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New insights into the mechanism of alcohol-mediated organ damage via its impact on immunity, metabolism, and repair pathways: A summary of the 2021 Alcohol and Immunology Research Interest Group (AIRIG) meeting

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Abstract

On November 19th, 2021, the annual Alcohol and Immunology Research Interest Group (AIRIG) meeting was held at Loyola University Chicago Health Sciences Campus in Maywood, Illinois. The 2021 meeting focused on how alcohol misuse is linked to immune system derangements, leading to tissue and organ damage, and how this research can be translated into improving treatment of alcohol-related disease. This meeting was divided into three plenary sessions: the first session focused on how alcohol misuse affects different parts of the immune system, the second session presented research on mechanisms of organ damage from alcohol misuse, and the final session highlighted research on potential therapeutic targets for treating alcohol-mediated tissue damage. Diverse areas of alcohol research were covered during the meeting, from alcohol's effect on pulmonary systems and neuroinflammation to epigenetic changes, senescence markers, and microvesicle particles. These presentations yielded a thoughtful discussion on how the findings can lead to therapeutic treatments for people suffering from alcohol-related diseases.

Keywords

brain; cardiovascular disease; ethanol; inflammation; liver; lung; myopathy; pneumonia; skin; stem cell

Introduction

Alcohol misuse is a deadly yet preventable cause of death; annually, 95,000 people die in the United States from alcohol misuse, and 3 million people die worldwide (Centers for Disease Control and Prevention, 2021). Alcohol misuse is defined by excessive daily consumption, total consumption, or both, specifically daily consumption of more than 4 drinks per day for men or more than 3 drinks per day for women, or excess total consumption of more than 14 drinks per week for men or more than 7 drinks per week for women (Centers for Disease Control and Prevention, 2021). This disorder can lead to chronic health consequences, such as liver and digestive diseases, and increased susceptibility to infectious diseases, cancer, and cognitive decline, resulting in more than $249 billion in economic costs in the United States (Centers for Disease Control and Prevention, 2018). While much progress has been made in investigating how alcohol misuse leads to these diseases, many questions remain unanswered surrounding alcohol-mediated tissue and organ injury and their impact on immune and inflammatory pathways. Thus, the 2021 AIRIG meeting was convened to highlight recent work on alcohol's impact on inflammation and the immune system, and the mechanisms by which this leads to organ damage.

Dr. Elizabeth J. Kovacs, Director of the Burn Research and Alcohol Research Program in the Department of Surgery at the University of Colorado Denver, Dr. Mashkoor A. Choudhry, Director of the Alcohol Research Program at Loyola University Chicago's Stritch School of Medicine, and Dr. Vivian Gahtan, Chair of the Department of Surgery at Loyola University Chicago, opened the 2021 AIRIG meeting with welcoming remarks, and expressed appreciation to the attendees, the speakers for sharing their research, and the organizers for making this conference a reality during the COVID-19 pandemic. They also acknowledged the National Institute on Alcohol Abuse and Alcoholism (NIAAA) for providing financial support through its R13 meeting grants (R13 AA020768) and H. Joe Wang, PhD, for virtually attending the meeting. Dr. Wang held a helpful discussion and answered questions regarding current and upcoming NIAAA funding opportunities for junior investigators and trainees.

Alcohol, host immunity, and organ damage

Presenters from four different institutions gave oral presentations on the effect of alcohol on host immunity and organ damage. This first plenary session was chaired by Dr. Rebecca McCullough (University of Colorado Denver, Anschutz Medical Campus) and Dr. Abigail Cannon (Loyola University Chicago, Health Sciences Center).

Inflammation and the microbiome: implications for TBI and co-occurring psychiatric conditions (Dr. Lisa A. Brenner, University of Colorado Denver, Anschutz Medical Campus)

