Chemico-Biological Interactions 325 (2020) 109131

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Potential role of gut microbiota, the proto-oncogene PIKE (*Agap2*) and cytochrome P450 CYP2W1 in promotion of liver cancer by alcoholic and nonalcoholic fatty liver disease and protection by dietary soy protein

Martin J. Ronis[a,*], Kelly E. Mercer[b], Kartik Shankar[b], Casey Pulliam[a], Kim Pedersen[a], Magnus Ingelman-Sundberg[c], Simonetta Friso[d], Derrick Samuelson[a], Luis Del Valle[a], Chris Taylor[a], David A. Welsh[a]

[a] Louisiana State University Health Sciences Center, New Orleans, USA
[b] Arkansas Children's Nutrition Center, USA
[c] Karolinska Institute, Sweden
[d] University of Verona, Italy

ARTICLE INFO

Keywords:
Ethanol
NASH
Liver cancer
Soy
Microbiota
CYP2W1

ABSTRACT

We have previously demonstrated promotion of diethylnitrosamine (DEN) initiated liver tumorigenesis after feeding diets high in fat or ethanol (EtOH) to male mice. This was accompanied by hepatic induction of the proto-oncogene PIKE (*Agap2*). Switch of dietary protein from casein to soy protein isolate (SPI) significantly reduced tumor formation in these models. We have linked EtOH consumption in mice to microbial dysbiosis. Adoptive transfer studies demonstrate that microbiota from mice fed ethanol can induce hepatic steatosis in the absence of ethanol suggesting that microbiota or the microbial metabolome play key roles in development of fatty liver disease. Feeding SPI significantly changed gut bacteria in mice increasing alpha diversity ($P < 0.05$) and levels of *Clostidiales spp*. Feeding soy formula to piglets also resulted in significant changes in microbiota, the pattern of bile acid metabolites and in inhibition of the intestinal-hepatic FXR/FGF19-SHP pathway which has been linked to both steatosis and hepatocyte proliferation. Moreover, feeding SPI also resulted in induction of hepatic PPARα signaling and inhibition of PIKE mRNA expression coincident with inhibition of steatosis and cancer prevention. Feeding studies in the DEN model with differing dietary fats demonstrated tumor promotion specific to the saturated fat, cocoa butter relative to diets containing olive oil or corn oil associated with microbial dysbiosis including dramatic increases in *Lachnospiraceae* particularly from the genus *Coprococcus*. Immunohistochemical analysis demonstrated that tumors from EtOH-fed mice and patients with alcohol-associated HCC also expressed high levels of a novel cytochrome P450 enzyme CYP2W1. Additional adoptive transfer experiments and studies in knockout mice are required to determine the exact relationship between soy effects on the microbiota, expression of PIKE, CYP2W1, PPARα activation and prevention of tumorigenesis.

1. Introduction

HCC is the world's second leading cause of cancer mortality. It is found 3-times more frequently in men than women [1–3]. Incidence of HCC has increased in the U.S. since the 1970s from 1.6 to 4.9/100,000 [1]. One-year survival rates remain below 50% [1]. Fatty liver diseases produced by alcohol (EtOH) abuse (alcoholic steatohepatitis, ASH) and associated with obesity, type II diabetes, metabolic syndrome and high levels of dietary fat (nonalcoholic steatohepatitis, NASH) are well-known risk factors for HCC. ASH and NASH account for 36–67% of all HCC cases [4,5]. The mechanisms whereby EtOH/high fat consumption cause HCC remain incompletely understood and there are currently few clinical strategies to treat HCC in patients with ASH/NASH other than by liver transplant. Epidemiological data suggest that consumption of > 80 g/d of alcohol over 10 years increases the risk of HCC 5-fold in Western populations [6]. Moreover, obesity rates have also recently risen dramatically with an estimated 5% increase in HCC risk for each unit of body mass index (BMI) [5]. There is evidence that EtOH and dietary fat may initiate tumors as a result of generation of reactive oxygen species and as a result of increased activation of environmental

* Corresponding author. Department of Pharmacology & Experimental Therapeutics, Louisiana State University Health Sciences Center, 1901 Perdido Str, New Orleans, 70112, USA.
E-mail address: mronis@lsuhsc.edu (M.J. Ronis).

https://doi.org/10.1016/j.cbi.2020.109131
Received 13 June 2019; Accepted 6 May 2020
Available online 14 May 2020

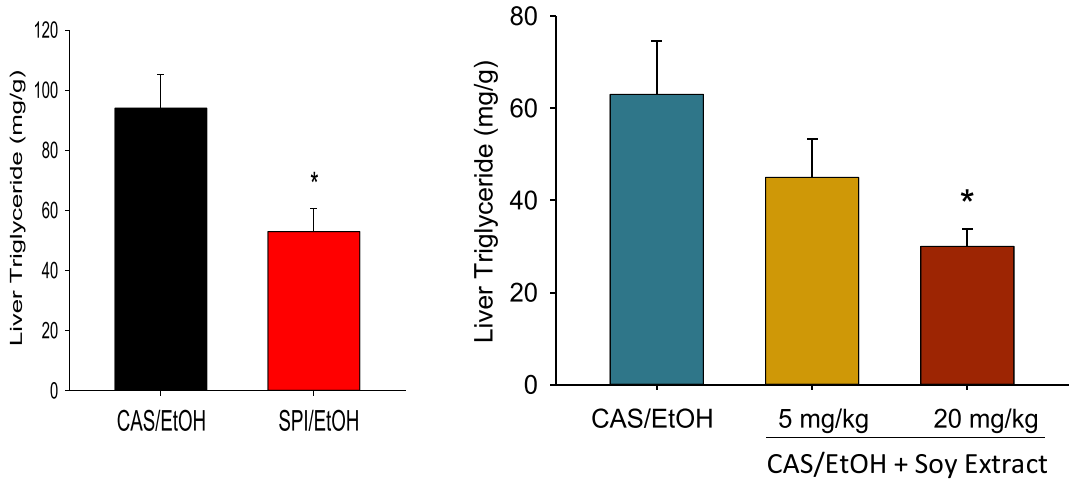

Fig. 1. Hepatic triglyceride concentrations in male mice fed casein (CAS) or SPI-containing Lieber DeCarli ethanol (EtOH) diets or the casein diet + soy phytochemical extract in the NIAAA chronic + binge model of alcohol exposure. Data are mean ± SEM for N = 5–6/group, *P < 0.05 vs CAS/EtOH.

