Research Report

Firstly, of the IgA type autoantibodies formed against gut innate immunity proteins, glycoprotein 2 [GP2] and CUB and zona pellucida-like domain-containing protein-1 [CUZD1] referred as PAbs together was tested in a large cohort of patients with alcoholic liver cirrhosis (LC) and chronic liver diseases (CLD) but without cirrhosis as a controls (n=290, primary sclerosing cholangitis [PSC]: 69, primary biliary cholangiopathy [PBC]: 102 and chronic hepatitis C virus [chrHCV]: 119) by indirect immunofluorescence test system (IFTT) (CIBD Mosaic, EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). Interestingly, ssignificantly more PSC patients were positive for PAbs (43.5%) compared to healthy controls(0%) or patients with LC or other CLD (5.7%, $p<0.001$ for both). In PSC, frequencies of anti-GP2 and anti-CUZD1 antibody positivity were equal. Anti-GP2 antibody was exclusively IgA, while anti-CUZD1 was both IgA and IgG isotypes. Anti-GP2 IgA but none of the anti-CUZD1 Ig isotypes predicted faster disease progression during prospective follow-up (median:87 [9-102] months). Anti-GP2 IgA-positivity was associated with shorter time to orthotopic liver transplantation and/or liver-related death[*p*LogRank<0.01], and remained an independent predictor after adjusting for Mayo risk score(HR:5.01 [1.13-22.23], *p=0.034*). In our study, both liver-related death and OLTx were considered as equal endpoints, since they represent the development of end-stage liver disease as the result of the progressive fibrosis. Novel finding regarding anti-GP2 IgA proved highly specific because none of the other serological response to other bacterial antigens (e.g. phosphopeptidomannan [ASCA], different endotoxins [EndoCab], multiple Gram-negative and Gram-positive proteins [OMP] or TTB-5/ FtsZ [ANCA]) were not associated with disease progression in PSC. Supporting clinical evidence for the anti-GP2 IgA - fibrosis linkage in PSC might be the observation, that anti-GP2 IgA was reported to be more prevalent in CD patients with stricturing disease. This work was awarded 1st Prize at *FALK Symposia* (Gut-Liver Interactions: From IBD to NASH Innsbruck, Austria, March 11-12, 2016. P33) and also presented as an oral presentation *at UEGW 2016* (Session Title: 215 - Mechanisms of Primary Sclerosing Cholangitis, Session Type: Free Paper, Session Date: October 17, 2016. OP032).

Formation of anti-GP2 antibody represents the breakdown of tolerance towards GP2 protein. Glycoprotein 2, an innate immunity protein released from the exocrine pancreas into the intestinal lumen, is also expressed on the apical membrane surface of M-cells. There, GP2 can interact with FimH-positive bacteria bearing type-1 fimbriated pyli. Thus, GP2-mediated transcytosis is necessary for the initiation of antigen-specific mucosal immune responses against this type of bacterial antigen. To explore the possible link between mucosal immunity and the development of severe phenotype in PSC, we developed (1) an in-house ELISA system to measure the serum levels of total secretory (s)IgA. The total sIgA levels were significantly elevated, namely three-fold higher, compared to healthy controls (median [IQR], 96 [73-148] vs. 30 [21-42] µg/ml, *p<0.001*). These results indicate an elevated retro-transport of sIgA from the gut mucosal surface in PSC. An additional two-fold elevation of total sIgA levels were found in anti-GP2 IgA-positive cases (149 [86-240] vs. 89 [71-118] µg/ml, *p=0.021)* that was not seen in the presence of other serological

anti-microbial antibodies or ANCA. These results suggest that retro-transport is specifically further enhanced in patients with anti-GP2 IgA-positivity. Thereafter (2) we developed a flow-cytometry subtyping assay (IgA1/A2 and secretory component[SC]) to characterize further the anti-GP2 IgA antibody in patients with PSC. The presence of SC on GP2 IgA antibodies was high: a total of 68.4% of PSC patients.

