncology (which is currently replacing the term personalised oncology) is the perspective, strategy, and promise to develop better suited tumor therapies and improve the definition of patients that are likely to benefit from these therapies (or not). Response to tumor therapy largely resides on the diagnostic identification of molecular tumor characteristics and is driven by basic and translational molecular tumor research, which is taking giant leaps forward, also in basic liver cancer research. In other tumor diseases, such as breast, lung and colon cancer, malignant lymphoma and melanoma, we are witnessing significant clinical improvement, as seen for example by the enormous numbers of clinical trials, novel molecular tests and treatments and tremendous improvements of bench – bedside – bench research. Despite HCC showing no fundamental differences to these other tumor entities and being an important and growing health burdon we are not witnessing a comparable development in liver cancer; no single targeted therapy or predictive diagnostic assay has reached approval or clinical practice for HCC so far. The reasons are obvious and need to be tackled in order to prevent HCC from becoming an orphan tumor disease without participating in novel research findings and progress of precision oncology: HCC is the only relevant cancer, which does not mandatorily require histological confirmation for further specific, especially non-surgical treatment. This leads to an estimated 50–70% of nonresectable HCCs, in which diagnosis is established only by imaging without availability and analysis of (biopsy) tissue. Leaving aside many resulting and relevant practical clinical problems and diagnostic deficiencies this unique deficiency has lead to the situation that cancer tissue is also not mandatorily available in most clinical trials, even phase III trials relevant for clinical approval. Lack of trial associated research and failure of promising phase III trials (e.g. Sunitib and Brivanib) has been the disastrous consequence that is burdening the field in competition to other tumor diseases. In none of these trials tumor based parameters that guide response and define potential responsive subgroups could be analysed. This situation is fundamentally different to other tumor diseases, where mandatory tumor tissue availability has guided development of predictive diagnostics, inclusion criteria, analysis of resistance parameters, and even post hoc rescue of overall non-superior trial results, as exemplified by the worldwide applauded IPASS trial in NSCLC. Numerous important bedside – bench translational research projects have been the consequence.
In comparison to other (successful) tumor diseases the necessary steps and improvements will be explained. They involve strenghthening interdisciplinary diagnostics, especially mandatory tumor biopsy, and the implementation of strategic oncological planning for HCC.

Chances and limitations of Personalized Medicine

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Personalized Medicine (PM) is defined as the management of patient's disease or disease predisposition supported by molecular analyses in order to achieve optimal medical outcome for the individual, thereby improving the quality of life and health, and potentially reducing overall healthcare costs (http://www.personalizedmedicinecoalition.org).
In current practice, *Personalized Medicine* is trying to integrate comprehensive knowledge on individual (risk) factors such as the genetic make-up or environmental conditions in diagnostic decisions or therapeutic interventions. In addition, it contributes to discovery and clinical testing of novel medical products such as new drugs and biomarkers.
At the core of Personalized Medicine lies a thorough characterization of patients, including established clinical examinations, imaging technologies, and molecular methods. In this presentation, general principles required for PM-focused research and translation in academic centers are outlined and specific examples for both opportunities and limitations are provided.

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Macrophages and targeted therapies

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Monocytes and macrophages fulfil critical decisive roles in inflammatory and malignant liver diseases, because their activation and differentiation crucially determine the perpetuation or restriction of inflammation: pro-inflammatory M1 macrophages promote inflammation but also antitumoral responses, whereas anti-inflammatory M2 macrophages and myeloid-derived suppressor cells (MDSC) restrict local and systemic inflammation but promote tumor growth. Experimental murine models of liver injury highlighted the importance of hepatic macrophages, so-called Kupffer cells, for driving inflammatory responses by releasing proinflammatory cytokines and chemokines (e.g., TNF, IL-6, MCP-1) as well as activating hepatic stellate cells (HSC), which then promote liver scarring. Recent studies in mice demonstrate that these actions are only partially conducted by liver-resident macrophages, but largely depend on the recruitment of monocytes into the liver, namely of the inflammatory $Gr1^+$ ($Ly6C^+$) monocyte subset as precursors of tissue macrophages. The modification of macrophage functionality, particularly in the liver, appears to be a promising novel therapeutic target for inflammatory disorders, fibrosis and cancer. Monocyte-derived macrophages can be targeted by modulating chemokine – chemokine receptor pathways that control their migration to the injured liver. For instance, the infiltration of proinflammatory monocytes into injured murine liver can be specifically blocked by novel anti-MCP-1 directed agents. Moreover, hepatic macrophages can be targeted by nano-sized drug carrier systems, which are often also biofunctionalized with antibodies or peptides. Prior *in-vitro* data demonstrated that human macrophages can be skewed towards M1- or M2-effector macrophages by distinct biofunctionalized gold nanorods. In experimental murine models, gold nanorods efficiently target hepatic macrophages, but nanoparticle modifications of their surface chemistry considerably affect the phenotype and functionality of inflammatory monocytes and Kupffer cells in the liver, thereby impacting the hepatic response towards acute or chronic injury. The recently identified cellular and molecular pathways for monocyte subset recruitment, macrophage differentiation and interactions with other hepatic cell types in injured liver may therefore represent interesting novel targets for future therapeutic approaches in liver fibrosis.

Cell cycle structures as targets for anticancer treatment

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Changes in the composition or expression levels of basic regulators of the cell cycle are of critical importance for the development of cancer. Specifically mechanisms which dysregulate the activity of cyclin kinase complexes which regulate the transition of cells from the G1 phase into S phase and from G2 into M phase have been shown to result in molecular changes that lead to genetic instability. We have previously shown that the regulation of the cyclin E protein by mechanisms of proteolytic turnover and through direct inhibition by cylin kinase inhibitors is critically important to maintain homeostasis in the liver. Dysregulation in these complexes leads to expansion of tumorigenic liver stem cells and formation of tumors like HCC and CCC. Based on these findings we have then started to develop new treatments or targets for new treatments to fight hepatobiliary cancers. Specifically we defined the p27 protein as a new target for tumor treatments, identified the Notch signalling pathway as a central regulator of cyclin E expression and CCC formation and identified mTOR signalling as a regulator of cancer stem cell differentiation. Aspects of this work will be presented during the meeting.

Session II

Targeted therapies in liver cancer and metabolic liver disease

Inflammatory signaling pathways in HCC development

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Hepatocellular carcinoma (HCC) arises almost exclusively in a setting of chronic hepatic inflammation, induced e.g. by viral hepatitis, alcoholic and non-alcoholic fatty liver disease and other pathogens. However, the knowledge on this unique association between inflammation and cancer development has not yet translated into a chemopreventive strategy in patients with chronic liver disease, since it is presently unclear which inflammatory signaling molecules might be the best candidates for future drug targeting approaches.
The cytokine tumour necrosis factor (TNF) represents a key messenger involved in regulating hepatocarcinogenesis. TNF activates several different intracellular signaling cascades controlling cell death, inflammation and stress responses, such as the Caspase-cascade, the NF-kappaB, the JNK and the p38 MAPKinase pathways. Within the complex TNF-dependent signaling axes, certain molecules, namely members of the NF-κB activating IKK complex (IKK1, IKK2 and NEMO) as well as the molecule TAK1, represent important signalling nexus, since they integrate signals from numerous upstream receptors and control several downstream signaling pathways. Therefore, this presentation will focus on the complex role of the IKK complex and TAK1 in the regulation of liver cancer development with a special emphasis on data generated in genetically modified mouse models.

HCC gene profiling: Role for future targeted therapies in liver cancer

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Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most lethal neoplasm causing an estimated 600,000 deaths annually1. In the United States the incidence of HCC has doubled over the past two decades, and despite the recent improvements in treatment and diagnostics, only 30–40% of patients with HCC are eligible for curative treatments. Our recent genomics analyses identified *COP1* and *CSN5* in a screen for survival genes in human HCC3. Both genes regulate p53 activity via proteasome-dependent degradation. Here we addressed whether targeting of *COP1* or *CSN5* can provide a novel therapeutic modality against human HCC. Silencing of each gene by small interfering (si) RNA inhibited proliferation of HCC cells and increased apoptotic cell death through the restoration of p53 function. Systemic delivery of the modified target siRNAs by stable-nucleic-acid-lipid-particles (SNALP) remarkably suppressed neoplastic growth and increased survival without eliciting immune response in an orthotopic xenograft mouse model. Analysis of COP1 knockdown signature revealed that antitumor effect *in vivo* was driven by a p53-dependent apoptosis. The study suggests that p53 ubiquitination pathway is an attractive target for treating HCC and provides an important new step towards the potential clinical application of siRNA utilizing SNALP technology.

Targeted therapies for liver cancer – Today and tomorrow

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Systemic therapy of HCC is a challenge for the treating physician not only because the severity of the disease but also because of limited treatment options. While systemic chemotherapy has failed to demonstrate clinical benefit in patients with HCC, sorafenib, a multi receptor tyrosine kinase inhibitor is the only targeted therapy, which has shown to improve patients survival by less than three months. Multiple other targeted therapies have been evaluated since the approval of sorafenib for the treatment of HCC. However, until today none of the drugs tested have shown a clear benefit for patients with HCC in phase II and more importantly in phase III trials. Based on these disappointing results new and/or alternative treatment options are needed in for the treatment of patients with HCC. We have been evaluating immune based therapies in HCC. Different immune based therapies are currently being evaluated in HCC. Such treatments aim at enhancing tumor-specific immune responses, blockade of cytokines and chemokines, targeting immune cells with suppressor function and finally inhibition of immune checkpoint inhibitors. While some of these treatments have already shown limited clinical effects in early clinical trials, combination of immune based therapies, with molecular targeting therapies and local ablative therapies may provide an effective treatment option for patients with HCC and will be discussed.

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Targeted therapies in NASH

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Non-alcoholic fatty liver disease (NAFLD) has emerged as leading liver disease in the western world. Whereas simple steatosis has an excellent prognosis, the inflammatory variant i.e. NASH, which appears in approximately 10% of affected individuals leads to cirrhosis and liver cancer.
It is well-recognized that the combination of clinical exam, imaging studies and/or serology-based laboratory values may indicate the presence of fatty liver on the one hand, or end-stage liver disease, on the other, but only histologic evaluation of a liver biopsy will provide the additional information on the presence and extent of ongoing necroinflammatory injury and fibrosis. Identification of the NASH patient subgroup, however, is mandatory as only those patients are considered relevant for a potential therapy. Various treatments have been studied in the last years, often based on the currently considered pathophysiology of this disease. Surprisingly, although very effective in the treatment of type 2 diabetes, metformin has not proven effective, although NASH is often accompanied by insulin resistance. So far, only treatments with vitamin E and pioglitazone have demonstrated some efficacy based on rather stringent clinical and histological criteria. NASH has been demonstrated to be associated with increased circulating levels of certain biomarkers such as C-reactive protein, ferritin or interleukin-1 receptor antagonist. Future treatment studies have to take this into consideration to advance clinical care and treatment possibilities of these patients. Only a better understating of disease pathophysiology and a more precise identification of the inflammatory phenotype (i.e. NASH) ideally based on new imaging techniques ± biomarkers will allow us to obtain effective therapies in the future. Treatment of the most common liver disease in our societies is unfortunately still in its infancy and currently we can only offer “lifestyle modification” for our patients which is far away from a satisfactory treatment strategy.

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Molecular pathogenesis of pruritus: New targets for interventional therapies

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Pruritus is a common symptom in cholestatic liver diseases, undergoes diurnal variations and is usually reported as most intense in the late evening and early night[1]. It is mainly localized on the palms, soles and limbs, but can also be generalized. Primary skin lesions are not observed, scratching-induced excorations and prurigo nodularis are quite common[2]. Extreme itch may lead to suicidal ideations.
The molecular pathogenesis of pruritus in cholestasis remains poorly understood[2]. Itch perception is induced by stimulation of an itch-specific subgroup of mechano-insensitive C-nociceptors in the cutis/subcutis. Unmyelinated C-fibres transmit the signals from the skin through the dorsal root ganglia to a second neuron in the dorsal horn of the spinal cord, crossing to the contralateral side and projecting via the spinothalamic tract to the ventromedial nucleus of the thalamus and the primary sensory cortex, supplementary motor area, anterior cingulate cortex and inferior parietal lobe. Guideline-supported therapeutic approaches (see below) with the anion exchange resin, cholestyramine, the PXR agonist, rifampicin, the opioid antagonist, naltrexone, or the serotonin reuptake inhibitor, sertraline, allow the conclusion that pruritogens in cholestasis (i) are biotransformed (or their formation is modulated) in liver and/or intestine, (ii) are secreted into bile, (iii) show characteristics fitting with an enterohepatic circulation, and (iv) may interact with the endogenous opioid and serotoninergic systems. Bile salts, histamine, endogenous opioids, serotonine, steroid metabolites and various other compounds which accumulate in cholestasis have been made responsible for induction of pruritus in the past, although evidence for a key role of these agents was weak at best[2].
Recent experimental evidence unravelled the lysophospholipase D, autotaxin (ATX), and its product, lysophosphatidate (LPA), as potential mediators of cholestatic pruritus[3, 4]. By screening serum of pruritic patients for activation of neuronal cell lines we identified LPA as a potent stimulus[3, 4]. Serum levels of ATX correlated closely with itch intensity and responded to therapeutic interventions[3, 4]. LPA injected intradermally into mice, induced scratch responses[4]. These data suggest a new model for the pathogenesis of pruritus in cholestasis in which LPA and ATX play a critical role and may serve as potential targets for future therapeutic interventions. An additional factor X in bile is under investigation.

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Session III

Targeted therapy in cholestatic and autoimmune liver diseases

Is targeted therapy a future option for antifibrotic therapy?