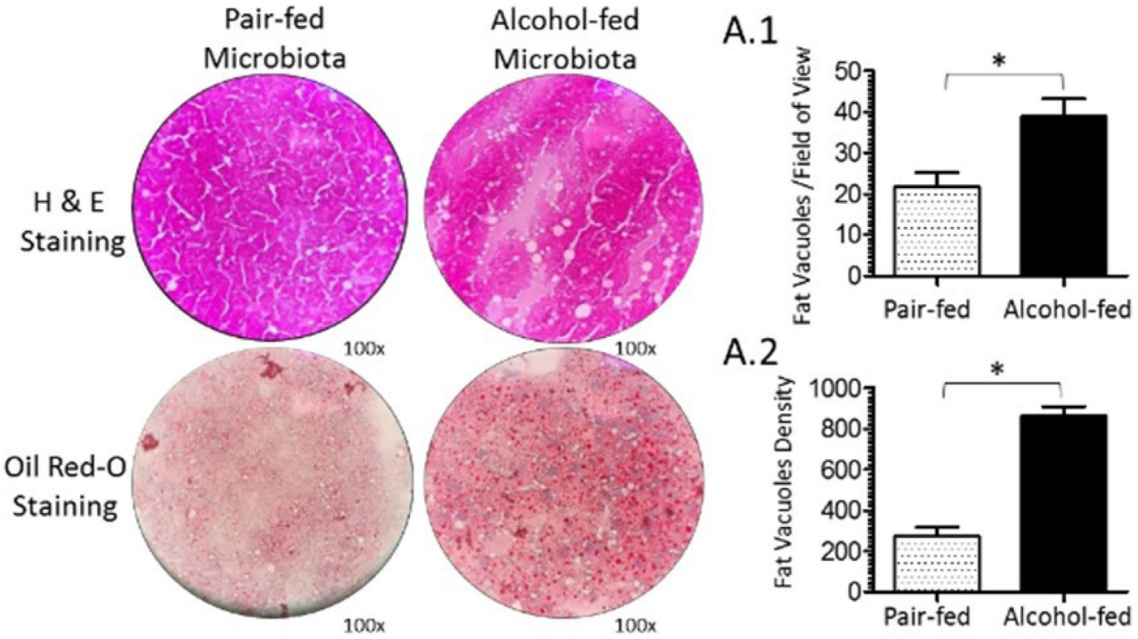

Fig. 2. Accumulation of lipid droplets in livers of antibiotic-treated mice subsequently treated with microbiota from cecum of mice fed EtOH Lieber DeCarli diets in the absence of alcohol vs. mice subsequently treated with microbiota from pair-fed mice in adoptive transfer experiments using a modified NIAAA chronic + binge model ( ). Data are mean ± SEM for N = 5–6/group, *Effects of EtOH microbiota statistically significant P < 0.05.

pro-carcinogens such as nitrosamines found in well-cooked meats and cigarette smoke by the major EtOH and dietary fat-inducible cytochrome P450 enzyme CYP2E1 [6–9]. EtOH and FA metabolism by CYP2E1 also produce reactive metabolites including acetaldehyde and lipid peroxides. EtOH disrupts of one-carbon metabolism which may further contribute to initiation [2]. Alcohol use and dietary fat consumption may also synergize with hepatitis C and other initiating factors such as cigarette smoking in development of HCC [10,11]. However, a major role for EtOH/dietary fat appears to be to act as tumor promoters [4,6]. Chronic EtOH consumption in experimental animals results in continuing liver injury with the appearance of inflammation and fibrosis [8,12]. Many groups have reported increased hepatocyte proliferation after chronic alcohol consumption as a repair response [12–14]. A similar pattern of progressive injury accompanied by increased hepatocyte proliferation has been observed in animal models of high fat-driven NASH [15–17]. The hepatic regenerative response associated with fatty liver disease is influenced by dietary EtOH/fat concentration, length of exposure, fat type, nutritional status and hormonal milieu. It has been suggested that the pro-proliferative signals associated with the regenerative response are responsible for tumor promotion [6,18–20].

We have demonstrated that EtOH and dietary fat act as hepatic tumor promoters in mice where carcinogenesis was initiated by treatment with a single dose of diethylnitrosamine (DEN) on postnatal day (PND) 13 [18–21]. Tumor development was coincident with development of steatosis, elevation of ceramide/sphingosine synthesis and development of inflammation and fibrosis [18–20]. Development of ASH was additionally linked to depletion of hepatic retinoids [18]. These models replicate the progression of HCC in fatty liver diseases observed clinically. We have previously shown that tumors in the DEN/EtOH mouse model were β-catenin positive [18]. In addition, increased β-catenin signaling has also been reported in a DEN/high fat mouse model of NASH-driven HCC [20].

There is epidemiological data to suggest that Asians are at lower risk of development of alcoholic HCC. In the U.S. there is a reported 5-fold increased risk of HCC among chronic or excessive drinkers [1]. In

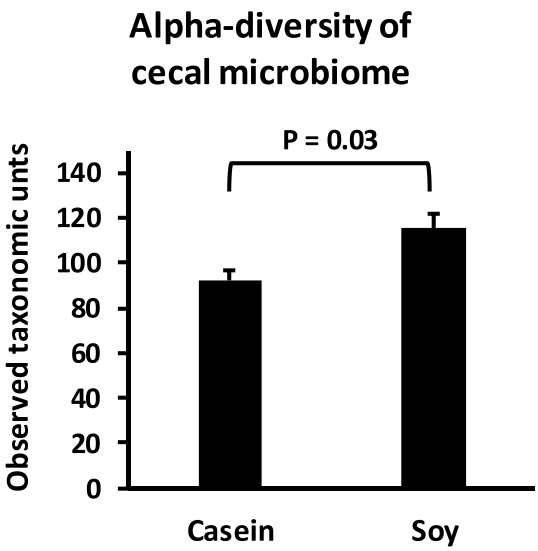

Fig. 3. Alpha diversity is significantly increased in cecal microbiome of male mice fed SPI-EtOH Lieber DeCarli diets compared to mice fed casein-EtOH Lieber DeCarli diets in the NIAAA chronic + binge model of alcohol exposure. Data are mean ± SEM for N = 5–6/group.

Table 1
Liver tumor and Agap2 expression in DEN-treated male mice fed low fat diet or high fat diets for 30 Weeks.

Diet Group	Adenoma + HCC Incidence	Adenoma + HCC Multiplicity	Agap2 mRNA
Chow (Saline)	–	–	0.57 ± 0.04[a]
Low Fat	45[a]	0.5 ± 0.2[a]	1.00 ± 0.16[b]
Corn Oil	60[a]	3.0 ± 1.5[a]	1.23 ± 0.18[b]
Cocoa Butter	100[b]	11.3 ± 3.5[b]	4.59 ± 0.81[c]
Olive Oil	60[a]	1.5 ± 0.6[a]	2.24 ± 0.2[b]
Corn Oil + DHA	72[a,b]	5.0 ± 2.0[a]	0.86 ± 0.08[b]

Incidence: % mice with tumors; Multiplicity: Number of tumors/mouse. Data are mean ± SEM for N = 9–10/group. a < b < c statistically significant P < 0.05. Previously published in part [23].

contrast, epidemiological studies in Asia report only a 1.6–1.8-fold increase in risk [22,23]. We have published data showing that substitution of soy protein isolate (SPI) for casein as the protein source in high fat liquid diets with EtOH results in inhibition of tumor promotion coincident with blockade of EtOH-induced Wnt-β-catenin activation and of hepatocyte proliferation [18,19]. However, anti-tumor and anti-proliferative effects of SPI appear to involve pathways in addition to Wnt signaling. We observed protective effects of SPI against high fat-induced tumorigenesis in the DEN model even in the absence of changes in nuclear β-catenin expression [20]. SPI blocks development of steatosis in rodent models of both ASH and NASH and anti-cancer effects may simply reflect inhibition of multiple pathways triggered by excess triglyceride accumulation. The anti-steatotic effects of feeding SPI appear to be associated with activation of PPARα and increased FA degradation [20,24–26]. However, it appears that the protective effects of SPI on EtOH-associated tumor promotion are not mediated via the isoflavone genistein which has been implicated in some of the anti-cancer properties of soy [27]. Studies included in the current report were designed to determine if protein/peptide or phytochemical components of SPI other than isoflavones play a role in protection against EtOH-induced steatosis by feeding SPI.

