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A Mechanistic Insight Into The Role Of Gut Microbiota In The Pathogenesis Of Primary Sclerosing Cholangitis

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Primary sclerosing cholangitis (PSC) is one of the four major autoimmune diseases of the liver and is characterised by inflammation and fibrosis of the biliary tree. PSC is unique amongst the autoimmune liver conditions in that it is comorbid with inflammatory bowel disease (IBD) in 60-80% of patients, which most resembles a type of ulcerative colitis (UC) (Clin Liver Dis 2017 9(5):107-110). Disease onset may be insidious, and the clinical course is often variable. There is progressive inflammatory and obliterative destruction of bile ducts that can lead to cirrhosis, portal hypertension, recurrent biliary sepsis and ultimately decompensated disease (Gastroenterology 2003 125:1364–1369). Furthermore, large duct PSC is considered a premalignant condition, with an increased risk of hepatobiliary cancers: cholangiocarcinoma, gallbladder cancer and hepatocellular carcinoma in cirrhotic patients and colonic adenocarcinoma, requiring regular surveillance (Clin Liver Dis (Hoboken) 2014 3(4):83-85, J Hepatol 2002 36:321-327, Gastrointest Endosc. 2002 56(1):48-54).

PSC should now be considered a unique subtype of IBD alongside Crohn's disease and non-PSC-UC. The colitis of PSC is characterised by different clinical features to that of non-PSC IBD alone, such as rectal sparing (52% vs 6%), backwash ileitis (51% vs 7%), pancolitis (87 vs 54%) with PSC being predominately a right sided colonic disease (Gut 2005 54:91–96). To explain PSC and comorbid IBD, it has previously been hypothesised that gut primed adaptive and innate immune cells are responsible for both colonic and liver inflammation, with the assumption that these cells can infiltrate both niches due to the shared expression of unique homing signals and immune receptors (J Exp Med 2004 200(11):1511-7, J Crohns Colitis. 2017 11(9):1124-1134).

There has been a growing interest in the role of a colonic dysbiosis in the pathogenesis of PSC. The first indication that the gut microbiome might potentially play a role in the pathogenesis of PSC was seen in rat models of small bowel bacterial overgrowth, which resulted in a hepatic injury as a result of surgically-created self-filling jejunal blind loops (Hepatology 1991 13(4):766-72). Furthermore, positive bacterial cultures have been demonstrated from bile and bile ducts from 21 out of 36 explanted livers of patients with PSC compared to no positive cultures from explants from patients with primary biliary cholangitis (PBC) (J. Hepatol 1998 28:426–32).

There have been multiple studies in the past four years demonstrating a gut dysbiosis in PSC and PSC-IBD compared to healthy controls and non-PSC IBD (J Crohns Colitis 2015 9(4):342-8, Gut 2016 65:1681-1689, Aliment Pharmacol Ther 2016 43(7):790–801, Gut 2017 66(4):611-619, Gut 2017 66:386–388, World J Gastroenterol 2017 23(25):4548-4558, United European Gastroenterol J. 2018 6(1):112-122, United European Gastroenterol J 2018

6(1):112-122). However, to date these have failed to provide a mechanistic understanding of pathogenesis. It is therefore of great relevance that Nakamoto and colleagues have now provided a potential mechanistic insight into the role of the microbiome in the pathogenesis of PSC.

In their study, Nakamoto and colleagues used humanised mouse models with the aim of developing a greater understanding of how gut dysbiosis contributes to PSC pathogenesis, with particular focus on how specific microorganisms could be implicated. They took faecal samples from patients with PSC and UC (PSC-UC), non-PSC-UC (UC) and healthy controls (HC) to create gnotobiotic mice (PSC-UC mice, UC mice and HC mice), and confirmed that the dominant microbial taxonomy was preserved in each model.

Examination of T cell profiles of the livers and colons of the gnotobiotic mice demonstrated potent Th17 (IL-17+ CD4+ T helper cell) priming in the colon of HC mice and crucially, in the liver in 60% of the PSC-UC mice only, with amyloid A and IL1B also upregulated in both their colons and livers. One important observation was that a faecal transplant alone from a PSC-UC patient to all gnotobiotic mice was insufficient to induce hepatic histological or serological change. The group therefore questioned whether gut dysbiosis could promote hepatobiliary injury, and demonstrated this using a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-fed mouse model (a commonly used model for PSC). DDC-fed HC mice demonstrated increased disease activity (significantly raised serum bilirubin and alkaline phosphatase (ALP) representing cholestatic injury, and liver fibrosis (higher percentage Sirius Red positive area compared to germ free (GF) mice) but this was seen to a significantly greater degree in the DDC-fed PSC-UC mice, with an associated Th17 response (IL17+ CD4+ T helper cells) in the liver.