Dr. Brenner opened the session by presenting research evaluating the effects of probiotic supplementation on psychiatric conditions in military veterans. Many veterans returning from Operation Enduring Freedom/Operation Iraqi Freedom/Operation New Dawn experienced symptoms associated with mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD). Interventions aimed at addressing symptoms associated with these physical and psychological exposures are needed. Based on the

following overarching theses, research has been initiated to explore the interventional use of probiotics: 1) elevated inflammation increases the risk of developing persistent post-concussive (PPC) and PTSD symptoms; 2) chronic inflammation and impaired immunomodulation perpetuate symptoms among those with mTBI and PTSD; 3) exacerbation of inflammation is associated with increased severity of PPC and PTSD symptoms; and 4) administration of an anti-inflammatory/immunoregulatory probiotic is likely to influence an individual's gut microbial community, decrease intestinal permeability, decrease systemic inflammation, and dampen autonomic responsivity and PPC and PTSD symptoms (Brenner et al., 2020). Toward this end, Brenner and colleagues conducted a randomized clinical trial with an anti-inflammatory/immunoregulatory probiotic, *Lactobacillus reuteri* DSM 17938, and findings supported the feasibility, acceptability, and safety of supplementation, as well as identification of C-reactive protein (CRP) as a potential viable inflammatory marker (Brenner et al., 2020). Moreover, a significant impact of *L. reuteri* DSM 17938 was noted on the Trier Social Stress Task, a well-documented biomarker of the autonomic stress response (Brenner et al., 2020), with those on placebo supplementation having significantly increased heart beats per minute. To further work in this area, members of this team are currently engaged in a Phase IIa RCT of *Lactobacillus rhamnosus* GG (*LGG*; ATCC 53103) for PTSD (ClinicalTrials.gov; Identifier: NCT04150380). Immunomodulatory probiotics, such as *L. reuteri* and *LGG*, may have the potential to decrease exposure-induced inflammatory responses, while being highly accessible, low-cost, self-sustaining (e.g., portable), and, based on previous safety and tolerability trials, without serious side effects.

Broken barriers: the effects of alcohol on the lung epithelium (Dr. Michael Koval, Emory University School of Medicine)

Dr. Koval began the discussion on how alcohol contributes to organ damage by talking about his research on chronic alcohol use disorder. Chronic alcohol use disorder (AUD) is a significant risk factor for acute respiratory distress syndrome (ARDS), in part due to alcohol affecting the ability of the lung to properly regulate fluid balance, rendering it susceptible to airspace flooding in response to insults such as ventilator-induced injury, infectious pneumonia, and sepsis (Yeligar et al., 2016). Alveolar epithelial cells, in the distal airspaces where gas exchange occurs, control lung fluid balance. Dr. Koval discussed how healthy alveolar epithelial cells maintain fluid balance by regulating paracellular permeability (influx), which is normally balanced by sodium-driven fluid efflux. In response to alcohol exposure, paracellular alveolar permeability increases (leak), which otherwise healthy patients with AUD can counterbalance by increased efflux. However, subsequent lung injury causes alveolar leak to rise to dramatically increased levels that cannot be restored by efflux, increasing susceptibility to ARDS. Critically, alcohol exposure sensitizes lung epithelial cells to increased barrier disruption following SARS-CoV-2 infection, suggesting that AUD is a risk factor for increased severity of pneumonia associated with COVID-19. Paracellular permeability is regulated by tight junctions (TJs), a complex of transmembrane paracellular channel proteins (claudins) linked to the actin cytoskeleton by cytosolic scaffold proteins (Schlingmann et al., 2016). In the alveolar epithelium, TJs mainly consist of claudin-18 connected to actin by zonula occludens-1 (ZO-1). TJs of alveolar epithelial cells from rats fed an alcohol diet are leaky and also have claudin-18

projections perpendicular to cell—cell junctions (called TJ spikes). TJ spikes are also formed by human lung epithelial cells exposed to alcohol. The Koval Laboratory has found that chronic exposure to alcohol increases claudin-5. Claudin-5 binds claudin-18, which inhibits claudin-18:ZO-1 interactions, which then induces TJ spikes to form and increases paracellular leak. This effect is antagonized by a claudin-5 peptide mimetic, Ac-EFYDP-NH_2, reducing TJ spike formation and improving barrier function (Schlingmann et al., 2016), suggesting a potential approach to treat AUD-associated lung syndrome. TJ spikes are not sites of increased barrier dysfunction, as determined by measuring local disruptions to TJ barrier function with the XPerT assay (Lynn et al., 2021). Instead, TJ spikes are associated with actin filaments anchored by adjacent β-catenin-containing plaques and a pool of junction-associated dynamin-2. The architecture of TJ spikes suggests that they may be specialized structures acting as a signaling hub organized by claudin-18. Current studies by Dr. Koval are underway to further define the molecular composition of TJ spikes and to define roles for them in regulating impairment of alveolar epithelial physiology due to AUD.