One possible additional factor in development of HCC in ASH and NASH and in protection by SPI may be effects related to the microbiome and the microbiome –associated metabolome. Alterations in the mucosa-associated colonic bacterial microbiota by chronic alcohol consumption was first reported in rats by Mutulu et al. [28]. Bacterial overgrowth and intestinal microbial dysbiosis was subsequently described in the mouse Tsukomoto-French intragastric model after three weeks of alcohol consumption accompanied by decreases in *Firmicutes*, beneficial bacteria including *Lactobacillus, Pediococcus, Leuconostoc* and *Lactococcus* and by increases in *Bacteriodetes* [29]. One consequence of microbial dysbiosis is a significant alterations in the microbial metabolome. Alcohol consumption has been reported to suppress production of the short chain fatty acid (SFA) butyrate, reduce the levels of taurine-conjugated bile acids and increase levels of more toxic unconjugated and glycine-conjugated bile acids and significantly reduce intestinal amino acid metabolism [30–33]. Changes in the microbial metabolome and microbial metabolism of EtOH to acetaldehyde have been linked to disruption of the intestinal epithelial barrier, intestinal inflammation and changes in intestinal FXR signaling and production of FGF15/19 [30–33]. In addition, increased intestinal permeability results in translocation of pathogen-associated molecular patterns (PAMPs) such as endotoxin, peptidoglycan and bacterial DNA which may contribute to development of hepatic inflammation [29]. Microbial dysbiosis and alterations in microbial metabolism of bile acids have also been suggested to play a role in NASH progression to HCC [34]. Probiotic treatment/fetal transplants have been proposed as potential therapies for ASH and NASH [35,36]. It has also been postulated that protective effects of soy on lipid homeostasis and steatosis are the result of beneficial changes in gut microbiota populations and altered bile acid metabolism and signaling [35,36]. Soy feeding was reported to increase microbial diversity in Golden Syrian hamsters including elevation in

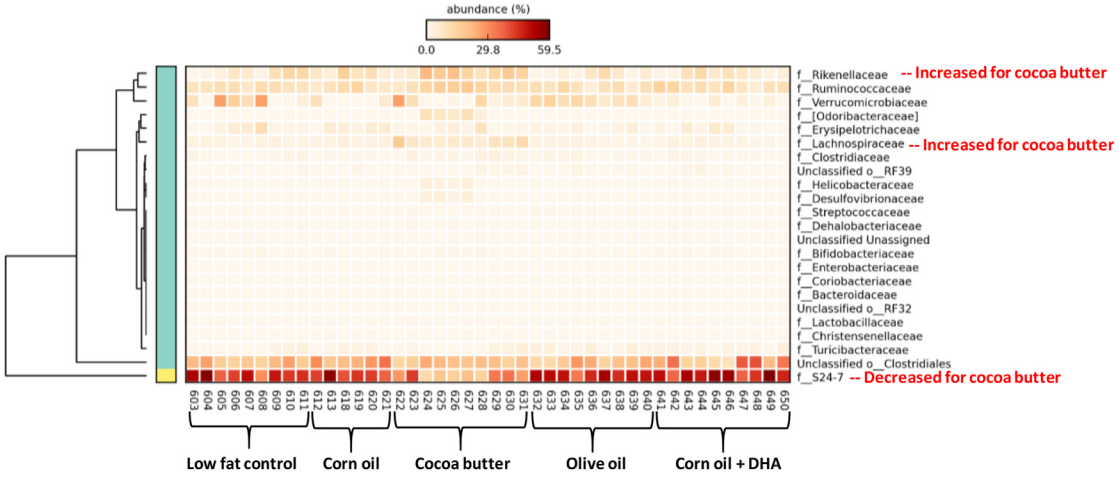

Fig. 4. MIIseq data from DEN-treated male mice fed low or high fat diets of differing composition for 30 weeks (23). Each lane represents data from a single mouse.

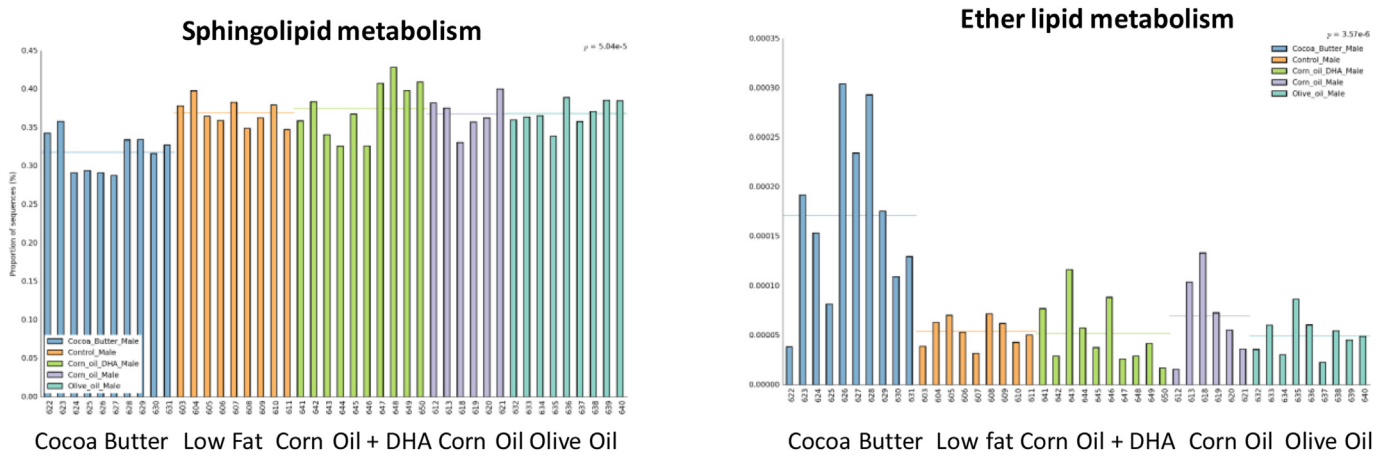

Fig. 5. Differences in the predicted microbiome –associated metabolome by KEGG analysis of MIIseq data from DEN-treated male mice fed low or high fat diets of differing composition for 30 weeks (23). Each lane represents data from a single mouse. Cocoa butter profiles differ from other diet groups $P < 0.05$. KEGG analysis suggest that these changes in microbiome composition results in significant suppression of microbial sphinogolpid metabolism and significant increases in ether lipid metabolism relative to other high fat diets with less potent tumor promotion effects (Fig. 5). RNAseq analysis of global hepatic gene changes in livers from DEN/cocoa butter fed mice relative to mice fed olive oil, corn oil or corn oil + DHA revealed elevated expression of a proto-oncogene *Agap2* (PIKE) which we have verified by real time RT-PCR (Table 1) induction of which occurred prior to tumor development in livers from mice 15 weeks of cocoa butter feeding [23]. Subsequent RT-PCR analysis of livers from DEN-treated mice fed high fat and EtOH Lieber DeCarli liquid diets with or without SPI substitution for casein revealed similar significant upregulation in this model and suppression in SPI-fed vs. casein-fed mice [23].