This high percentage of SC strongly indicates that after secretion to the gut lumen, anti-GP2 IgA antibodies are also retro-transported across the mucosal epithelium. Retro-transported sIgA molecules, however, are not “lonely” particles, but rather exist in partnership with their antigens. Since antigen-coupled sIgA molecules are those that have high affinity to their epithelial receptors, anti-GP2 IgA antibodies grab GP2-coated FimH-positive bacteria as companions. In this way enhanced retro-transport of anti-GP2 IgA might increase microbial overload in the mucosal compartment and perpetuate antigen induced signalling. Memory T lymphocytes primed in the inflamed gut and homing to the biliary tract via aberrantly expressed adhesion molecules plays a fundamental role in the extension of gut inflammation to the biliary tract. Interestingly, FimH has been recently identified as a ligand of Toll-like receptor (TLR)-4. Sustained TLR4 activation leads to enhanced fibrosis through TGF-beta signaling. At the same time, FimH elicits an immune response with enhanced type I Interferon production that has been linked to disease amplification in autoimmunity. These mechanisms could serve an explanation of how the breakdown of tolerance towards GP2 in the gut is associated with the development of enhanced fibrosis, and thus disease progression in the liver. The GP2 – FimH axis deserves further exploration in the pathogenesis of PSC and might also be a highly intriguing issue from the therapeutic point of view. GP2 has a high structural and functional homology with uromodulin (Tamm-Horsfall protein in the urinary tract). Recombinant vaccine against adhesion protein of FimH is already under development in recurrent urinary tract infections. Furthermore mannose-derived FimH antagonists, also hold promise as a novel treatment for UTIs and Crohn’s disease. These results were published in a full length paper (*Sci Rep. 2018 Jan 10;8(1):399.*).

Unfortunately, type 4 aGP2 abs were not more frequent in patients with alcoholic liver cirrhosis as we expected previously. Thus we were carrying-on aGP2 abs project in the PSC population and took part in a European validation cohort assessment directed by Prof. Dirk Roggenbuck (Institute of Biotechnology, Faculty Environment and Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Senftenberg, Germany). Anti-$GP2_{1\text{-}4}$ IgA and IgG were detected in 212 PSC patients of four European university hospitals and 145 controls comprising 95 patients with cystic fibrosis and 50 healthy subjects. We were also active participant in the development of a new IIFT system for additional subtype anti-GP2 abs (transfected HEp2 cells expressing all the four isotypes [1-4] of GP2 proteins) as well. This test system differs significantly the currently used ELISA or IIFT techniques that only use isotype 4 of GP2 as an antigen. A full-length paper has just been published (*Front Immunol. 2018 Aug 28;9:1959.)* GP2 isoforms were stably expressed as glycosylphosphatidylinositol-anchored molecules in the membrane of HEp-2-cells and used as autoantigenic targets in IIFT. Combined $aGP2_1$ and $aGP2_4$ IgA testing with a sensitivity of 66.0% and a specificity of 97.9% resulted in the best diagnostic performance (Youden index: 0.64) regarding all aGP2 and

combinations thereof. Combined aGP2$_1$ and aGP2$_4$ IgA analysis is preferred to single aGP2 isoform for sensitive PSC autoantibody testing. Clinical findings of the validation cohort support our previous findings. Positivity for aGP2$_1$ and aGP2$_4$ IgA was associated with cirrhosis in PSC and could be used for risk stratification. Having this new IIFT not only for aGP2 abs, type 4 but also for type 1,2 and 3, we are to retest serum samples of our cirrhotic patient cohort in a newly awarded Horizon 2020 project *(MICROBiome-based biomarkers to PREDICT decompensation of liver cirrhosis and treatment response, Grant Agreement number: 825694 — MICROB-PREDICT — H2020-SC1-BHC-2018-2020/H2020-SC1-2018-Single-Stage-RTD)* in which our group will lead the work package 1 (WP1) and having an ancillary study titled "Gut Barrier Failure: A Piece in the Puzzle of Acute-on Chronic Liver Failure Syndrome Development?". We are to apply a semi-quantitative real-time PCR to assess GP2 mRNA synthesis normalized to the housekeeping gene b-actin in ileum and colon biopsies of patients with alcoholic cirrhosis, PSC or IBD (CD and UC as well) with healthy controls. In parallel, detection of GP2 (positive staining of enterocytes) in ileum and colon biopsies by IIFT will also be performed.

