Mechanisms, Biomarkers and Targets for Therapy in Alcohol-associated Liver Injury: From Genetics to Nutrition: Summary of the ISBRA 2018 Symposium

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Abstract

The symposium "Mechanisms, Biomarkers and Targets for Therapy in Alcohol-associated Liver Injury: From Genetics to Nutrition" was held at the 19th Congress of International Society for Biomedical Research on Alcoholism on September 13th, 2018 in Kyoto, Japan. The goal of the symposium was to discuss the importance of genetics and nutrition in alcoholic liver disease (ALD) development from mechanistic and therapeutic perspectives. The following is a summary of this session addressing the gene polymorphisms in ALD, the role of zinc in gut-liver axis perturbations associated with ALD, highlighting the importance of dietary fat in ALD pathogenesis and hepatic n6 and n3 PUFA oxylipin pattern associated with ethanol-induced liver injury, and finally deliberating on new biomarkers for alcoholic hepatitis and their implications for diagnosis and therapy. This summary of the symposium will benefit junior and senior faculty currently investigating alcohol-induced organ pathology as well as undergraduate, graduate and post-graduate students and fellows.

Keywords

1. Introduction

Alcohol consumption is an important social, economic, and health problem (Axley et al., 2019). Heavy alcohol consumption results in a wide range of multi-organ pathology, including alcoholic liver disease (ALD) which is a major cause of alcohol-related morbidity and mortality in the United States and Worldwide (Farooq and Bataller, 2016;Rehm et al., 2017). ALD is one of the main causes of chronic liver diseases worldwide and accounts for up to 48% of all cirrhosis-associated deaths in the United States (Lucey, 2019). The clinical spectrum of ALD ranges from fatty liver to alcoholic hepatitis, fibrosis and cirrhosis, which may progress even further to hepatocellular carcinoma (Farooq and Bataller, 2016;Roth and Qin, 2019). ALD has limited prevention and therapeutic options (Farooq and Bataller, 2016;Singh et al., 2017;Osna et al., 2017). Therefore, understanding the molecular mechanism(s) that mediate the development and progression of alcohol-induced organ pathology, as well as identifying new biomarkers, therapeutic targets and promising novel therapeutic agents to prevent, manage, or reverse the disease progression, is of paramount importance.

The advanced stages of ALD develop only in a subset of long-term heavy drinkers. Genetic polymorphisms, epigenetic changes, intestinal dysbiosis, alterations in methionine metabolism, various environmental factors (e.g., smoking and dietary habits), are some of the factors that influence the pathogenesis and progression of ALD (Osna et al., 2017;Sugimoto and Takei, 2017;Kourkoumpetis and Sood, 2019). Gender and drinking patterns are among other well-known risk factors for ALD (Osna et al., 2017). The goal of the symposium was to discuss the newer insights into the importance of genetics and

nutrition in ALD development from mechanistic and therapeutic perspectives, and to analyze new biomarkers and their implications for diagnosis and therapy for ALD.

Overall, this scientific session reiterated that ALD is a complex disease and emphasized the important role that dietary and genetic factors play in the pathogenesis of ALD. The session was comprised of the following presentations:

i. Relevance of Genetics in Alcoholic Liver Disease;

ii. Impaired Phospholipid Methylation Results In Decreased Lipid Droplet Lipolysis: Role In Hepatic Steatosis;

iii. Hepatic ω−6 and ω−3 PUFA Oxylipin Patterns Associated with Ethanol-Induced Liver Injury in Mice;

iv. Paneth Cell Dysfunction Mediates Alcohol-Induced Dysbiosis and Hepatitis in Mice: Role of Zinc Deficiency and

v. Biomarkers for Acute Alcoholic Hepatitis: Implications for diagnosis and therapy

The highlights of these presentation were that: (i) The novel mutations identified in Genome-wide association studies (GWAs) of ALD patients were all in genes involved in lipid metabolism/processing; (ii) The specialized cellular organelles that store lipids, lipid droplets, are altered structurally and functionally by the alcohol-induced methylation defects which impairs the breakdown of the stored lipids that ultimately contributes to the development of alcoholic steatosis; (iii) A targeted lipidomic approach revealed that the dietary fats generate distinct hepatic n6 and n3 PUFA oxylipin pattern which may contribute to exacerbation of alcoholic liver injury; (iv) Cellular zinc deficiency mediates the deleterious effects of alcohol on Paneth cells dysfunction which results in the development of alcohol-induced intestinal dysbiosis, endotoxemia and hepatitis and (v) urine 3-hydroxypropyl-mercapturic acid (HPMA) and blood *P. gingivalis* IgA levels may serve as good biomarkers for severe acute alcoholic hepatitis (AAH).

2.   Summary of Presentations at the Symposium

2.1.   Relevance of Genetics in Alcoholic Liver Disease - Devanshi Seth, PhD

It is unknown why only a proportion of heavy and prolonged alcohol misusers develop cirrhosis and others who drink similar levels don't. In addition to the amount, type and pattern of alcohol intake, factors such as female gender and ethnicity increase the risk for ALD/alcoholic liver cirrhosis (ALC). For example, with 10 standard drinks per day, the relative risk of ALD in females reaches 10.69 compared to 4.38 for males and inheritance of risk for ALD is ~3-fold higher in monozygotic twins than dizygotic twins (Hrubec and Omenn, 1981). Furthermore, ALD-related mortality varies considerably among ethnicities, supporting a genetic basis for this disease. The role of genetics has been investigated for specific genes known to operate during ALD pathogenesis. The best examples how genetic mutations can alter the risk of ALD, comes from mutations altering enzyme kinetics in the alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). ADH1B rs1229984 induces ADH activity and acetaldehyde formation, whereas ALDH2*2 rs671

reduces ALDH activity impairing its ability to clear acetaldehyde. Both increase the accumulation of toxic acetaldehyde, resulting in profound rise in arterial blood flow to the face causing flushing and nausea. These SNPs are associated with reduced risk of alcoholism in carriers of one or both mutations and confer protection against alcohol use disorders including ALD. East Asians are doubly protected as these SNPs are particularly prevalent in this population. Other candidate gene studies regulating alcohol metabolism (ADH, ALDH, CYP2E1), inflammation (TNFα, IFNγ, IL10, IL1B, CD14), oxidative stress (CYP2E1, GST, MnSOD), ECM and fibrosis (TGFβ, angiotensin, CTGF, leptin, TIMPs, MMPs) (Stickel and Osterreicher, 2006) remain largely unconfirmed due to underpowered and flawed study designs.