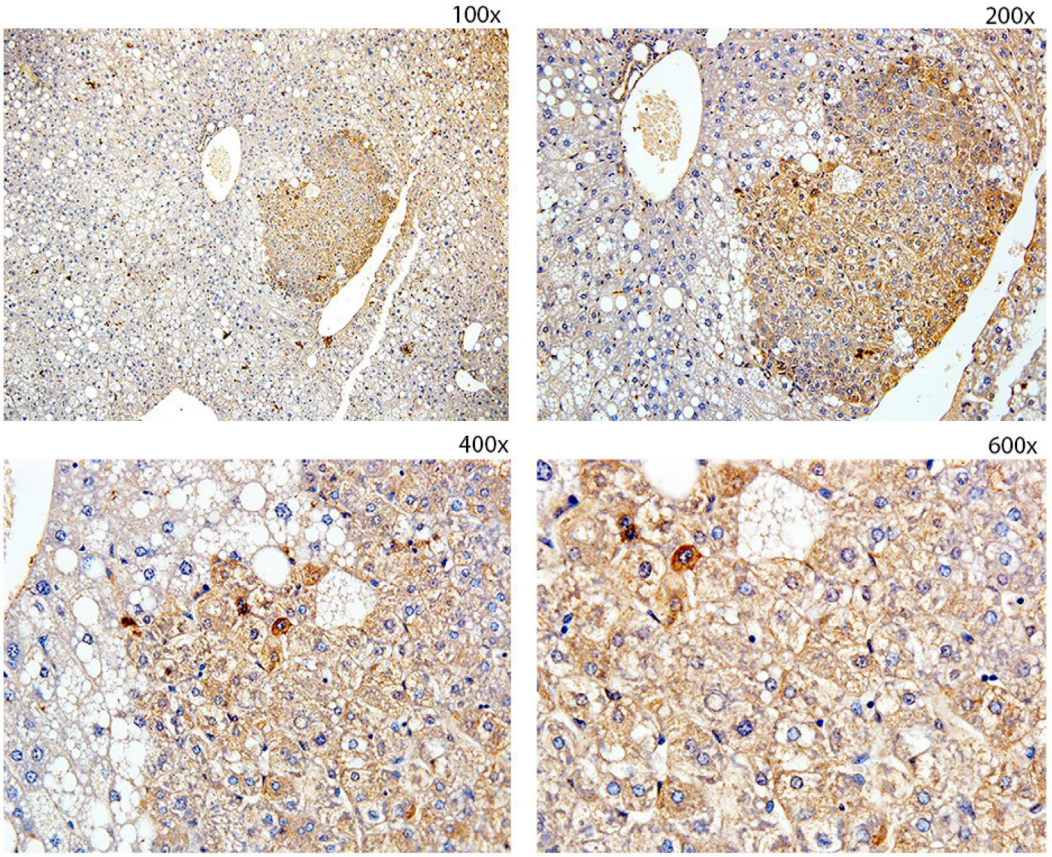

Fig. 6. Representative positive CYP2W1 staining of a hepatic adenoma in a DEN/EtOH treated male mouse relative to the surrounding non-tumored tissue.

Bifidobacteria and *Clostridales* in comparison to feeding milk protein [37]. This was accompanied by reductions in serum triglycerides [37]. Similar increases in *Bifidobacter* have been reported in women fed soy bars [38]. Improved body composition and lipid homeostasis were also reported in soy-fed female low-fit rats and in obese OLETF rats accompanied by a lower ratio of *Firmicutes* to *Bacterioides* and increases in *Lactobacillus* [39]. We have analyzed microbiome composition in mice fed EtOH liquid diets with casein or SPI as the protein source or fed high fat diets with differing fat composition and conducted adoptive transfer studies to examine the role of microbial dysbiosis in development of ASH/NASH and the role of altered microbiome composition in the protective effects of SPI.

CYP2W1 is an orphan CYP enzyme, with a well conserved sequence between humans, rats and mice, which was first cloned from the

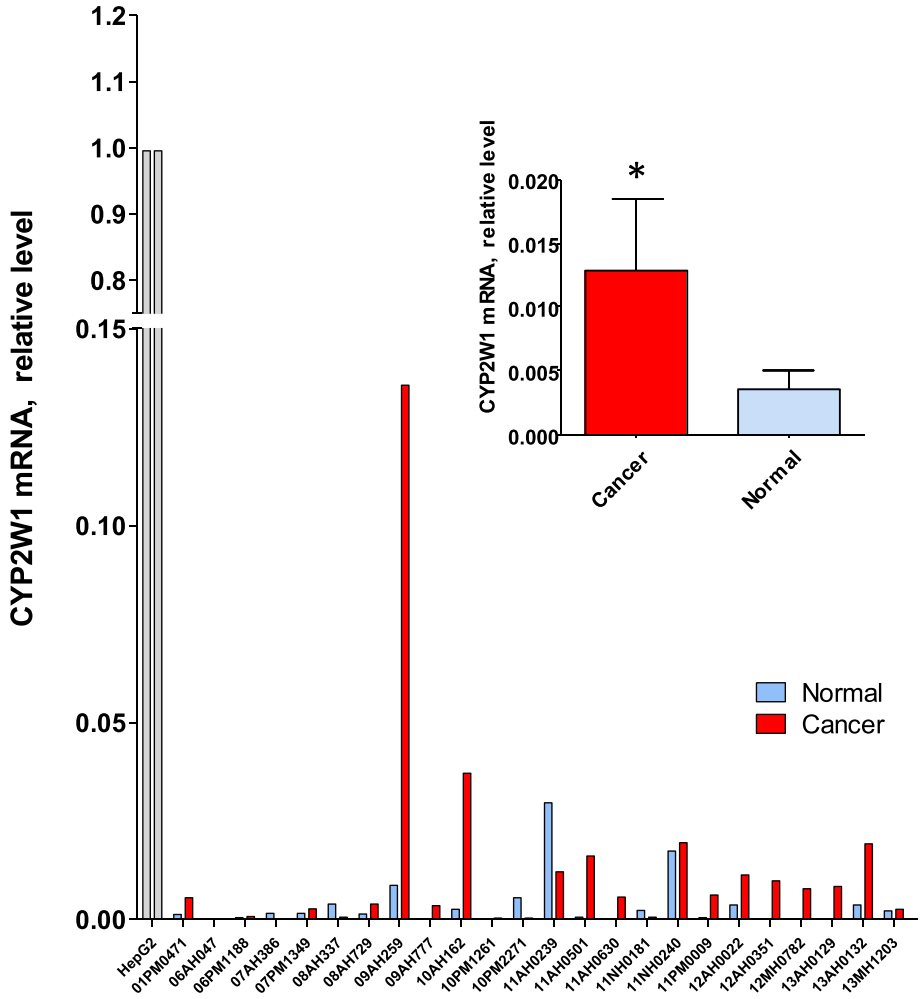

Fig. 7. Expression of CYP2W1 mRNA in hepatic tumor and non-tumor surrounding tissue from HCC patient samples in the Karolinska Institute archive relative to expression in the HepG2 cell line from which CYP2W1 was originally cloned. * Statistically significant $P < 0.05$ tumor vs. non-tumor in 24 patients.

hepatoma HepG2 cell line [40]. Neither the CYP2W1 mRNA nor protein were detected in adult human livers or other adult tissues but was observed in human colon tumors and in fetal intestine and colon [41]. The orthologues of human CYP2W1 were detected in the fetal GI tract of rats and mice [40,41]. Increased rates of tumor growth have been reported in subcutaneous CYP2W1 +ve colon cancer cell xenografts compared to xenografts using cells where CYP2W1 expression was abolished [42]. A variety of potential endogenous CYP2W1 substrates have been identified using recombinant CYP2W1 which might be metabolized to regulate tumor growth. These include retonoids, free fatty acids, lysophospholipids and arachidonic acid [43–45]. CYP2W1 has been found to be inducible in colon cancer cell lines by linoleic acid and its derivatives which is consistent with these compounds being CYP2W1 substrates [41]. Since CYP2W1 appears to be specific to tumors, it has been suggested that it may also serve as both a cancer biomarker and as a therapeutic target for prodrugs which can be converted by CYP2W1 to cytotoxic metabolites [40–42]. A recent series of studies using the chloromethylindoline duocarmycin analogue ICT2706, which is activated by CYP2W1 to a DNA alkylating agent, demonstrated abolition of tumor growth in CYP2W1 positive human colon cancer xenografts whereas CYP2W1 negative xenografts were resistant [46]. One small previous study using an antibody against CYP2W1, suggested that in addition to expression in colon tumors, CYP2W1 is also over-expressed in HCC [47]. We have conducted studies to examine if CYP2W1 is over-expressed in tumors from our DEN/EtOH mouse model and in human HCC compared to surrounding tissues.

