Original research

Microbiota tryptophan metabolism induces aryl hydrocarbon receptor activation and improves alcohol-induced liver injury

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ABSTRACT

Objective Chronic alcohol consumption is an important cause of liver-related deaths. Specific intestinal microbiota profiles are associated with susceptibility or resistance to alcoholic liver disease in both mice and humans. We aimed to identify the mechanisms by which targeting intestinal microbiota can improve alcohol-induced liver lesions.

Design We used human associated mice, a mouse model of alcoholic liver disease transplanted with the intestinal microbiota of alcoholic patients and used the prebiotic, pectin, to modulate the intestinal microbiota. Based on metabolomic analyses, we focused on microbiota tryptophan metabolites, which are ligands of the aryl hydrocarbon receptor (AhR). Involvement of the AhR pathway was assessed using both a pharmacological approach and AhR-deficient mice.

Results Pectin treatment modified the microbiome and metabolome in human microbiota-associated alcohol-fed mice, leading to a specific faecal signature. High production of bacterial tryptophan metabolites was associated with an improvement of liver injury. The AhR agonist Ficz (6-formylindolo (3,2-b) carbazole) reduced liver lesions, similarly to prebiotic treatment. Conversely, inactivation of the *ahr* gene in alcohol-fed AhR *knock-out* mice abrogated the beneficial effects of the prebiotic. Importantly, patients with severe alcoholic hepatitis have low levels of bacterial tryptophan derivatives that are AhR agonists.

Conclusions Improvement of alcoholic liver disease by targeting the intestinal microbiota involves the AhR pathway, which should be considered as a new therapeutic target.

Significance of this study

What is already known on this subject?

- The intestinal microbiota is a causal factor of alcohol-induced liver lesions in mice and humans.
- Pectin is able to prevent alcohol-induced liver injury in mice by altering the intestinal microbiota.
- The protective effect of pectin is associated with an improvement of gut barrier function.

What are the new findings?

- Moderate amount of pectin can cure liver disease in a mouse model of alcoholic liver disease by changing microbiota.
- Pectin increases the production of tryptophan metabolites, which are aryl hydrocarbon receptor (AhR) ligands, by the microbiota, improving gut barrier function.
- Pharmacological activation of the AhR by Ficz, an exogenous AhR ligand, is sufficient to simulate the effect of pectin.
- AhR deficiency blocks the beneficial effect of pectin, suggesting that microbial products, via the AhR pathway, can reverse alcohol induced liver injury.

How might it impact on clinical practice in the foreseeable future?

- Therapeutic options in alcohol-induced liver injury are limited. Our results show that targeting intestinal microbiota using moderate amount of pectin can reverse alcohol-induced liver injury through the AhR pathway. Modifying intestinal microbiota to increase its production of AhR ligands or AhR ligand administration could be new therapeutic targets for alcoholic patients.

INTRODUCTION

Chronic alcohol consumption is a major cause of liver-related deaths.[1] Severe alcoholic hepatitis (sAH) is a life-threading form of alcoholic liver disease (ALD), with few therapeutic options.[2] Recent studies have shown that specific microbiota profiles are associated with susceptibility or resistance to alcohol-induced liver lesions in both mice and humans, opening new therapeutic options.[3–6] Moreover, the production of cytolysin by *Enterococcus faecalis* has been specifically shown to be involved in ALD development in 30% of sAH patients and its eradication by phagotherapy improves liver injury in a mouse model of ALD.[7 8] Aside from deleterious bacteria, it is also relevant to identify bacteria that can protect patients from developing alcohol-induced liver lesions and to understand the molecular mechanisms involved in such protective effects.

Several studies have reported that modulation of intestinal microbiota (IM) composition by faecal microbiota transfer[4 5 9] or treatment with *Akkermansia muciniphila*,[10] *Roseburia intestinalis*,[11] other probiotics or fibre/prebiotics[12 13] can improve liver injury in mouse models of ALD. Molecular mechanisms by which microbiota alterations can improve alcohol-induced liver lesions are poorly understood and involve changes in the gut barrier and bacterial metabolites.

Disruption of the gut barrier correlates with endotoxemia and the severity of ALD in humans and mice.[4 5 14] This disruption is associated with a decrease in the production of mucus and antimicrobial peptides and the disruption of tight junctions.[15–17] Modulation of the IM by faecal transfer, prebiotics or probiotics restores a leaky gut.[4 6 13 15] Moreover, changes in IM composition in patients with ALD induce modifications in microbiota-associated metabolites, including short chain fatty acids and bile acids,[12] and are involved in the severity of alcohol-induced liver injury.[5 18 19] Among microbiota-associated metabolites, tryptophan-derived indoles, produced by a large number of bacteria, including *Bacteroides,* are ligands of the aryl hydrocarbon receptor (AhR). AhR signalling improves the function of the intestinal barrier by increasing local expression of interleukin (IL-22)[20 21] and, consequently, increases expression of antimicrobial proteins.[22] Moreover, the abundance of Bacteroidetes and level of plasma tryptophan decrease after acute alcohol administration in humans, suggesting impaired tryptophan metabolism.[23]

Here, we aimed to identify the mechanisms by which targeting the IM with a prebiotic can improve alcohol-induced liver lesions. We used mice which were transplanted with the IM of patients with sAH (FMT), to work in the context of the human microbiota. We used the prebiotic fibre, pectin, to alter the IM. We and others have already shown that pectin can modify the mouse IM and prevent ALD by improving the leaky gut-barrier. However, the molecular mechanisms involved in this process and the effects of pectin in the context of the human IM are still unknown.[4 6] We now demonstrate that pectin reshapes the microbiome in the context of the human microbiota and not only prevents but also reverses alcohol-induced liver injury in mice. Metabolomic studies showed that changes in the microbiota composition also induced alterations in bacterial tryptophan metabolism, leading to the high production of indole derivatives, which activate the AhR. Pharmacological treatment of mice with an AhR agonist simulating the effect of pectin on the liver and reversed ALD, whereas inactivation of the *ahr* gene in knock-out mice abrogated the effects of the beneficial microbiota in alcohol-fed mice. The results observed in the humanised mice are also supported by a decreased level of AhR agonists in patients with sAH, suggesting that AhR may be a new therapeutic target in ALD.

MATERIAL AND METHODS

Mice

Female C57BL/6J mice (Janvier laboratory, Le Genest, France) were kept in humidity and temperature-controlled rooms, on a 12-hour light-dark cycle. Mice had access to a chow diet and water ad libitum before the study. Body weight and food intake were measured three times a week. All our experimental procedures were validated by the ethical committees and the French veterinary minister (2015052715405651_v2 (APAFIS#729) and 2017042314557080 v1(APAFIS#4788).