Since ALD is a complex multifactorial disease, and its susceptibility is controlled by many genes, each gene mutation makes a small overall contribution towards the disease phenotype. In complex traits, simultaneous search for global mutations using GWAs has identified novel mutations in unsuspected genes. The only GWAs in ALD so far included a moderate size retrospective cohort of drinkers with and without cirrhosis (Buch et al., 2015). Interestingly, SNPs identified in this study (PNPLA3, TM6SF2, MBOAT7) have also been reported in nonalcoholic fatty liver disease (NAFLD)/steatohepatitis (NASH) (Romeo et al., 2008;Kozlitina et al., 2014;Mancina et al., 2016).

Patatin-like phospholipase domain containing 3 (PNPLA3) variant rs739409: C>G on chromosome 22 is the single most replicated variant in liver diseases. It is a non-synonymous single nucleotide mutation altering a highly conserved amino acid isoleucine to methionine at residue 148. PNPLA3 encodes for adiponutrin, a transmembrane protein highly expressed in the liver and adipose tissues. Previous independent studies reported a strong association of I148M with ALC (Seth et al., 2010;Tian et al., 2010), increasing the ancestry adjusted odds ratio by 1.79 per G allele (Seth et al., 2010). Our meta-analysis of 10 studies (excluding the GWAS by Buch et al), showed a significant correlation with the entire spectrum of ALD, with the magnitude of association increasing with disease severity (Salameh et al., 2015). PNPLA3 has lipogenic transacetylase and triglyceride hydrolase activities and is strongly linked with lipid droplets. It is suspected that I148M promotes hepatic intracellular lipid accumulation by reducing the breakdown of TG trapped in the lipid droplets (Pirazzi et al., 2012).

Another non-synonymous single nucleotide variant in the transmembrane 6 superfamily 2 (TM6SF2) rs58542926: C>T (E167K) on chromosome 19, was modestly associated with an increased the risk of ALD (Buch et al., 2015). This variant, also first identified in NAFLD, is associated with hepatic triglyceride content (Kozlitina et al., 2014), lipid transportation and as an independent risk factor for liver fibrosis. In vitro, the TM6SF2 inhibition was associated with increased cellular triglyceride concentration and lipid droplet content and its overexpression reduced lipid droplet size and number (Mahdessian et al., 2014).

Buch *et al* also reported a variant rs641738: C>T in the Membrane Bound O-AcylTransferase domain-containing 7 gene (MBOAT7) as moderately associated with ALC (Buch et al., 2015). MBOAT7 is expressed in the liver and encodes an enzyme with lysophosphatidylinositol acyltransferase (LPIAT1) activity. It is implicated in neutrophil

mediated anti-inflammatory processes and inflammation-driven liver fibrogenesis. Again, this variant is associated with hepatic fat in NAFLD (Mancina et al., 2016).

Most recently, association between a variant rs72613567:T>A in the Hydroxy-Steroid 17-beta Dehydrogenase 13 (HSD17B13) gene, on chromosome 4, was reported to be associated with reduced hepatic transaminases in ALC and NAFLD (Abul-Husn et al., 2018). Previous association with plasma liver enzymes in a large functional genomic approach, was reported by the same group (Chambers et al., 2011). HSD17B13 rs72613567:TA is associated with reduced risk of all stages of liver diseases, including ALD and NAFLD. Most interestingly, the T allele mitigated the risk conferred by the risk allele of PNPLA3 (Abul-Husn et al., 2018).

It is worth noting, that all polymorphisms identified so far in ALD/are shared with NAFLD/ NASH, indicating that in addition to histopathology, disease progression and other common risk factors such as obesity and diabetes, both diseases also have a common genetic basis. These shared risks indicate non-specificity to alcohol-induced liver disease.

Most intriguingly, all reported polymorphisms are involved in lipid metabolism/processing perhaps supporting inflammation and fibrogenesis during ALD progression. Despite widespread recognition that liver steatosis is the earliest and most consistent morphologic feature in heavy drinkers, the mechanism of how excessive alcohol consumption-induced liver fat accumulation plays a crucial role in disease chronicity remains unclear.

2.2. Impaired Phospholipid Methylation Results In Decreased Lipid Droplet Lipolysis: Role In Hepatic Steatosis - Kusum K. Kharbanda, PhD

With the aim of identifying factors that contribute to fatty liver, Kharbanda laboratory characterized lipid droplets (LDs). These are specialized cell organelles that store fat and are composed of a core of neutral lipid including triglycerides and cholesteryl esters. The neutral lipid core of a LD is shielded from the surrounding cytosol by a phospholipid monolayer consisting mostly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Bartz et al., 2007;Chitraju et al., 2012). The LD surface is further decorated with LD associated proteins whose functions vary widely and include roles in lipid metabolism, trafficking, and signaling (Fujimoto et al., 2004;Khan et al., 2015;Turro et al., 2006). Kharbanda laboratory and others have consistently observed that Wistar rats fed the ethanol Lieber DeCarli diet for 4–5 weeks exhibit dramatic increases in hepatic lipid accumulation as compared to the rats fed the control diet (Kharbanda et al., 2007). An increase in both LD size and number as revealed by BODIPY-staining was observed in the livers of ethanol-fed rats (Kharbanda et al., 2012;Listenberger et al., 2018). Differences in hepatic lipid metabolism following ethanol feeding were also detected by examining the ability of the isolated hepatocytes to catabolize accumulated fat. Hepatocytes of rats fed ethanol for 4–5 weeks displayed an approximately 50% decrease in the rate of lipolysis as compared to hepatocytes isolated from control diet fed animals (Listenberger et al., 2018). As the liver exports triglycerides and cholesterol only as constituents of very low-density lipoprotein (VLDL) particles, its secretion is one of the major mechanisms for removing liver fat. Since up to 70% of the triglycerides packaged and secreted by hepatocytes in VLDLs are derived via lipolysis of preformed triglyceride stores in LDs (Gibbons et al., 2002;Salter et al.,