Regarding autoimmune liver diseases we took part in a multicenter study organized by Radboud University Medical Center, Nijmegen, The Netherlands. We found that the dose of predniso(lo)ne to induce remission in patients with autoimmune hepatitis (AIH) is less relevant than assumed. An initial predniso(lo)ne dose below 0.50 mg/kg/day substantially decreases unnecessary exposure to predniso(lo)ne in patients with AIH. The first manuscript of this collaborative work has just been published *(Clin Gastroenterol Hepatol. 2019 Jan 5. pii: S1542-3565(19)30008-4.)*

Secondly, we tested IgA type autoantibodies related to gut barrier failure (anti-filamentous actin [AAA] and anti-gliadin [AGA] in a large cohort of patients with liver cirrhosis (LC, n= 260) and PSC (n=69) by ELISA. Gut barrier failure and the consequential pathological bacterial translocation (BT) are characteristic features of alcoholic cirrhosis and also PSC play an important role in the progression of liver disease. We hypothesized that serological hallmarks of gut barrier dysfunction are associated with accelerated progression of liver disease in alcoholic cirrhosis and PSC in the development of specific complications and liver-related death.

A total of 28.4%, 28.0%, 9% and 20.9% of PSC patients were positive for AAA IgA, AAA IgG, AGA IgA and AGA IgG, respectively. Frequencies of AAA IgA and AAA IgG ($P < 0.001$, for both) and AGA IgG ($P = 0.01$, for both) but not AGA IgA were significantly higher compared to both of the healthy and the diseases control groups (ulcerative colitis, UC, n=172). In survival analysis, AAA IgA-positivity was revealed as an independent predictor of poor disease outcome after adjusting either for the presence of cirrhosis [HR = 5.15 (1.27-20.86), $P = 0.022$ or for the Mayo risk score (HR = 4.24 (0.99-18.21), $P = 0.052$]. AAA IgA-positivity was significantly associated with higher frequency of anti-microbial antibodies ($P < 0.001$ for EndoCab IgA and $P = 0.012$ for anti-OMP Plus IgA) and higher level of the enterocyte damage marker (median I-FABP$_{\text{AAA IgA pos } vs. \text{ neg}}$: 365 *vs* 166 pg/mL, $P= 0.011$), but not with serum LBP level. Presence of IgA type AAA identified PSC patients with progressive disease. Moreover, it is associated with enhanced mucosal immune response

to various microbial antigens and enterocyte damage further highlighting the importance of the gut-liver interaction in PSC. These results were published in a full length paper (*World J Gastroenterol. 2017 Aug 7;23(29):5412-5421.*). This work was presented *at UEGW 2016* (Session Title: 215 - Mechanisms of Primary Sclerosing Cholangitis, Session Type: Free Paper, Session Date: October 17, 2016. OP034) and was awarded 1st Prize.

Elevated concentrations of the gut failure markers IgA-AAA (62.7 vs. 4.4%) and IgA-AGA (27.7 vs. 2.6%) were more often observed in cirrhosis as compared to healthy controls ($p<0.001$ for both). In addition, serum I-FABP was increased in cirrhosis as compared to controls (741 vs. 244 pg/mL, $p<0.001$) and correlated with serum levels of IgA-AAA and IgA-AGA. IgA-AAA positivity was associated with alcoholic liver disease, liver disease scores and decompensated clinical stage (all $p<0.001$). Serological markers of BT were more often found in patients with elevated IgA-AAA compared to those without (72.3 vs. 13.5 % for IgA-EndoCab and 85.2 vs. 20.5% for IgA-anti-OMP, $p<0.001$ for both). In patients with compensated disease stage (n=131) the risk of decompensation was higher in patients with elevated IgA-AAA (HR [95%CI]: 1.85 [1.06-3.24]), as was the risk of liver-related mortality (HR: 2.66 [1.27-5.56]). Such associations were not observed for IgG-AAA and IgA/IgG-AGAs. In the overall cohort, IgA-AAA remained an independent predictor of liver-related death (HR_{adj}: 1.96 [1.08-3.55]) when adjusting for important clinical variables (MELD score, etiology, clinical stage). Elevated serum concentrations of IgA antibodies against filamentous-actin indicate patients with an unfavorable outcome in cirrhosis, which may be related to intestinal damage beyond being related to bacterial translocation. IgA-AAA might be considered as a novel serologic marker of the disease progression. This work was presented as an oral presentation in *UEGW 2017 (Session Alcoholic liver disease: Anything new?, 31th of October)* and also selected for a presentation award in *FALK Symposia (Hamburg, 25-26 January, 2018, Liver-Gut-Microbe Interactions).* Based on our research work in this field we got a possibility to present a state-of-art introduction lecture in *UEGW 2017 with a title of New insights of microbiota: Gut and liver aspects)*.