Treatments

In all our experiments, pectin was given at D21, as a curative treatment, using an alternative Lieber DeCarli diet containing different concentrations of pectin from apple (0.4%, 1%, 2% and 6.5%, w/w, Sigma-Aldrich, Saint Quentin Fallavier, France). To treat mice by an AhR agonist, the 6-formylindolo (3,2-b) carbazole (Ficz; Sigma-Aldrich) was resuspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich), diluted in olive oil (Sigma-Aldrich) and administered intraperitoneally. Ficz (1 µg/mouse) treatment was injected three times during the last week when mice were exposed to the maximum dose of alcohol (5%) and until euthanisation. Control mice received DMSO vehicle diluted in olive oil intraperitoneally alone for the Ficz treatment group.

Faecal microbiota transfer

Mice received faeces from alcoholic patients with sAH as previously described.[5 24] Two set of independent experiments were performed, a first set with faeces from two different patients (F_1 and F_2) and a second set with faeces from one patient (F_3). Briefly, faeces from human patients were recovered and immediately stored at 4°C in an anaerobiosis generator (Genbox, Biomérieux, Capronne, France) to favour the preservation of anaerobic bacteria. All samples were processed within 24 hours. Faeces were rapidly diluted 100-fold in Brain Heart Infusion (BHI, Becton Dickinson) supplemented with 0.5 mg/mL L-cysteine (Sigma-Aldrich, St-Louis, Missouri, USA) and 20% skim milk (Becton Dickinson) (vol/vol) and stored in aliquots at −80°C. This ready-to-use faecal suspension was used for FMT to mice.

Mice were fasted 1 hour and then subjected to bowel cleansing by oral-gastric gavage with PEG (polyethylene glycol, Macrogol 4000, Fortrans, Ipsen Pharma, France). Four hours later, mice received the human faeces by oral gastric gavage (200 µL of resuspended faeces prepared as described above). Mice were then allowed free access to food and water. FMT was repeated twice a week for 4 weeks. Bowel cleansing was only performed on day 1.

Patients

Two groups of patients were included in the study: patients with sAH and alcoholic patients without alcoholic hepatitis and without cirrhosis (noAH). All patients were admitted to the hepatogastroenterology department of Antoine-Béclère University Hospital, Clamart, France. Alcoholic patients were eligible for inclusion if they had consumed at least 50 g of alcohol per day over the previous year, were negative for hepatitis B surface antigens, and seronegative for antibodies against hepatitis C virus. Exclusion criteria were gastrointestinal bleeding, bacterial infection, hepatocellular carcinoma or other carcinoma, acute pancreatitis, other severe associated disease, diabetes mellitus, dyslipidaemia, presence of anti-HIV antibodies and antibiotic or probiotic intake in the last 3 months. A standardised questionnaire was used, to collect information about alcohol consumption.[25] sAH was suspected in patients with a Maddrey score >32 and was confirmed by a liver biopsy (histological score for AH ≥6 with neutrophilic infiltration).[26 27] Faeces from three independent patients with sAH were used for the faecal transfer in mice.

All the participants provided written informed consent.

Statistical analyses

Results are represented as the mean±SEM. Statistical comparison was performed by first testing the normality of the data using the Shapiro-Wilk test of normality and then performing unpaired Mann-Whitney, unpaired t-test, Kruskal-Wallis or analysis of variance tests as appropriate (Graphpad Prism, Graphpad Software, La Jolla, California, USA); $p<0.05$ was considered to be statistically significant.

See the online supplemental section for the sources of materials and detailed methods.

RESULTS

Altering the IM reverses alcohol-induced liver lesions

We tested whether altering the IM can reverse the progression of alcohol-induced liver lesions using mice which were transplanted

with the IM of patients with sAH (FMT) as we have previously shown that the IM worsens alcohol-induced liver lesions in this model.[5] Pectin, a dietary fibre known to favour the growth of specific bacterial genera, such as *Bacteroides*,[4 28] which are reduced by alcohol intake,[27] was used to alter the IM. Conventional (Alc) and FMT mice from three independent patients with sAH (Alc F_1, Alc F_2, Alc F_3) were fed alcohol using the Lieber DeCarli diet, as described previously[4] (figure 1A). The clinical characteristics of the donors with sAH are presented in online supplemental table 1. Principal coordinate analysis showed that alcohol, human microbiota transfer, and