2. Materials and methods

2.1. In vivo mouse models

Two models of tumor promotion in DEN mouse models were utilized. In the first model, male C57/BL6 mice were injected i.p. with 10 mg/kg DEN on PND13 and were fed standard rodent chow until PND 65. Mice were then pair-fed 35% fat Lieber DeCarli liquid diets (Dyets Inc., Bethlehem, PA) with or without substitution of EtOH for carbohydrate calories up to 28% total calories and with either casein or SPI as the sole protein source for 16 weeks as previously described [18]. In the second model male C57/BL6 mice were also injected i.p. with 10 mg/kg DEN on PND13. In this case mice were weaned on PND 28 onto a low (12%) mixed fat pelleted casein-based AIN-93G diet or onto pelleted diets in which fat was substituted for carbohydrate at 35% of calories and the fat source was either cocoa butter (saturated fat), olive oil (monounsaturated fat), corn oil (polyunsaturated fat) or 30% corn oil + 5% decosohexaenoic acid (DHA, w-3 enriched). These diets were fed for 30 weeks as previously described [21]. In addition, more acute hepatic effects of EtOH were examined in modified versions of the NIAAA chronic binge model alcohol model [48]. Male C57/BL6 mice age 8 weeks were pair-fed (5%) Lieber DeCarli diets with casein or SPI as the protein source or with casein plus a phytochemical soy extract (Samsara Herbs, Soy Isoflavone Extract, 40% concentrated extract, Nik Trade #78342342) for 10 d and given a 4 g/kg binge 6 h prior to sacrifice. In addition, groups of male C57BL/6 mice were pair-fed (5%)

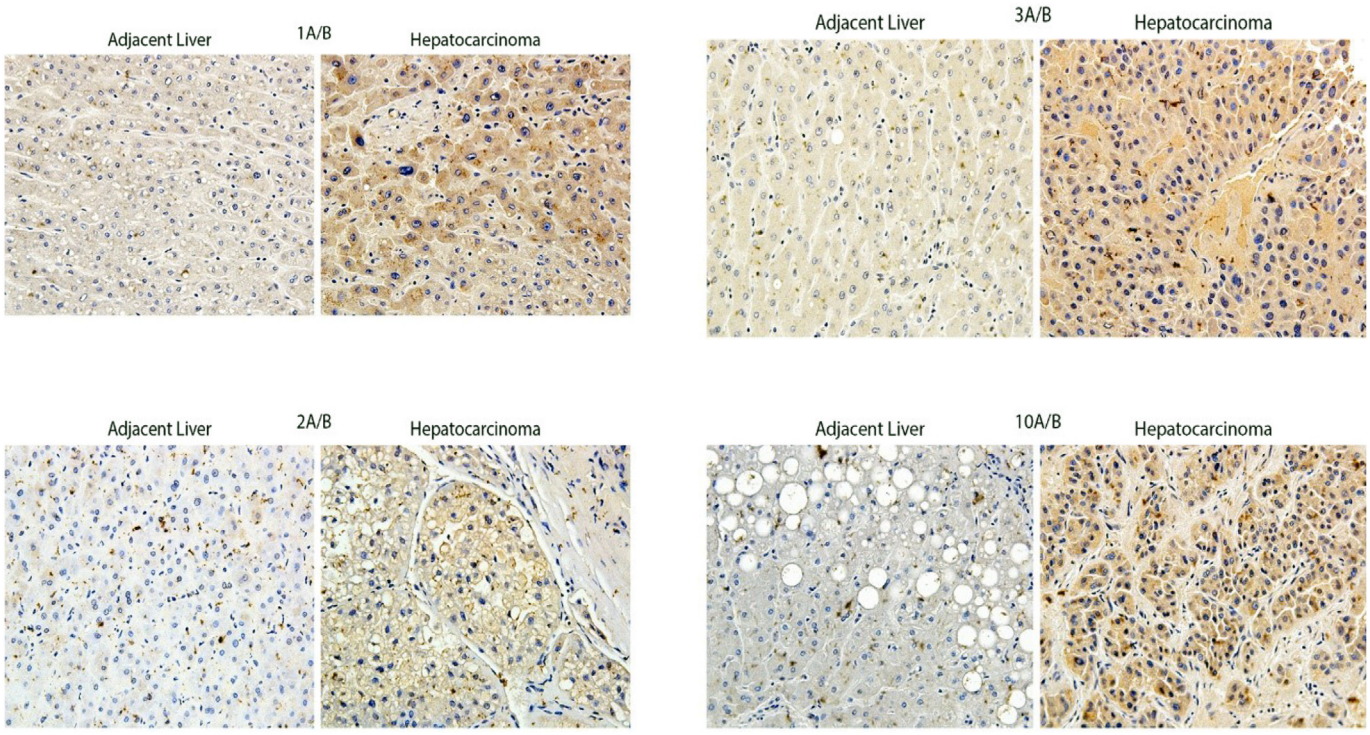

Fig. 8. Positive CYP2W1 staining of resected HCC in the liver of 4 patients diagnosed with alcohol-associated HCC relative to the surrounding non-tumored tissue.

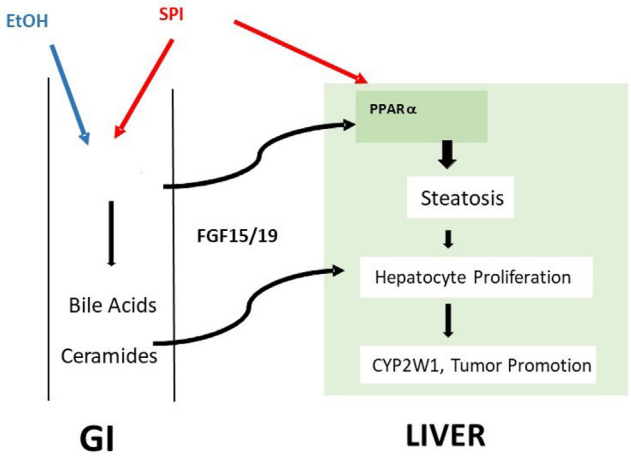

Fig. 9. Hypothesized relationship between EtOH exposure, SPI feeding, composition of the gut microbiota and the microbiome-associated metabolome, signaling via PPARα, *Agap2* and FGF19 hepatic steatosis and promotion of hepatic tumorigenesis.

Lieber DeCarli diets with casein for 13 d with 4 g/kg binges at d 5 and d 10 and sacrificed 24 h later or were subject to adoptive transfer of cecal microbiota from EtOH-treated or pair-fed mice after 14 d administration of an antibiotic cocktail as described previously [49]. All animal studies were approved by IACUC committees at the University of Arkansas for Medical Sciences or LSUHSC – New Orleans.

2.2. Clinical samples of HCC and surrounding non-tumored tissue

Resected HCC and surrounding non-tumored tissue was obtained from an archival tissue bank of patients at the Karolinska Institutet, Stockholm, Sweden and from a tissue bank of livers of HCC patients at the University of Verona, Italy diagnosed with ASH. Samples were collected under IRB approved protocols.

2.3. Pathological, biochemical and microbial analysis

Formalin fixed lobes from DEN-treated mice were H&E stained and scored for the presence of adenomas and HCC by certified veterinary pathologists at UAMS and the LSU School of Veterinary Medicine as described previously [18,23]. Unstained fixed liver sections from mice from the DEN/EtOH model and from human HCC-ASH patients also underwent immunohistochemical staining for the presence of CYP2W1 using a highly specific rabbit polyclonal antibody to the C-terminal of human CYP2W1 (Ab 852, from M.I-S, 40–42). Frozen HCC and surrounding non-tumored tissue from the Karolinska Institutet archive were used for mRNA extraction and real time RT-PCR analysis of CYP2W1 mRNA expression [50]. Expression of PIKE (*Agap2*) mRNA from frozen DEN-treated mouse liver was quantified by RNAseq and real time RT-PCR as described previously [23]. Steatosis in fixed liver sections from mice fed Lieber DeCarli diets was measured biochemically or via quantification of Oil Red O staining [13,15]. MIIseq DNA sequencing of the 16S rRNA gene sequences of mouse cecal samples and bioinformatics was conducted as previously described [23,49]. 16S rRNA sequences were curated using Quantitative Insights Into Microbial Ecology (QIIME 1.91) and R package Phyloseq scripts and microbiome metabolome patterns by KEGG analysis [49].