1998;Wiggins and Gibbons, 1992), the reduction in lipolysis observed in their recently published study (Listenberger et al., 2018) is consistent with our previous report showing a decreased *in vivo* VLDL secretion rate in the ethanol-fed rats compared to control rats (Kharbanda et al., 2009).

Interestingly, triacylglycerol hydrolase (TGH), a major lipase in hepatocytes that has been shown to mobilize LD triglycerides for VLDL assembly (Gilham et al., 2003;Wang et al., 2010;Wei et al., 2010), was not altered by ethanol exposure (Listenberger et al., 2018). To understand the reason for reduced lipolysis, isolated populations of LDs were prepared by differential centrifugations and the composition of these specialized structures examined. An increase in the amount of triglyceride in the hepatic LDs isolated from ethanol fed rats was detected which was consistent with the greater overall accumulation of triglyceride in the livers of these animals (Listenberger et al., 2018). Additionally, the amount of PC relative to PE was significantly decreased in the LD fractions from ethanol fed rats compared to hepatic LDs from control rats (Listenberger et al., 2018). This difference is likely due to the reduction in the generation of PC from PE via the phosphatidylethanolamine methyltransferase (PEMT)-catalyzed methylation because of the lowering of the hepatocellular SAM:SAH ratio in the ethanol fed (2.49±0.22) compared to control (4.40±0.59) rats as reported before (Barak et al., 2003;Kharbanda et al., 2007). In addition, LD fractions from the livers of ethanol fed rats contained elevated levels of perilipin 2 and 3 (Listenberger et al., 2018). These specific LD proteins have been associated with the onset of alcohol-induced hepatic steatosis (Carr and Ahima, 2016;Ikura and Caldwell, 2015) facilitating over-accumulation of lipid in the liver by preventing lipolysis (Chang et al., 2010;Carr et al., 2014;Carr et al., 2012).

How these anti-lipolytic proteins are recruited to the LD surface is largely unknown. Many organelles use special lipids (e.g., phosphoinositides, phosphatidylserine, phosphatidic acid) for mediating protein targeting to membranes (Walther and Farese, 2012;Thiam et al., 2013). We were interested in examining whether LDs utilize a similar strategy for recruiting surface proteins. Since the phospholipid ratio (PC and PE) and surface protein association (perilipins 2 and 3) in hepatic LDs are both altered by ethanol exposure (Listenberger et al., 2018), we considered the possibility that these major LD surface phospholipids were playing a role in recruiting LD proteins. To test this conjecture, we utilized an *in vitro* binding assay previously developed to explore perilipin 2 targeting to lipid droplet surfaces (Sletten et al., 2014).

Large unilamellar vesicles (LUVs), that mimicked the PC:PE ratios observed in the livers of control and ethanol-fed rats, were constructed and incubated with perilipin 2 from the cytosolic fraction of perilipin 2 overexpressing HEK 293 cells (Listenberger et al., 2007) as previously described (Sletten et al., 2014). Next, unbound perilipin 2 was separated from the LUVs by sucrose gradient centrifugation and the amount of perilipin 2 that fractionated with the LUVs of each phospholipid composition was determined. These experiments showed that the relative levels of PC and PE influenced the amount of perilipin 2 bound to LUVs (Listenberger et al., 2018). Specifically, perilipin 2 association increased as the ratio of PC to PE decreased. This difference was significant for PC:PE ratios that modeled the hepatic lipid droplets of control (3.5:1) and ethanol fed (2.5:1) rats.

Collectively, these studies reveal significant differences in the overall composition of hepatic LDs from control and ethanol fed rats. Especially relevant were the alterations in the relative amounts of PC and PE, along with association of specific LD proteins in hepatic LDs from ethanol fed rats. Modeling the changes to the phospholipid composition of LDs in cultured cells and *in vitro* experiments revealed similar changes in the association of specific LD proteins led to the conclusions that changes to the LD phospholipid composition impact protein association and LD function to impair lipolysis and subsequent VLDL secretion contributing to the development of alcoholic steatosis.

2.3. Hepatic ω−6 and ω−3 PUFA Oxylipin Patterns Associated with Ethanol-Induced Liver Injury in Mice - Irina A. Kirpich, PhD

A primary focus of Kirpich laboratory is to understand the role of different types of dietary fatty acids (FAs, *e.g.,* saturated *vs.* unsaturated; and ω−6 polyunsaturated fatty acids (PUFA) vs. ω−3 PUFA) in gut-liver axis alterations associated with ALD. Kirpich laboratory investigated the potential mechanisms by which dietary FAs may exacerbate or attenuate alcohol-induced intestinal and liver injury. They postulated that oxidized PUFA metabolites (oxylipins), among other critical factors, play significant roles in ALD pathogenesis. Oxylipins are diverse bioactive lipid mediators synthesized from ω−3 and ω−6 PUFAs and may exert pro-inflammatory or dual anti-inflammatory and pro-resolution of inflammation activities (Moghaddam et al., 1996;Gabbs et al., 2015). Previous studies using a mouse model demonstrated that the combination of ethanol and a diet with a high content of linoleic acid (LA) exacerbated liver injury through increased lipoxygenase (LOX) pathway production of oxidized LA metabolites (OXLAMs) that shift RAW cells/macrophages toward an M1 pro-inflammatory phenotype (Warner et al., 2017). However, less is known regarding the role of oxidized PUFA metabolites generated via other metabolic pathways (*e.* CYP and soluble epoxide hydrolase [sEH]). In addition, the role of lipid mediators derived from ω−3 PUFAs such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the ALD pathogenesis is understudied.