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Liver fibrosis is the consequence of years or decades of chronic liver disease and leads to the development of cirrhosis and hepatocellular carcinoma for which at present the only curative treatment is transplantation. We urgently require medicines that are able to prevent the progression of fibrosis and ideally promote regression of fibrotic extracellular matrix and recovery of normal liver architecture and function. Fibrosis is a complex pathophysiological process in which multiple cell types including immune cells, endothelial and epithelial cells and (myo)fibroblasts cross-talk to regulate inflammation, matrix deposition, angiogenesis and regenerative responses. Many of the molecular events implicated in fibrosis are normal physiological processes that are fundamental to liver homeostasis and immunity. As liver fibrosis is a slowly progressing (and likely slowly regressing) pathology, it is anticipated that treatments will be long-term and possibly life-long in order to prevent recurrence. Global (systemic/untargeted) modulation of fibrotic processes may therefore in the longer term lead to significant side-effects that impact on health and vitality. It is therefore imperative that we identify therapeutic targets that can selectively modulate pathways that are critical for fibrogenesis but that will not have a negative impact on immunity and tissue homeostasis.

We have focused on the liver myofibroblast as a therapeutic target, principally because these cells are rare in the normal liver but proliferate in diseased liver where they function as the major cell type responsible for deposition and maintenance of fibrotic extracellular matrix. The major cellular source of liver myofibroblasts is the hepatic stellate cell (HSC); with contributions from portal fibroblasts and circulating fibrocytes. We show that targeted apoptotic clearance of HSC-derived myofibroblasts is able to attenuate progressive fibrosis in the context of ongoing liver injury. Technologies exploiting myofibroblast-selective surface receptors for targeted delivery of drugs and/or imaging tools will be described. We also suggest that HSC-derived myofibroblasts may suppress compensatory hepatocyte proliferation and identify a specific paracrine signaling pathway responsible for this effect. Serotonin mainly released by platelets triggers 5-HT2B receptor signaling in myofibroblasts, which via an ERK/JunD pathway stimulates expression of TGFβ1, which can then act as an autocrine stimulator of fibrosis and a paracrine repressor of epithelial cell proliferation. We demonstrate that antagonism of 5-HT2B is both anti-fibrogenic and pro-regenerative. Since serotonin is pro-regenerative for hepatocytes (via 5-HT2A) and does not stimulate TGFβ1 expression in Kupffer cells we propose that antagonism of 5-HT2B signaling offers an attractive means of targeting the fibrogenic actions of myofibroblasts. We have additionally investigated alternative pathways for control of NF-κB signaling in myofibroblasts that has identified sulphasalazine and angiotensin II blockers as potential anti-fibrotics. Data will be presented for more refined targeting of a phosphorylation event on the RelA subunit of NF-κB which delivers targeted apoptosis of myofibroblasts and attenuation of fibrosis in disease models using therapeutic protocols.

Etiopathogenesis of primary biliary cirrhosis: Implications for therapy

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There have been significant advances both in humans and experimental models that relate to the etiopathogenesis of primary biliary cirrhosis. Many of these advances are based on the rigorous definition of the antimitochondrial response, the serologic signature of PBC. First, it is well established that AMA are directed against members of the 2-oxoacid dehydrogenase complexes (2-OADC), among which the major epitopes are within the lipoylated domains of the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). Second, autoreactive CD4+ and CD8+ T cells can be detected in PBC peripheral blood, regardless of the AMA status, and the infiltration of autoreactive T cells in the liver and periductular spaces is one of the most prominent immune features. Autoreactive T cells of both subtypes recognize PDC-E2 sequences overlapping with the AMA epitopes. An increase in cytotoxic T cell precursors in the blood in the early stages of the disease compared to the advanced ones and a 10-fold increase of specific liver $CD8^+$ T cells compared to peripheral blood have been demonstrated. Third, additional data on the immunobiology components of PBC autoimmunity has been recently obtained in $CD4^+CD25^{high}$ natural regulatory T cells which appear to be numerically reduced in PBC. PBC bile duct cells manifest unique features during apoptosis while co-culture experiments do not support a direct role for these cells in determining their immune – mediated injury. Apoptotic cells are phagocytosed by BECs and consequently are an exogenous source of autoantigens in cholangiocytes, possibly through anti-CD16. As a result, the impact of putative changes in apoptosis and autophagy specific to BEC remains to be fully determined in PBC. Fifth, the innate immune compartment has been recently investigated in PBC with promising results. PBC monocytes manifest an increased response to pathogen associated stimuli, as indicated by higher levels of pro-inflammatory cytokines. Further, the hyper-IgM associated with PBC is secondary to an aberrant innate immune response, potentially induced by stimulation of toll like receptor 9 by bacterial CpG-B. The female preponderance may hold an important key to PBC etiology. X-linked genes determine gender-related characteristics at different levels while also regulating the immune function, particulalry to maintain tolerance. Major X chromosome defects such as those leading to Turner's syndrome or premature ovarian failure are commonly characterized by autoimmune comorbidities (particularly thyroid disease) and, less frequently, cholestasis. Our group first determined a significantly higher frequency of monosomy of the X chromosome in peripheral leukocytes (particularly those of the adaptive immune response, i.e. T and B cells) in women PBC compared to age-matched control women. Monosomy frequency correlated with age in all three groups, as expected but monosomic cells were not microchimeric cells. We further demonstrated that the X loss in PBC affected was not random but affected more frequently one parentally-inherited chromosome. These data and observations will be put in the context of the key mechanisms, including the role of TLRs in modulating these responses. Finally using several strains of congenic mice, unique models of PBC have been developed. The collective data from these studies form the basis for future therapeutic interventions.

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Targeted therapies in cholestatic liver diseases – Role of bile acids and bile acid receptors

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Over the past two decades our improved understanding of the molecular mechanisms of bile formation and cholestasis has opened new perspecitives for targeted therapies of these complex disorders. Cholestatic liver diseases encompass a wide spectrum of disorders of different etiologies characterized by impaired heptobiliary excretory function ultimately resulting in accumulation of bile acids (BAs) and other cholephils. The etiology and pathogenesis of several chronic cholestatic disorders/cholangiopathies such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are still poorly understood and several subphenotypes of disorders such as PBC and PSC are recognized which may require separate/specific treatments.

The accumulation of potentially toxic BAs leads to hepatocellular damage followed by inflammation, fibrosis, and finally – depending on the disease severity and duration – may culminate in liver cirrhosis and hepatocellular or cholangiocellular cancer. To handle potentially toxic cholephils under physiological and pathological conditions, the liver possesses a complex network of BA-activated nuclear receptor (NR)-regulated pathways that coordinate BA homeostasis and bile secretion to limit their concentrations and prevent hepatic as well as systemic accumulation. The most relevant BA-activated NRs for regulation of hepatobiliary homeostasis, bile secretion and thereby understanding and treating cholestasis, include the farnesoid X receptor (FXR, NR1H4), pregnane X receptor (PXR, NR1I2) and vitamin D receptor (VDR, NR1I1). Apart from BAs other biliary constituents such as bilirubin can also activate NRs such as the constitutive androstane receptor (CAR, NR1I3). Furthermore other NRs such as glucocorticoid receptor (GR, NR3C1) and fatty acid-activated peroxisome proliferator-activated receptors PPARs, in particular PPARα (NR1C1) and PPARγ (NR1C3) as regulators of inflammation, fibrosis and energy homeostasis, may also impact on biliary homeostasis and cholestatic liver injury. Due to their capability to control hepatic BA metabolism, hepatic inflammation and fibrosis, NRs in general and BA-activated NRs in particular have emerged as promising therapeutic targets in many liver diseases including cholestatic disorders.

Many drugs used as treatments for cholestasis act via NRs and stimulation of their target genes. Ursodeoxycholic acid (UDCA) which is currently used as therapeutic standard in cholestasis has multiple beneficial mechanisms which may be mediated to at least in part by NRs (e.g., GR, PXR). Although these various mechanisms of action of UDCA have been studied in detail in last decades, the complete picture underlying the beneficial effects of UDCA remains to be determined. Apart from UDCA, other already available drugs may exert their benficial effects in cholestasis via NR activation (e.g., rifampicin via PXR; fibrates via PPARα, budesonide via GR). The most promising future BA-based therapeutic options for targeted therapy of cholestatic liver diseases include 24-*nor*ursodeoxycholic acid (*nor*UDCA) and bile acid receptor/farnesoid X receptor (FXR) agonists (e.g., obeticholic acid (OCA), already sucessfully tested in PBC).

*nor*UDCA is a side chain-shortened C_{23} homologue of UDCA which possesses one less methylene group in its side chain and is more resistant to conjugation with taurine or glycine than UDCA, but instead is secreted into bile mostly in unchanged form. The secreted *nor*UDCA undergoes absorption by cholangiocytes, returns to the liver and is resecreted into bile. Such cholehepatic shunting leads to a bicarbonate-rich hypercholeresis and may also result in improved targeting to the liver and dieseae bile ducts ('ductular targeting'). *nor*UDCA (but not "conventional" UDCA) reversed sclerosing cholangitis in the *Mdr2 (Abcb4)*$^{-/-}$ cholangiopathy model within 4 weeks of treatment. Its possible therapeutic mechanisms include (i) amelioration of bile hydrophobicity by biliary enrichement with hydrophilic *nor*UDCA and its metabolites, (ii) flushing of injured bile ducts by stimulation of bile flow and bicarbonate-rich choleresis, which dilutes toxic biliary content and reinforces the bicarbonate umbrella protecting against potentially toxic bile acids, (iii) induction of alternative bile acid detoxification (phase I and II enzymes) and elimination routes for bile acids, and (iv) direct anti-inflammatory and anti-fibrotic properties. Notably, tauro-*nor*UDCA which lacks cholehepatic hepatic shunting with stimulation of bicarbonate secretion also looses the therapeutics effects. A recent comprehensive gene expression and metabolomic profiling revealed profound alterations in fatty acid and triglyceride metabolism, including a restoration of elevated short-chain and medium-chain fatty acids and reduced long-chain fatty acids resulted in a less lipotoxic lipid profile in the *Mdr2*$^{-/-}$ cholangiopathy model by *nor*UDCA. *nor*UDCA also targets the inflammatory cross talk between cells involved in inflammation and fibrogenesis in sclerosing cholangitis. As such, *nor*UDCA represents a multi-targeted therapeutic approach, targeting hepatocytes, cholangiocytes and Kupffer cells. Such a multi-targeted therapeutic approach may be essential for the treatment of a complex multifactorial disease such as PSC, as well as other cholangiopathies such as PBC. As a result of the very encouraging experimental data in preclinical (P)SC models, *nor*UDCA has undergone further clinical development for PSC. Phase I clinical trials have been successfully completed and a multicenter Phase II dose-finding trial testing *no*rUDCA in PSC has been initiated.

Another interesting opportunity for targeted therapy in cholestasis are agents directed at the bile acid receptors TGR5 and FXR. TGR5 is a G-protein coupled bile acid receptor at a plasma membrane, while FXR is a nuclear hormone receptor, and both receptors are involved in the regulation of metabolism and inflammation through bile acids. Notably, some TGR5 polymorphisms have recently been associated with pathogenesis of PSC and ulcerative colitis. A range of TGR5 and FXR are selective antagonists as well as dual TGR5/FXR ligands are now available and some of them have already been tested in preclinical models and disorders such as PBC. It is important to emphasize, that neither UDCA nor *nor*UDCA are FXR or TGR5 ligands.

The protective effects of FXR were demonstrated in several animal models. A non-BA synthetic FXR agonist GW4064 and BA-derived 6α-ethyl derivative of CDCA (also known as 6-ECDCA or INT-747 or obeticholic acid (OCA)) have beneficial effects in mouse models of chemically-induced liver injury (ANIT and estradiol-induced) or in bile duct-ligation (BDL). We have recently tested some FXR and/or TGR5 ligands in the *Mdr2 (Abcb4)*$^{-/-}$ cholangiopathy model. A dual ligand with high affinity to FXR (INT-767, but not the clincial lead compound INT-747/OCA) was able to cure bile duct injury in these mice. Subsequent studies in FXR knock-out mice revealed that these effects were mediated exclusively by FXR and not by TGR5. The

therapeutic mechanisms involved suppression of bile acid synthesis and direct anti-inflammatory and antifibrotic effects and silencing of the reactive cholangiocyte phenotype. Notably, similar to *nor*UDCA this therapeutic effect was also linked to generation of a bicarbonate-rich choleresis which appears to be a common denominator for successful treatment of cholangiopathies in general.

FXR agonists have already entered clinical trials. Combination therapy of UDCA with the INT-747 in phase II clinical trial in PBC patients not responding to UDCA showed substantial reduction of biochemical parameters of liver damage and cholestasis such as ALT and ALP after short and long-term administration. In line with the results obtained with combination therapy, INT-747 monotherapy in PBC patients also achieved a significant reduction of cholestasis. Dose dependent itching was reported to be the most common adverse event in patients receiving higher doses of INT-747. Since pruritus represent a common symptom of PBC that may lead to severe disability in suffering patients, subsequent clinical trials have excluded patients suffering from pruritus due to the disease. A multicenter, placebo-controlled, randomized phase III clinical trial, testing INT-747 in PBC patients who have not non-responded to standard UDCA is currrenty under way.

In conclusion, we witness a revolution of expanding use of BA-targeting therapies in cholestatic liver diseases. The translation of expanding knowledge on NRs and novel insights into BA (patho)biology should result in optimization of the currently available therapies with careful selection of patients' subgroups benefiting from such novel targeted therapeutic approaches.

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Session IV

Viral hepatitis and liver transplantation

Viral life cycle analysis for the identification of antiviral therapies – HCV as a model

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Licensing of the first directly acting antiviral drugs which target the HCV NS3-4A protease has improved treatment options for HCV patients. However, these drugs complement rather than replace the IFN-based regimens and triple therapy is not effective in all patient populations. Moreover, viral genotype-specific efficacy of these drugs demands more efforts to develop well tolerated treatments with a high barrier to resistance and efficacy across all viral genotypes.