2.4. Statistical analysis

Data are presented as mean ± SEM. Adenoma and HCC incidence wefre determined using Fisher's Exact Test. Tumor multiplicity, biochemical and mRNA expression data were determined by One-way ANOVA with Mann-Witney U rank-sum test or Neuman-Keuls test for post hoc comparisons. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Potential role of steatosis, the microbiome and Agap2 in tumor promotion and the protective effects of SPI in ASH/NASH livers

We examined the development of steatosis in the NIAAA binge-on-

chronic model of ASH in mice fed Lieber DeCarli diets where casein protein was swapped for SPI or where a phytochemical extract of soy was added to the casein diet. As shown in Fig. 1, both SPI and the extract were effective in suppressing triglyceride accumulation after EtOH feeding and the effects of the extract were dose-dependent ($P < 0.05$).

Moreover, data from an adoptive transfer experiment utilizing a modified NIAAA model [50].demonstrated that cecal microbiota isolated from EtOH-fed mice were capable of inducing steatosis in antibiotic-treated mice pair-fed control liquid diets in the absence of EtOH (Fig. 2). This implicates microbial dysbiosis produced by EtOH in hepatic triglyceride accumulation and downstream events leading to development of HCC. One possible mechanism underlying the anti-steatotic effects of feeding.

SPI may be related to positive effects on microbial composition which have previously been documented in rodent models and clinical studies [37–39]. In preliminary studies using the NIAAA model, we have observed significantly increased alpha diversity in the cecal microbiome of EtOH-fed mice fed SPI compared to casein (Fig. 3). Moreover, we observed significant increases in *Clostidiales spp*. which have been implicated in mucus biosynthesis, butyrate production and Treg cell-mediated anti-inflammatory responses [51,52]. Additional evidence for a potential role for microbial dysbiosis in tumor promotion in fatty liver disease comes from an additional study of the importance of dietary fat type in hepatic tumor promotion in the mouse DEN model [23]. We have previously shown selective promotion of liver tumors in mice treated with DEN and fed high fat pelleted diets made with a highly saturated fat source, cocoa butter relative to other monounsaturated or polyunsaturated fat sources (Table 1). This was accompanied by specific effects of cocoa butter feeding on composition of the cecal microbiota. In particular there was an increase in *Rikenellaceae* and *Lachnospiraceae* (especially Copprococcus, 23) and a reduction in S24-7 (Fig. 4).

3.2. Potential role of CYP2W1 in ASH-Associated tumor promotion

Immunohistochemical analysis of fixed livers from the DEN/EtOH mouse model using a rabbit polyclonal antibody specific for human CYP2W1 revealed expression in tumors but not the surrounding liver (Fig. 6). Real time RT-PCR analysis of CYP2W1 mRNA expression in HCC and surrounding non-tumored liver from patients in the Karolinska Institute tissue archive demonstrated highly variable expression lower than that found in HepG2 cells from which it was originally cloned but overall higher expression in tumors than surrounding tissue (Fig. 7). Subsequent immunohistochemical analysis of HCC specifically from 10 patients diagnosed with ASH from the University of Verona tissue bank demonstrated a similar HCC-specific expression pattern of CYP2W1 protein to that seen in the DEN/EtOH mice with little or no expression in surrounding non-tumored liver (Fig. 8).

4. Discussion

We have previously demonstrated that both EtOH and high fat liquid diets significantly promote hepatic tumorigenesis in the male mouse DEN model with EtOH the most potent factor [18]. In addition, we have demonstrated that switch of dietary protein from casein to SPI results in suppression of tumorigenesis in both ASH and NASH models [19,20]. However, whereas inhibition of β-catenin signaling is implicated in SPI protection against EtOH-associated tumor promotion [19], we found that SPI also protected against high fat-induced tumor promotion in DEN-treated mice even when nuclear β-catenin levels remained elevated [20]. These data imply additional mechanisms underlying cancer prevention by SPI feeding. One potential additional mechanism may be prevention of steatosis and downstream effects on ceramide/sphingosine metabolism and development of inflammation [19,20]. We previously observed prevention of triglyceride accumulation by feeding SPI in several rodent models of fatty liver disease associated with activation of PPARα signaling [20,24–26]. However, which component of SPI might be responsible and possible involvement of additional pathways was unclear. We have previously published data showing that genistein, the major isoflavone phytochemical bound to SPI does not protect against tumorigenesis in the mouse DEN/EtOH model [27] nor do isoflavones fed at levels found in SPI induce PPARα-regulated genes in rats despite *in vitro* studies showing induction at higher doses [26]. Our current data suggest that prevention of steatosis by SPI is associated with a phytochemical component other than isoflavones. Data from previous studies of gut microbiota following EtOH exposure [28–30] and our adoptive transfer study [50] suggest that microbial dysbiosis plays a key role in development of alcoholic steatosis and liver pathology. Interestingly, PPARα is expressed in the GI tract in addition to liver and mediates expression of antimicrobial peptides and anti-inflammatory cytokines [53]. It is possible that activation of PPARα in the GI plays a role in beneficial effects of SPI feeding on microbiome phenotype and hepatic triglyceride accumulation after alcohol consumption (Fig. 9). In separate studies in the neonatal piglet we have recently published data demonstrating differential effects of feeding cow's milk based infant formula (largely casein) and soy-based infant formula on composition of the intestinal microbiome with additional effects on microbial metabolism of primary and secondary bile acids [54]. Bile acid signaling has been shown to modulate the intestinal FXR-FGF19 axis which in turn affects hepatic lipid metabolism [30,54]. Suppression of FGF19 expression in soy formula fed piglets and recent studies by Grace Gao linking FGF19 signaling with control of hepatocyte proliferation [55] suggest that this pathway may also play a role in prevention of hepatic tumor promotion by feeding SPI (Fig. 9). Additional adoptive transfer studies with microbiota from SPI fed mice and studies in PPARα knockout mice and FGF19 transgenic mice are required to confirm a role for these pathways in regulation of hepatic tumor promotion in ASH/NASH.

Our data also indicate a potential role for expression of the proto-oncogene *Agap2* and expression of CYP2W1 in tumors may play a role in tumor promotion in fatty liver disease.

Agap2 is a PI3-kinase enhancer which acts to enhance downstream pro-proliferative signaling via the Akt and Erk signaling pathways [23]. CYP2W1 includes retinoids as its substrates [43–45]. Alcohol abuse and EtOH treatment of rodent models results in hepatic retinoid depletion. Over-expression of CYP2W1 in tumors may further deplete local retinoid concentrations. Previous studies have demonstrated inhibition of retinoid signaling is linked to β-catenin activation [56] and the tumors in the DEN/EtOH mouse model are β-catenin positive [18]. In addition, other studies have suggested negative cross-talk between retinoids and sphingosine-1-phosphate in regulation of hepatocyte proliferation [57]. Additional studies in knock out and transgenic animals are required to determine the relative importance of *Agap2* and CYP2W1 pathways in hepatic tumor promotion.

Funding

Funded in part by R21 CA169389 (M.J.R.) and start-up funding from LSUHSC – New Orleans (M.J.R.) and United States Department of Agriculture (Agricultural Research Service Project 6026-51000-010-05S) (K.M).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2020.109131.

References

[1] S.F. Altekruse, K.A. McGlynn, M.E. Reichman, Hepatocellular carcinoma incidence, mortality and survival trends in the United States from 1975 to 2005, J. Clin. Oncol. 27 (2009) 1485–1491.

[2] T.R. Morgan, S. Mandayam, M.M. Jamal, Alcohol and hepatocellular carcinoma, Gastroenterology 127 (2004) S87–S96.

[3] R. Agabio, I. Campesi, C. Pisanu, G.L. Gessa, F. Franconi, Sex differences in substance use disorders: focus on side effects, Addiction Biol. 21 (2016) 1030–1042.

[4] A. Marengo, et al., Liver cancer: connections with obesity, fatty liver, and cirrhosis, Annu. Rev. Med. 67 (2016) 103–117.

[5] R.J. Wong, et al., Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S, Hepatology 59 (2014) 2188–2195.

[6] F. Stickel, D. Schuppan, E.G. Hahn, H.K. Seitz, Cocarcinogenic effect of alcohol in hepatocarcinogenesis, Gut 51 (2002) 132–139.