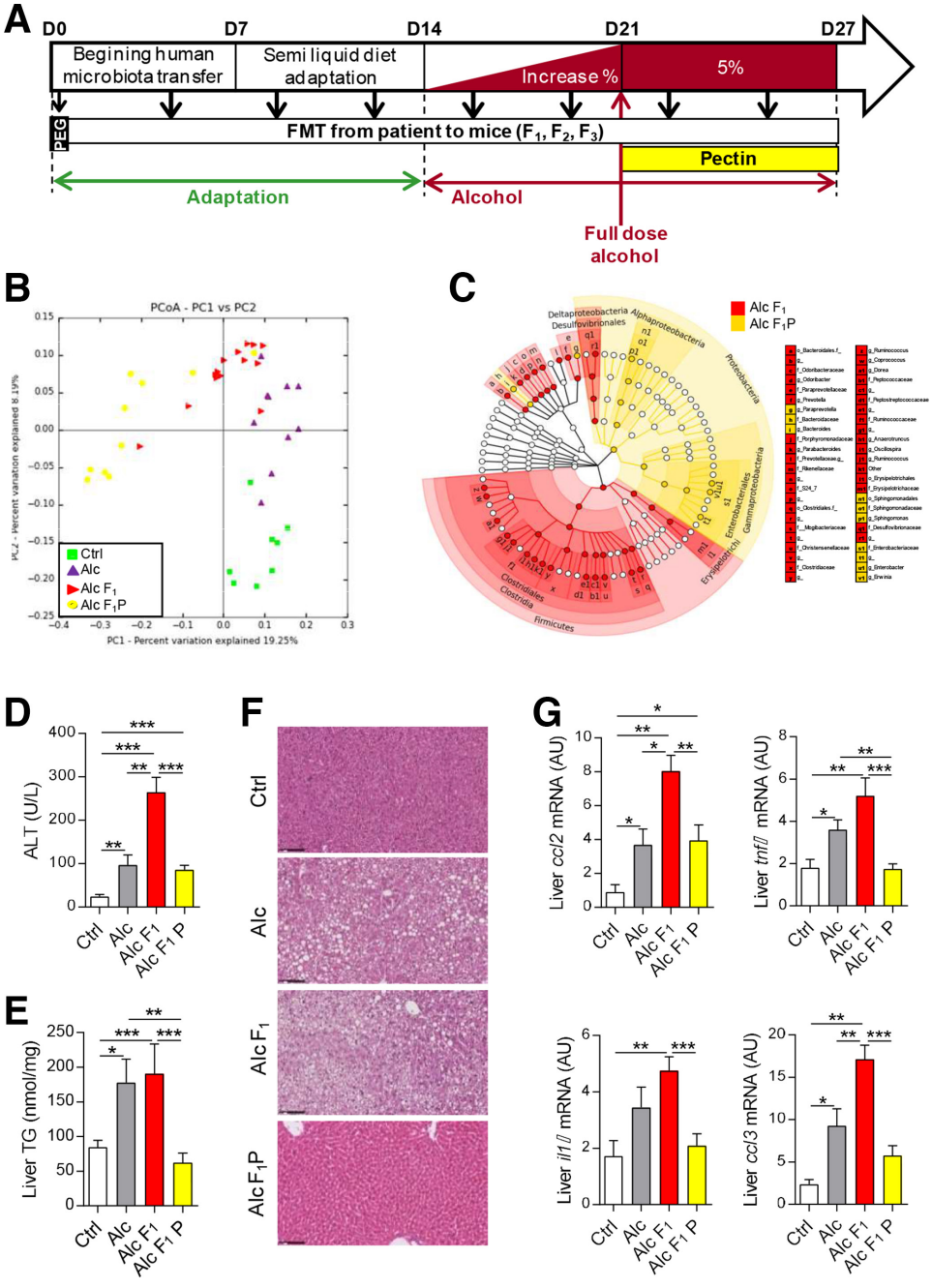

Figure 1 Altering the intestinal microbiota using pectin reverses alcohol induced liver lesions. (A) Experimental design: mice were progressively adapted to a semiliquid, Lieber DeCarli (LDC) diet, then an ethanol diet (1%–3%), and finally fed a 5% ethanol diet for 1 week. Pectin was introduced in the diet at the same time as the 5% ethanol. Microbiota analysis: (B) PCoA plot, showing the unweighted UniFrac distance (p<0.001, R=0.58, analysis of similarities (ANOSIM) test, 10 000 permutations, using the first 5 PC); (C) LDA effect size cladograms showing the taxa most differentially associated with Alc F_1 (red) or Alc F_1 P mice (yellow) (Wilcoxon rank-sum test). Circle sizes in the cladogram plot are proportional to bacterial abundance. The circles represent, going from the inner to outer circle: phyla, genus, class, order and family. (D) Alanine transaminase (ALT) level in Ctrl (n=8), Alc (n=8), Alc F_1 (n=12) and Alc F_1P (n=10) mice. (E) Liver triglyceride quantification in Ctrl (n=8), Alc (n=8), Alc F_1 (n=12) and Alc F_1P (n=10) mice. (F) Representative images of liver sections stained with H&E, scale bar 100 µm. (G) Liver mRNA levels determined by qPCR: *ccl2*, *tnfα*, *il1β* and *ccl3* normalised to that of the *gapdh* gene in Ctrl (n=4), Alc (n=5), Alc F_1 (n=12) and Alc F_1P (n=10) mice. Results (D–G) are shown as the mean±SEM. Significant results for *p<0.05, **p<0.01 and ***p<0.001 were determined by Mann-Whitney tests unless stated otherwise. Alc, alcohol-fed mice; Ctrl, control-fed mice; Alc F_1, alcohol-fed mice humanised with the microbiota from a patient with severe alcoholic hepatitis (patient F_1); Alc F_1 P, alcohol-fed mice humanised with microbiota from a patient with sAH (patient F_1) and treated with 6.5% pectin; LDA, linear discriminant analysis.

pectin treatment induced changes in IM composition (unweighted Unifrac, ANOSIM, r=0.59, p<0.001, figure 1B and online supplemental figure1A,C). These changes included an increase in the *Bacteroides* genus in the pectin-treated group (figure 1C and online supplemental figure1B,D).

FMT alcohol-fed mice developed liver lesions during the first week of alcohol intake, as shown by liver TG accumulation and an increase in ALT levels and several markers of inflammation (online supplemental figure2A–E). At this point, mice were treated with pectin in order to alter IM. Pectin did not modify the alcohol absorption (figure 1A and online supplemental figure 2F). Changing microbiota by using pectin, reversed alcohol-induced liver lesions in FMT mice fed alcohol. These mice (Alc F_1P) had lower levels of ALT (figure 1D), liver TG (figure 1E), steatosis (figure 1F) and liver inflammation markers (CC chemokine ligand (*ccl2 and ccl3*), tumour necrosis factor and *il1β*)(figure 1G) than Alc F_1 and Alc mice. We obtained similar modifications of the IM and recovery of alcohol-induced liver lesions in FMT mice using faeces from two other independent sAH patients (Alc F_2 and Alc F_3, online supplemental figures 3 and 4).

Altering the IM using high-dose of fibre may be associated with poor tolerance (bloating, abdominal distension).[29] We, therefore, tested the efficacy of lower doses of pectin on ALD. Two percent pectin induced similar changes of the alcohol-induced liver lesions and gut barrier function while improving treatment tolerance (online supplemental figure 4). Pectin treatment induced dose-dependent changes in the IM (online supplemental figure 5A–D). Among the specific changes observed in the linear discriminant analysis effect size analysis, Alc F_3 P2 showed an increase in the abundance of the Bacteroidetes phylum, a decrease in the abundance of the Firmicutes phylum, and an increase in the abundance of *Bacteroides* and *Lactobacillus* genera, similar to that of Alc F_3 P6.5 relative to Alc F_3 (online supplemental figure 5D). However, the increase in the abundance of Proteobacteria and Enterobacteriaceae observed in the Alc F_3 P6.5 mice was not observed in the Alc F_3 P2 mice (online supplemental figure 5E).

Altering the IM improves gut-barrier function in alcohol-fed human microbiota associated mice

Disruption of the intestinal barrier correlates with the severity of liver injury in ALD.[4–6 15 30] Alcohol-induced gut barrier disruption results in a decrease in the level of the antimicrobial peptides regenerating islet-derived protein (*reg3β* and *reg3γ*) and mucus production.[4 6] Restoration of these functions is required to improve alcohol-induced liver injury.[4 6 16 31] Altering the IM improved liver injury through improvement of the gut barrier function, as shown by an increase in *reg3β* and *reg3γ* messenger ribonucleic acid (mRNA) levels in the colon and ileum, and the proportion of goblet cells (online supplemental figure 5A–G). This was associated with an improvement of intestinal permeability, as shown by an increase in tight junction proteins (Zona occludens (*ZO-1*) and *occludin*) as shown by mRNA levels and immunofluorescence in the colon and ileum (online supplemental figure 6H,I and online supplemental figure 7) and a decrease of bacterial translocation into the liver (online supplemental figure 6J). These results show that altering the microbiota improves the gut barrier and reverses alcohol-induced liver injury, despite ongoing heavy alcohol consumption.