HCV is a highly variable plus-strand RNA virus of the family *Flaviviridae*. Viral strains are grouped into seven genotypes that differ from each other by more than 30% at the nucleotide level. The variability of HCV permits immune evasion and facilitates persistence. It is also a substantial challenge for development of specific antiviral therapies effective against all HCV genotypes and for prevention of drug resistance.

In this respect host factors crucial for virus replication have emerged as alternative targets for novel therapies, because their use is likely conserved among HCV isolates and genotypes. For instance, cell entry of all major HCV genotypes is mediated by a minimal set of four cellular entry factors. Therefore, current efforts for the development of novel therapeutics not only target viral enzymes but also host factors essential for virus replication. Detailed cell culture studies have revealed numerous HCV replication co-factors and established novel models to test efficacy of antiviral compounds across different viral genotypes. In the light of these developments, possible future treatment options of chronic Hepatitis C as well as the antiviral mechanism of selected inhibitors will be discussed.

Personalized therapies for hepatitis C – The end is at hand?

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In view of the inconvenience and high rate of significant side-effects of IFN-based therapy, all-oral regimens will certainly become a first choice for a number of patients in the very near future. A key lesson from the body of available data is that those patients who do not respond well to IFN-α-based therapies, especially previous null-responders to pegIFN-α and ribavirin therapy, do not respond well to many interferon-free regimens as well, independently from whether these regimens include ribavirin or not. Exemplarily, the combination regimen of the NS3-4A inhibitor ABT-450/r, the NNI ABT-333, and ribavirin resulted in SVR rates of 100% and 47% in HCV genotype 1 treatment-naïve patients and previous null-responders, respectively. In addition to previous treatment outcome, *IL28B* genotype appears to be a predictor of success of some (but not of all) interferon-free regimens, although its impact is significantly attenuated compared to pegIFN-α and ribavirin dual therapy. An explanation of these results might be that an appropriate endogenous immune response against HCV is still required for the final clearance of residual, possibly drug-resistant virus escaping DAA combinations. In addition to these host-associated determinants of treatment outcome, there clearly exist virus-associated factors which obviously attain significant importance in the upcoming era of interferon-free therapy. For all-oral regimens (and for IFN-α-based triple therapy approaches) based on either NS3-4A inhibitors or NS5A inhibitors, lower SVR rates have consistently been observed in HCV genotype 1a *vs.* 1b patients, a difference which can be explained by the significantly lower genetic barrier of resistance mutations against these drugs at defined positions in the HCV genotype 1a *vs.* 1b genome.
Whereas a high chance of cure in general is realistic in patient populations with favorable baseline characteristics, further improvement is still relevant in terms of side-effects and convenience of available treatment options. However, in difficult-to-cure patients such as patients with liver cirrhosis or HCV genotype 1a patients with previous null-response to pegIFN-α/ribavirin, most thus far investigated combinations of two DAAs ± ribavirin cannot prevent viral breakthrough in a proportion of patients. Hence, it will be key to define DAA combination partners which could replace IFN-α for a successful all-oral therapy of these patients. Perhaps, optimized DAA regimens combining compounds with a very high antiviral activity (e. g. potent NS3-4A or NS5A inhibitors) together with a very high barrier to resistance (e. g. potent NIs like sofosbuvir or cyclophilin inhibitors) with or without ribavirin might be successful even in difficult-to-cure HCV-infected patients.

Strategies for individualized organ tolerance

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Liver transplantation has become a highly successful therapeutic option for many patients with severe liver diseases. One of the major problems – besides donor organ shortage and recurrence of underlying diseases – is the requirement for life-long treatment of the patient with immunosuppressive drug. This treatment is associated with many side effects, which accumulate over time (nephrotoxicity, metabolic changes, osteoporosis, hypertension etc.). This causes marked morbidity and even mortality in transplant recipients in the long-term.

Because of these problems, the “manipulation” of the patient’s immune system in a way that it “learns” to accept the allogeneic organ as “self” has been the holy grail in transplantation research and medicine from its beginning. Misfortunately, this has not been achieved so far. Because of some “protolerogenic” features of the liver – as compared to other transplanted organs, like kidney, heart, or lung – liver transplantation may be the best clinical setting to attempt the induction of allograft tolerance (or at least “near-tolerance” with only very minimal requirement of immunosuppressive drugs).

While tolerance will probably not be achievable by the use of the immunosuppressive drugs that are available currently or in the near future, targeted cell therapy may hold promise in this context. Several researchers worldwide are testing various cell types for their immunoregulatory effects in the organ transplant setting. These include different types of regulatory T cells (T_{reg}), but also macrophages (M_{reg}) as well as B cells, and mesenchymal stem cells (MSC). For the effect of these cells, the type and dosing of initial immunosuppressive medication is highly critical in order to avoid blocking the regulatory effects of those cells.

Currently, a number of clinical studies in which cell therapy approaches are used for achieving individualized organ tolerance are being conducted: A European consortium is performing a study with different types of cells in combination with a unified immunosuppressive protocol (ONE Study). In liver transplantation, there are currently ongoing single center studies using T_{reg} (e.g., San Francisco) or using MSC (e.g., Regensburg).

Because of the requirement of using donor- or recipient-derived cells for individualized treatment, and because of many administrative/regulatory require-ments, these trials are extremely difficult to perform, very expensive, and will take long time from initial safety studies via optimization of immunosuppressive protocols towards broad clinical use.

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POSTER ABSTRACTS

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Effect of radiation on erythropoietin (EPO) gene expression in a rat liver

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Introduction: Hypoxia is one of a hallmark of radiation and Liver plays a key role in erythropoietin during hypoxia in the body. In the present work, we analyzed erythropoietin (EPO) gene expression changes in the rat liver after radiation.

Methods: We exposed rats to single dose x-irradiation (25-Gy) that was focused on the liver. Tissues were collected for mRNA, Protein and Immunohistochemistry analysis.

Results: At mRNA level, an early and quick increase of EPO (up to 2-fold increase) gene expression was detected with maximum of 3 hours in the liver of irradiated animals followed by a gradual decrease over the next 48 hours. In the liver, a parallel up-regulation of the hypoxia-inducible factor-2 (HIF-2) gene was observed (up to 1.5-fold increase), while HIF-1 gene expression remained unaltered after radiation.
Furthermore, a similar pattern of increase in EPO and HIF-2 (3h) was also observed in isolated hepatocytes after irradiation. In addition to that, treatment with cytokines (IL-1β, IL-6, TNF-α) showed significant difference of gene expression (HIF-1 and HIF-2) in hepatocytes after x-irradiation.

Discussion/Conclusion: These results suggest that EPO may have an important role in healthy liver after radiation and during hypoxia main regulator of EPO is Hif-2. According to these findings EPO can be considered as a targeted therapy for radiation induced liver injury.

2

Lipocalin-2 is a biomarker for rat fatty liver model triggered by dietary fructose

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Introduction: Fat accumulation in the liver is associated with metabolic syndrome; and through NASH, it could progress to hepatic cirrhosis and carcinoma. Since lipocalin-2 (LCN2) synthesis in the liver is influenced by inflammatory and metabolic processes, we aimed to examine LCN2 as a potential biomarker in two rat models of diet-induced fatty liver.

Methods: Fatty liver was induced in male Sprague Dawley rats fed either with liquid Lieber-DeCarli (LDC) diet or LDC + 70% kcal fructose (L-HFr) for 4 or 8 weeks. Chow-fed animals served as controls. (Immuno)histochemistry for liver tissue was performed to study inflammation, fat degeneration, and LCN2 localization. LCN2 was assessed in liver and serum at mRNA and protein levels using RT-PCR, Western blot, and ELISA. Hepatic mRNA for leptin-receptor (*LEP-R*) and inflammatory mediators were determined. Furthermore, serum fasting levels of triglycerides and leptin were studied by colorimetric assay, and radioimmunoassay, respectively.

Results: Treated rats developed hepatic fat deposit accompanied with mild periportal inflammation, featuring a fatty liver. Contrary to chow or LDC diet, fructose-supplemented regimen revealed significantly up-regulated hepatic LCN2 mRNA confirmed by the enhanced hepatic and serum LCN2 protein ($p < 0.001$). LCN2 expression was detected mainly in ED1+macrophages and granulocytes predominantly in the liver of fructose-ingested animals. Ballooned hepatocytes didn't conserve LCN2 positivity during fixation step. Despite of significant increased mRNA levels of hepatic *MCP1, CCR2, TNF-alpha, NF-κB,* and *STAT3* in L-HFr group; the increase of LCN2 was the highest. Additionally, the magnitude of hepatic *LEP-R* mRNA, and fasting serum leptin and triglycerides was substantially higher in rats fed with L-HFr than LDC or control group.

Conclusion: The present study suggests LCN2 as a biomarker for fructose-induced fatty liver.

miR-125b regulates the Lin-28/IGF-II axis during hepatocellular carcinogenesis

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Background: MicroRNA (miRNA), involved in posttranscriptional regulation of gene expression, play an important role in cell proliferation and differentiation. Previous data have shown that miR-125b expression was shown to be involved in liver carcinogenesis. Here, we focused on the role of miR-125b in development of hepatocellular carcinoma (HCC).

Methodology: A Cre-expressing adenoviral vector was applied to Alb-SV40 T-Ag transgenic mice in order to induce liver carcinogenesis. Expression levels of miR-125b were determined at different time points of murine tumorogenesis and in human hepatoma cell lines. Putative miR-125b binding sites were fused to the luciferase reporter and reporter assays were carried out with miR-125b treated hepatoma cells.

Principal findings: During development of mouse HCC, the expression of miR-125b progressively decreased. In agreement miR-125b was reduced in human hepatoma cells in comparison to normal liver. Overexpression of miR-125b in Hep3B and Pop10 cells resulted in a pronounced reduction of cell growth. Screening of putative miR-125b target transcripts by various algorithm calculations identified various pathways involved in proliferation and apoptosis. Reporter assays of 3'-UTR-regions of the putative targets identified miR-125b binding sites in lin-28 mRNA. Since lin28 is known to effect synthesis of the mitogen IGF-II, the miR-125b/lin28 axis is suggested to be involved in HCC pathogenesis by IGF-II mediated regulation of cell growth.

Conclusions: Expression of miR-125b is down-regulated during progression of hepatocarcinogenesis leading to up-regulation of lin-28 that in turn triggers enhanced cell growth and proliferation.

4

Preservation of transporter function in 3D primary human hepatocyte cultures allows liver toxicity studies

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Introduction: Primary human hepatocytes (hHeps) in 2D culture are the "gold standard" for *in vitro* toxicity studies. Since these conditions do not reflect tissue *in vivo* properties, hepatocytes rapidly lose their biotransformation capacity. To mimic *in vivo* conditions, we developed a 3D culture system maintaining hepatocytes polarity and function.

Methods: hHeps were isolated with two-step collagenase perfusion (according to ethical guidelines) and seeded in collagen I gels in 96-well-plates. As quality criteria for our system we measured cell viability, production of glucose and urea, functionality of hepatocyte specific transporters, namely multidrug resistance-associated protein-1 (MRP1) and permeability glycoprotein (P-gp) transporter, Cytochrome P450 (CYP) expression and performed toxicity assays (Acetaminophen and Diclophenac).

Results: The survival of hHeps embedded into collagen I gels was demonstrated by resazurin conversion and life-dead staining. In addition we detected increased glucose and urea production in 3 D versus 2D, as well as higher transporter activity for MRP1 and P-pg. In line with these findings, hHeps showed intoxication in 3D with Acetaminophen and Diclophenac, while under 2D conditions the effect was less pronounced.

Discussion/Conclusion: Our data reveal an improvement of hHeps biotrans-formation capacity in our system. The observed intoxication of hHeps in 3D is most likely due to the preservation of functional drug transporter, which may explain Acetaminophen and Diclophenac intoxication observed in some patients. In summary our data highlight the specific role of *in vitro* 3D cultivation, reflecting tissue *in vivo* properties and which is suitable for detailed *in vitro* toxicity assays.

Investigations on the oxidative stress response in primary hepatocytes from adolescent and aged rats

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Introduction: Various studies indicate that the accumulation of reactive oxygen species (ROS) and associated cellular damage play an important role in ageing of various organs, including the liver. Especially high concentrations of ROS in the mitochondria lead to oxidative damages, which have been proposed as the major cause of cellular ageing and death. Conceivably, the decrease of cytosolic and/or mitochondrial glutathione (GSH) concentrations as well as altered enzyme activities could be involved in the progressive redox imbalance of the ageing cell. Our approach is to investigate age-dependent cellular responses to oxidative stress using primary hepatocytes from adolescent (6–8 months) and aged (23–25 months) rats.

Methods: Hepatocytes were isolated from livers of male Wistar rats by in situ collagenase perfusion. After seeding on collagen-coated dishes, cells were cultured for up to 48 h and then treated with ethanol. Total cellular GSH and ROS levels were determined fluorimetrically. In order to determine mitochondrial GSH levels, cells were trypsinized and mitochondria-enriched and cytosolic fractions were separated by several centrifugation steps.

Results: Treatment with different ethanol concentrations lead to an initial decrease of total GSH compared to untreated cells in both age groups. However, differences in the detectable amount of ROS could be observed: In hepatocytes of aged rats, an induction of ROS was detected immediately after addition of ethanol. In contrast, ROS levels were initially attenuated in adolescent rat hepatocytes. First measurements of the basal mitochondrial GSH as well indicate a difference between adolescent and aged rats.

Discussion/Conclusion: Our first results reveal that there is an age-difference in the oxidative stress response in primary rat hepatocytes of the two age groups. Whether or not altered expression of enzymes involved in ROS detoxification is responsible for the described age-related differences will be one of the subjects of our future studies.

This study was supported by BMBF 0315891.