Using a targeted lipidomic approach, the pattern of ω−6 and ω−3 PUFA metabolites associated with liver injury caused by high dietary LA and ethanol consumption was examined. In this study a chronic-binge experimental mouse model of ALD (10+1 NIAAA model), which mimics the pathology of human alcoholic hepatitis (AH). Mice were fed Lieber–DeCarli liquid diets containing either saturated fat (SF, enriched in beef tallow/medium chain triglycerides) or unsaturated fat (USF, enriched in corn oil/LA). These data support a role for the CYP/sEH axis in ALD development and/or progression. CYPs metabolize numerous PUFAs and generate a variety of different signaling lipids, including epoxy-FAs, which are rapidly hydrolyzed by sEH to their corresponding vicinal diols, dihydroxy-FAs (Figure 1A). While epoxy-FAs possess potent cytoprotective, anti-oxidant and anti-inflammatory properties and mediate the resolution phase of inflammation (Gilroy et al., 2016), diols have long been considered to be less active than their parent epoxides. However, recent evidence demonstrated significant biological activity (Greene et al., 2000;Bettaieb et al., 2013;Fang et al., 2006;Oltman et al., 1998;Sisemore et al., 2001;Thompson and Hammock, 2007). In this study, the levels of epoxy-FAs were, in general, similar across all precursors and among all experimental groups; however, there

were significant changes in their hydroxylated metabolites (diols, Figure 1B, adapted from (Warner et al., 2018)). One important observation was the increased levels of the LA dihydroxy metabolites, 9,10-DiHOME and 12,13-DiHOME, and α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) -derived 8,9-DiHETrE and 11,12-DiHETrE, in USF+ethanol-fed mice (the group with increased hepatic neutrophil-mediated inflammation and injury) compared to USF-fed control and SF+ethanol-fed animals. Further studies will be necessary to elucidate the functional significance of the increase in these dihydroxy-FAs in the context of ALD.

EPA and DHA are precursors to numerous metabolites, including resolvins, protectins, and maresins, that possess anti-inflammatory and pro-resolving properties (Lopez-Vicario et al., 2016;Serhan, 2014;Serhan et al., 2008). Ethanol-fed mice had altered hepatic concentrations of several hydroxy- metabolites of EPA and DHA. For example, 5-HEPE (LOX product), which is known to reduce the inflammatory response of macrophages (Wang et al., 2017), was increased in the USF+ethanol group *vs.* USF alone. 18-HEPE (a cyclooxygenase product and precursor to the resolvin E series (RvE), was significantly increased in both the SF+ethanol and USF+ethanol groups compared to pair-fed controls. Several DHA metabolites related to the pro-resolving lipid mediators, such as 10S,17S-dihydroxy-docosahexaenoic acid (10S,17S-DiHDoHE, a protectin D1 isomer), and 4,17-DiHDoHE, were increased by ethanol in the USF+ethanol fed mice. Interestingly, elevated levels of pro-resolving molecules in the USF+ethanol group were associated with marked liver inflammation and injury, suggesting that the resolution of inflammation likely was initiated as a natural/adaptive response to inflammation in these mice. It is possible that the deleterious effects of pro-inflammatory oxylipins outweigh the benefits of anti-inflammatory and pro-resolving bioactive lipid mediators and tip the balance toward liver damage. In a preliminary study, we found that mice treated with resolvin D1 (a DHA metabolite) had attenuated ethanol and LPS-induced liver injury (data not shown) suggesting beneficial effects of resolvins in ALD, but more research into this area is needed.

In summary, a complex diet-specific pattern of pro- and anti-inflammatory metabolites associated with ethanol-induced liver pathology was identified which may contribute to specific mechanisms of liver injury exacerbation or attenuation, and which may represent potential biomarkers and/or therapeutic targets for ALD.

2.4. Paneth Cell Dysfunction Mediates Alcohol-Induced Dysbiosis and Hepatitis in Mice: Role of Zinc Deficiency - Zhanxiang Zhou, PhD

Increasing evidence suggest that intestinal dysbiosis and gut-derived pathogen-associated molecular patterns (PAMPs) contribute to the pathogenesis of ALD through induction of hepatic inflammation (Hendrikx and Schnabl, 2018). However, the mechanisms involved in the development of intestinal dysbiosis and PAMP translocation are still not fully defined. Intestinal antimicrobial peptides (AMPs) play a critical role in regulating microbiota homeostasis and limiting bacterial translocation (Chung and Raffatellu, 2018). Paneth cells are professional AMP-producing innate immune cells and, of note, a major source of an important type of AMPs, α-defensins (Adolph et al., 2018). It is estimated that α-defensins contribute to about 70% of total bactericidal activity of the small intestine. Moreover, given

