

PISA

PATHOBIOLOGY FOR INVESTIGATORS, STUDENTS & ACADEMICIANS

Meeting Abstracts

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Session 1: Metabolic Stress and Regenerative Therapeutics

Abstract 001

Targeted Deletion of Hepatocyte ERBB3 (HER3) Attenuates MASLD Progression and Reprograms the PPAR γ –HNF4 α Metabolic Axis

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Background and Aims: MASLD progression involves hepatocyte reprogramming characterized by increased PPAR γ -driven lipid storage and reduced HNF4 α -controlled hepatocyte gene expression. However, the hepatocyte membrane receptor that regulates this transcriptional switch remains elusive. Our previous studies show that while pharmacologic pan-ERBB inhibition modulates this switch resolving hepatic steatosis in murine MASLD models, hepatocyte-specific EGFR deletion fails to reproduce this benefit with compensatory ERBB3 activation. Here, we investigated whether hepatocyte ERBB3 drives MASLD progression and regulates the PPAR γ –HNF4 α metabolic switch. **Methods:** Hepatocyte-specific ErbB3 mice (ERBB3-KO^{Hep}) were challenged with a fast-food diet for 5 months. MASLD phenotypes were assessed by histology, biochemical assays, and immunoblotting, ERBB3–PI3K (p85) co-immunoprecipitation and multi-omics analysis. Therapeutic relevance was tested using lipid nanoparticle-delivered siERBB3 *in vivo* and siRNA-mediated ERBB3 knockdown in primary human MASH hepatocytes. **Results:** ERBB3 signaling was activated in both human and murine MASLD livers. ERBB3-KO^{Hep} mice were protected against FFD-induced steatosis, with reduced liver triglyceride and improved glucose homeostasis. ERBB3 deletion attenuated PI3K–AKT–mTOR signaling, suppressed PPAR γ expression and its lipogenic program and restored HNF4 α expression. This was accompanied with reduced lipogenesis, lipid uptake, and lipid droplet stabilization, and increased autophagy/lipophagy associated markers. ERBB3 loss also suppressed fibrogenic programs, stellate cell activation and collagen deposition. Therapeutic ERBB3 silencing via LNP in mice with established MASLD, and ERBB3 knockdown in primary human MASH hepatocytes reduced lipid accumulation that was associated with decreased PPAR γ expression and downstream lipogenic reprogramming. **Conclusions:** Our study uncovered that ERBB3, a largely understudied ERBB receptor in liver, has a central role in hepatocyte lipogenic reprogramming during MASLD progression by regulating the PPAR γ –HNF4 α switch. ERBB3 inhibition restores hepatocyte metabolic identity, and attenuates fibrosis, revealing ERBB3 as a promising therapeutic target for MASLD.

Abstract 002

Investigating the Role of TM6SF2 E167K in ER Stress and Unfolded Protein Response Signaling in MASH

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Background: Metabolic dysfunction-associated steatohepatitis (MASH) is a progressive form of metabolic dysfunction-associated steatotic liver disease (MASLD) in which excessive lipid accumulation in the liver leads to inflammation and damage to hepatocytes. One key cellular process that may contribute to this progression is endoplasmic reticulum (ER) stress. When hepatocytes experience lipotoxicity, excessive fatty acids disrupt ER homeostasis, leading to activation of the unfolded protein response (UPR). At first, the UPR activates adaptive mechanisms to restore ER homeostasis by increasing protein folding machinery and promoting the degradation of misfolded or damaged proteins. However, persistent lipotoxicity will overwhelm adaptive mechanisms and shift UPR signaling toward pro-apoptotic and pro-inflammatory pathways, contributing to hepatocyte death, fibrosis, and MASH progression. Previous studies have identified genetic variants in genes such as TM6SF2, PNPLA3, and GCKR that increase susceptibility to MASLD and MASH. However, how these genetic variants alter ER stress responses and UPR signaling remain unknown. We hypothesize that hepatocytes with different genetic backgrounds may respond differently to lipotoxicity-induced ER stress. Specifically, we are testing if ****TM6SF2** E167K** variant increases hepatocyte susceptibility to lipotoxicity, resulting in greater ER stress and altered UPR signaling. To test this, we will compare ER stress responses and UPR signaling in iPSC-derived hepatocytes carrying the ****TM6SF2** E167K** variant to a healthy control and CRISPR-corrected isogenic line.

We previously observed increased CHOP mRNA expression in a MASH line carrying the TM6SF2 variant, and the corrected isogenic line will help determine whether this difference is specifically caused by the TM6SF2 variant. Gene expression of key UPR signaling will be assessed using qPCR along with protein-level analyses. By determining how **TM6SF2** E167K variant alters ER stress responses during lipotoxicity, our study aims to provide insight into how genetic differences contribute to MASH progression and identify potential therapeutic targets for genetically susceptible patients with MASH.