The role of natural killer cells in host defense against alcohol-associated bacterial pneumonia (Dr. Derrick Samuelson, University of Nebraska)

Dr. Samuelson continued the discussion on the impact of alcohol on the immune system and the host by discussing his research on alcohol-associated bacterial pneumonia. Alcohol use is an independent risk factor for the development of bacterial pneumonia, which is, in part, due to impaired mucus-facilitated clearance, macrophage phagocytosis, and recruitment of neutrophils (Happel & Nelson, 2005). Alcohol consumption is also known to reduce peripheral natural killer (NK) cell numbers and to compromise NK cell cytolytic activity, especially NK cells with a mature phenotype (Zhang & Meadows, 2009). However, the role of innate lymphocytes, such as NK cells during host defense against alcohol-associated bacterial pneumonia, is essentially unknown. Dr. Samuelson's laboratory has previously shown that indole supplementation mitigates increases in pulmonary bacterial burden and improves pulmonary NK cell recruitment in alcohol-fed mice that were dependent on aryl hydrocarbon receptor (AhR) signaling (Samuelson et al., 2021). However, whether NK cells are required for optimal host defense in indole-supplemented mice or whether indole works directly or indirectly on NK cells, remains to be answered. By employing a binge-on-chronic alcohol-feeding model, they sought to define the role and interaction of indole and NK cells during pulmonary host defense against alcohol-associated pneumonia. Data demonstrating that alcohol dysregulates NK cell effector function and pulmonary recruitment via alterations in two key signaling pathways were presented. Specifically, it was found that alcohol increases transforming growth factor beta (TGF-β) signaling, while suppressing AhR signaling. They further demonstrated that NK cells isolated from alcohol-fed mice have a reduced ability to kill *Klebsiella pneumoniae*. NK cytolytic capacity was improved following indole treatment, but was drastically blunted by exogenous TGF-β. Likewise, NK cell migratory capacity to chemokines was significantly altered by alcohol. NK cells isolated from alcohol-fed mice exhibited preferential migration in response to CXCR3 chemokines, but exhibited reduced migration in response to CCR2, CXCR4, and CX3CR1 chemokines. NK cell migratory capacity was improved following indole treatment and impaired by exogenous TGF-β. Together, these data suggest that alcohol disrupts NK cell-specific TGF-β and AhR signaling pathways, leading to decreased pulmonary

recruitment and cytolytic activity, thereby increasing susceptibility to alcohol-associated bacterial pneumonia.

Alcohol amplifies neuroinflammatory IL-6 pathway signaling in the pre-frontal cortex (Jessica A. Cucinello-Ragland, PhD candidate in the laboratory of Dr. Scott Edwards, Louisiana State University Health Sciences Center)

Graduate students also presented their work throughout the sessions. First, Jessica A. Cucinello-Ragland, a PhD candidate in Dr. Edwards' lab, discussed their latest research investigating the role of alcohol in inflammatory signaling in the brain. Chronic alcohol exposure elicits strong neuroimmune responses in the brain via the reduction of anti-inflammatory signaling and the amplification of pro-inflammatory signaling. This enhanced inflammatory response leads to neuronal damage and apoptosis, glial activation, and several behavioral alterations, ultimately contributing to both the development and maintenance of AUD. One such alcohol-induced neuroimmune response is the enhancement of Interleukin-6 (IL-6) levels in the brain (Alfonso-Loeches & Guerri, 2011). While alcohol can induce a neuroimmune response throughout the central nervous system, the prefrontal cortex represents a significant neural substrate that mediates the development of addiction (Koob & Volkow, 2010). Using the chronic intermittent ethanol vapor exposure paradigm in adult male mice, alcohol dependence was induced and confirmed via the two-bottle choice task prior to sacrifice (Winters et al., 2021). Regional dissections were then used to quantify prefrontal cortical levels of the IL-6 pathway proteins IL-6, Glycoprotein 130, phosphorylated Signal Transducer and Activator of Transcription 3 (STAT3), and Suppressor of cytokine signaling 3 (SOCS3). Alcohol dependence induced an increase in GP130 levels and STAT3 phosphorylation, as well as a decrease in SOCS3 levels. These results indicate that alcohol dependence increases IL-6 signaling in the prefrontal cortex, potentially inducing a shift to the more pro-inflammatory trans-signaling pathway. Future work will investigate alterations in IL-6 pathway proteins in additional addiction-related brain regions.