Altering the IM modifies its functions and the faecal metabolome

We next explored the functional impact of altering the IM using pectin by generating the predicted metagenome using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.[32] A total of 4,977 Kyoto Encyclopedia ofGenes and Genomes (KEGG orthologs were assigned to 146 metabolic pathways and 115 structural complex modules. Pectin-treated mice showed a higher number of bacterial genes involved in carbohydrate, lipid, and amino-acid metabolism (figure 2A). Conversely, control mice showed a higher number of bacterial genes involved in amino-acid, energy and cofactor and vitamin metabolism. We obtained similar predicted metagenome profiles in mice transplanted with the IM of the two other independent patients (F_2 and F_3) (online supplemental table 2).

We further studied whether such changes in the bacterial pathways induce alterations in the faecal metabolome by performing targeted metabolomic profiling. Principal component analysis and heatmaps showed that altering the IM induced a specific faecal metabolomic profile (figure 2B,C). Enrichment analysis using metabolom analyst led to the identification of 52 pathways that were modified between Alc F_1 and Alc F_1 P mice (false-discovery rate <0.05) (figure 2D and online supplemental table 3). These pathways belong to the metabolism of amino acids (lysine, tyrosine, tryptophan, valine, leucine, isoleucine and beta-alanine), carbohydrates (starch and sucrose, pentose and glucose interconversion, and ascorbate) lipids and vitamins (biotin and ascorbate). Many of the changes in the faecal metabolomic pathways belong to those highlighted by the predicted bacterial metagenome (figure 2A,D). These results were also confirmed when using lower doses of pectin (online supplemental figure 8).

Among the amino acids of which the levels were modified by pectin, we specifically identified a decrease in the levels of tryptophan and indole, precursors of microbiota-derived tryptophan metabolites (figure 2E). We then performed specific metabolomic profiling of tryptophan metabolites, as we observed an increase in the abundance of *Bacteroides*, taxa that can metabolise tryptophan into indole derivatives, in pectin-treated mice. We observed a decreased level of indole-3-acrylic acid in alcohol-fed conventional and FMT mice as compared with control mice and a decrease in overall AhR agonists (sum of 3-indoxyl sulfuric acid, 5-Methoxy-3-indoleacetic acid, indole-3-acetic acid, indole-3-acrylic acid, indole-3-aldehyde, indole-3-lactic acid, indole-3-propionic acid) in alcohol-fed FMT mice. These changes were restored after pectin treatment with an overall increase in total AhR agonists in pectin treated mice (figure 2F).

Activation of the AhR pathway improves alcohol-induced liver injury

Altering the IM with pectin in the FMT mouse model of ALD reverses alcohol-induced injury and is associated with changes in the microbiota and tryptophan metabolism. We, therefore, studied the role of the AhR pathway, which can be activated by bacterial tryptophan metabolites. We analysed the expression of cytochrome P (*cyp1a1*) and AhR repressor (*ahrr*), target genes of AhR activation in the colon. Their expression in the colon (figure 3A), together with that of *il22*[20] and *il17* (figure 3B), which are also controlled by AhR activation,[33] increased after altering the IM by pectin treatment. We then assessed the direct involvement of AhR in the improvement of alcohol-induced injury by treating mice with an AhR agonist, 6-formylindolo (3,2-b) carbazole (Ficz). Treatment of Alc mice with Ficz increased the expression of AhR target genes *Cyp1a1* and stearoyl-coenzyme A desaturase (*Scd1*) in the liver (figure 3C) and decreased alcohol-induced liver lesions, with a decrease in ALT, liver TG and inflammatory marker levels (figure 3D,E). Treatment with Ficz also increased antimicrobial peptide levels in the colon and ileum (figure 3) but only the expression of *Reg3γ* in the ileum reached statistical