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Inhibition of inflammation in NASH by chronic ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE): Development of sodium salt UDCA-LPE for oral use

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Introduction: **U**DCA-LPE is designed for treatment of non-alcoholic steatosis hepatitis (NASH). UDCA-LPE when administered intraperitoneally (i.p.) is cytoprotective in inhibiting liver injury in acute hepatitis and ameliorating steatosis and inflammation in NASH mice. UDCA-LPE is hydrolyzed to UDCA and LPE raising a possibility that orally administered UDCA-LPE may not be effective. We here analyzed hepatic concentrations of UDCA-LPE and UDCA in NASH models, and test whether water-soluble sodium salt UDCA-LPE (Na-UDCA-LPE) could be used orally.

Methods: Ob/Ob mice were i.p. injected with 30 mg/kg UDCA-LPE twice a week for 4 months. C57/BL6 mice fed with high fat diet (HFD) were treated with UDCA-LPE the same way by i.p. for 6 months or by oral gavage for the last 3 months on HFD. Assays for liver injury and inflammation were performed. UDCA-LPE and UDCA levels were analyzed by liquid-chromatography/mass spectrometry (LC/MS). To mimic oral gavage, Na-UDCA-LPE was used to inject directly into the lieum.

Results: UDCA-LPE lowered serum liver enzymes when given i.p. in Ob/Ob and HFD-fed mice, but was not effective when orally administered. However, inflammation and fibrosis (Sirius red and α-SMA staining) was improved by UDCA-LPE in these chronic models. LC/MS analyses of these livers showed an absence of UDCA-LPE, but marked increases of UDCA. UDCA-LPE was detectable in the liver after a single i.p., but not by oral gavage or by-pass to the ileum. Na-UDCA-LPE injected into the ileum delivered UDCA-LPE to the liver with an optimum dose of 100 mg/kg. Pharmacokinetics showed maximal liver and serum UDCA-LPE concentrations respectively at ~ 400 ng/g and ~ 10 μg/ml after 1.5 h.

Conclusions: UDCA-LPE inhibited hepatic inflammatory and fibrosis markers by chronic i.p. or oral gavage, whereby UDCA-LPE was readily hydrolyzed to UDCA. By by-passing the stomach, Na-UDCA-LPE was absorbed to the liver. Formulations of Na-UDCA-LPE are being developed to optimize an oral use of UDCA-LPE.

Targeting death receptors pathway of apoptosis for the treatment of chronic hepatitis and hepatocellular carcinoma

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Introduction: Mice with hepatocyte-specific ablation of Nemo in hepatocytes ($Nemo^{\Delta hepa}$) developed spontaneous hepatitis characterized by increased hepatocyte apoptosis which chronically triggers the development of hepatocellular carcinoma (HCC). However, the molecular mechanisms that trigger spontaneous hepatocyte apoptosis in $Nemo^{\Delta hepa}$ mice remain unknown. Thus, we investigated whether the modulation of death receptor pathways such as TRAIL, TNF-R1, and Fas-R might prevent the progression of cell death-associated chronic liver injury in $Nemo^{\Delta hepa}$ mice, with the view to translate these results into potential therapy of liver disease by inhibiting apoptosis.

Methods: We blocked TRAIL, TNF-R1 and FasR in $Nemo^{\Delta hepa}$ by generating $Nemo^{\Delta hepa}/TRAIL^{-/-}$, $Nemo^{\Delta hepa}/TNF\text{-}R1^{-/-}$ and $Nemo^{\Delta hepa}/Fas\text{-}R^{-/-}$ double knockout mice, respectively, and analyzed the progression of chronic liver inflammation to tumor development.

Results: Ablation of TNF-R1 and Fas-R in $Nemo^{\Delta hepa}$ mice significantly improved serum transaminases and hepatocyte apoptosis in these mice compared to $Nemo^{\Delta hepa}/TRAIL^{-/-}$ and $Nemo^{\Delta hepa}$. Interestingly, reduced apoptotic cell death accompanied with a strong reduction in JNK activation was observed after deletion of TNF-R1. Infiltration of immune cells – monocytes, macrophages and neutrophils was reduced in $Nemo^{\Delta hepa}/TNF\text{-}R1^{-/-}$ and $Nemo^{\Delta hepa}/Fas\text{-}R^{-/-}$. Compensatory proliferation and cell cycle parameters such as Cyclin A, Cyclin D and p21 were significantly less activated in $Nemo^{\Delta hepa}/TNF\text{-}R1^{-/-}$ livers compared with $Nemo^{\Delta hepa}/TRAIL^{-/-}$ and $Nemo^{\Delta hepa}$ animals. Moreover, markers of liver fibrosis such as collagen IA1 and αSMA and indicators of tumor initiation and development – liver *vs* body weight ratio, number of nodules and $CD\text{-}34^{+}$ area- were significantly decreased in absence of TNF-R1 and Fas-R compared to $Nemo^{\Delta hepa}$ and $Nemo^{\Delta hepa}/TRAIL^{-/-}$ mice.

Discussion/Conclusion: Our present data demonstrate that blockage of TNF-R1 and Fas-R but not TRAIL is beneficial in the prevention of chronic liver injury in $Nemo^{\Delta hepa}$ mice. Our results open a new therapeutic opportunity for the treatment of chronic liver disease by modulating death receptor-mediated apoptosis.

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Upregulation of Myc-associated zinc finger protein (MAZ) in the pathogenesis of chronic liver disease

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Introduction: Myc-associated zinc finger protein (MAZ) is a ubiquitously expressed transcription factor that has been shown to be involved in the regulation of various biological processes and diseases, including inflammation and cancer. Further, MAZ was identified as a mediator of PPARgamma, which is known to play a critical role in hepatic fibrosis and the activation of hepatic stellate cells, respectively. Beyond that, MAZ is a regulator of VEGF which plays a main role during hepatic fibrogenesis and inflammation. Only few studies have assessed hepatic MAZ expression and found increased expression in hepatocellular carcinoma compared to non-tumorous hepatic tissue.

The aim of this study was to get deeper insight into the expression of MAZ in chronic liver disease.

Methods and results: Hepatic MAZ expression was significantly induced in mice subjected to bile-duct ligation or fed a NASH inducing diet. Beyond that, quantitative real time PCR revealed increased MAZ expression in patients with cirrhosis due to chronic alcoholic abuse or chronic infection with hepatitis B or C. In contrast, no MAZ induction was detected in a murine model of hepatic steatosis without significant inflammation and fibrosis. According to this, induction of cellular lipid accumulation in primary human hepatocytes (PHHs) *in vitro* did not alter MAZ expression. Besides, PHHs were stimulated with various pro-inflammatory cytokines as well as endotoxin which did not lead to significant changes of MAZ expression. However, MAZ expression was significantly up-regulated in hepatic stellate cells during *in vitro* activation.

Conclusion: Together, these data indicate that activated hepatic stellate cells are the cellular source of increased MAZ expression in chronic liver disease. Further studies have to unravel the functional role of MAZ in (activated) HSCs but the known biological effects of this transcriptional regulator suggest it as an attractive therapeutic target for the treatment of chronic liver disease.

Anti-lipidemic drugs show beneficial influence on lipid metabolism in a human *in vitro* model of steatosis hepatis

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Introduction: Fatty liver diseases have become pivotal in surgical interventions and organ transplantation. For the reduction of the hepatic lipid content, drugs such as Fenofibrate (FEN) are already therapeutically used. Epidemiologic studies suggest that beside these anti-lipidemic drugs natural compounds like Genistein (GEN) have a beneficial influence on energy metabolism and consequently could also be able to improve hepatic lipid levels.
Our aim was to establish a human in vitro steatosis model in order to investigate the effects of anti-lipidemic compounds on hepatic energy metabolism.

Methods: Primary human hepatocytes (PHH) were isolated from human liver resectates using a two-step collagenase perfusion technique. PHH were treated with free fatty acids for 24 h. Fenofibrate (FEN, positive control) and GEN were then applied for 24 h. Lipid accumulation was displayed by Oil-Red-O staining. Lipotoxicity was investigated by measurement of cell viability (AST, LDH and XTT assay). Real time-PCR and Western blot analysis were used for investigation of known targets in insulin resistance and energy metabolism.

Results: Steatotic PHH showed a significant increase in the lipid level accompanied by a slight lipotoxic effect compared to control PHH. Additionally insulin resistance was observed by impaired phosphorylation levels of insulin-responsive downstream targets on transcript and protein level, respectively. Neither GEN nor FEN lowered hepatic lipid content significantly in this setting. However, FEN acted as PPARalpha agonist whereas GEN slightly increased active PPARalpha and additionally decreased active SREBP1c, implying a beneficial impact on hepatic lipid catabolism and an inhibition of lipid anabolism, respectively.

Discussion/Conclusion: In summary, our data show that the human in vitro model of NAFLD is a reliable reproduction of *in vivo* conditions regarding fat accumulation, lipotoxicity and insulin resistance. Testing of anti-lipidemic drugs revealed that FEN and GEN were able to improve pathophysiological changes of steatotic PHH and showed a benefical influence on signaling pathways of the lipid metabolism.

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Dietary induced obesity, glucose intolerance and non-alcoholic steatohepatitis are improved by orally applied xanthohumol

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Introduction: Non-alcoholic fatty liver disease (NAFLD) is considered as hepatic manifestation of the metabolic syndrome and is frequently associated with obesity and diabetes. In a subset of cases hepatic steatosis progresses to steatohepatitis (NASH), which may lead to liver fibrosis and finally to liver cirrhosis and cancer. There is growing interest in phenolic compounds and their presumed role in the prevention of various degenerative diseases. Xanthohumol, the principal prenylated flavonoid of the female inflorescences of the hop plant, has been most extensively studied as cancer chemopreventive agent. In addition, anti-inflammatory properties have been demonstrated in various organs including the liver.
The aim of this study was to investigate the effect of xanthohumol in a dietary murine model of obesity, hyperglycemia and non-alcoholic steatohepatitis.

Methods and results: Feeding a high fat diet (HFD) to male 129S1/SvImJ mice for 24 weeks induced significant body weight gain, an impaired glucose tolerance and elevated fasting blood glucose levels. Supplementation of the HFD with 0.5% (w/w) xanthohumol resulted in only low serum levels of free xanthohumol, whereas concentration in the liver reached markedly higher levels. While xanthohumol supplementation did not affect food consumption it almost completely blunted body weight gain and suppressed the diet-induced rise of leptin serum levels. Furthermore, xanthohumol prevented an increase of blood glucose levels and markedly improved glucose tolerance. Moreover, HFD-induced increase of hepatic triglyceride and serum transaminases levels were significantly lower in mice which received XN in addition to the HFD. Additionally, elevated hepatic expression of pro-inflammatory (TNF, MCP-1) and pro-fibrogenic (TGF-beta, TIMP-1) genes in response to HFD-feeding was significantly blunted by xanthohumol.

Discussion/Conclusion: Xanthohumol prevented nutritionally induced obesity and diabetes as well hepatic steatosis, inflammation and fibrogenesis in mice. Previous studies have shown the safety of even long term application of hop extracts in man, and thus, our data indicate the potential of xanthohumol as a functional nutrient to fight the development of the metabolic syndrome and fatty liver diseases.

miR-198 acts as a tumor suppressor in hepatocellular carcinogenesis by regulating expression of cellular adhesion proteins

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Introduction: Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths, worldwide. MicroRNAs, inhibiting gene expression by targeting various transcripts, are involved in genomic dysregulation during hepatocellular tumorigenesis. In previous studies, microRNA-198 (miR-198) was shown to be significantly downregulated in HCV-positive hepatocellular carcinoma (HCC). Herein, the function of miR-198 in hepatocellular carcinoma cell growth and gene expression was studied.

Methods: First, transcription levels of miR-198 as well as of the liver specific transcription factors HNF1α and HNF4α were determined in different hepatoma cell lines. Putative regulation of HNF1α and HNF4α by miR-198 was investigated by miR-198 transgenic expression and RNA interference experiments. Consecutively, gene expression profiling in response to miR-198 overexpression in the hepatoma cell line Pop10 was performed by Affymetrix microarray hybridisation. After data interpretation by different spotfire based software, real-time PCR and western blotting analysis was used for evaluation of miR-198 affected transcript and protein expression.

Results: Both, transcription factors HNF1α and HNF4α as well as miR-198 were down-regulated in different hepatoma cells. Importantly, we could show that miR-198 expression is induced by these two liver-specific transcription factors. Gene expression profiling after miR-198 overexpression revealed a prominent dysregulation of several signal transduction pathways such as insulin and TGF-β signaling. In particular, bioinformatic analysis and subsequent comprehensive transcript and protein analyses demonstrated that the expression of the adhesion proteins E-cadherin and claudin-1 is highly affected by miR-198 overexpression. This is of particular interest because these proteins, involved in adherence, are decreased during HCC progression. Furthermore, RNA interference silencing and miR-198 overexpression revealed that the miR-198/claudin-1 and E-cadherin axis affects hepatoma cell migration.

Discussion/Conclusion: In conclusion, miR-198 acts as a tumor suppressor by repression of motogenic pathways, diminishing cell growth and migration.

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Prediction of clinical outcome in chronic hepatitis due to HCV genotype 4 after combination therapy with pegylated interferon α-2a and ribavirin

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Introduction: Combined therapy using interferon alfa (IFN) and ribavirin (RIB) represents the standard treatment in patients with chronic hepatitis C. However, the percentage of responders to this regimen is still low, while its cost and side effects are elevated. Therefore, the possibility to predict patient's response to the above treatment is of paramount importance. **Aim of this work** is to estimate the clinical and prognostic role of TNF-αR (P55), IL-1ra and TNF receptors related to inflammatory cytokines and, GH and insulin hormones metabolized in the liver in HCV infection, cirrhotic and non-cirrhotic. Also to find their significance as noninvasive biochemical markers that may correlate with HCV infection on predicting the outcome of interferon α-2a therapy in patients with chronic HCV infection.

Methods: 54 patients infected by HCV genotype 4 were enrolled in this study. They were classified into two groups according to the liver histology. Group A of 42 chronic compensated HCV patients with no cirrhosis, Group B of 12 chronic HCV patients with established cirrhosis and 12 healthy controls. Patients were treated by Pegylated IFN α-2a (180 µg for group A and 130 µg for group B) once weekly and 1200 mg ribavirin/day in two doses. Tested parameters have been done by ELISA method before and after treatment for group A, group B and control group.