Before drafting a final paper of our results we would like to further clarify our findings and also confirm the hypothesis proposed below. The evaluation of the below hypothesis will also be a part of the Horizon 2020 project. Namely, we hypothesize that immune response to F-actin should be considered as a serologic hallmark of the overwhelming damage-associated molecular patterns (DAMPs) due to cell death or injury. Enhanced pathologic BT (various pathogen-associated molecular patterns [PAMPs], like lipopolysaccharide [LPS]) from gut tract induces exaggerated pro-inflammatory response not only in the intestinal tract but also in remote organs and results in tissue injury. PAMP and DAMP signals have different downstream pathways; however PAMP induced subsequent DAMP signalling is also known phenomena and has also been described previously. E.g. LPS is able to induce TNF-α production leading to hepatocyte damage. Sustained hepatocyte damage exacerbates liver injury that is a prerequisite of liver fibrosis. We suppose that enhanced IgA-AAA formation in alcoholic cirrhosis is a consequence of augmented release or cell surface exposition of cytoskeletal F-actin by damaged cells (hepatocytes and/ or enterocytes). F-actin is the 2nd most

abundant protein in eukaryotic cells and has a fundamental function in cells' vital activity. Appearance of F-actin on cell surfaces or its release into the systemic circulation is considered as a DAMP signal. High-mobility group box 1 protein (HMGB1) is a well known, while F-actin is a lesser-characterized cytoskeletal DAMP in the field of hepatology. Recently DNGR-1 (CLEC9A) has been described as a novel dendritic receptor for dead cells and recognises cell surface F-actin. DNGR-1 signalling does not couple to down-stream activation of NF-κβ but Syk-SFK. F-actin antigen is cross-presented to cytotoxic CD8+ T-cells. (*Sancho D, Nature. 2009; 458: 899-903; Ahrens S, Immunity. 2012; 36: 635-45 and Hanč P, Immunity. 2015; 42: 839-49.*). Our results potentially open the door for future studies on a more general role of this specific DAMP in inflammation and immunity in cirrhosis. Mapping of the binding sites on DNGR-1 and on F-actin may lead to new strategies to target, block, or stimulate the receptor. To confirm our hypothesis the following measurements are planned: evaluation of plasma F-actin level and its scavenger proteins (e.g. gelsolin, vitamin D binding protein). As a proof of concept we expect that in patients with IgA-AAA positivity, serum F-actin level would be increased, while levels of scavenger proteins decreased compared to those with IgA-AAA negativity. It is a further concern whether IgA type immune reaction to cytoskeletal F-actin is beneficial (tolerance) response of the host to the overwhelming harm or it is an active play-actor of the injurious processes.