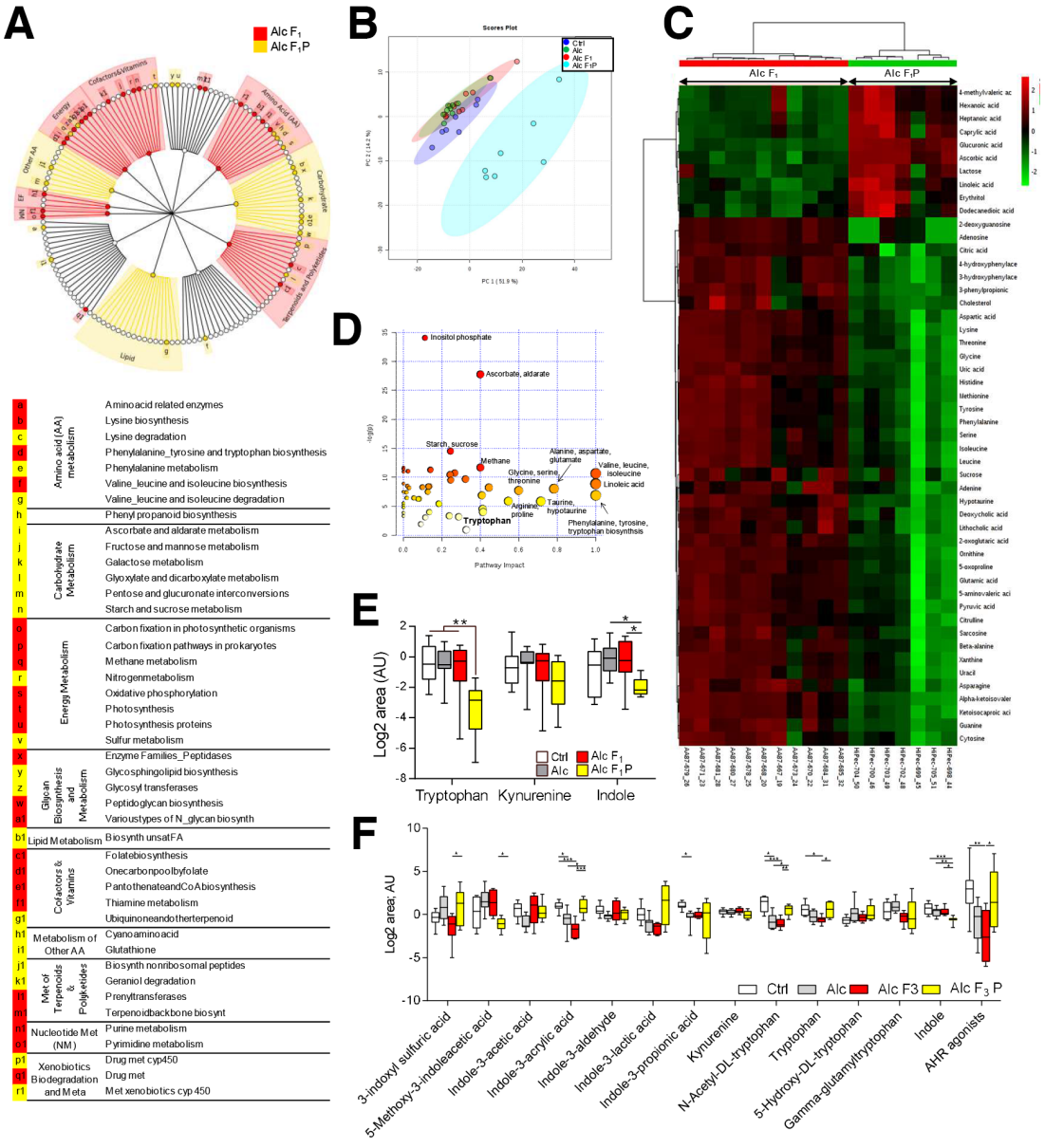

Figure 2 Functions of the intestinal microbiota and faecal metabolome are modified by pectin treatment. (A) LEfSe cladograms of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway contributions of predicted metagenomic data in Alc F_1 and Alc F_1P mice (Wilcoxon rank-sum test). In (A): Ctrl n=8, Alc n=10, Alc F_1 n=14, and Alc F_1P n=10 mice per experiment. (B) PCA ordination plot on all faecal metabolomic data (147 metabolites). (C) Heatmap showing the first 60 metabolites ranked by t-tests between Alc F_1 (red) and Alc F_1P (green). (D) Metabolic enrichment analysis showing the most altered pathways in Alc F_1P mice relative to Alc F_1 mice. All matched pathways are displayed as circles. The colour and size of each circle are based on the p value and pathway impact value, respectively. The graph was obtained by plotting the –log of p values from the pathway enrichment analysis on the y axis and the pathway impact values, derived from the pathway topology analysis, on the x-axis. (E) Relative faecal levels of tryptophan, kynurenine and indole. In (B–E): Ctrl n=8, Alc n=10, Alc F_1 n=11 and Alc F_1P n=7 mice per group. (F) Tryptophan metabolites quantification in faeces. Aryl hydrocarbon receptor (AhR) ligands (methyl-indole 3-acetic acid, indole 3-propionic acid, indole 3-aldehyde, indole 3-acrylic acid, and 3-indoxyl sulfuric acid). In f): Ctrl n=8, Alc n=9, Alc F_3 n=6, and Alc F_3P n=8 mice per group. *p<0.05, **p<0.01, ***p<0.001 by the ANOVA or Kruskal-Wallis test with Tukey or Dunn correction for multiple comparisons, as appropriated. ANOVA, analysis of variance; Alc, alcohol-fed mice; Alc F_1 and Alc F_3, alcohol-fed mice humanised with microbiota from a patient with sAH (patients F_1 or F_3); Alc F_1P and Alc F_3P, alcohol-fed mice humanised with microbiota from a patient with sAH (patients F_1 or F_3) and treated with 6.5% pectin (F_1) or 2% pectin (F_3); LEfSe, linear discriminant analysis effect size.

significance, simulating the effects of pectin. We next tested whether AhR signalling mediates the effects of pectin using alcohol-fed mice deficient for AhR (AhR KO). Although pectin treatment still restored ALT levels in alcohol-fed AhR KO mice, it could neither alleviate steatosis (figure 4A,B) nor restore *cyp1a1* and *ahrr* mRNA levels in the colon (figure 4C). Accordingly, restoration of *il22*, *reg3β*, and *reg3γ* mRNA levels in the colon by pectin treatment was also abrogated (figure 4D). Overall, these data show that the effects of pectin are, at least partially, mediated by AhR pathways.

Tryptophan metabolism is impaired in patients with sAH

We next explored the relevance of impaired tryptophan metabolism in the context of human disease and analysed faecal and