[7] Ronis MJJ, K. Lindros, M. Ingelman-Sundberg, The CYP 2E1 family, in: C. Ioneddes (Ed.), Cytochrome P450: Biochemical and Toxicological Aspects, CRC Press, 1996, pp. 211–239.

[8] T.M. Badger, M.J.J. Ronis, H.K. Seitz, E. Albano, M. Ingelman-Sundberg, C.S. Lieber, Alcohol metabolism: role in toxicity and carcinogenesis, Alcohol Clin. Exp. Res. 27 (2003) 336–346.

[9] J.S. Kang, H. Wanibuchi, K. Morimura, F.J. Gonzalez, S. Fukushima, Role of CYP2E1 in diethylnitrosamine-induced hepatocarcinogenesis in vivo, Canc. Res. 67 (2007) 11141–11146.

[10] A. Tagger, F. Donato, M.L. Ribero, R. Chiesa, G. Portera, U. Gelatti, et al., Case-control study on hepatitis C virus (HCV) as a risk factor for hepatocellular carcinoma: the role of HCV genotypes and the synergism with hepatitis B virus and alcohol. Brescia HCC Study, Int. J. Canc. 81 (1999) 695–699.

[11] H. Vandenbulcke, C. Moreno, I. Colle, J.F. Knebel, S. Francque, T. Sersté, et al., Alcohol intake increases the risk of HCC in hepatitis C virus-related compensated cirrhosis: a prospective study, J. Hepatol. 65 (2016) 543–551.

[12] Ronis MJJ, A. Butura, S. Korourian, K. Shankar, J. Badeaux, E. Albano, M. Ingelman-Sundberg, T.M. Badger, Cytokine and chemokine expression associated with steatohepatitis and hepatocyte proliferation in rats fed ethanol via total enteral nutrition, Exp. Biol. Med. 233 (2008) 344–355.

[13] J.N. Baumgardner, K. Shankar, T.M. Badger, Ronis MJJ, Undernutrition enhances alcohol-induced hepatocyte proliferation in the liver of rats fed via total enteral nutrition, Am. J. Physiol. 293 (2007) G355–G364.

[14] C.T. Shearn, C.F. Pulliam, K. Pedersen, K. Meredith, K.E. Mercer, L.M. Saba, D.J. Orlicky, M.J. Ronis, D.R. Petersen, Knockout of the Gsta4 gene in male mice leads to an altered pattern of hepatic protein carbonylation and enhanced inflammation following chronic consumption of an ethanol diet, Alcohol Clin. Exp. Res. 42 (2018) 1192–1205.

[15] J.N. Baumgardner, K. Shankar, L. Hennings, T.M. Badger, Ronis MJJ, A new rat model for nonalcoholic steatohepatitis utilizing overfeeding of diets high in polyunsaturated fat by total enteral nutrition, Am. J. Physiol. 294 (2008) G27–G38.

[16] J.N. Baumgardner, K. Shankar, L. Hennings, T.M. Badger, M.J.J. Ronis, N-acetylcysteine attenuates progression of liver pathology in a rat model of non-alcoholic steatohepatitis, J. Nutr. 138 (2008) 1872–1879.

[17] M.J.J. Ronis, K.E. Mercer, B. Engi, P. Zimniak, C.T. Shearn, T.M. Badger, D.R. Petersen, Global deletion of glutathione-S-transferase A4 exacerbates developmental nonalcoholic steatohepatitis, Am. J. Pathol. 187 (2017) 418–430.

[18] K.E. Mercer, L. Hennings, N. Sharma, K. Lai, M.A. Cleves, R.A. Wynne, T.M. Badger, M.J.J. Ronis, Alcohol consumption promotes diethylnitrosamine-induced hepatocarcinogenesis in male mice through activation of the Wnt/β-catenin signaling pathway, Canc. Prev. Res. 7 (2014) 675–685.

[19] K.E. Mercer, C. Pulliam, L. Hennings, K. Lai, M. Cleves, E. Jones, R.R. Drake, M. Ronis, Soy protein isolate protects against ethanol-mediated tumor progression in diethylnitrosamine-treated male mice, Canc. Prev. Res. 9 (2016) 466–475.

[20] K.E. Mercer, C.F. Pulliam, K.B. Pedersen, L. Hennings, M.J. Ronis, Soy protein isolate inhibits hepatic tumor promotion in mice fed a high-fat liquid diet, Exp. Biol. Med. 242 (2017) 635–644.

[21] K.B. Pedersen, C.F. Pulliam, A. Patel, F. Del Piero, T. Watanabe, U.D. Wankhade, K. Shankar, C. Hicks, M.J. Ronis, A high saturated-fat diet results in alterations of the intestinal microbiome and promotes liver tumorigenesis specifically in male mice, Carcinogenesis 40 (2019) 349–359.

[22] R.H. Luo, Z.X. Zhao, X.Y. Zhou, Z.L. Gao, J.L. Yao, Risk factors for primary liver carcinoma in Chinese population, World J. Gastroenterol. 11 (2005) 4431–4434.

[23] T. Shimazu, S. Sasazuki, K. Wakai, A. Tamakoshi, I. Tsuji, Y. Sugawara, et al., Alcohol drinking and primary liver cancer: a pooled analysis of four Japanese cohort studies, Int. J. Canc. 130 (2012) 2645–2653.

[24] T.M. Badger, M.J.J. Ronis, G. Wolff, S. Stanley, M. Ferguson, K. Shankar, Consumption of soy protein isolate reduces hepatosteatosis in obese yellow Agouti (Avy) mice, but does not alter the coat color phenotype, Exp. Biol. Med. 233 (2008) 1242–1254.

[25] Ronis MJJ, T.M. Badger, Y. Chen, J. Badeaux, Dietary soy protein isolate attenuates metabolic syndrome in rats via effects on PPAR, LXR and SREBP-1c signaling, J. Nutr. 139 (2009) 1431–1438.

[26] Ronis MJJ, Effects of soy diet and isoflavones on cytochrome P450 expression and activity, Drug Metabol. Rev. 48 (2016) 331–341.

[27] K.E. Mercer, C.F. Pulliam, L. Hennings, K. Lai, M.A. Cleves, E.E. Jones, R.R. Drake, M.J.J. Ronis, Diet supplementation with soy protein isolate, but not genistein, protects against alcohol-induced tumor progression in DEN-treated male mice, Adv. Exp. Med. Biol. 1032 (2018) 115–126.

[28] E. Mutlu, A. Keshavarzian, P. Engen, C.B. Forsyth, M. Sikaroodi, P. Gillevet, Intestinal dysbiosis: a possible mechanism of alcohol-induced endotoxemia and alcoholic steatohepatitis in rats, Alcohol Clin. Exp. Res. 33 (2009) 1836–1846.

[29] P. Stärkel, S. Leclercq, P. de Timary, B. Schnabl, Intestinal dysbiosis and permeability: the yin and yang in alcohol dependence and alcoholic liver disease, Clin. Sci. (Lond.) 132 (2018) 199–212.

[30] P. Hartmann, K. Hochrath, A. Horvath, P. Chen, C.T. Seebauer, C. Llorente, et al., Modulation of the intestinal bile acid/farnesoid X receptor/fibroblast growth factor 15 axis improves alcoholic liver disease in mice, Hepatology 67 (2018) 2150–2166.

[31] K. Brandl, P. Hartmann, L.J. Jih, D.P. Pizzo, J. Argemi, M. Ventura-Cots, et al., Dysregulation of serum bile acids and FGF19 in alcoholic hepatitis, J. Hepatol. 69 (2018) 396–405.

[32] P. Chen, Y. Miyamoto, M. Mazagova, K.C. Lee, L. Eckmann, B. Schnabl, Microbiota and alcoholic liver disease, Alcohol Clin. Exp. Res. 40 (2016) 1791–1792.