Thirdly, we studied the impact of pattern recognition receptor (PRR) genetic variants to BT. In Crohn's diseases it is well a known fact that the presence of NOD2/CARD15 mutation is associated with enhanced anti-microbial antibody formation such as *anti-Saccharomyces cerevisiae* or anti-outer membrane porin C antibodies in most clinical studies. This association however has not been assessed in cirrhosis so far. Thus we examined the effect of various *NOD2* and *TLR2 and 4* SNPs on the IgA type anti-microbial antibody formation. The frequency of anti-OMP IgA and EndoCab IgA did not differ between patients with various PRR genotypes; neither in the entire cohort nor in the subgroup of patients with/or without ascites. These results confirm that in cirrhosis acquired component of BT is more dominant than the functional genetic polymorphisms in the development of BT *(Liver Int. 2018 Jul;38(7):1242-1252.)*

Regarding alcoholic liver cirrhosis and development of bacterial infections we took part in a multicenter study organized by Hospital Clinic, University of Barcelona and European Foundation of Chronic Liver Failure (EF-Clif), Barcelona, Spain. We found that infections caused by bacteria resistant to the main antibiotic families (MDR bacteria) are constitute a prevalent, growing and complex healthcare problem in patients with decompensated cirrhosis and acute-on-chronic liver failure across Europe, negatively impacting on prognosis. Strategies aimed at preventing the spread of antibiotic resistance in cirrhosis should be urgently evaluated. The first manuscript of this collaborative work has just been published *(J Hepatol. 2018 Nov 2. pii: S0168-8278(18)32511-X.)*

We analyzed different parameters of von Willebrand factor (VWF), including detailed multimer distribution by densitometry and platelet adhesion, together with adisintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) activity and antigen

and C-reactive protein (CRP) levels in patients with stable (*ST, n = 99*) cirrhosis, acute decompensation (AD, *n* = 54) and controls (*n* = 92). Characteristic changes of VWF parameters were revealed. VWF antigen, ristocetin co-factor as well as collagen-binding activities were elevated in both cirrhotic groups in a stepwise manner. There was a decrease in high and an increase in low molecular weight multimer ratios in the majority of ST cirrhosis. However, in 24 out of 54 AD patients, ultra-large VWF multimers (ultra-large molecular weight multimers [ULMWM]) were found. ADAMTS13 activity in ST and AD patients without ULMWM was similar to controls (median [interquartile range; IQR]%: 98 [67-132] and 91 [60-110] vs. 106 [88-117], respectively). The presence of ULMWM in AD patients was associated with low ADAMTS13 activity [33 (24-49)%] and high CRP level [23 (7.1-83.6) mg/L]. Adhesion of normal platelets showed a stepwise increase in the presence of cirrhotic plasmas, reaching the highest level in AD patients with ULMWM. In patientswith AD, highly increased VWF and reduced ADAMTS13 activity along with the presence of ULMWM are possible markers and contributors of the disease progression (*Thromb Haemost. 2018 Aug;118(8):1397-1408*).

Lastly, to accomplish our aim that was the identification of a new auto-antigen target of the IgA type atypical perinuclear anti-neutrophil cytoplasmic antibody (atypical P-ANCA) we start a collaborative project with Prof. Dirk Roggenbuck (Institute of Biotechnology, Faculty Environment and Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Senftenberg, Germany). As a results we were able to develop a new system for this purpose. After separation of whole cell proteins isolated from neutrophils, we used two dimensional electrophoresis and Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF-MS) based protein identification of the spots that displayed Western blotting signals with anti-neutrophil cytoplasmic antibody (ANCA)-positive sera. Chitinase 3-like protein 1 (CHI3L1) was identified as a novel neutrophil autoantigenic target. Our results have recently been published *(J Crohns Colitis. 2019 Feb 7. doi: 10.1093/ecco-jcc/jjz012. [Epub ahead of print])*. The establishment of the method has also been performed in the Laboratory Medicine Department (Prof. Dr. Antal-Szalmas Péter) to be able to seek potentially new targets in other gastroenterology and liver diseases associated with enhanced IgA type atypical P-ANCA or other ANCAs wit unknown target. Thereafter we developed an enzyme-linked immunosorbent assays (ELISAs) system to assess the prevalence of IgG, IgA, and secretory IgA (sIgA) to chitinase 3-like protein 1 (CHI3L1). To date patients with IBD and celiac disease and also healthy controls were assessed. Patients with alcoholic liver cirrhosis and autoimmune liver diseases are under evaluation.