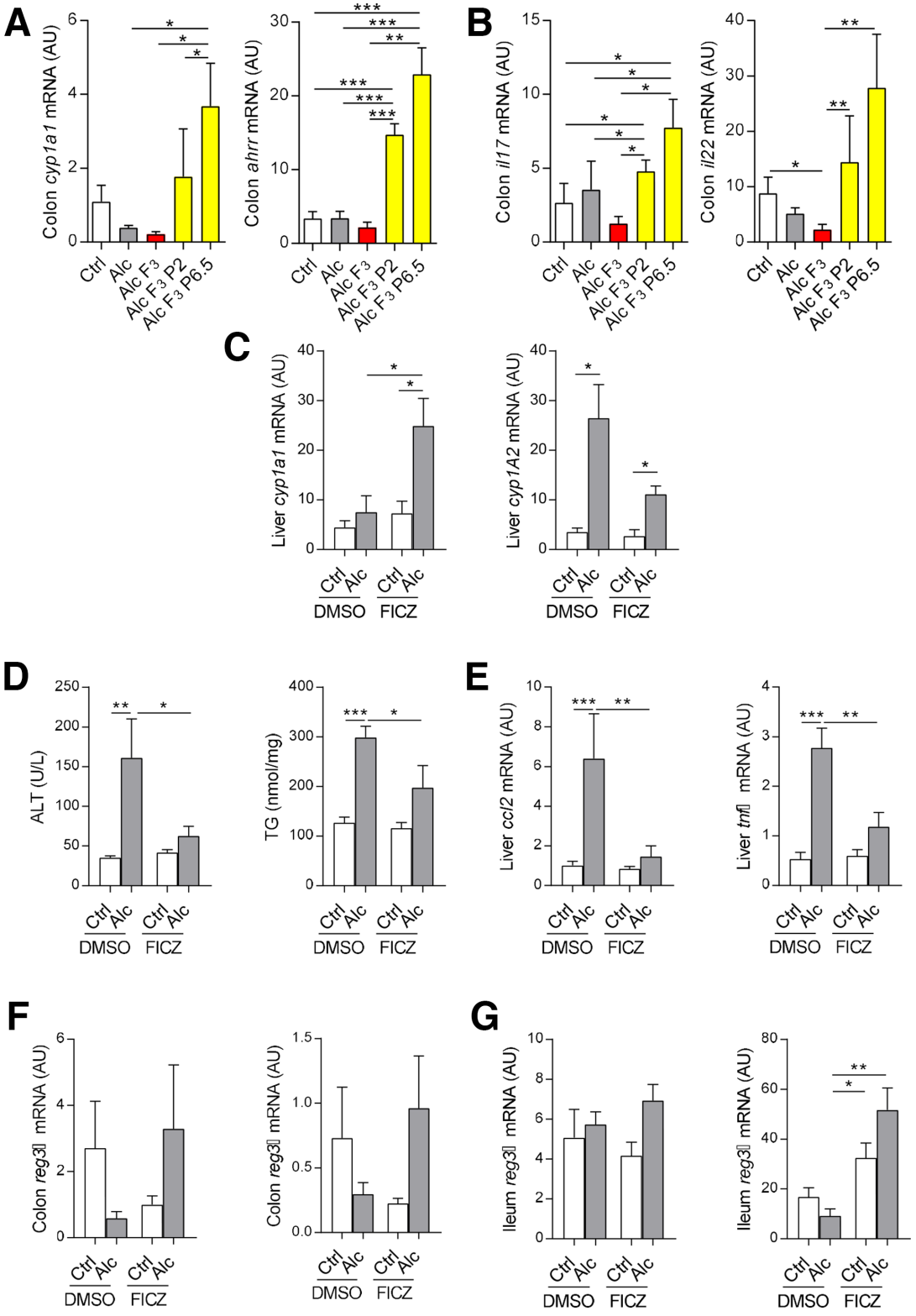

Figure 3 AhR activation reverses alcohol-induced liver lesions. (A, B) Alc F_3, alcohol-fed mice humanised with the microbiota from a patient with sAH (patient F_3); Alc F_3 P2, Alc F_3 P6.5, alcohol-fed mice humanised with the microbiota from a patient with sAH (patient F_3) and treated with two or 6.5% pectin. Colon mRNA levels were determined by qPCR: (A) *cyp1a1* and *ahrr,* (B) *il17* and *il22*, normalised to that of the *18s* gene, in Ctrl (n=7), Alc (n=8), Alc F_3 (n=4), Alc F_3 P2 (n=15), and Alc F_3 P6.5 (n=16) mice. (C–G) DMSO or Ficz, mice treated with DMSO or Ficz. (C) Liver *cyp1a1* and *scd1*, normalised to that of the *gapdh* gene. (D) ALT level and liver triglyceride quantification in Ctrl DMSO (n=7), Alc DMSO (n=12), Ctrl Ficz (n=8), and Alc Ficz (n=8) mice. (E) Liver mRNA levels determined by qPCR: *ccl2* and *tnfα*, normalised to that of the *gapdh* gene, in Ctrl DMSO (n=6), Alc DMSO (n=11), Ctrl Ficz (n=8), and Alc Ficz (n=7) mice. (F) Colon mRNA levels determined by qPCR: *reg3β* and *reg3γ*, normalised to that of the *gapdh* gene. (G) Ileum mRNA levels determined by qPCR: *reg3β* and *reg3γ*, normalised to that of the *gapdh* gene, in Ctrl DMSO (n=8), Alc DMSO (n=12), Ctrl Ficz (n=8), and Alc Ficz (n=10) mice. *p<0.05, **p<0.01, ***p<0.001. AhR, aryl hydrocarbon receptor; Alc, alcohol-fed mice; Ctrl, control-fed mice; sAH, svere alcoholic hepatitis.