Binge-ethanol exposure differentially influences senescence markers in the aged brain and BV-2 cells (Paige Anton, PhD candidate in the laboratory of Dr. Rebecca McCullough, University of Colorado Denver, Anschutz Medical Campus)

The final talk of the session, by Paige Anton, presented recent data from the laboratory of Dr. McCullough exploring the effects of ethanol on the aged brain. Binge alcohol consumption is becoming more common among aged (>65 years of age) adults. Although binge drinking in midlife is suggested to increase the risk for cognitive decline and dementia development during advanced aging, the distinct effects of binge consumption on the aged brain are not well understood (Järvenpää, Rinne, Koskenvuo, Räihä, & Kaprio, 2005). Aged adults may be more sensitive to alcohol-related neuroinflammation and injury due to microglia senescence. During advanced aging, microglia obtain an arrested growth state that is accompanied by enhanced production of pro-inflammatory cytokines and associated neurodegeneration and cognitive dysfunction (Angelova & Brown, 2019). Ethanol exposure promotes cellular senescence in peripheral organs, yet the influence of ethanol exposure on inflammation and cellular senescence, including microglia senescence, in the aged brain is not well characterized (Aravinthan et al., 2013). To address these gaps in the literature, they examined senescence and inflammation markers in the hippocampi of young and aged mice

after chronic binge ethanol exposure. Briefly, young (3 months old) and aged (18 months old) female C57BL/6 mice were exposed to ethanol (3 g/kg) or vehicle gavages every other day over the course of 3 weeks for a total of 10 gavages. Hippocampal tissue was isolated 18 h after final gavage for qRT-PCR analysis of pro-inflammatory cytokine (*Il-1b, Tnf-a, Mcp-1*) and senescence marker (*cyclin d1, p16, p21*) mRNA expression. To investigate the distinct effects of ethanol on microglia senescence *in vitro*, murine immortalized microglial cell line (BV-2) was exposed to 50 mM ethanol for 24 h and then stimulated with or without 1 ng/mL lipopolysaccharide (LPS) for 4 h. RNA was isolated to measure expression of pro-inflammatory cytokine and senescence marker mRNA. Binge ethanol exposure significantly increased hippocampal pro-inflammatory cytokine and senescence marker mRNA expression in aged but not young animals. In BV-2 cells, ethanol and LPS challenge increased pro-inflammatory cytokine expression but decreased cellular senescence markers, compared to vehicle-treated cells. Although binge-ethanol exposure amplified age-related increases in cellular senescence and inflammation in the hippocampus, these data do not indicate the specific impact of advanced age and ethanol on microglia phenotype. The *in vitro* data suggest that while ethanol and LPS exposure promotes inflammatory responses in BV-2 cells, they do not mimic the age- and ethanol-related changes in cellular senescence observed in the hippocampus. Due to the emerging role of senescence of microglia in age-related neurodegeneration, further mechanistic work is needed to understand the influence of ethanol exposure on the microglia senescence state *in vivo*.

Patterns of alcohol misuse, metabolism, and differentiation

The second plenary session focused on topics related to patterns of alcohol misuse, the effects of alcohol on metabolism, and differentiation. This session was chaired by Dr. Sanjay Maggirwar (School of Medicine at The George Washington University) and De'Jana Parker (North Carolina Central University).

Binge drinking and young adults: a dangerous combination (Dr. Mariann R. Piano, Vanderbilt University)

Dr. Piano opened the second session by presenting recent research exploring the effects of binge drinking on cardiovascular function. Excessive alcohol use is one of the four main risk factors for chronic disease and has been a primary public health concern among college and young adult populations. Binge drinking is the most common pattern of excessive alcohol use. In 2020, 85 million people in the United States reported at least one episode of binge drinking over the past month, and the rate of binge drinking was highest among young adults (18—25 years; 31.4%) (Substance Abuse and Mental Health Services Administration, 2021). Compared to previous young adult generations, the prevalence and intensity of binge drinking have increased, which may be associated with greater adverse health effects, including subclinical cardiovascular disease. An overarching aim of Drs. Piano's and Shane Phillips's research is that repeated binge drinking episodes (≥4/5 drinks for females/males respectively, on one occasion over the last 30 days, for at least 2 years) may be a triggering event that leads to subclinical vascular dysfunction, such as microvascular dysfunction and increased arterial stiffness. Microvascular dysfunction refers to abnormal microvascular vasodilation and constriction responses to physiologic and pathophysiologic