Results: There was a significant increase of serum insulin ($p < 0.01$) of group A after treatment compared to group A before treatment, group B and control group. On the other hand, serum TNF-R (P55) showed significant decrease ($p < 0.05$) in group A after treatment compared to group A before treatment, group B and control group. TNF-αR (P55) showed positive correlation with sALT and sAST. Also, serum GH level decreased in group A after treatment compared to the other studied groups; but, this decrease was not statistically significant.

Discussion/Conclusion: Pegylated IFN α-2a and ribavirin are effective combination in treatment of chronic HCV genotype 4. Insulin and TNF-αR (P55) correlate with HCV infection and could be used as a marker of peg-INF α-2a and ribavirin response while IL-1ra and GH are of no value.

The effects of Smad7 in progression of hepatocellular carcinoma

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Introduction: Hepatocellular carcinoma (HCC) is the most frequent primary liver tumor. The transforming growth factor-β (TGF-β) signaling pathway plays a critical role in cancer progression. Smad7, a TGF-β target gene, is a physiological feedback inhibitor of TGF-β signaling pathway and has been reported to act a tumorigenic mediator in several tumors. However, the role of Smad7 in HCC is unknown so far. This study investigated the potential effects of Smad7 in HCC.

Methods: Expression of Smad7 was analyzed in 146 HCC patients by real time-RT PCR and immunohistochemistry to correlate expression patterns with clinical characteristics. Functional studies on Smad7 related to cell migration, invasion and colony formation were conducted in two HCC cell lines, Huh7 and FLC-4.

Results: Q-PCR analysis revealed that 65.8% patients showed overexpression of Smad7 (> 1.2 folds), 17.8% unchanged (0.8–1.2 folds) and 16.4% down regulated (< 0.8 fold). In vitro, transwell assay showed that both Huh7 and FLC-4 cells migration were inhibited by adenovirus Smad7 overexpression.

Discussion/Conclusion: The results suggest that Smad7 might play a role in the development of HCC. Detailed molecular mechanisms of Smad7 in HCC need further investigated, particularly in animal models.

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The efficacy of terlipressin vs. octreotide in the treatment of variceal bleeding

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Introduction: Standard therapy for variceal bleeding includes early using of vasoactive drugs. In our department we are using equally terlipressin and octreotide for all patients admitted with variceal bleeding. The aim of this study was to compare the efficacy of this two agents as therapy for controlling acute variceal bleeding and also to asses the effects of standard doses of both drugs on mortality.

Methods: We conducted a two years retrospective case-control study on a group of 279 cirrhotic patients hospitalized in our department with variceal bleeding. We excluded patients who received other hemostatic therapies (banding, sclerotherapy or Blackmore balloon) and patients with malignancies. Patients received either treatment with terlipressin 4 mg/24 h or octreotide 50 µg/h for 5 days. We assed the efficacy of this two drugs on controlling variceal bleeding, prevention rebleeding, and in-hospital mortality and 6 weeks and 1 year mortality.

Results: Terlipressin group included 123 patients and octreotide group 156 patients. Both groups were comparable for age, gender, etiology of cirrhosis, hemoglobin level at presentation, Child-Pugh class, MELD score and hemodynamic parameters. Control of bleeding was 65.85% in terlipressin group and 63.46% in octreotide group ($p = 0.261$). Rebleeding occurred in 9.75% patients in terlipressin group and 9.61% patients in octreotide group ($p = 0.366$). In hospital mortality was similar in both groups 14.63% terlipressin vs. 14.10% sandostatin, ($p = 0.512$). Kaplan-Meier curve identified no significant difference in mortality between this two drugs in 6 weeks, and 1 year. Hospital stay (days) was the same in the two groups. No side effects requiring intervention occurred in either group.

Discussion/Conclusion: The efficacy of terlipressin was not inferior to octreotide for the control of variceal bleedindg and the mortality was similar in both groups.

Identification of gene clusters linked to hepatic fibrogenesis

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Introduction: Quantitative trait loci (QTLs) analysis allows the identification of genetic loci linked to fibrosis progression. Furthermore, interacting genes and gene networks can be modelled. Recently we identified QTLs on chromosomes (chr) 4, 5, 7, 12, and 17 in a genetic reference panel of CCl_4 treated BXD recombinant inbred lines linked to hepatic fibrosis. Here we provide an experimental outline to dissect these loci and identify risk genes underlying fibrosis progression.

Methods: Hepatic expression profiles from 30 BXD lines were generated after fibrosis induction by CCl_4 (12 injections IP; 0.7 mg/kg) using Affymetrix Mouse Gene 1.0 ST arrays (> 34,000 probes). First, we mapped expression levels of genes located in pQTL regions to identify regulatory loci of gene expression (eQTLs) during fibrogenesis. We determined locally (*cis*)-regulated quantitative trait genes (*cis*QTG) within a maximum distance of 10 Mb to the regulatory locus. Then three selection criteria were chosen to select candidate genes: **A)** correlation with fibrosis phenotype data; **B)** comparison of gene regulation in different experimental groups (CCl_4 vs. NaCl); **C)** non-synonymous single nucleotide polymorphisms (nsSNPs).

Results: eQTL mapping revealed 61 *cis*QTG in pQTL regions. Overall, 19 *cis*QTGs correlated significantly ($p < 0.05$) to fibrosis phenotypes, 21 *cis*QTGs contained nsSNPs and 35 *cis*QTGs were differentially regulated in healthy animals. In summary, we determined 41 *cis*QTGs that fulfilled at least one of the criteria **A-C**. We identified five gene clusters located on chr 4, 5, 7, and 12, which showed similarities in gene regulation during fibrogenesis.

Discussion/Conclusion: QTL mapping of expression and phenotype data within the BXD panel allowed us to identify novel regulatory networks. These networks include known fibrogenic molecules *(Cxcl10*, *Nr1h2*, *Tnc*), which supports our selection criteria. Our experimental set-up further provides a basic experimental framework for the discovery of gene networks that drive complex liver diseases and might guide specific therapeutic interventions.

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Peroxisome proliferator-activated receptor-gamma inhibits mouse liver regeneration after partial hepatectomy via HGF/c-met/ERK1/2 pathway

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Introduction: Peroxisome proliferator-activated receptor-gamma (PPARγ) is a nuclear receptor demonstrated to play an important role in various biological processes. However, the role of PPARγ on liver regeneration remains unclear and controversial. Aim of this study was to determine the effect of PPARγ on liver regeneration upon partial hepatectomy in mice and to elucidate the underlying molecular mechanisms.

Methods: Eight- to ten-week-old female C57BL/6J mice were subjected to 2/3 partial hepatectomy by removal of the left and median lobe of the liver. Before surgery, mice were either treated with the PPARγ agonist rosiglitazone, the PPARγ antagonist GW9662 alone or in combination with the c-met inhibitor SGX523. Serum and tissue specimens were harvested and parameters, such as liver-to-body-weight ratio, serum transaminase, alkaline phosphatase, glucose, cholesterol, triglyceride levels, were assessed. To elucidate liver regeneration and hepatocyte proliferation, immunohistochemical analysis of PPARγ, Ki67 and PH3 was performed. Components of PPARγ specific signaling pathway during liver regeneration were identified by western blot (PPARγ, STAT3, CyclinD1, HGF, c-met, and ERK1/2) or RT-PCR (Tnfα and Il6).

Results: Our results indicate that liver regeneration after 2/3 partial hepatectomy is being inhibited by rosiglitazone and accelerated by PPARγ antagonist GW9662. Inhibition of c-met abrogates GW9662 induced liver regneration and hepatocyte proliferation. In addition, STAT3 was found to be phosphorylated shortly after partial hepatectomy while hepatic cyclin D1 expression was delayed in rosiglitazone-treated mice. Similarly, HGF, phosphorylated c-met and phosphorylated ERK1/2 protein levels were significantly down-regulated after rosiglitazone treatment with oppositional tendency after GW9662 treatment.

Discussion/Conclusion: These data support the concept that PPARγ does inhibit liver growth and hepatocellular proliferation by inhibition of the HGF/c-met/ERK1/2 pathway. This pathway may represent a potential target during liver regeneration in response to liver disease and these findings could thus impact on the future development of molecular therapies in patients with liver disease.

Non-invasive quantification of hepatic fibrosis in mouse models by magnetic resonance relaxometry (MRR)

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Introduction: To date studies in mouse models of hepatic fibrogenesis have been hampered by the need for tissue specimens to quantify liver fibrosis. Hence, the development of non-invasive methods of quantifying hepatic scarring is crucial to monitor potential antifibrotic therapies. Our **aim** was to develop non-invasive magnetic resonance-based quantification of liver fibrosis in mice with different aetiologies of fibrogenesis.

Methods: We analyzed 13 BALB/cJ mice after toxic induced liver fibrosis (1.4 mg CCl_4 /kg/wk i.p. for 6 weeks), 23 *Abcb4* knockout (*Abcb4*$^{-/-}$) mice with biliary fibrosis and 27 controls. To assess hepatic fibrosis, we ascertained relative collagen areas after Sirius red staining and collagen contents by hydroxyproline. Magnetic resonance relaxometry (MRR) was performed on a 9.4 Tesla animal scanner (Bruker Biospec 9.4/200) with a circular polarized receive/transmit coil. The relaxation times T1, T2 and T2* and signal intensities were acquired with turbo spin echo and multiple gradient echo techniques.

Results: Compared to untreated wild-type mice, animals treated with CCl_4 and *Abcb4*$^{-/-}$ mice display significantly ($p < 0.01$) enhanced hepatic collagen contents. CCl_4-challenged mice show increased T2* (7.0 range 5.0–11.4 vs. 8.0 range 7.1–8.7 ms; $p < 0.05$) relaxation times as compared to controls. Furthermore, T2* times correlate ($p < 0.05$) with relative collagen areas in CCl_4-treated animals. T1 relaxation times do not differ between CCl_4-treated and controls. In contrast, *Abcb4*$^{-/-}$ mice display enhanced T1 (923.8 range 717.9–1248.8 vs. 1073.7 range 681.4–1507.8 ms; $p<0.001$) but no differences in T2 and T2* relaxation times compared to controls.

Discussion/Conclusion: Our study demonstrates the feasibility of MRR as a non-invasive method to discriminate between fibrotic and non-fibrotic liver tissue in mice independent of the aetiologies of hepatic injury. Additional studies are currently being performed to develop algorithms allowing refined differentiation of fibrosis stages and assessment of fibrosis resolution to provide a tool for the monitoring of antifibrotic therapies *in vivo*.

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Divergent TGF-beta regulation of the putative therapeutic target microRNA-29 in liver stellate and hepatoma cells

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Introduction: The miR-29 family members, targeting genes involved in apoptosis and gene methylation, are downregulated during hepatocarcinogenesis. In addition to their tumorsuppressor function in hepatocellular carcinoma (HCC), they act in stellate cells (HSC) as antifibrotic mediators. Herein, we studied the promoter regulation of miR-29 in hepatic stellate and hepatoma cells.

Methods: pri-miRNA29a/b1 expression was determined by Real-Time PCR in hepatoma Huh7 cells after TGF-beta or phorbolester stimulation. The miR-29a/b1 promoter was analyzed by rVista-2.0 bioinformatics. For investigation of transcription factor binding, nuclear extracts from Huh7 and HSC were prepared and electrophoresis mobility shift assays (EMSA) were carried out. Transcriptional miR-29a/b promoter regulation was studied by reporter assays. Putative binding sites were analyzed after comprehensive site-specific mutation and recombinant expression of the mutated promoter constructs.

Results: Quantification of cellular levels of primary transcripts of the miR-29a/b1 gene in addition to the mature processed forms of the miR-29a and miR-29b revealed a pronounced upregulation in TGF-beta or phorbolester stimulated Huh7, but a downregulation in HSC. Promoter analyses identified putative Ap-1 binding sites in the upstream regulatory domain of the miR-29 promoter. An Ap-1 binding site close to the TATA-box was highly conserved between species. Reporter assays and EMSA pointed out that TGF-beta and phorbolester controlled pri-miR-29 expression through Ap-1 activation in hepatoma cells but not in stellate cells. Reporter assays with numerous site-specific mutation constructs, as well as binding studies and supershift analyses using pan-fos antibodies confirmed the central function of the transcription factor Ap-1 in miR-29 regulation in liver cancer cells. In contrast, in HSC miR-29 repression by TGF-beta stimulation led to smad promoter binding.

Discussion/Conclusion: miR-29a/b gene is controlled by a complex signaling network, in which the transcription factor Ap1 acts as a central transcriptional inducer in hepatoma cells, but Smad as a main repressing factor of miR-29 a/b1 expression in stellate cells.

A human model for studying the relationship between Kupffer cell activation and hepatic tissue damage

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Introduction: Hepatic tissue damage can occur due to surgical interventions, infections or drug induced liver injury (DILI). Kupffer cells (KC) play not only an important role in initiating tissue damage but also in regenerative processes. KC activation consequently lead to a release of different cytokine patterns that support inflammation or induce tolerance reactions.
The present study aimed to establish a human *in vitro* model which enables the detection of cell-cell communication between KCs activated by damaged or stressed hepatocytes.

Methods: Primary human hepatocytes (PHH) and KCs were isolated from human liver resectates using a two-step collagenase perfusion technique. The isolated KCs were characterized by CD68 immunofluorescent staining and the initial intracellular reactive oxygen intermediates (ROI) level using the DCF-assay. KC viability was controlled by the XTT-assay. Direct KC activation was investigated by LPS treatment using as positive control. To simulate the activation of KC following hepatocyte damage, KCs were incubated with supernatants of PHH previously incubated with drugs, respectively.