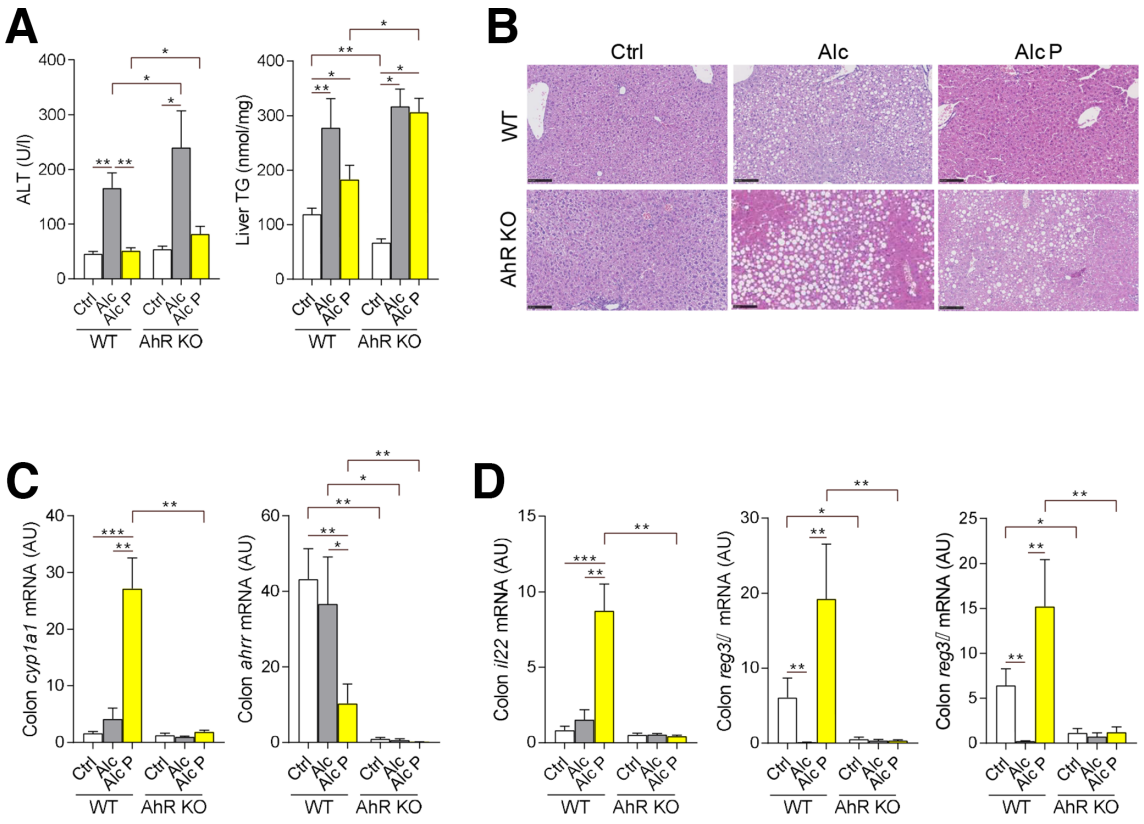

Figure 4 KO of AhR partly blocks the liver protective effects of pectin. (A–D) WT, wild type mice; AhR KO, AhR deficient mice; Alc P2, alcohol-fed mice treated with 2% pectin. (A) ALT levels and liver triglyceride quantification in WT Ctrl (n=8), WT Alc (n=5), WT Alc P (n=8), AhR KO Ctrl (n=4), AhR KO Alc (n=4) and AhR KO Alc P (n=5) mice. (B) Representative images of liver sections stained with hematoxylin-eosin, scale bar 100 µm. (C, D) Colon mRNA levels determined by qPCR: *cyp1a1*, *ahr*, *il22*, *reg3β* and *reg3γ*, normalised to that of the *18s* gene, in WT Ctrl (n=8), WT Alc (n=5), WT Alc P (n=8), AhR KO Ctrl (n=4), AhR KO Alc (n=4) and AhR KO Alc P (n=5) mice. Results are shown as the mean±SEM. Significant results for *p<0.05, **p<0.01, and ***p<0.001 were determined by Mann-Whitney U tests unless stated otherwise. AhR, aryl hydrocarbon receptor; Alc, alcohol-fed mice; Ctrl, control-fed mice; *p<0.05, **p<0.01, ***p<0.001.

serum samples of alcoholic patients with (sAH) or without alcoholic hepatitis (noAH) (online supplemental table 4). There were no differences in the faecal levels of tryptophan, kynurenine or AhR agonists between alcoholic patients, regardless of the severity of the liver injury (noAH or sAH) (data not shown). There was also no difference in the serum level of kynurenine between alcoholic patients (figure 5A). Conversely, serum levels of tryptophan and AhR agonists were lower in sAH patients than in noAH patients (figure 5A). We also found negative correlations between serum levels of Trp and AST ($r=-0.6$, $p<0.01$), bilirubin ($r=-0.7$, $p<0.001$), prothrombin time ($r=-0.7$, $p<0.001$) and MELD score ($r=-0.6$, $p<0.001$) (figure 5B). This suggests that tryptophan metabolism is impaired in patients with alcoholic hepatitis and that the modulation of AhR could be a new therapeutic target.

DISCUSSION

The IM plays a role in the pathophysiology of ALD and bacterial composition contributes to the severity of liver injury, independently of alcohol intake.[2 5] Bacteria interact directly with the host and indirectly through a large panel of bacterial metabolites.[34] Impairment of several bacterial metabolic functions has been shown to exacerbate ALD, including that of bacterial synthesis of saturated long-chain fatty acids,[35] bile acids[5 19 27] and tryptophan.[36] In ALD, disruption of the intestinal barrier correlates with the severity of liver lesions.[4 15 30] The role of the IM in the development of a leaky gut is associated with decreased levels of antimicrobial Reg 3 peptides and decreased mucus production.[4 16] Altering the IM using probiotics or prebiotics in murine models can prevent ALD by modulating these functions.[6 10 37–39] Specifically, pectin, a fibre that modulates the IM, can prevent alcohol-induced liver injury by improving gut barrier function.[4] Nevertheless, a preventive effect of such a treatment is not relevant for patients with alcohol-use disorders that have ALD. Therefore, we addressed the effect of pectin in a mouse model of ongoing alcohol administration after the onset of liver injury. We focused on bacterial indole derivatives, as the molecular mechanisms by which fiber-induced changes of the IM improve ALD have not been elucidated.

Here, we show that pectin, used as a curative treatment, is able to reverse alcohol-induced liver injury in the context of the human microbiota. We used FMT transplanted with the faeces of alcoholic patients with sAH. Liver injury in these mice is worse than that of wildtype alcohol-fed mice. Improvement of liver lesions is associated with improved gut barrier function, including the restoration of mucus production and antimicrobial Reg 3 peptide levels. Pectin, as a dietary fibre, is known to favour the growth of specific bacterial genera, such as *Bacteroides*.[4 28] In alcoholic patients, sAH is associated with a decrease in the abundance of Bacteroidetes and changes in IM function.[5 40] A similar decrease in the abundance of Bacteroidetes has also been observed in animal models of ALD.[4] Here, we show that pectin induces an increase in the abundance of *Bacteroides*, regardless of the effective dose.