stimuli, whereas increased arterial stiffness refers to a decrease in elastic properties of the arterial wall. In this cross-sectional study, they found that binge drinking was associated with microvascular dysfunction and increased arterial stiffness in young healthy adults (n = 49; 18—30 years). Compared to abstainers and moderate drinkers (the consumption of no more than 2/3 drinks per sitting for females/males, no more than 1—2 times per week over the last 5 years), binge drinkers had a reduced vasodilation response to flow (14—18% reductions at higher flow gradients), and this response was further reduced following an exposure to high pressure (Hwang, Bian, et al., 2020). This binge-induced microvascular dysfunction was reversed by the addition of tetrahydrobiopterin, an antioxidant and important co-factor for nitric oxide production. They also found carotid-femoral pulse wave velocity (cfPWV), a measure of arterial stiffness, was 0.6 m/s and 0.5 m/s greater in binge drinkers and moderate drinkers respectively, compared to abstainers (Hwang, Piano, et al., 2020). Higher cfPWV values were correlated with higher levels of 24-h urinary norepinephrine, a marker of sympathetic activity. Collectively, these findings indicate that repeated binge drinking in young adults is associated with signs of premature/subclinical cardiovascular disease, which may be mediated by oxidative stress, nitric oxide bioavailability, or sympathetic neural mechanisms.

Alcohol-mediated metabolic dysregulation: epigenomic adaptations (Dr. Liz Simon, Louisiana State University Health Sciences Center)

At-risk alcohol use is associated with increased incidence of myopathy, one of the earliest alcohol-associated pathological tissue changes. Older adults who consume alcohol are susceptible to falls, bone fractures, and other unintentional injuries. Higher incidence of immobilization, prolonged bed rest, and overall skeletal muscle disuse represent the leading cause of injury-related deaths in the aging population, with at-risk alcohol use representing a significant risk factor. In her talk, Dr. Simon presented data on how alcohol impairs functional muscle mass. Studies in a preclinical chronic alcohol administration model in non-human primates show that both *in vivo* and *in vitro* alcohol decreases differentiation potential of muscle stem cells (myoblasts) to mature muscle myotubes and decreased expression of muscle regulatory factors implicated in myogenesis. Using an *in vitro* approach, her lab demonstrated that alcohol increases Class IIA histone deacetylase expression, and TMP195, a class IIA HDAC inhibitor, restoring differentiation of alcohol-treated myoblasts (Adler, Molina, & Simon, 2019). In addition, alcohol altered myoblast bioenergetic function by decreasing glycolytic capacity, which was associated with decreased myoblast differentiation (Levitt, Chalapati, Prendergast, Simon, & Molina, 2020). Experiments are ongoing to elucidate whether decreased glycolytic enzyme activity is due to alcohol contributing to the changes in skeletal muscle regenerative capacity. Based on the data that alcohol significantly dysregulates muscle metabolic function, Dr. Simon's laboratory next investigated whether alcohol alters myotube extracellular vesicle bioactive cargo that regulates intercellular and inter-organ communication, to alter metabolic regulation. Results indicate that chronic *in vivo* alcohol administration differentially regulates microRNAs (miRs) implicated in metabolic regulation but does not alter EV numbers or size. Using a preclinical rodent model, they found that alcohol significantly decreases quadriceps muscle weight and fiber area, increases TGF-β and promotes a profibrotic milieu, and delays the increase in expression of markers of myogenic

differentiation 14 days after recovery following hind limb immobilization. Additionally, alcohol and immobilization increased expression of histone deacetylase 4 and runt-related transcription factor 1 (*Runx1*), a neuromuscular junction (NMJ) marker. Overall, the results indicate that alcohol dysregulates muscle stem cell function, and extracellular matrix and neuromuscular junction remodeling. Whether this synergizes with aging and accelerates dysfunctional muscle mass, increasing the risk of muscle-related frailty, and interventions to improve functional muscle mass in people with at-risk alcohol use, is actively being investigated.