Results: The isolated KC yield was $1.1 \pm 0.8 \times 10^6$ cells/g liver tissue with a purity of > 80% determined by counting CD68 positive cells. In general, most of the KC isolations showed high initial ROI levels, which could be in some cases related to the different donor livers. The stimulation of KCs with LPS leads to a concentration-dependent increase in ROI formation. The incubation of KCs with supernatants from drug-treated PHH increased mitochondrial activity measured by the XXT-assay and the formation of ROIs compared to untreated KCs.

Discussion/Conclusion: In summary, most of the isolated KCs showed high basal ROI levels, which are most likely linked to the isolation process and to the quality of the donor liver tissue. However, LPS and drug-damaged hepatocytes led to an activation of KCs. The KC activation and reaction depends strongly on the donor liver tissue and the patients' original disease state.

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Increased lipid metabolisms in hepatocellular carcinoma cells

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Introduction: Tumor cells gain a survival/growth advantage by adapting their metabolism to respond to environmental stress, a process known as metabolic transformation. Increased glucose and lipid uptake as well as de novo lipogenesis are believed to be involved in oncogenesis.

The aim of this study was to investigated the expression of regulators of hepatic lipid metabolisms in hepatocellular carcinoma (HCC).

Methods and results: Quantitative RT-PCR analysis revealed a significantly increased expression of acetyl-CoA carboxylase in 4 human HCC cell lines (Hep3B, Huh7, HepG2, PLC) compared to primary human hepatocytes (PHH). Also expression of fatty acid synthase (FASN) and stearoyl-coenzyme A desaturase 1 (SCD1), which are key regulators of *de novo* lipogenesis, as well as diacylglycerol acyltransferase (DGAT), the last enzyme of triglyceride assembly, was markedly increased in HCC cells compared to PHH. Further, increased expression of acyl-Coenzyme A oxidase 1 (ACOX1) indicated enhanced peroxisomal oxidation of free fatty acids, while lipoprotein lipase (LPL) expression was down regulated in HCC cells compared to PHH.

Summary and Conclusion: HCC cells reveal marked alterations of lipid metabolisms, indicative of increased *de novo* lipogenesis (from glucose), and lipid utilization and storage. Targeted inhibition of key factors of lipid metabolisms appears as promising for HCC prevention and treatment.

Common *NPC1L1* variants in gallstone diseases: Combined analysis of genetic risk and cholesterol homeostasis

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Introduction: Individuals with gallstone disease (GSD) secret increased amounts of cholesterol into bile[1]. Since NPC1L1 mediates sterol uptake in hepatocytes and enterocytes[2], we now investigate common *NPC1L1* polymorphisms – known determinants of serum cholesterol[3] – for their association with GSD.

Methods: Serum surrogate markers of cholesterol synthesis (lathosterol and desmosterol) and transport (sitosterol and campesterol) were measured by GC-MS in a German cohort of 99 patients with GSD (42 males, age 39–84 years) and 127 controls (56 males, age 31–89 years). Three *NPC1L1* polymorphisms (rs17655652, rs2072183, rs41279633)[2] were genotyped in Germans as well as in a Romanian cohort of 235 sibs with GSD (30 males, age 24–80 years) and 260 controls (19 males, age 21–78 years).

Results: We detected a potential association between GSD and the *NPC1l1* variant rs41279633. German individuals with genotype [GG] were at increased GSD risk as compared to [TT] carriers (OR = 3.7, 95% CI: 1.0–14.1, p = 0.042). Moreover, we identified a departure from Hardy-Weinberg equilibrium in German cases ($p < 0.01$) and a trend for an association in the Romanian cohort (common OR = 1.4, p = 0.06). However, serum markers of cholesterol homeostasis were not affected by genotypes, and the non-parametric linkage analysis sib-pairs did not provide evidence for linkage between *NPC1L1* and GSD.

Discussion/Conclusion: Our results point to a potential association between the *NPC1L1* variant and GSD. Apparently, the increased gallstone risk is not reflected by distorted systemic cholesterol homeostasis. Further studies are warranted to investigate the intestinal and/or hepatobiliary effects of additional rare *NPC1L1* variants on GSD and to develop novel tailored therapies for patients at increased GSD risk.

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A target/ligand-based study of secondary plant ingredients as serine/threonine kinase inhibitors in Wnt/beta-catenin signaling

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Introduction: Casein kinase-2 represents an important kinase in differentiation, proliferation and cell transformation processes. This kinase is part of the Wnt/beta-catenin pathway and plays a critical role in gastrointestinal tumorigenesis.

Methods: For the target/ligand predictions, the *in silico* studies were performed with the genetic algorithm of AutoDock 3.05. Thereby, the Wnt targets (CK-1/2, GSK-3beta, CDK2/cyclinA) were modeled on the basis of their crystallographic structures and were optimized with MOE 2005.06. The flavonoids/anthranoids were modeled with Spartan 5.0. The relevance of the hydroxylation grade and deposits toward CK-2 were subsequently verified by an *in vitro* phosphorylation assay.

Results: The natural flavonoids/anthranoids offered potent CK-2 inhibitors with *in vitro* IC_{50}-values of 0.5 to 20 µM. A two-sided and even better a triangular stabilization via hydrogen bonds facilitated inhibition in the lower micro-molar to upper nano-molar range (e.g. apigenin 500 nM and emodin 300 nM). A more side by side hydroxylated core tended, in the majority of cases, to be obstacle due to the presence of intra-molecular forces. An exception was the fully hydroxylated myricetin (700 nM). Here the hydrogen bonds of the internal hydroxyl of ring-B alternated between the vicinal hydroxyls. As a result, one hydroxyl was always free for inter-molecular interactions. Therefore, the inhibitory capability depended not only on the number, but also on the position of the existing hydroxyls. Beyond the increasing number of polar interactions decreased here the *in silico* selectivity toward CK-1 and GSK-3beta. This was understandable due to the more hydrophophilic ATP-competitive cavities.

Discussion/Conclusion: Comparing the potency and selectivity of flavonoids with natural anthranoids, we can conclude that the potency as well as the selectivity and further issues, like a second binding-site of CK-1 and CK-2 are much similar. The elucidation of molecular details of specific target/ligand interactions discloses further possibilities for improving target-orientated signalling pathway interference.

Cellular phenotyping of hepatocytes in a genetic reference population identifies novel candidate genes and potential therapeutic targets in fibrogenesis

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Introduction: The aim of our study was to identify risk factors and fibrogenic drivers by quantitative trait locus (QTL) mapping of cellular susceptibility *in vitro* and *in vivo*. We assessed hepatocellular susceptibility to pro-fibrogenic TGF-beta signalling in cultured primary hepatocytes from inbred mouse strains C57BL/6J and DBA/2J, which are the parental strains of the genetic reference population BXD and differ in fibrosis susceptibility (Hillebrandt et al. Nat Genet. 2005), and in 20 BXD lines.
Resulting QTL loci were analyzed for their impact on gene expression following short-term liver damage *in vivo*.

Methods: Hepatocytes of BXD lines (at least 6 animals per line) were isolated, cultivated in serum free medium and stimulated with 5 ng/ml recombinant TGF-beta for 48 hours.
Cellular damage was quantified by measuring the release of lactate dehydrogenase into the tissue culture supernatant as percentage of total LDH. Differences between untreated and treated cells were used as trait in genome wide linkage analysis.
Resulting QTL areas were scrutinised for genomic loci (SNPs) that were associated with gene expression in response to 24 hours of ethanol damage or 6 weeks of carbon tetrachloride (CCl_4) injections.

Results: QTL mapping identifies chromosome (Chr) 11q 83–89 Mb as a modifier of cellular susceptibility in BXD mice. Gene expression analysis in BXD livers *in vivo* in response to short-term ethanol damage or CCl_4 injections reveals five single nucleotide variants within the peak area that have a regulatory impact on other genes during acute liver damage. Amino acid exchanges near expression-associated polymorphisms in the QTL area indicate candidacy of *Expi* and *Msi2*. *Expi* is adjacent to a SNP showing regulatory activity following ethanol injury; its sequence reveals functional homology with "WAP-motif" genes involved in inflammation and innate immune response; *Msi2* is a haematopoietic stem cell marker.

Discussion/Conclusion: Cellular phenotyping of hepatocyte susceptibility to a pro-fibrogenic cytokine followed by QTL analysis of the resulting damage indicates the involvement of *Expi*, a short “alarm peptide” involved in protease inhibition and/or the innate immune response, in liver fibrogenesis. Being a secreted, soluble signalling molecule, *Expi* is highly amenable to pharmacological intervention.

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Y-box protein-1 is a potential regulator of liver progenitor cells expansion

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Introduction: Chronic liver diseases may progress to liver cirrhosis, thus concomitantly increase the risk of hepatocellular carcinoma (HCC). Liver cirrhosis has three pathological characteristics, including fibrosis, regenerating nodules and remodeling vessel system. Given a critical role of liver progenitor cells (LPC) in liver regenerating nodules, weinvestigate the role of the multifunctional Y-box protein-1 (YB-1) in LPCs.

Methods: 69 patients were enrolled in the study, including 44 chronically HBV-infected (33 non-cirrhotic, 11 cirrhotic), 11 hepatocellular carcinoma (HCC) patients and 5 healthy controls. YB-1 expression was detected by immunohistochemistry (IHC). Cellular localization of YB-1 was determined by immunofluorescence staining. The protein expression of YB-1 in BMOL cells, a murine liver progenitor cell (LPC) line, was examined by immunoblotting analysis.

Results: By IHC, pronounced YB-1 positive staining showed in patients with chronic HBV-infection and HCC. Confocal microscopy analysis revealed that cellular localization of YB-1 protein demonstrated in hepatocytes, cholangiocytes and LPCs, but rarely in activated hepatic stellate cells. Semi-quantitative IHC assessment showed significantly elevated YB-1 IHC scorein cirrhotic patients when compared to those without cirrhosis ($p < 0.05$). No correlation between inflammation and YB-1 IHC score was displayed in chronically HBV-infected patients. Furthermore, an inverse correlation between YB-1 expression and Snail/S100A4, two mesenchymalcell markers, was found in sequential tissue slides of cirrhotic patients, indicating a potential effect of this transcript factor in epithelial to mesenchymaltransition (EMT). *In vitro*, TGF-β incubation induced EMT-like change of BMOL cells. During this course, YB-1 protein expression was down-regulated, suggesting a potential effect of YB-1 in TGF-β mediated EMT.

Discussion/Conclusion: The current results suggest that YB-1 might be an important mediator in liver regeneration.

Novel in vitro models of alcohol mediated hepatic fibrosis and liver injury

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Introduction: Only a fraction of drinkers develops significant hepatic inflammation, and even less progresses to significant hepatic fibrosis. There is a strong evidence for combined effects of alcohol and non-alcoholic fatty liver disease on the progression of hepatic fibrosis and liver injury. Still, the mechanisms of mutual interaction for these combined pathophysiological effects are not well understood.

The aim of this study was to establish *in vitro* models to study direct effects of alcohol on hepatocytes and indirect hepatocyte-mediated effects on hepatic stellate cells (HSC), the central mediators of liver fibrogenesis.

Methods and results: Primary human hepatocytes (PHH) were incubated with clinically relevant alcohol concentrations (0.1–2%) for 48 h, which did not affect cellular viability or mitochondrial activity but led to dose-dependent hepatocellular lipid accumulation as assessed by analysis of triglyceride content and free fatty acid (FFA) levels. Subsequently, human HSC were incubated with conditioned media (CM) from alcohol stimulated hepatocytes, which significantly induced proliferation, and pro-inflammatory (IL-8, RANTES) and pro-fibrogenic (TGF-beta) gene expression compared to HSC incubated with CM from control PHHs. At higher concentrations (> 5%) alcohol in dose-dependent manner induced cytotoxicity and apoptosis in PHHs. However, in PHHs in which hepatocellular steatosis had been induced by pre-incubation with FFAs, toxic alcohol effects occurred already at lower concentrations, which was also associated with higher ROS production and pro-inflammatory and pro-fibrogenic gene expression.

Conclusion: Our *in vitro* data indicate the release of soluble factors as a potential mechanism for development and progression of fibrogenesis in alcoholic liver disease. The identification of these factors may lead to novel prognostic markers. Furthermore, we developed a novel model to study the interactive effects of alcoholic and non-alcoholic fatty liver injury, which may be used to identify and test novel therapeutic targets for treatment of liver injury.

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Change in hepatic chemokines gene expression during acute-phase response

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CXC- and CC-chemokines are known to attract inflammatory cells and play an important role in transmigration process during inflammation. Liver cells are able to synthesize and secrete several chemokines. The aim of this study was to investigate hepatic gene expression of CXC-, CC-chemokines in a model of aseptic abscesses and on isolated hepatocytes. Acute-phase rat and mice (wild type and IL-6-ko) model of local inflammation was generated by injecting intramuscular turpentine oil (TO) in each hind limb. Animals were sacrificed at different time points after TO-injection. Blood and livers were taken and stored. Hepatocytes were isolated from normal rat liver and treated with acute-phase cytokines. Liver tissue was used for immunohistochemistry, western blot and RNA isolation. RNA from liver tissue and isolated hepatocytes was studied by PCR. By means of immunohistology, no increase in MPO^+ and $ED1^+$ cells was observed in the liver after TO-injection. However, parallel to serum increase of CXCL1, CXCL2 and CXCL8, a fast (2 h) time dependent upregulation in the gene expression of CXCL1, CXCL2, CXCL5 and CXCL8 was observed in the rat liver after TO-treatment. The induction in gene expression of CXCL1 and CXCL8 was the fastest and strongest among all other studied chemokines which also remained upregulated until 48 h. In contrast, gene expression of CXCL10 and CCL2 showed mild downregulation in the liver during APR. Similar pattern of changes was detected in the liver of wild type mice. The liver of IL-6-ko mice however showed no significant change of chemokines gene expression. Treatment of rat hepatocytes with cytokines increased the expression of all chemokines. In addition, IL-1β upregulated the gene expression of CXCL10. These results indicate that CXC-chemokines (with exception of CXCL10 in this model) behave as positive acute-phase proteins and increase in their gene expression in TO-induced APR is IL-6 dependent.