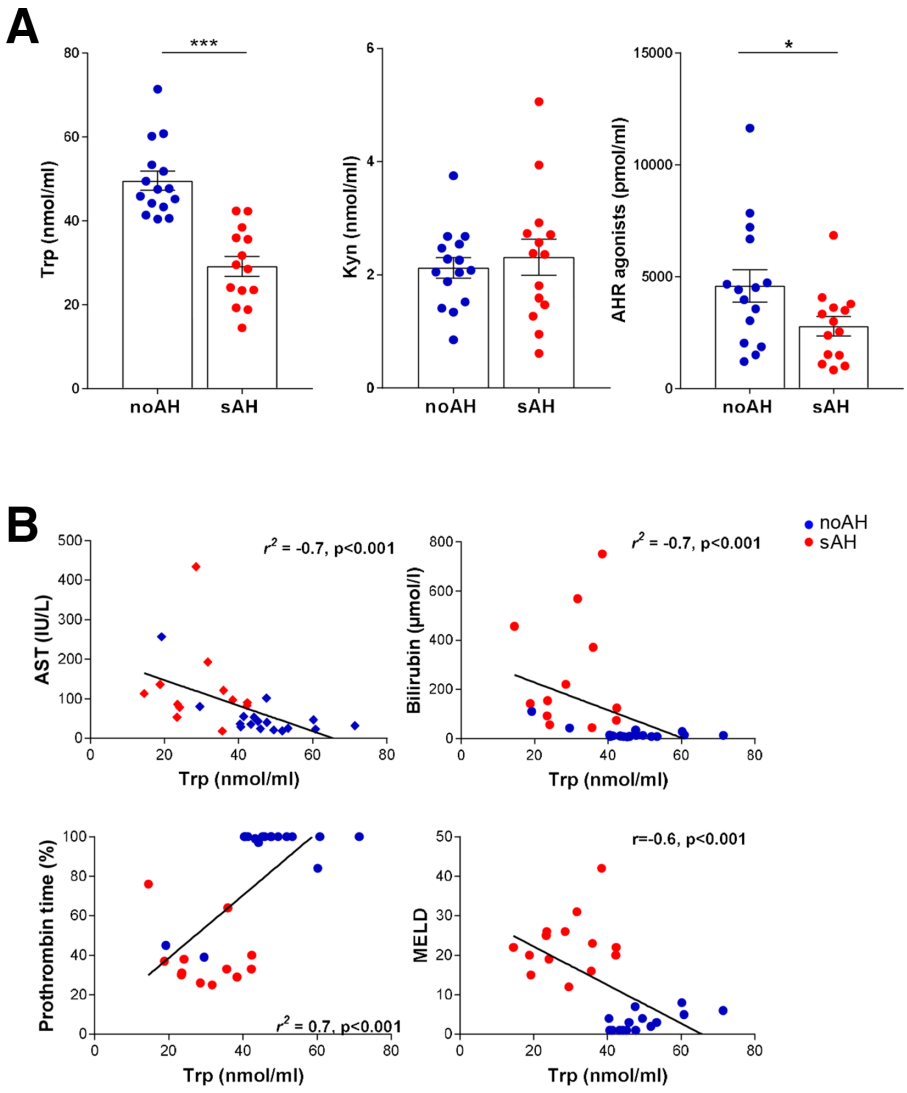

Figure 5 Tryptophan metabolism is reduced in patients with severe alcoholic hepatitis (sAH) and correlates with disease severity. (A) Serum concentrations of tryptophan, kynurenine and AHR agonists (tryptamine, indole, indole 3-acetic acid, indole 3-acetaldehyde and indoxyl sulfate) in sAH patients (n=14) and patients without sAH (noAH, n=15). (B) Spearman correlation of the serum tryptophan and AST, bilirubin, and prothrombin time levels and MELD score. *p<0.05, ***p<0.001.

A high dose of pectin (6.5%) was also associated with an increased abundance of Proteobacteria, which could pose safety concerns, as several species of this phylum are considered to be opportunistic pathogens.[41] Moreover, fermentable fibre (including pectin) has been reported to induce an increase in the abundance of Proteobacteria and hepatocarcinoma in several animal models (Toll like receptor (TLR5, TLR4) and Lipocalin 2 deficient mice). This is due to the inability of the innate immunity in the gut to prevent the translocation of Proteobacteria species.[42] However, in our study, the lower dose of pectin (2%) used to improve intestinal tolerance to a diet rich in fibre abrogated Proteobacteria overgrowth and achieved the beneficial effects that we observed on alcohol-induced liver lesions with a pectin-enriched diet. The amount of pectin to administrate in patients to match the minimal effective dose described in our study (2%) would be of 40 g/day. Of note, the recommended daily dose of fibre intake ranges between 30–38 g/day in men and 21–25 g/day in women.[43] Nevertheless, the amount of fibre consumed by humans is dependent on their diet. It has been suggested that omnivores consumed less than 23 g of fibres/day, vegetarians significantly more (37 g/day) and vegans the most (47 g/day).[44] Patients with chronic liver disease (viral and alcoholic cirrhosis) have a lower intake of vegetables which are rich in fibres.[45] Moreover, a high-fibre diet has been related to regression of non-alcoholic fatty liver disease[46] and recent epidemiological data showed that dietary fibre intake, especially soluble fibres (such as pectin), is inversely associated with the risk of several chronic diseases and with mortality.[47] Studies investigating pectin administration in different conditions used up to 60 g/day and reported good tolerance.[48] The main side effect was bloating but individual sensitivity to develop side effects is highly variable.[49] These data suggest that a moderate amount of pectin may be a promising and safe alimentary complement in the management of alcoholic patients.

We further analysed intestinal metabolites to investigate the mechanisms by which pectin-induced modifications of the IM reduce alcohol-induced gut and liver injuries. The microbiota reshaped by pectin harboured more genes involved in amino acid and xenobiotic metabolism. This metagenomic prediction was confirmed by the quantification of faecal metabolites. We specifically identified a decrease in tryptophan levels and an increase in the level of indole derivatives. These metabolites are

only produced by the IM from tryptophan.[36] Several microbiota-derived tryptophan metabolites are able to activate the AhR, thus playing a key role in gut homeostasis through the regulation of anti-microbial peptide and mucus production by IL-22.[21] Moreover, it has been previously shown that IL-22 is downregulated in alcohol-fed mice and oral treatment with recombinant IL-22 or bacteria that produce this cytokine prevents alcohol-induced liver injury.[36 50] AhR activation has been shown to improve inflammatory bowel disease[51] and metabolic syndrome.[36 52 53] Moreover, hepatic AhR activation prevents HSC activation and the expression of genes required for liver fibrogenesis by disrupting the interaction of Smad3 with β-catenin.[54] Here, we show that the improvement of mucus and anti-microbial peptide production by pectin is associated with the restoration of AhR-responsive gene expression, including that of IL-22, Cyp1a1, and ahrr. Conversely, low levels of *reg3β*, *reg3γ* and mucus production in untreated alcohol-fed mice correlated with lower levels of *il22*, *Cyp1a1*, and *ahrr*.

We also treated mice with an AhR agonist, Ficz, to address the direct involvement of AhR in the effects of pectin. Ficz treatment was sufficient to mediate a reduction in alcohol-induced injury. In contrast, pectin treatment of AhR-deficient alcohol-fed mice had only a minimal effect on alcohol-induced liver lesions suggesting that the effects of pectin treatment in our model of ALD are not solely medicated by AhR. Indeed, pectin induces broad changes at the microbial and metabolomic level and other mechanisms independent of AhR could also mediate the effect observed in our study.

The relevance of impaired tryptophan metabolism in the context of human disease was confirmed by reduced serum levels of tryptophan and AhR agonists in patients with sAH. Conversely, there were no differences in the faecal levels of tryptophan, kynurenine or AhR agonists between alcoholic patients, regardless of the severity of the liver injury (noAH or sAH). However, it has been reported that patients with alcoholic hepatitis have lower levels of faecal indole-3-acetic acid and indole-3-lactic acid than healthy patients who do not consume alcohol.[36] These discrepancies suggest that alcohol induces impairment of tryptophan metabolism independently of liver disease.

Our results provide the basis for further studies in patients with ALD that will aim to correct the AhR-ligand deficiency. Indeed, it has been recently shown that *Lactobacillus reuteri*, which is known to produce AhR agonists, improves ALD,[36] as well as treatment with a direct agonist, such as indole-3 acetic acid. Moreover, treatment with *Lactobacillus reuteri* can also improve metabolic syndrome[52] and colitis[55 56] in animal models. Indole-3-pyruvic acid, an AhR agonist, improves experimental colitis[57] and indigo, a tryptophan metabolite that activates the AhR, is effective in inducing remission in patients with ulcerative colitis.[58]

In conclusion, our study shows that alcohol-induced liver lesions can be reversed by modifying AhR-agonist production by the IM. As there is no treatment that can reverse alcohol-induced liver lesions other than liver transplant, modulation of the AhR pathways by supplementation with prebiotics, AhR ligand-producing bacteria or pharmacological AhR ligands, may hold promise in the development of new therapeutic approaches to ALD.

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