PSC-UC mice demonstrated increased serum levels of bacterial endotoxin (LPS) indicative of bacterial translocation. Furthermore, harvested livers and mesenteric lymph nodes (MLNs) of these mice at day 21 post inoculation, followed by in vitro culture showed the presence of bacterial clones in the MLNs of PSC-UC mice only, but not in the liver or spleen and no organs from specific pathogen-free (SPF), HC or UC mice. 16S rRNA sequencing identified *Klebsiella pneumoniae, Proteus mirabilis and Enterococcus gallinarum* from the MLNs from the PSC-UC gnotobiotic mice. These three bacteria were then inoculated into GF mice. The 3-mix gnotobiotic mice showed bacterial translocation to MLNs but not to liver on day 21 post oral bacterial inoculation. Fluorescence in-situ hybridization (FISH) staining confirmed bacterial DNA present in the liver after IV bacterial inoculation but not after oral

inoculation, confirming that failure of bacterial translocation to the liver through oral inoculation was not as a result of low sensitivity.

To compare these findings to human subjects, metagenomic analysis was undertaken on faecal samples collected from patients with PSC-UC (n=18), UC (n=16) and HC (n= 10), showing significant differences between HCs and PSC-UC and UC respectively. 16S qPCR of all faecal samples showed *Klebsiella pneumoniae* in 17/18 PSC-UC samples, 5/16 UC samples and 0/10 healthy control samples ($p<0.0001$ and $p<0.0002$, Fisher's exact test). *Enterococcus gallinarum* was higher in patients with PSC than those in other groups (12/18 PSC-UC, 3/16 UC, 0/10 HC). Furthermore, the group analysed faecal samples from 9 patients with autoimmune hepatitis (AIH) and 10 patients with PBC and analysed these with 16S qPCR – *Klebsiella pneumonia* was present in 3/9 AIH and 5/10 PBC, no samples with *Proteus mirabilis* and 1/9 AIH and 1/10 PBC of *Enterococcus gallinarum*. This could suggest a potential association with *Klebsiella* and hepatobiliary diseases.

The genera that were significantly different between each patient group (Linear Discrimination Analysis Effect Size) were *Megamonas, Ruminococcus, Lachnoclostridium* and *Clostridium* present in HCs, *Streptococcus, Klebsiella, Lactobacillus, Enterococcus* present in PSC-UC and *Veillonella* in patients with UC.

By assessing the 3-mix gnotobiotic mice alongside inoculating germ-free mice with bacteria either individually or in combination, Nakamoto and colleagues were able to establish that mono-colonisation with *Klebsiella pneumonia* or dual colonisation of *Proteus mirabilis* and *Enterococcus gallinarum* were less effective at Th17 induction (lower frequency of RORγT t+IL17+ cells) than 3-mix bacteria in liver ($p<0.0001$) and colon ($p=0.0004$). This suggests a cooperative induction of hepatobiliary inflammation by the microorganisms. Heat-killed 3-mix bacteria could also elicit Th17 induction from naïve CD4+ T cells.

Th17 differentiation was therapeutically targeted in DDC-fed 3-mix gnotobiotic mice with anti-IL-17A antibody administered intraperitoneally during DDC feeding, which failed to ameliorate the hepatobiliary injury. However, when the DDC-fed 3-mix gnotobiotic mice were treated daily with RORγT inverse agonist (RIA) for 2 weeks, a significant reduction in the number of Th17 cells was seen without affecting the numbers of Th1 cells (representing specific inhibition of Th17). Furthermore, RIA treatment also improved hepatocellular injury and fibrosis of the DCC-fed gnotobiotic mice (improved serum bilirubin and ALP).

FISH analysis was performed on the intestinal mucosa of 3-mix gnotobiotic mice. In comparison to SPF mice, bacterial DNA was detected underneath the intestinal epithelium in the 3-mix gnotobiotic mice, indicating bacterial invasion. This was also detected in mono-colonised *Klebsiella pneumoniae* gnotobiotic mice but not the *Enterococcus gallinarum* or *Proteus mirabilis* gnotobiotic mice. A monolayered organoid co-culture system was then employed to assess the interaction between bacteria and the epithelial surface. Two strains of *Klebsiella pneumoniae* were assessed (both derived from MLNs of PSC-UC mice). Both strains induced significant epithelial pore formation in the organoid model (assessed by scanning electron microscopy). Organoid RNA sequencing showed upregulation of genes related to apoptotic and inflammatory pathways. Fluorescein isothiocyanate (FITC)-conjugated dextran leakage analysis showed the gnotobiotic mice with epithelial pore-forming *Klebsiella* leaked FITC dextran into systemic circulation, indicating gut barrier dysfunction.