the facts that ALD is associated with zinc deficiency (Mohammad et al., 2012b) and Paneth cells are enriched in zinc (Giblin et al., 2006), the role of alcohol-induced zinc deficiency in Paneth cell function and its pathophysiological consequences necessitate investigation. The present study aimed to determine if chronic alcohol exposure perturbs AMP production by Paneth cells via zinc deficiency, and if α-defensin dysfunction mediates alcohol-induced intestinal dysbiosis, PAMP translocation, and hepatitis. Mouse models of chronic alcohol exposure, genetic α-defensin dysfunction, and dietary, experimental and genetic zinc depletions were conducted. Wild type mice after 8 weeks of alcohol consumption developed intestinal dysbiosis as showed by perturbed microbiome, PAMP translocation as indicated by elevated levels of plasma endotoxins, and hepatic inflammation as indicated by increased cytokine expression and inflammatory cell infiltration. Meanwhile, the expression and bactericidal activity of Paneth cell AMPs were significantly reduced in the ileum of the alcohol-fed mice. The mechanistic link between Paneth cell AMP reduction and alcohol-induced dysbiosis as well as hepatitis was further determined using the matrix metallopeptidase 7 knockout ($Mmp7^{-/-}$) mice that are deficient in active α-defensins. The $Mmp7^{-/-}$ mice showed more severe intestinal dysbiosis, endotoxemia and hepatitis along with reduced intestinal bactericidal activity, compared to the wild type mice after 8 weeks of alcohol feeding. Importantly, elevations of hepatic lipocalin 2 (LCN2), an immune mediator, and KC, a chemokine that attracts neutrophil infiltration, were observed to be a direct result of PAMP translocation instead of alcohol intoxication. The expression levels of both LCN2 and KC were significantly higher in the alcohol-fed $MMP7^{-/-}$ mice compared to the alcohol-fed wild type mice. Lipopolysaccharide (LPS) treatment directly induced LCN2 and KC production in hepatocytes, whereas ethanol or ethanol metabolites did not. Antibiotic treatment reduced PAMP translocation and prevented LCN2 and KC induction in these mice. To test if restoring Paneth cell antimicrobial activity would reverse the detrimental effects of alcohol, recombinant human defensin 5 (rHD5) was administrated to the alcohol-fed mice for the least 2 weeks of the 8-week feeding. rHD5 treatment effectively attenuated alcohol-induced intestinal dysbiosis and hepatic inflammation. Intriguingly, rHD5 upregulated Paneth cell AMP expression. To determine the role of zinc deficiency in alcohol-induced Paneth cell dysfunction, a mouse model of chronic alcohol feeding plus dietary zinc deficiency and an *ex vivo* model of zinc chelation of intestinal crypts was utilized. Dietary zinc deficiency exaggerated chronic alcohol-impaired Paneth cell bactericidal activity in association with downregulation of AMP gene expression. Ex vivo experiments showed that chelation of zinc from Paneth cell-secreted AMPs led to a reduced bactericidal activity. Bacterial killing experiment further showed that addition of zinc to rHD5 enhanced the bactericidal activity of both oxidized and reduced forms of rHD5, suggesting a role of zinc coordination in bactericidal activity of α-defensins. Moreover, we found that a Paneth cell-predominant zinc transporter, ZIP8, was significantly decreased by chronic alcohol feeding; the reduction was through acetaldehyde rather than zinc deficiency *per se*. To define the role of ZIP8 in Paneth cell zinc homeostasis and AMP activity, a Paneth cell-specific ZIP8 knockout mouse model ($Zip8^{PC-/-}$) was generated. Knock out of Paneth cell ZIP8 led to decreased cellular zinc levels in Paneth cells and reduced production of α-defensins. As a result, $Zip8^{PC-/-}$ mice exhibited more severe hepatic inflammation. Taken together, our study suggest that Paneth cell AMP dysfunction is a pathophysiological factor in the development of alcohol-induced intestinal dysbiosis, endotoxemia and hepatitis,

whereas cellular zinc deficiency may mediate the deleterious effects of alcohol on Paneth cells. The study also suggests that rHD5 has the potential to be a new class of therapeutics for treating alcoholic hepatitis.

2.5   Biomarkers for Acute Alcoholic Hepatitis: Implications for diagnosis and therapy - Craig J. McClain, MD

Biomarkers are increasingly used in evaluating and treating acute alcoholic hepatitis (AAH). Some biomarkers, such as the discriminant function (DF) and the model for end-stage liver disease (MELD) are used for inclusion of patients into treatment arms of clinical trials for severe AAH. Traditionally, DF >32 and MELD 20 have been criteria for inclusion in these studies (Crabb et al., 2016;Mitchell et al., 2017). These scoring systems and a variety of other static biomarkers utilize liver-related tests such as bilirubin and INR that are elevated with increasing severity of liver injury or non-hepatic factors such as creatinine that also tend to increase with very severe liver injury. Multiple other static scoring systems have been developed using logistic regression models for these types of laboratory tests and demographic factors such as age (Bissonnette et al., 2017).

The sources of biomarkers are typically serum, plasma or urine. Less commonly, saliva, breath analysis and cell-based functional assays are used. Easily-obtained specimens are optimal. Biomarkers can serve multiple functions including disease diagnosis, disease severity or prognosis, mechanisms of disease, response to therapy, or they can be a component of personalized medicine. Our group has been particularly interested in biomarkers that can also serve as indications of mechanisms of disease. We highlight below two of our recent observations on mechanistic biomarkers.

Endoplasmic Reticulum (ER) stress-Unfolded Protein Response (UPR) has recently been established as an important mechanism contributing to hepatic injury in AAH; however, the mediators and mechanisms of alcohol-induced ER stress and liver injury in AAH are not fully understood. Alcohol consumption and metabolism increase lipid peroxidation and generate acrolein, a reactive and toxic aldehyde which is known to cause ER stress and damage hepatocytes (Mohammad et al., 2012a). Acrolein exposure can also come from exogenous sources, such as cigarette smoke, fried foods, or environmental contact. Acrolein is primarily metabolized by the enzyme glutathione-S-transferase Pi (GSTP), and unless cleared, acrolein can form irreversible covalent adducts with proteins, thereby interfering with their function. Our preliminary *in vivo* (mice) data demonstrate that alcohol consumption results in significant hepatic acrolein generation and accumulation of acrolein-protein adducts, which correlates with ER stress, apoptosis and liver (Chen et al., 2016). Also, our data show that alcohol consumption decreases GSTP levels in mice livers, suggesting that GSTP downregulation may lead to a pathogenic accumulation of acrolein. Further, our pilot research showed that removal of acrolein by the scavenger hydralazine, significantly attenuated alcohol-induced liver injury in mice (Chen et al., 2016).