[33] J.M. Ridlon, D.J. Kang, P.B. Hylemon, J.S. Bajaj, Gut microbiota, cirrhosis, and alcohol regulate bile acid metabolism in the gut, Dig. Dis. 33 (2015) 338–345.

[34] F. Shen, R.D. Zheng, X.Q. Sun, W.J. Ding, X.Y. Wang, J.G. Fan, Gut microbiota dysbiosis in patients with non-alcoholic fatty liver disease, Hepatobiliary Pancreat. Dis. Int. 16 (2017) 375–381.

[35] I.A. Kirpich, N.V. Solovieva, S.N. Leikhter, N.A. Shidakova, O.V. Lebedeva, P.I. Sidorov, et al., Probiotics restore bowel flora and improve liver enzymes in human alcohol-induced liver injury: a pilot study, Alcohol 42 (2008) 675–682.

[36] A. Horvath, B. Leber, B. Schmerboeck, M. Tawdrous, G. Zettel, A. Hartl, et al., Randomised clinical trial: the effects of a multispecies probiotic vs. placebo on innate immune function, bacterial translocation and gut permeability in patients with cirrhosis, Aliment. Pharmacol. Ther. 44 (2016) 926–935.

[37] D.N. Butteiger, A.A. Hibberd, N.J. McGraw, N. Napawan, J.M. Hall-Porter, E.S. Krul, Soy protein compared with milk protein in a western diet increases gut microbial diversity and reduces serum lipids in golden Syrian hamsters, J. Nutr. 146 (2016) 697–705.

[38] H. Huang, H.B. Krishnan, Q. Pham, L.L. Yu, T.T. Wang, Soy and gut microbiota: interaction and implication for human health, J. Agric. Food Chem. 64 (2016) 8695–8709.

[39] M.R. Panasevich, C.M. Schuster, K.E. Phillips, G.M. Meers, S.V. Chintapalli, U.D. Wankhade, et al., Soy compared with milk protein in a Western diet changes fecal microbiota and decreases hepatic steatosis in obese OLETF rats, J. Nutr. Biochem. 46 (2017) 125–136.

[40] M. Karlgren, A. Gomez, K. Stark, J. Svärd, C. Rodriguez-Antona, E. Oliw, M.L. Bernal, y Cajal S. Ramón, I. Johansson, M. Ingelman-Sundberg, Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1, Biochem. Biophys. Res. Commun. 341 (2006) 451–458.

[41] E. Choong, J. Guo, A. Persson, S. Virding, I. Johansson, S. Mkrtchian, M. Ingelman-Sundberg, Developmental regulation and induction of cytochrome P450 2W1, an enzyme expressed in colon tumors, PLoS One 10 (4) (2015) e0122820.

[42] K. Stenstedt, M. Hallstrom, I. Johansson, M. Ingelman-Sundberg, P. Ragnhammar, D. Edler, The expression of CYP2W1: a prognostic marker in colon cancer, Anticancer Res. 32 (2012) 3869–3874.

[43] Y. Zhao, D. Wan, J. Yang, B.D. Hammock, P.R. Ortiz de Montellano, Catalytic activities of tumor-specific human cytochrome P450 CYP2W1 towards endogenous substrates, Drug Metab. Dispos. 44 (2016) 771–780.

[44] Z.L. Wu, C.D. Sohl, T. Shimada, F.P. Guengerich, Recombinant enzymes overexpressed in bacteria showbroad catalytic specificity of human cytochrome P450 2W1and limited activity of human cytochrome P450 2S1, Mol. Pharmacol. 69 (2006) 2007–2014.

[45] Y. Xiao, F.P. Guengerich, Metabolomic analysis and identification of a role for the orphan human cytochrome P450 2W1 in selective oxidation of lysophospholipids, J. Lipid Res. 53 (2012) 1610–1617.

[46] S. Travica, K. Pors, P.M. Loadman, S.D. Shnyder, I. Johansson, M.N. Alandas, H.M. Sheldrake, S. Mkrtchian, L.H. Patterson, M. Ingelman-Sundberg, Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins, Clin. Canc. Res. 19 (2013) 2952–2961.

[47] K. Zhang, L. Jiang, R. He, B.L. Li, Z. Jia, R.H. Huang, Y. Mu, Prognostic value of CYP2W1 expression in patients with human hepatocellular carcinoma, Tumour Biol. 35 (2014) 7669–7673.

[48] B. Gao, M.J. Xu, A. Bertola, H. Wang, Z. Zhou, S. Liangpunsakul, Animal models of alcoholic liver disease: pathogenesis and clinical relevance, Gene Expr. 17 (2017) 173–186.

[49] D.R. Samuelson, J.E. Shellito, V.J. Maffei, E.D. Tague, S.R. Campagna, E.E. Blanchard, M. Luo, C.M. Taylor, Ronis MJJ, P.E. Molina, D.A. Welsh, Alcohol-associated intestinal dysbiosis impairs pulmonary host defense against Klebsiella pneumoniae, PLoS Pathog. 13 (2017) e1006426.

[50] P. Nolé, B. Duijndam, A. Stenman, C.C. Juhlin, M. Kozyra, C. Larsson, M. Ingelman-Sundberg, I. Johansson, Human cytochrome P450 2W1 is not expressed in adrenal cortex and is only rarely expressed in adrenocortical carcinomas, PLoS One 6 (2016) e0162379.

[51] J.P. Jacobs, L. Lin, M. Goudarzi, P. Ruegger, D.P. McGovern, A.J. Fornace Jr., J. Borneman, L. Xia, J. Braun, Microbial, metabolomic, and immunologic dynamics in a relapsing genetic mouse model of colitis induced by T-synthase deficiency, Gut Microb. 8 (2017) 1–16.

[52] Y. Furusawa, Y. Obata, K. Hase, Commensal microbiota regulates T cell fate

decision in the gut, Semin. Immunopathol. 37 (2015) 17–25.
[53] I. Manoharan, A. Suryawanshi, Y. Hong, P. Ranganathan, A. Shanmugam, S. Ahmad, et al., Homeostatic PPARα signaling limits inflammatory responses to commensal microbiota in the intestine, J. Immunol. 196 (2016) 4739–4749.
[54] K.E. Mercer, S. Bhattacharyya, M.E. Diaz-Rubio, B.D. Piccolo, L.M. Pack, N. Sharma, M. Chaudhury, M.A. Cleves, S.V. Chintapalli, K. Shankar, Ronis MJJ, L. Yeruva, Infant formula feeding increases hepatic cholesterol 7α hydroxylase (CYP7A1) expression and fecal bile acid loss in neonatal piglets, J. Nutr. 148 (2018) 702–711.
[55] B. Kong, R. Sun, M. Huang, M.D. Chow, X.B. Zhong, W. Xie, Y.H. Lee, G.L. Guo, A novel fibroblast growth factor 15 dependent- and bile acid independent-promotion of liver regeneration in mice, Hepatology 68 (2018) 1961–1976.
[56] A. Yanagitani, S. Yamada, S. Yasui, T. Shimomura, R. Murai, Y. Murawaki, K. Hashiguchi, T. Kanbe, T. Saeki, M. Ichiba, Y. Tanabe, Y. Yoshida, S. Morino, A. Kurimasa, N. Usuda, H. Yamazaki, T. Kunisada, H. Ito, Y. Murawaki, G. Shiota, Retinoic acid receptor alpha dominant negative form causes steatohepatitis and liver tumors in transgenic mice, Hepatology 40 (2004) 366–375.
[57] T. Nowatari, S. Murata, K. Nakayama, N. Sano, T. Maruyama, R. Nozaki, K. Fukunaga, N. Ohkohchi, Sphingosine-1-phosphate has anti-apoptotic effects on liver sinusoidal endothelial cells and proliferative effect on hepatocytes in a paracrine manner in human, Hepatol. Res. 45 (2015) 1136–1145.