MRP and P-gp transporter are preserved in a continuous 3D flow culture of primary human hepatocytes

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Introduction: *In vitro* toxicity assays are performed with primary human hepatocytes (PHH) cultured under 2D conditions in contrast to the *in vivo* situation where PHH are in 3D and surrounded by a continuous circulatory system. Conventional 3D flow cultures like bioreactors require a large number of cells, thereby limiting their application in high-throuput toxicity screenings. The main objective of this work was to down-scale the 3D culture system and to include a continuous flow to maintain the cells over long times in culture close to their *in vivo* situation.

Methods: PHH were isolated by a two-step collagenase perfusion technique from liver sections according to the ethical guidelines and seeded in collagen I gels in µ-slide $VI^{0.4}$. Flow rates were monitored by light microscope. To validate our cell culture model we compared cell viability, function of specific hepatic transporter such as multidrug resistance-associated protein 1 (MRP1) and permeability glycoprotein (P-gp) transporter with static 2D and 3D culture systems.

Results: By testing different flow speeds, we identified the optimum flow rate enabling consistent medium exchange and applying low shear stress onto the PHH. Resazurin conversion and live-dead staining demonstrated that the viability of the PHH was not affected by their embedment into collagen I gel. Furthermore, the flow cultivation preserved cell viability over the culture time. In line with these improved culture conditions, we observed a higher transporter activity for MRP1 and P-gp than in 2D static cultures.

Discussion/Conclusion: Our data clearly show an improvement of PHH viability with an increase in transporter activity *in vitro*, indicating that our flow culture system is reflecting the *in vivo* tissue properties. The observed increase in transport activity in the flow cultures will be crucial in the future for hepatotoxicity studies.

The research was supported by SET Foundation.

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Nuclear localization of iron transport proteins in rat liver and spleen indicates nuclear iron sequestration during acute-phase response

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Liver is the major organ for iron metabolism. During acute phase response, the hepatic iron concentration increases together with an increase in hepatic expression of iron import proteins. However, less is known about the cellular localization of iron transport proteins. Current study aimed to compare cellular localization of iron-transport-protein ferroportin-1- (Fpn-1) and of other iron import proteins in rat liver after experimental tissue damage induced by injecting turpentine-oil- (TO) in both hind-limbs.

Immunohistochemistry of DMT-1 and TfR2 (iron import proteins) were mainly detected in the nucleus of control and TO-injected rat's liver while TfR1 (iron import protein) was clearly localized in plasma membrane. Fpn-1 (the main iron export protein) was mostly found in the nuclei of liver cells. Western-blot analysis of liver nuclear and cytoplasmic fractions confirmed immunohistochemical results. Compared to controls, increased hepatic cytoplasmic and nuclear iron content was observed in TO-injected animals.

Our results demonstrate the differential cellular localization of iron transport proteins in rat liver during acute phase response. The nuclear localization of iron transport proteins may suggest transient storage of iron within the nuclei which help tissue reduce stress and/or might be used to satisfy the high metabolic load known for the acute phase response.

Why targeting vascular endothelial growth factor is not sufficiently effective?

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Introduction: Existing database shows that inhibition of vascular endothelial growth factor (VEGF) signaling may affect tumor growth through several mechanisms. However, clinical studies involving patients with hepatocellular carcinoma (HCC) present various and often disappointing results. We hypothesized that VEGF inhibiting may not be equally effective due to different HHC types.

Methods: Human HCC cells lines Hep 3B, Hep G2, and Sk-hep-1 were cultivated in modified media seeded onto well plates. VEGF-targeting drug Sorafenib 0.05 mg/ml added in study group cultures. General cells count and nuclei morphology were visualized with the TUNEL-staining protocol and cells viewed with a fluorescence microscope (magn. x400). The number of apoptotic cells calculated in percentage of total nuclei. Apoptosis related cytokines were analyzed by Western blotting.

Results: Sorafenib related changes become evident in Hep G2/Hep 3B cell lines after 48 hours of treatment leading to a significant time-dependent reduction of cell numbers of 67.9–83.2% ($p < 0.01$). Cells became sparse, rounded, and detached from the dishes representing morphologic signs of apoptosis. This correlated with activation of caspase-9, caspase-3, and caspase-6. However, Sk-hep-1 cell culture responded much worse with only 36.7–43.7% reduction during same time interval.

Discussion/Conclusion: VEGF-targeted therapy may act through parallel mechanisms that have more or less important role depending on tumor type. In certain malignancies VEGF-targeted therapy has significant activity, whereas in other has no clinical benefit. Our study gives explanation to the fact of variations in clinical response rate of VEGF-targeted therapy. Different subtypes of HCC have different sensitivity to VEGF-targeted therapy.

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Plasminogen K1-5 targets cell adhesion molecules resulting in abolished intrahepatic metastasis

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Introduction: Plasminogen K1-5 (PlgK1-5) is well known as an angiostatic factor, targeting endothelial cells and tumor angiogenesis. Recent studies indicate that the effectiveness of PlgK1-5 can also be expanded to tumor cells themselves but the molecular events have not yet been clarified. To further analyze the effects of PlgK1-5, we investigated effects on cell adhesion and metastasis.

Materials and methods: *In vitro*, Hepa129 and SVEC4-10 endothelial cells were treated with AdPlgK1-5 and AdLacZ as control and analyzed for altered cell adhesion. Expression of ICAM, VCAM and PECAM were determined using LightCycler and ELISA technique.
Hepa129 hepatomas were induced in healthy and fibrotic C3H mice. Five days after tumor cell implantation, animals were treated with adenoviruses coding for PlgK1-5 or LacZ as control. 10 days later, animals were sacrificed and tumors explanted. Tumor growth was determined.

Results: Analysis of antitumoral effects *in vitro* showed that PlgK1-5 inhibited Hepa129 cell adhesion to SVEC4-10 endothelial cells by 52% compared to the controls. In correspondence, ICAM and VCAM expression was reduced by 39% and 26% in PlgK1-5 treated Hepa129. In SVEC4-10, PlgK1-5 reduced VCAM and PECAM expression by 31% and 45% compared to the controls.
Intrahepatic tumor growth and metastasis was completely abolished (-98%) in healthy and fibrotic mice after treatment with AdPlgK1-5 compared to the controls. Analysis of gene expression showed that ICAM, VCAM and PECAM were reduced in tumor bearing livers by 21–48% compared to the controls.

Discussion: PlgK1-5 not only affects tumor angiogenesis but also intrahepatic metastasis by reducing cell adhesion of tumor cells to the endothelium. Interestingly, effects were stronger in fibrotic livers, without any toxic side effects caused by adenovirus application. This makes PlgK1-5 a promising tool not only for primary tumors, but also for the inhibition of metastatic spread in the (fibrotic) liver.

Progression of chronic liver diseases in children

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Introduction: Fibrosis in chronic diffuse liver diseases leads to serious consequences. The speed and laws of fibrogenesis in chronic diffuse liver diseases in children are not described enough in literature. The aim of research is to define the speed and directions of fibrogenesis in children with chronic diffuse liver diseases.

Methods: We conducted a prospective observational research; 342 patients with chronic liver diseases participated in it. The age of children is between 1 month and 18 years. The diagnosis was made on the base of clinical-biochemical data, serologic markers of viral hepatitis, determination of nucleic acids, morphologic study. We've analyzed the results of pair biopsies and the level of hyaluronic acid. Statistical processing of results is done with the help of “STATISTICA6” program.

Results: We determined the speed of progression of liver fibrosis in children: chronic hepatitis C – 0.15 ± 0.17; chronic hepatitis B – 0.31 ± 0.25; Wilson disease – 0.54 ± 0.32 from the age of 7 years; α1-antitrypsin deficiency – 1.03 ± 1.24; autoimmune liver disease – 2.26 ± 1.99 stage per year. The lowest speed of progression is typical for chronic hepatitis C (0.15 ± 0.17 stage per year), the highest one – for autoimmune liver disease (2.96 ± 1.99; $p < 0.05$). We observed the reverse development of fibrosis in some patients as a result of etiotropic therapy: 65% of patients with chronic hepatitis B and 12.5% of patients with chronic hepatitis C.

Discussion/Conclusion: We determined the marker of high speed of fibrogenesis (> 1 stage per year) – the exceeding of the highest limit of norm of hyaluronic acid by > 10 times (specificity – 100%, sensitivity – 92.4%). Some patients with chronic hepatitis B (65%) and chronic hepatitis C (12.5%) displayed the decrease of fibrosis stage as a result of antiviral therapy.

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Strain-dependent differences in the susceptibility to non-alcoholic steatohepatitis

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Introduction: Epidemiological studies clearly indicate a strong variation in the natural course of non-alcoholic fatty liver disease (NAFLD) in different ethnic groups. NAFLD encompasses a wide clinico-pathologic spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). The latter is widely considered as the first relevant pathophysiological step in the progression of NAFLD.

The aim of this study was to assess whether there are differences in the susceptibility to NASH between different mouse strains.

Methods and results: Male C57BL/6, 129/SvJ and BALB/C mice (8 weeks old) were fed with high-fat diet (HFD), which has been shown to induce NASH that closely resembles pathopyhsiological changes observed in human NASH (Matsuzawa et al. Hepatology 2007). HFD induced similar hepatic steatosis in all 3 mouse lines after 12 weeks of feeding. However, the increase of serum transaminases and hepatic proinflammatory gene expression (TNF, IL-1, MCP-1) in response to HFD-feeding were significantly higher in 129/SvJ and BALB/C mice than in C57BL/6, while 129/SvJ and BALB/C mice did not differ significantly regarding these parameters. Also the liver-to-body weight ratio, profibrogenic gene expression (Collagen I, TIMP-1, TGF-beta) and histological fibrosis were significantly higher in 129/SvJ than in C57BL/6 mice after HFD. However and interestingly, profibrogenic gene expression and fibrosis were even more increased in BALB/C than in 129/SvJ mice.

Conclusion: There is significant mouse strain-dependent variation in the susceptibility to NASH development and progression, and interestingly, these differences selectively affect hepatic inflammation and fibrosis. Identifying the underlying molecular mechanisms of the strain dependent differences may provide new insights in the pathophysiological progression of NASH in humans.

Primary human hepatocytes isolated from fatty liver: A promising tool for investigation of steatohepatitis

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Introduction: Non-alcoholic fatty liver disease is a growing disease in Western societies. It leads to pathophysiological changes in liver tissue accompanied by inflammatory processes. Thereby a development from steatohepatitis through to cirrhosis is possible. These cause decreased liver regeneration as well as less detoxification capacity. Our aim was to establish a long-term steatosis model based on primary human hepatocytes (PHH) in order to investigate lipid-induced steatohepatitis related inflammatory processes.

Methods: PHH were isolated from resected human liver using a two-step collagenase perfusion technique. To induce steatosis PHH were treated with a free fatty acids (FFA) diet up to 5 days. Lipid content was displayed by Oil-Red-O staining. The investigation of the initial lipid content led to classification in two groups, high- (HLCH) and low-lipid-content hepatocytes (LLCH). Toxic effects of FFA were evaluated by SRB, XTT, AST and LDH assay. As a marker for inflammatory reactions the intracellular oxidative stress level was analyzed by DCF assay.

Results: LLCH showed a continuous increase in lipid amount whereas HLCH incipiently revealed an increase and then stagnation on a plateau. Measurement of the protein content using the SRB assay showed a general increase in the first days after isolation probably due to regenerative processes. The mitochondrial activity measured by XTT assay showed an increase in control PHH while steatotic PHH showed a decrease. In both groups the FFA treatment was not accompanied by cytotoxic effects up to 4 d. Investigation of reactive oxygen species (ROS) formation revealed increasing ROS levels in FFA treated HLCH in contrast to LLCH.

Discussion/Conclusion: In summery, a continuously decreasing mitochondrial activity connected with an elevated ROS formation was only observable in FFA-treated HLCH. Both effects can be valued as a sign of proceeding lipotoxicity comparable to observations in *in vivo* steatohepatitis. Further investigations are needed to evaluate lipotoxic effects of prolonged FFA treatment.

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Inverse association of vitamin D with depression in patients with chronic liver disease

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Introduction: Patients with chronic liver disease (CLD) frequently exhibit vitamin D deficiency (Arteh et al. Dig Dis Sci 2010). Vitamin D has been implicated in depression, and serum 25-hydroxyvitamin D levels often correlate inversely with depressive symptoms (Ganji et al. Int Arch Med 2010). Vitamin D receptors (VDR) are confirmed to be involved in depression pathophysiology in brain (Eyles et al. J Chem Neuroanat. 2005). We investigate the potential of vitamin D supplementation in ameliorating depressive symptoms in CLD.

Methods: Currently 72 CLD patients have been recruited (51% male, age 20–76 years). Patients are allocated to the intervention or control group when serum 5-hydroxyvitamin D levels are < 30 ng/ml or ≥ 30 ng/ml, respectively. The intervention group receives 20,000 IU 25-hydroxyvitamin D per week for six months. Patients are followed up at 3, 6 and 12 months to assess depression severity using the validated Beck Depression Inventory (BDI-II), and to measure serum 25-hydroxyvitamin D levels via chemiluminescence immunoassay.

Results: In total, 80% of patients have serum 25-hydroxyvitamin D levels < 30 ng/ml (mean 19.4 ± 10.8 ng/ml) and 32% (*n* = 23) have depressive symptoms. A significant inverse correlation between serum vitamin D levels and severity of depressive symptoms is demonstrated in patients with known depression ($p = 0.027$) but not in patients without depression ($p = 0.729$). Initial results show reduced severity of depressive symptoms in CLD patients (*n* = 6) after 3 months of vitamin D supplementation (median BDI score 4; range 0–26) compared to baseline (median 11; range 0–22). In contrast, the BDI score in the control group did not differ markedly between baseline and 3 months (median 7 and 9, respectively).