Based on these compelling observations, we extended our *in vitro* and mouse studies to humans with AAH. We obtained human urine specimens from individuals with AAH of varying severity and selected suitable matched non-alcoholic control specimens. We compared urine HPMA (3-hydroxypropyl-mercapturic acid, a metabolite of acrolein) levels

in healthy controls and AAH patients, as a single collective group, or as divided into non-severe (MELD   19) and severe (MELD   20) groups (Fig. 2). HPMA levels were significantly higher in AAH patients as a group compared to healthy controls (Fig. 2A). There was no statistical difference found in HPMA values between non-severe AAH patients and healthy controls. When we compared HPMA levels in healthy volunteers and severe AAH patients, we found significant elevation of 3.23-fold in HPMA with a moderate effect size, adjusted $R^2=0.427$ (Fig. 2B); with age as a covariate, the effect size was augmented to $R^2=0.465$. We postulate that urine HPMA may be a good non-invasive biomarker for severe AAH, and that acrolein likely plays an etiologic role in AAH. These findings highlight the importance of lipids and lipid peroxidation products in AAH.

Translocation of bacteria/bacterial proteins across the gastrointestinal tract plays a critical mechanistic role in AAH. Bacterial infection is frequently observed in patients with AAH (discussed elsewhere in this manuscript). *P. gingivalis* is a major periodontal pathogen that plays a predominant role in chronic periodontitis, and it can also affect remote organs such as the liver. We evaluated the role of *P. gingivalis* in the development/progression and severity of disease in patients with AAH (Zhou et al., 2019).

Plasma specimens from 47 AAH patients (16 moderate [MELD < 20] and 31 severe [MELD   20]) and 22 healthy controls (HC) were collected. Antibody tests for IgG, IgM and IgA against two *P. gingivalis* strains were performed.

Severe AAH patients showed significantly higher plasma levels of IgG, IgA and IgM against both *P. gingivalis* strains (W83 and 33277) compared to HC (Zhou et al., 2019). Moderate AAH patients also had significantly-elevated anti-*P. gingivalis* IgA concentrations for both strains compared to HC (Fig. 3). These significantly-elevated plasma anti-*P. gingivalis* IgG, IgA and IgM in severe AAH provide preliminary data that *P. gingivalis* could be a novel risk factor in the development/progression or severity of AAH, and we postulate that *P. gingivalis* IgA levels serve as biomarkers for severity of AAH. This study highlights the potential importance of the oral microbiome in AAH, and it presents a unique therapeutic target.

In summary, the advent of molecular tools, such as proteomics and metabolomics as well as large well-characterized specimens/data sets from AAH trials should provide new biomarkers that may serve multiple functions, ranging from disease severity/prognosis to mechanisms and comparison diagnostics.

3.   FINAL SUMMARY

Overall, this symposium provided an update on the mechanistic and therapeutic viewpoint on the role of genetics and nutrition in the development of ALD which is schematically highlighted in Figure 4. The following summarizes the take-home message of the session's presentations: (i) GWAS of ALD patients have identified novel mutations in many genes which are involved in lipid metabolism/processing; (ii) Alcohol-induced methylation defects alter the structure and function of the specialized cellular organelles that store lipids, lipid droplets, which impairs the breakdown of the stored lipids that ultimately contributes to the development of alcoholic steatosis; (iii) A targeted lipidomic approach revealed that the

dietary fats generate distinct hepatic n6 and n3 PUFA oxylipin pattern which may contribute to exacerbation of alcoholic liver injury; (iv) Cellular zinc deficiency mediates the deleterious effects of alcohol on Paneth cells dysfunction which results in the development of alcohol-induced intestinal dysbiosis, endotoxemia and hepatitis and (v) urine 3-hydroxypropyl-mercapturic acid (HPMA) and blood *P. gingivalis* IgA levels may serve as good biomarkers for severe acute alcoholic hepatitis (AAH).

Acknowledgments

Abbreviations

HPMA	3-hydroxypropyl-mercapturic acid
ALDH	acetaldehyde dehydrogenase
AAH	acute alcoholic hepatitis
ALD	alcoholic liver disease
ALC	alcoholic liver cirrhosis
ADH	alcohol dehydrogenase
ALA	α-linolenic acid
AMPs	antimicrobial peptides
CTGF	connective tissue growth factor
CYP	cytochrome P450
DF	discriminant function
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA	fatty acids
GWAs	genome-wide association studies
GST	glutathione S-transferase
GSTP	glutathione-S-transferase Pi
HSD17B13	hydroxy-steroid 17-beta dehydrogenase 13

LUVs	large unilamellar vesicles
LA	linoleic acid
LDs	lipid droplets
LCN2	lipocalin 2
LOX	lipoxygenase
LPIAT1	lysophosphatidylinositol acyltransferase
MnSOD	manganese superoxide dismutase
MBOAT7	membrane bound O-acyltransferase domain-containing 7 gene
MMP	metallopeptidase
MELD	model for end-stage liver disease
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
OXLAMs	oxidized LA metabolites
PNPLA3	Patatin-like phospholipase domain containing 3
PAMPs	pathogen-associated molecular patterns
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine methyltransferase
PUFA	polyunsaturated fatty acids
rHD5	recombinant human defensin 5
RvE	resolvin E series
SF	saturated fat
she	soluble epoxide hydrolase
TIMPs	tissue inhibitors of metalloproteinases
TM6SF2	transmembrane 6 superfamily 2
TGHL	triacylglycerol hydrolase
UPR	unfolded protein response
USF	unsaturated fatty acids
VLDL	very low-density lipoprotein