Alcohol impairs alveolar macrophage mitochondrial bioenergetics and phagocytosis through changes in hyaluronic acid dynamics (Kathryn Crotty, PhD candidate in laboratory of Dr. Samantha M. Yeligar, Emory University)

Next, Kathryn Crotty, from Dr. Samantha Yeligar's laboratory, discussed their recent research exploring the role of the mechanism of alcohol-induced alveolar macrophage impairment. Excessive alcohol use augments the risk of pneumonia and acute respiratory distress syndrome, leading to increased morbidity and mortality. Alveolar macrophages (AM) are responsible for engulfing and clearing pathogens in the lower respiratory tract. However, *in vivo* mouse models have demonstrated that alcohol-induced mitochondria redox imbalance impairs the ability of AM to phagocytose pathogens (Liang, Harris, & Brown, 2014; Morris, Harris, Brown, & Yeligar, 2021). Oxidative stress also alters the molecular dynamics of the extracellular matrix polysaccharide, hyaluronic acid (HA), which has been implicated in pulmonary immunity and inflammation (Hällgren, Samuelsson, Laurent, & Modig, 1989). *In vitro* experiments were performed using the MH-S mouse AM cell line, treated with or without 0.08% ethanol or 25 nM HA for 3 days. To delineate how ethanol affects HA, expression of key HA-binding and signaling proteins was measured by qRT-PCR and western blotting techniques. Ethanol-exposed AMs showed increased expression of mRNA and protein for both HA synthase 2 (HAS2), which is involved in high molecular weight (HMW) HA (>1000 kD) synthesis, and cluster of differentiation 44 (CD44), which is involved in HA internalization and recycling. Mitochondrial bioenergetics and fuel flexibility were measured using an extracellular flux bioanalyzer. High molecular weight HA impaired mitochondrial bioenergetics compared to untreated and low molecular weight HA- (<200 kD) treated MH-S cells. Ethanol and HMW HA altered basal respiration, mitochondria-linked ATP respiration, maximal respiration, and spare respiratory capacity in MH-S cells. Further, ethanol modified metabolic substrate dependency, flexibility, and capacity of utilizing the glutaminase and fatty acid oxidation pathways in MH-S cells. Overall, ethanol-induced changes in HA could alter mitochondrial bioenergetics and fuel metabolism through deranged HA binding and signaling pathways. These data support modified HA dynamics as a mechanism for increased risk of respiratory infections in people with alcohol use disorders. Identifying the underlying mechanisms of HA dysregulation could potentially uncover novel targets for therapeutic intervention in alcohol-induced pulmonary immune dysfunction.

Episodic alcohol exposure in mice attenuates chondrogenic differentiation of mesenchymal stem cells within fracture callus (Dr. Farah Sharieh, research associate in the laboratory of Dr. John J. Callaci, Loyola University Chicago)

Bone fracture repair is a dynamic, regenerative process that involves many steps, including mesenchymal stem cell differentiation into chondrocytes and osteoblasts to form a fracture callus. A healthy fracture callus is required for normal bone healing. However, fracture nonunion, which is incomplete healing of bone that necessitates a surgical intervention, is a common problem. One of the known risk factors for fracture nonunion is alcohol abuse (Chakkalakal, 2005). The Callaci laboratory has previously reported that ethanol-exposed rodents with a surgically created tibia fracture display deficient fracture callus formation (Roper, Abbasnia, Vuchkovska, Natoli, & Callaci, 2016). They have also shown that *in vitro* ethanol treatment of rat mesenchymal stem cells (MSCs) inhibits both chondrogenic and osteogenic differentiation (Sharieh, Eby, Roper, & Callaci, 2020). These findings led to the hypothesis that alcohol exposure may delay or prevent fracture healing by inhibiting normal mesenchymal stem cell chondrogenic differentiation within the developing fracture callus. To test this hypothesis, Dr. Sharieh utilized three novel transgenic mouse lines with a fluorescent reporter driven by MSC (PRX1) and chondrogenic (*Col2a1, Col10a1*) gene expression-specific promotor regions, to determine which stage(s) of chondrogenic differentiation may be affected by ethanol exposure. Surgically induced mid-shaft tibia fracture was performed for both sham and ethanol-treatment groups, and the effects of ethanol on pre-, early- and late-mesenchymal stem cell chondrogenic differentiation in the callus at 4, 6, and 9 days post-injury were investigated. Effects of ethanol on callus cell proliferation and apoptosis were also studied. Data were presented showing that ethanol-treated rodents had reduced fracture callus area at 4, 6, and 9 days post-fracture. Ethanol-treated animals had reduced early (*Col2a1*) and late (*Col10a1*) chondrogenic gene expression in the developing fracture callus, but there was no effect of ethanol on MSC-specific (Prx-1) expressing cells. Ethanol also failed to alter apoptosis in the fracture callus at any of the examined time points. The ethanol-treated group had significantly fewer proliferative cells in the fracture callus at 9 days post-fracture but did not exhibit altered cell proliferation at earlier time points. These findings suggest that ethanol may inhibit both early chondrogenic differentiation and later chondrocyte maturation during fracture callus development. Ethanol has minimal effect on MSC accumulation at the site of fracture to form a callus in the early healing process of fracture, which suggests that alcohol perturbation of MSC differentiation may be a primary defect in alcohol-related deficient fracture healing.