To confirm the role of pore-forming *Klebsiella pneumoniae* in the development of liver dysfunction, a modified 3-mix gnotobiotic mice (m3-mix) including a non-pore forming strain of *Klebsiella pneumoniae* showed no mucosal barrier dysfunction, lower serum endotoxin (LPS) and lower levels of Th17 priming in an alternative model of cholestatic liver injury using taurocholic acid (TCA).

Finally, gnotobiotic mice generated using faecal samples from one PSC-UC patient harbouring both *Klebsiella pneumoniae* and *Enterococcus gallinarum* were treated with metronidazole and vancomycin. The Th17 response was significantly decreased in the liver and colon of these mice.

Comment

Nakamoto and colleagues have demonstrated for the first time a mechanism by which the gut microbiota (pathobionts) are firstly responsible for the physical disruption of the intestinal epithelial barrier leading to bacterial translocation, secondly gnotobiosis promoting hepatobiliary injury in mouse models of cholestasis and thirdly their ability to induce Th17 differentiation in the colon and liver with colonisation of the 3-mix bacteria but not by mono-colonisation with *Klebsiella pneumoniae*, suggesting a co-operative relationship between pathobionts in PSC-UC.

Inhibition of RORγT blocked the DDC-induced hepatobiliary injury, whereas IL-17A inhibition was ineffective. The group postulate this could be due to a residual population of Th17 cell

cytokines. This work suggests a potential therapeutic option for PSC with RORγT inhibition. VTP-43742 (an oral RORγT reverse inhibitor) has now undergone a phase IIA clinical trial for psoriasis in which there was a significant clinical improvement (Expert Opin Ther Pat. 2017 27(1):1-8). These findings suggest this agent could be explored as a therapy in other autoimmune conditions where RORγT is implicated, such as PSC. Furthermore, this work clearly provides credence to previous work demonstrating a potential role of vancomycin and metronidazole in the treatment of PSC (Aliment Pharmacol Ther. 2013 Mar;37(6):604-12) and hints at the urgent need to design treatments that could selectively modulate the microbiome or inhibit selective pathobionts.

There have now been several cohort studies to assess the faecal and/or mucosal microbiome in patients with PSC (J Crohns Colitis 2015 9(4):342-8, Gut 2016 65:1681-1689, Aliment Pharmacol Ther 2016 43(7):790–801, Gut 2017 66(4):611-619, Gut 2017 66:386–388, World J Gastroenterol 2017 23(25):4548-4558, United European Gastroenterol J. 2018 6(1):112-122, United European Gastroenterol J 2018 6(1):112-122). Most of these studies have demonstrated decreased microbial diversity in the microbiome of PSC patients compared to IBD and/or healthy controls. This is the first cohort where an increased abundance of *Klebsiella* species have been demonstrated. Although *Klebsiella pneumoniae* demonstrated pore-forming capacity with associated bacterial translocation, this does not explain the pathogenesis of PSC in patients where these bacteria have not been identified in the microbiota. Therefore further mechanistic work is now needed with other bacteria frequently found in the dysbiotic microbiome of patients with PSC, for example *Veillonella* (Gut 2016 65:1681-1689, World J Gastroenterol 2017 23(25):4548-4558) to gain a better understanding of potential pathogenic mechanisms linking the gut microbiome with the development of PSC colitis and PSC. This could include further metagenetic analysis of pathobionts, taking into account the community of bacteria present but also to include within-species comparative genomics to identify genes and potential virulence factors associated with pathogenic profiles. Further work should also consider the patients' disease profile, including the degree of cholestasis, fibrosis, colitis and the concomitant effect of medications given to patients (for example biologics/ursodeoxycholic acid). It will be important to explore the influence of host genetics in PSC and how this interacts with the microbiome and influences disease susceptibility.

In conclusion, Nakamoto and colleagues clearly demonstrate the role of the microbiota in PSC-UC disease pathogenesis with *Klebsiella pneumoniae* directly disrupting the epithelial barrier allowing bacterial translocation and in conjunction with other pathobionts eliciting a Th17 priming in the liver and MLNs. Further work is needed to replicate these findings in a

larger cohort of patients with PSC-IBD from other geographic regions in comparison to control groups, through further analysis of the luminal and mucosal microbiome with further mechanistic work on key pathobionts identified. These findings provide insight into potential therapies for PSC, in particular in relation to target a key transcription factor for Th17 differentiation, RORγT.