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The symposium "Mechanisms, Biomarkers and Targets for Therapy in Alcohol-associated Liver Injury: From Genetics to Nutrition" was held at the 19th Congress of International Society for Biomedical Research on Alcoholism on September 13th, 2018 in Kyoto, Japan. The goal of the symposium was to discuss the importance of genetics and nutrition in alcoholic liver disease (ALD) development from mechanistic and therapeutic perspectives. Overall, this scientific session reiterated that ALD is a complex disease and highlighted the important role that dietary and genetic factors play in the pathogenesis of alcoholic liver disease.
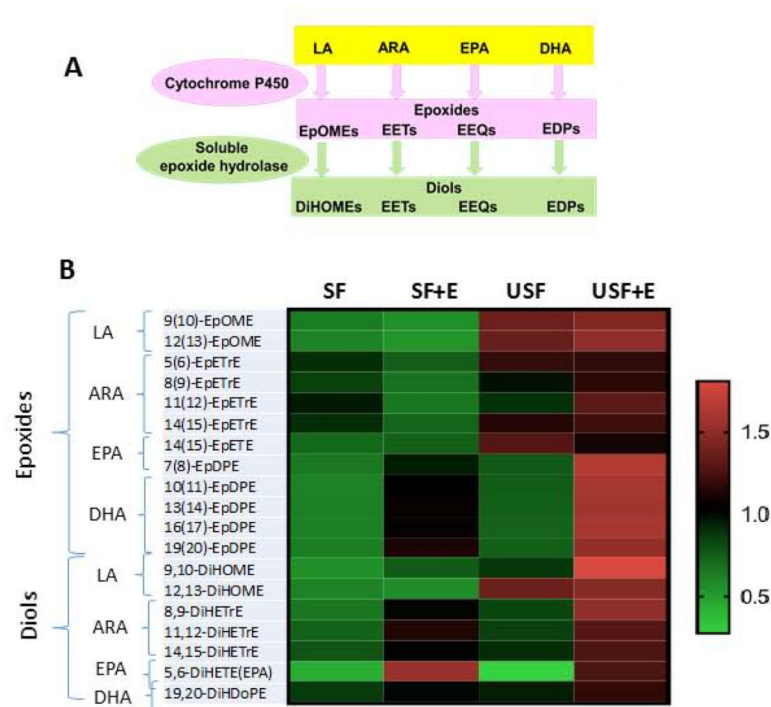
Figure 1.
Diet-specific alterations in oxylipin profiles in response to EtOH administration. A.
Schematic representation of the CYP and sEH metabolic pathways of epoxide and diol
production. B: Changes in the abundance of individual epoxides and diols between
experimental groups. Results are expressed as a matrix view (heat map) where rows
represent individual oxylipins and columns represent group distribution. The intensity of
each color denotes the standardized ratio between each sample value and the average levels
of each individual oxylipin across all samples. ARA, arachidonic acid; DHA,
docosahexaenoic acid; DiHDoPEs, dihydroxy-docosapentaenoic acids; DiHETEs,
dihydroxy-eicosatetraenoic acids; DiHOMEs, dihydroxy-octadecenoic acid; EPA,
eicosapentaenoic acids; EpDPEs, epoxy-docosapentaenoic acids; EpETE, epoxy-
eicosatetraenoic acids; EpETrEs=EETs, epoxy-eicosatrienoic acids; EpOMEs, epoxy-
octadecenoic acid; Et, ethanol; LA – linoleic acid, SF, saturated fat; USF, unsaturated fat. P
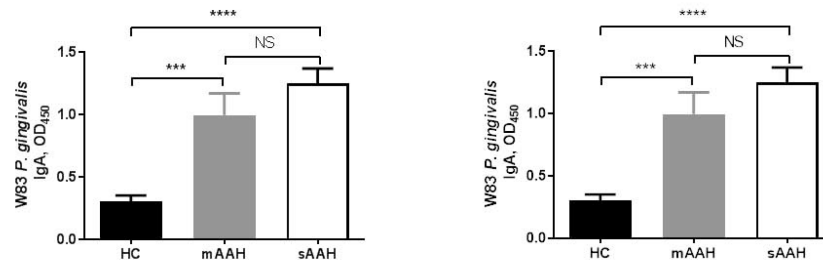< 0.05 a: SF vs SF+Et; b: USF vs USF+Et; c: SF+ET vs USF+Et; d: SF vs USF