Effectors and mechanisms of alcohol-mediated tissue injury

The final plenary session covered research exploring the effectors and mechanisms of alcohol-mediated tissue injury; the following talks were presented and chaired by Dr. Erin Lowery (University of Colorado School of Medicine) and Dr. Adam Kim (Cleveland Clinic).

The platelet-activating factor system and microvesicle particles as effectors for intoxicated thermal burn injury pathology (Dr. Jeffrey B. Travers, Wright State University)

The combination of ethanol intoxication and thermal burn injury is associated with significant pathology. Murine model systems have faithfully replicated many of the pertinent findings in humans, namely an acute pro-inflammatory response with multiple organ dysfunction followed by delayed immunosuppression. Murine studies have implicated cytokines, including IL-6, the tolllike receptor 4, and activation of myosin light-chain kinase (MLCK), in the gut with bacterial translocation. However, one major knowledge gap is how the signals from the skin keratinocytes, in response to the combination of ethanol and burn injury, result in the systemic responses. Dr. Travers' research group has been exploring the role of the lipid mediator platelet-activating factor (PAF) in the keratinocyte response to multiple environmental stressors. Of interest, systemic exposure to PAF can mimic much of the pathology associated with intoxicated thermal burn injury. They have recently reported that the combination of ethanol and burn injury in keratinocytes results in increased levels of PAF (Liu et al., 2020). Moreover, activation of the keratinocyte PAF receptor generates subcellular microvesicle particles (MVPs) in a process involving the enzyme acid sphingomyelinase (aSMase) (Liu et al., 2021). Of importance, they showed that MVPs carry high levels of PAF, which could provide a mechanism by which this highly labile lipid mediator is protected from inactivation of cell and serum acetylhydrolases (Harrison et al., 2018). Current *in vitro* and pre-clinical *in vivo* studies presented indicate that combining ethanol and thermal burn injury generates exaggerated levels of MVP. They propose that high levels of PAF-laden MVP released from skin in response to ethanol and thermal burn injury is an important effector in the toxicity associated with this clinically relevant combination. The confirmation of this novel hypothesis could result in new therapeutic targets, especially PAF and aSMase.

Phosphoproteomic analysis reveals pathways underlying the role of receptor interacting protein kinase 3 (RIP3) in alcohol-associated liver disease (Dr. Vaibhav Singh, postdoctoral fellow in laboratory of Dr. Laura Nagy, Cleveland Clinic)

Alcohol-associated liver disease (ALD) shows an increasing prevalence and is a major type of chronic liver injury. The therapeutic possibilities are limited and restricted to lifestyle intervention since specific drugs for ALD are unavailable so far. Evidence indicates a clear association between inflammation and hepatocellular death (Gao, Ahmad, Nagy, & Tsukamoto, 2019; Nagy, Ding, Cresci, Saikia, & Shah, 2016) in ALD. TNF-α has been involved as a major pathogenic driver of ALD (Shojaie, Lorga, & Dara, 2020). While TNF-α is hepatoprotective in healthy liver, exposure to chronic alcohol sensitizes hepatocytes to TNF-α-induced cell death. TNF-α, acting through TNF-receptor 1 (TNF-R1), can trigger cell death via multiple mechanisms such as apoptosis or necroptosis, depending on cellular contexts. While multiple forms of cell death are associated with ALD, the relationship between different cell death pathways and the progression of ALD are poorly understood. Earlier studies found that mice deficient in Rip3 ($Rip3^{-/-}$), a key effector of necroptotic cell death, are protected from liver injury following chronic ethanol feeding; however, the underlying mechanisms are poorly understood. Post-translational modifications (PTMs), in particular phosphorylation modifications, are crucial for regulating protein functions during programmed cell death, for example, during necrosome assembly and activation. The