Discussion/Conclusion: Preliminary evidence indicates an inverse association between vitamin D deficiency and depression severity. Moreover, supplementation with cholecalciferol appears to ameliorate depressive symptoms in patients with CLD, however the validity of these associations have yet to be confirmed.

Expression of Lipocalin2 in upper and lower parts of the liver after lung irradiation

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Introduction: During lung irradiation, as due to the concave anatomy of the lung recesses, the irradiation field included the upper part of the liver. So we decided to evaluate the difference of LCN2 expression in upper and lower liver after lung irradiation in comparison with the expression when liver was irradiated directly (previously showed).

Methods: A single dose of 25 Gray was administered percutaneously to the lung of randomly paired rats after a planning CT scan. Male Wistar rats were sacrificed 1, 3, 6, 12, 24 and 48 h after irradiation along with sham irradiated control. Immuno-fluorescence staining, Western blot, and RT-PCR were performed. As due to the concave anatomy of the lung recesses, the irradiation field included the upper part of the liver. The directly irradiated upper was therefore separated from the lower liver processed separately. Local gene expression of different acute-phase cytokines was determined in upper and lower liver.

Results: Upper liver showed an up-regulation up to 5-fold after lung irradiation but lower liver did not show any considerable increase. These results were further confirmed by Western blot analysis and immunofluorescence staining. While when Liver was directly irradiated it showed increase upto 600-fold. Acute phase cytokines (IL-6, IL-1β and TNF-α) showed a significant increase on transcript level in upper liver, while lower liver did not show any considerable increase.

Discussion/Conclusion: We observed a volume effect for LCN2 expression following liver and lung irradiation: Whole liver irradiation as demonstrated previously lead to a high LCN2 expression (600-fold) as compared to lung irradiation where only upper part of the liver was directly exposed and LCN2 expression increased was only 5-fold. Such a volume effect has also been observed for the development of clinical radiation-induced liver diseases (RILD).

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Another challenge for personalized therapy: Efficacy of therapeutic influence on Peroxisome Proliferator Activated Receptor-gamma is determined by PPARG Pro12Ala gene polymorphism

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Introduction: Multiple studies showed that Peroxisome Proliferator Activated Receptor-gamma – NR1C3 (PPARG) plays an important role in various biological processes including lipid and glucose metabolism. PPARG agonists have been used in treatment of different metabolic disorders and non-alcoholic steatohepatitis (NASH) decreasing steatosis, inflammation, and fibrosis.
The aim of the study was to clarify the perspectives for individualized therapy with thiazolidinediones.

Methods: 249 patients with hypertension, dyslipidemia, metabolic syndrome participated in the study. Among them 50 patients with NASH were selected to form study group. PPARG agonist Pioglitazone administered 30 mg daily during 50–51 weeks. Genetic polymorphism (Pro12, Pro12Ala, Ala12Ala) of PPARG gene determined by PCR. Genotypes were: Pro12 (n = 32, 64.0%); Pro12Ala (n = 14, 28.0%); Ala12 (n = 4, 8.0%) Liver biopsies performed prior and after study.

Results: Pioglitazone improved glycemic control and glucose tolerance ($p < 0.001$), normalized liver aminotransferase levels as it decreased AST by 42.1 ± 1.17% $p = 0.014$; ALT by 57.5 ± 1.37%, $p < 0.001$; decreased hepatic fat by 54.6 ± 2.09%, $p < 0.001$; and increased hepatic insulin sensitivity by 48.5 ± 1.63% $p = 0.006$. Administration of pioglitazone caused improvement in histologic findings with regard to steatosis, ballooning necrosis, and inflammation. In 4 (8%) Ala12 patients no reliable changes were observed, except glycemic control and glucose tolerance.
Reduction in fibrosis did not change significantly. Statistically insignificant weight gain and mild lower-extremity edema developed in 2 subjects with Pro12Ala genotype, no other side effects were observed.

Discussion/Conclusion: Administration of thiazolidinediones leads to metabolic and histologic improvement in most patients with NASH. However, individual response may be affected by Pro12Ala polymorphism of PPARG gene. This study shows that carriers of Ala genotype whilst comparatively rare among NASH patients are much less sensitive to PPARG agonists' therapy.

Lipopolysaccharide induces neovascularization and immunosuppression, and must be considered as therapeutic target

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Introduction: Previous studies showed that endotoxin – lipopolysaccharide (LPS) is both angiogenic and immunosuppressing, thus promoting metastatic growth (MG). However, the role of LPS as a therapeutic target is unclear. We hypothesized that anti-LPS therapy may decrease MG.

Methods: Murine model including 3 groups (25 each) of adolescent mice was used. Metastatic process was modeled by i/v injection of 200 µl spontaneously metastasizing mammary adenocarcinoma cell culture suspension. Control group (CG) animals received 200 µl sterile saline intraperitoneal (i.p.), experimental group 1 (EG1) – 200 µl suspension of 10 µg LPS per mouse, experimental group 2 (EG2) – same plus 20 µg at 0.5 ml anti-LPS monoclonal antibodies. MG evaluated hystochemically within lung metastases.

Results: EG1 showed significantly higher ($p < 0.001$) MG compared with the control. MG was characterized by 61.2% higher mitotic index (MI) in the EG1 and 42.3% lower apoptotic index (AI). MI/AI ratio in the EG1 was 3.2 times higher ($p < 0.001$) than control. LPS injection resulted in reliably ($p = 0.002$) higher levels of serum VEGF than in control with strong positive correlation ($r = 0.971$) between circulating VEGF and LPS levels. Addition of anti-LPS monoclonal antibodies significantly decreased MG, MI and increased AI with respective change of MI/AI ratio. VEGF becomes insignificantly higher than in control whilst LPS concentration decreased reliably ($p = 0.014$).

Discussion/Conclusion: Despite the well-established role of LPS as pro-inflammatory, pro-proliferator and pro-neovascularization factor, its role in carcino-genesis remains under evaluated.
Our findings show that targeted anti-LPS therapy may impact tumor growth due to prevention of neovascularization and inflammation as well as inducing apoptosis.

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Selective inhibition of cyclooxygenase-2 in vitro induces apoptosis and decreases proliferation of the human liver tumor cell

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Introduction: Recent studies showed that selective cyclooxygenase-2 (COX-2) inhibitors suppress growth of colon cancer cells and have chemopreventive potential during colon cancerogenesis. However, it is still debatable whether COX-2 contributes to the malignant growth and whether inhibition of COX-2 modifies the malignant potential of liver tumors. The aim of the study was to clarify the pro-apoptotic and anti-proliferative effect of selective COX-2 inhibition.

Methods: HCC cells lines Hep G2/Hep 3B were cultivated in modified media seeded onto well plates. Celecoxib 50 μmol/l added in study group cultures. Apoptosis related cytokines were analyzed by Western blotting. Apoptotic nuclei were visualized with the TUNEL-staining protocol and cells viewed with a fluorescence microscope (magn. x400). The number of apoptotic cells calculated in percentage of total nuclei.

Results: COX-2 inhibition related changes become evident in Hep G2/Hep 3B cell lines after 48 hours of treatment leading to a significant time-dependent reduction of cell numbers of up to 80% ($p < 0.05$). Cells became sparse, rounded, and detached from the dishes representing morphologic signs of apoptosis. This correlated with activation of caspase-9, caspase-3, and caspase-6 cytokines. However, exposure of cell cultures to 3 g/ml PgE_2 eliminated the COX-2 inhibiting and pro-apoptotic effect on cells. This indicates that the antineoplastic properties of COX-2 inhibiting are dependent on reduces conversion of arachidonic acid to PGE_2 attributable to COX-2 inhibition.

Discussion/Conclusion: Selective inhibition of COX-2 causes marked growth inhibition of human liver tumor cells, based on the induction of apoptosis and inhibition of proliferation. The mechanism by which COX-2 inhibiting-related apoptosis is realized is still unclear as well as involvement of other factors into antiproliferative effect of COX-2 inhibitors.

Upcyte® Hepatocytes show stable phenotype comparable to human hepatocytes: Possible clinical implications

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Introduction: A limited supply of much needed primary human hepatocytes is caused of donor organ scarcity. To address this problem, Medicyte has developed a new technology to induce proliferation of human hepatocytes by transducing the cells with genes enabling cell proliferation. Aim of this work was to elucidate gene expression and metabolic characteristics of these specially modified liver cells called upcyte® Hepatocytes at different stages of expansion.

Methods: Upcyte® Hepatocytes from different donors were cultured for approximately 20 Population Doublings (PD). At three different expansion stages, expression levels of different genes responsible for urea synthesis, blood coagulation and protein synthesis were measured by RT-PCR. Urea synthesis and ammonium chloride detoxification were measured photometrically. Furthermore, the secretion of albumin was measured in media by ELISA. Additionally we compared the expression levels of phase-I-enzymes and drug receptors after cultivation in 2D and 3D culture system. Data obtained from primary human hepatocytes exemplify as control.

Results: Upcyte® Hepatocytes grew in a linear fashion over time such that within two to three weeks, the cells proliferated from 6 million to about 120 million. Upcyte® Hepatocytes express enzymes necessary for urea synthesis, protein synthesis and coagulation, as observed by RT-PCR. The rate of urea production was lower in upcyte® Hepatocytes than in primary human hepatocytes. However, it was stable over the whole period of cell expansion (20 PDs). The two different culture systems indicated that the 3D cultured upcyte® Hepatocytes represent better the in vivo situation than the 2D cultured cells.

Discussion/Conclusion: In conclusion, our findings suggest application of the upcyte® technology to human hepatocytes induces proliferation without a remarkable functional loss. However, further elucidations will be necessary to test different metabolic characteristics of upcyte® Hepatocytes before clinical transfer for using them for a cell-based therapy of acute liver failure.

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Expression and function of Atrophin 2 in chronic liver disease

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Introduction: The atrophin gene family has been characterized after identification of atrophin as the cause of dentatorubral-pallidoluysian atrophy. Atrophin family proteins have been identified as nuclear receptor corepressors. They are implicated in the regulation of various biological processes including migration and orientation as well as apoptosis. Furthermore, altered atrophin expression has been shown in neurodegenerative disease and cancer. Atrophin 1 is not essential, whereas Atrophin 2 (ATN2) is crucial for normal mouse embryonic development.

The aim of this study was to analyze the expression and function of ATN2 in chronic liver disease.

Methods and results: Hepatic ATN2 expression was significantly increased in different murine models of chronic liver injury (chronic CCl_4 injury or dietary models of non-alcoholic steatohepatits [NASH]). Furthermore, we observed increased ATN2 expression in hepatic tissue of patients with NASH or liver cirrhosis of different origin. Moreover, ATN2 was significantly increased in primary murine and human hepatic stellate cells (HSC) during the course of *in vitro* activation. By transient transfection with siRNA we achieved more than 50% reduction of ATN2 in activated HSCs, and functional analysis revealed effects on attachment and proliferation. Because of the known association of ATN2 with cancer we analyzed ATN2 expression also in different human HCC cell lines and tissues and ascertained a marked upregulation compared to primary human hepatocytes and non-tumorous tissue.

Conclusion: Our data indicate ATN2 as a functionally relevant transcriptional regulator in activated HSCs. Furthermore, increased expression in HCC cells suggests that ATN2 also affects tumorigenicity. Herewith, ATN2 appears as a potential therapeutic target to inhibit the fibrosis and cancerogenesis in chronic liver disease.

Strain-specific differences modify hepatocellular carcinoma initiation in Abcb4-deficient mice

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Introduction: In patients, the development of hepatocellular carcinoma (HCC) is usually a consequence of advanced liver fibrosis but the mechanisms are still poorly understood. It has been shown that the initiation of HCC can be modelled in mice by the administration of a single dose of diethylnitrosamine (DEN), with HCC formation depending on interleukin (IL) 6 production (Naugler et al. Science 2007). Mice that lack the hepatocanalicular phosphatidylcholine transporter Abcb4 develop biliary fibrosis and can be used as a model to study tumor formation in injured liver. The aim of our study was to investigate HCC initiation in Abcb4-deficient mice of different genetic backgrounds.

Methods: Abcb4-deficient mice on the FVB/NJ genetic background were crossed to two distinct genetic backgrounds (Balb/cJ, C3H/HeN) for at least 10 generations. Congenic knockout and wild-type (wt) mice at the age of 16 weeks (when biliary fibrosis is established) were treated with DEN for 48 hours. Phenotypic differences were determined by analyzing hepatic apoptosis (TUNEL) and proliferation (Ki67) rates as well as inflammatory markers including IL6 expression.

Results: All parameters were significantly increased in Abcb4-deficient DEN-treated mice as compared to controls ($p < 0.05$) but differed markedly between the three congenic lines. In particular, IL6 expression (untreated wt = 1.00) increased from Balb-Abcb4 (4.37 ± 0.86) and FVB-Abcb4 (14.60 ± 2.96) to C3H-Abcb4 mice (53.44 ± 6.18); the same trend was observed for Ki67 staining (Balb-Abcb4: 3.91 ± 0.53; FVB-Abcb4: 7.45 ± 0.62; C3H-Abcb4: 10.96 ± 1.13% proliferating cells). Apoptosis rates were highest in Balb-Abcb4 (19.16 ± 2.41% apoptotic cells) whereas FVB-Abcb4-deficient mice showed the lowest apoptosis rates (1.96 ± 0.62% apoptotic cells).

Discussion/Conclusion: This study demonstrates that HCC initiation upon DEN challenge depends on pre-existing fibrosis and genetic background. As HCCs are known to occur spontaneously in FVB-Abcb4-deficient mice, the increased apoptosis rates in other congenics might confer a protective mechanism against HCC formation. The set of congenic mice with biliary fibrosis provides a basic experimental framework to dissect the genetic determinants that drive and/or modify HCC initiation and formation.

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