Figure 2:
Urine levels of the acrolein metabolite HPMA in healthy controls and patients with acute alcoholic hepatitis (AAH). A: HPMA levels in healthy controls and all patients with AAH, P = 0.01. B: HPMA levels in healthy controls and patients with non-severe and severe AAH. **P = 0.002 between control and patients with severe AAH, ***P = 0.001 between patients with non-severe and severe AAH. HPMA, 3-hydroxypropylmercapturic acid.
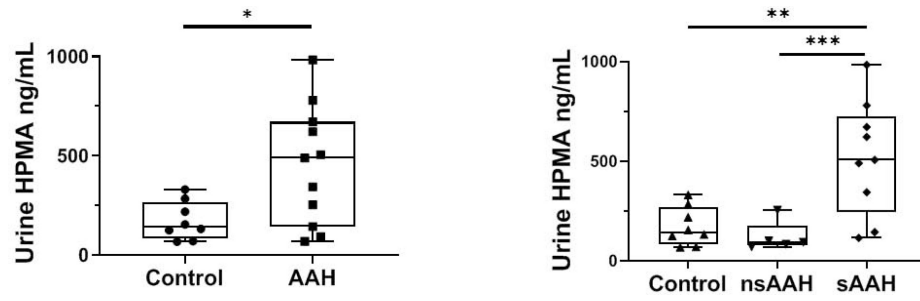
Figure 3.
Anti-*P. gingivalis* IgA and AAH severity. Anti-*P. gingivalis* Wi3IgA levels in HCs, patients with moderate AAH, and patients with severe AAH.
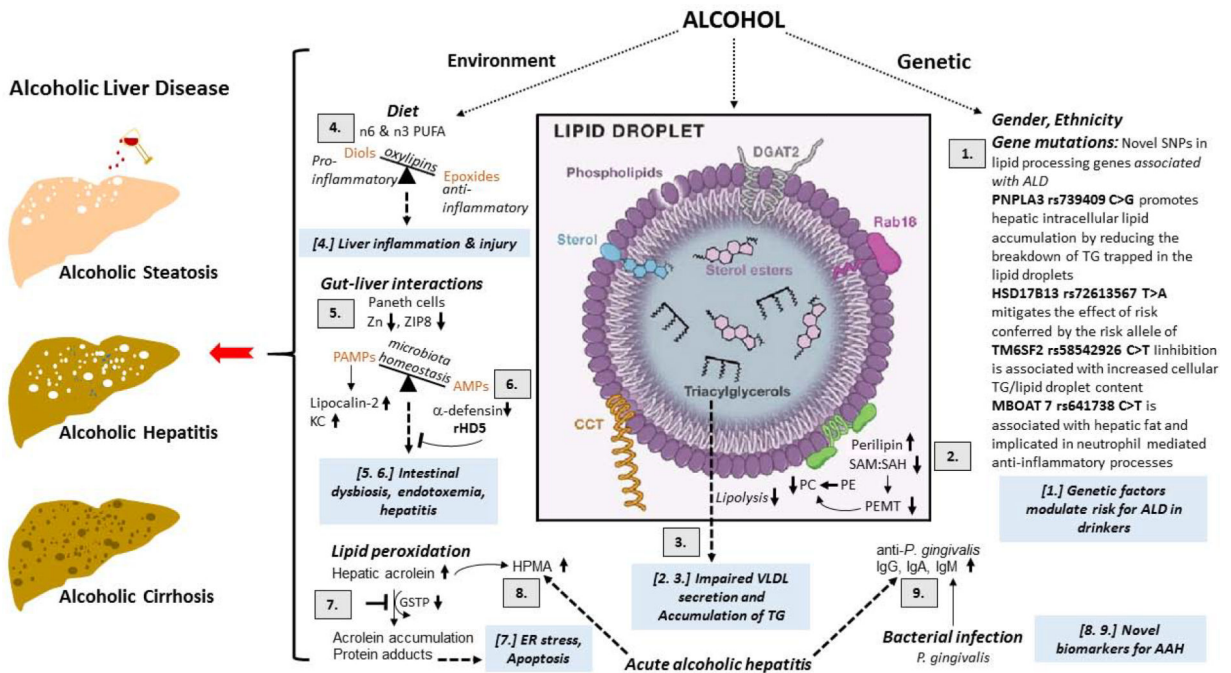
Figure 4.

Schematic of the update on the mechanistic and therapeutic viewpoint on the role of genetics and nutrition in the development of alcoholic liver disease (ALD).

[1.] GWAS identified SNPs in lipid processing pathways are associated with risk of progressing ALD. PNPLA3 rs739409 C>G promotes hepatic intracellular lipid accumulation by reducing the breakdown of TG trapped in the lipid droplets. HSD17B13 rs72613567 T>A mitigates the effect of risk conferred by the risk allele of PNPLA3. TM6SF2 rs58542926 C>T (E167K) inhibition is associated with increased cellular TG and lipid droplet content. MBOAT7 rs641738: C>T is associated with hepatic fat and implicated in neutrophil mediated anti-inflammatory processes.

[2.] Alcohol consumption decreases the phosphatidylethanolamine methyltransferase (PEMT)-catalyzed generation of the PC from PE that lowers the ratio of these two major classes of phospholipids on the lipid droplet (LD) surface monolayer. This change in the PC:PE ratio promotes the generation of enlarged LDs as well as recruits' specific proteins such as perilipins 2 to the LD surface.

[3.] Supersized LDs and increase in specific perilipins prevents the access of lipases to LD triglyceride stores to inhibit their lipolysis thus facilitating lipid over-accumulation in the liver contributing to the development of alcoholic steatosis.

[4.] Ethanol-induced liver injury is associated with distinct profile of bioactive n6 and n3 PUFA metabolites. CYPs metabolize numerous PUFAs and generate a variety of different signalling oxidised lipids (oxylipins), including anti-inflammatory epoxy-FAs, which are rapidly hydrolyzed by sEH to their corresponding vicinal diols, dihydroxy-FAs, which are less active or might be pro-inflammatory. Possibly the deleterious effects of pro-inflammatory oxylipins outweigh the benefits of anti-inflammatory and pro-resolving lipid mediators and tip the balance toward liver damage.

[5.] Chronic alcohol impairs zinc transporter, ZIP8, activity leading to zinc deficiency in Paneth cells. The consequent reduction in antimicrobial peptides (AMPs, a-defensin) allows for pathogen associated molecular patterns (PAMPS) translocation from the intestine to the liver to induce pro-inflammatory lipocalin-2 and KC chemokine production, thereby contributing to alcoholic hepatitis.

[6.] Restoring a-defensin by recombinant human defensin 5 (rDH5) restores AMP, reduces intestinal dysbiosis, endotoxemia and hepatitis and is potentially a new class of therapeutic for treating alcoholic hepatitis.

[7.] Alcohol consumption and metabolism increase lipid peroxidation and generate acrolein. The concomitant alcohol-reduced reduction in glutathione-S-transferase Pi (GSTP) impairs the clearance of acrolein facilitating its accumulation and protein adduct formation causing ER stress.

[8.] Acrolein metabolite 3-hydroxypropyl-mercapturic acid (HMPA) is increased in urine of acute alcoholic hepatitis (AAH) patients. Urine HPMA may be a good non-invasive biomarker for severe AAH. These findings highlight the importance of lipids and lipid peroxidation products in AAH.

[9.] P. gingivalis is a major periodontal pathogen that can also affect remote organs such as the liver. Plasma levels of IgG, IgA and IgM against *P. gingivalis* strains are increased in severe acute alcoholic hepatitis and can serve as biomarkers for severity of AAH.