investigation of the effect of chronic ethanol feeding on the hepatic phosphoproteome in C57BL/6 mice and RIP3 deficient ($Rip3^{-/-}$) mice, with the focus on TNF-α-mediated death receptor signaling (DR) pathways, could reveal the mechanisms of Rip3-dependent ethanol-induced liver injury. Modern nano-scale liquid chromatography-based mass spectrometry (MS) and pathway analysis can be advantageous to understand the interaction of chronic ethanol and RIP3 on hepatic phosphorylation events. The approach can quantitatively analyze the dynamic formation of TNF-induced signaling complexes of cell death to reveal the process of DR signaling. Identification of phosphorylation sites associated with phosphoproteins can help us better understand the molecular mechanisms involved in DR signaling. Furthermore, a quantification study of protein interactions in determining signaling consequences could clarify how death signals are integrated to direct a specific cell fate. Overall, phosphoproteomics could reveal a potential mechanism by uncovering novel targets in ALD.

Summary

The 2021 AIRIG meeting highlighted diverse work on alcohol's impact on various pathways, leading to cellular and tissue damage in multiple organs (Table 1). Binge drinking or chronic alcohol abuse leads to altered inflammatory pathways in the brain, such as amplified neuroinflammation via IL-6 after chronic ethanol exposure or increased pro-inflammatory cytokines and cell senescence in the hippocampus in aged mice after ethanol binge. In addition, data were presented on the impact of alcohol misuse on the immune system and its effectors. For example, natural killer cell functions decrease in alcohol-associated bacterial pneumonia, while alveolar macrophages have decreased capabilities to phagocytose and alter hyaluronic acid synthesis after ethanol exposure. There were multiple presentations that supported the notion that alcohol misuse affects tissue repair pathways. Chronic alcohol use disorder can sensitize lung epithelium to future damage by impairing junctional proteins. Alcohol can also induce muscle tissue dysfunction by decreasing the differentiation potential of muscle cells and halting myogenesis via epigenetic changes. Data were also presented showing that episodic alcohol use alters the bone repair process via impaired differentiation of chondrocytes from mesenchymal stem cells and inhibits maturation of chondrocytes after bone fracture. Lastly, potential novel clinical and therapeutic targets for alcohol-associated organ and tissue damage were also discussed. For example, PAF and microvesicle particles originating from keratinocytes are mediators of systemic inflammation after a combination of ethanol and burn injury, and the enzymes aSMase and PAF could be potential drug targets. In addition, phosphoproteomic analysis can be utilized to discover cell death and other novel pathways associated with alcohol-associated liver disease. For example, hepatocyte cell death is prevented in mice deficient in Rip3 ($Rip3^{-/-}$), a key mediator of necroptotic cell death, after chronic ethanol feeding. Moreover, a clinical trial is underway to determine whether modulating gut microbiome with a specific bacterial strain to manage inflammation in PTSD patients can be effective. Taken altogether, these data presented at the 2021 AIRIG symposium demonstrate how alcohol misuse impacts a wide variety of pathways in tissue and organ homeostasis, offering insight into several pathways for future studies. In addition, these studies present

many pathways to discover potential therapeutic targets for preventing and treating alcohol misuse diseases.

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Table 1

Alcohol's effects on multiple organs and tissues.

Brain	• ↑ IL-6; ↑ cell senescence in hippocampus
Lung	• ↑ Claudin → ↑ paracellular leak in lung epithelium
Vascular system	• ↑ microvessel stiffness and abnormal physiological response to stimuli → premature/subclinical cardiovascular disease in young adults
Skeletal system	• ↑ Class IIA HDAC and TMP195, a class IIA HDAC inhibitor; ↓ glycolytic capacity via alteration of miRs; ↓ myogenic differentiation markers → skeletal muscle dysfunction • ↓ Col2a1 and Col10a1 expression → ↓ mesenchymal stem cell differentiation → abnormal fracture healing
Blood	• ↑ TGF-β; ↓ Ahr signaling; ↓ NK cell function • ↑ HAS2 → ↑ hyaluronic acid → impaired mitochondrial bioenergetics → impaired macrophage function
Integumentary system	• ↑ keratinocyte PAF; ↑ MVP and aSMase → intoxicated thermal burn injury pathology

IL-6, interleukin-6; HDAC, histone deacetylase; Col2a1, collagen type II alpha 1; Col10a, Collagen Type X Alpha 1 Chain; TGF-β, transforming growth factor beta; Ahr, aryl hydrocarbon receptor; NK, natural killer; HAS2, hyaluronan synthase 2; PAF, platelet-activating factor; MVP, microvesicle particles; aSMase, acid sphingomyelinase.