Abstract 003

Selective LNP-siRNA Targeting of ZNRF3/RNF43 for Regenerative MASH Therapy

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Background: Metabolic dysfunction-associated steatohepatitis (MASH) is driven by intertwined metabolic dysfunction, inflammation, fibrosis, and impaired hepatocyte regeneration, yet most therapies target individual disease components rather than restoring the liver's intrinsic repair capacity. **Methods and Results:** We identified the WNT/ β -catenin negative regulators ZNRF3 and RNF43 as actionable regenerative targets. Hepatocyte-specific ZNRF3/RNF43 deletion (ZRdKO) in established MASH reactivated endogenous β -catenin signaling and drove coordinated regression of steatosis, inflammation, and fibrosis. Importantly, LNP-siRNA-mediated ZNRF3/RNF43 silencing recapitulated key therapeutic benefits, establishing pharmacological proof-of-concept for transient pathway targeting. A major challenge for β -catenin therapy is oncogenic risk. Our studies indicate that ZRdKO restored endogenous β -catenin activity without promoting tumor progression in MASH-HCC models and retained therapeutic activity in the surrounding diseased liver. Whereas oncogenic CTNNB1-N90 cooperated with MYC to induce extensive HCC, ZRdKO plus MYC failed to initiate tumors, supporting a therapeutic window for restoring regulated β -catenin signaling without recapitulating mutation-driven oncogenic activity. We also established an RNA drug-development pipeline targeting ZNRF3/RNF43. LNP screening identified candidates with highly efficient delivery to hepatocytes in steatotic and fibrotic livers. Notably, these LNPs showed minimal delivery to HCC lesions, providing an additional layer of therapeutic selectivity. In parallel, systematic screening in primary mouse hepatocytes and human liver cancer cells identified potent mouse and human ZNRF3/RNF43 siRNA candidates. Lead siRNA-LNP combinations are now advancing through in vivo validation and PK/PD studies to define target engagement, durability, dosing, and therapeutic window. **Conclusion:** Together, these studies support selective, transient restoration of endogenous regenerative signaling through ZNRF3/RNF43-targeted LNP-siRNA as a potential disease-modifying therapy for MASH.

Abstract 004

CCPG1-Mediated ER-Phagy Facilitates Gastric Epithelial Reprogramming Following Injury

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Background: Tissue repair requires differentiated cells to transiently relinquish specialized functions and adopt regenerative states following injury. In the gastric corpus, mature chief cells undergo this reprogramming by dismantling their secretory machinery, activating injury-associated gene programs, and re-entering the cell cycle. Because chief cells contain an extensive rough endoplasmic reticulum (ER), we hypothesized that selective ER remodeling through ER-phagy facilitates their transition to a regenerative state. **Methods:** Acute gastric epithelial injury was induced in adult mice using high-dose tamoxifen, a reversible model of epithelial regeneration. Gastric tissues were analyzed across the regenerative time course by molecular, histologic, and ultrastructural approaches. ER-enriched fractions were subjected to RNA sequencing and protein analyses, and autophagy-

associated cargoes were identified by p62/SQSTM1 immunoprecipitation coupled with mass spectrometry. The functional role of ER-phagy was evaluated using *Ccpg1*-deficient mice. **Results:** Gastric injury induced rapid cellular stress responses accompanied by extensive remodeling of the chief cell ER. Transmission electron microscopy revealed ER fragmentation and sequestration within autophagic vesicles, followed by progressive ER clearance during regeneration. RNA sequencing of ER-enriched fractions showed induction of stress-response, inflammatory, and tissue-remodeling programs together with loss of transcripts associated with mature secretory function. p62/SQSTM1-associated proteomic analysis identified multiple ER-localized proteins as autophagy-associated cargoes following injury. Importantly, CCPG1 deficiency resulted in persistent ER accumulation, impaired organelle remodeling, attenuated regenerative responses, and significantly reduced epithelial proliferation. These findings indicate that CCPG1-dependent ER-phagy facilitates progression through injury-induced epithelial reprogramming. **Conclusion/Significance:** Our findings identify ER remodeling as an integral component of gastric epithelial regeneration. CCPG1-dependent ER-phagy facilitates removal of differentiated secretory architecture as chief cells transition toward a regenerative state, linking selective organelle remodeling to epithelial plasticity, proliferation, and tissue repair.

Abstract 005

Chronic High-Fat Diet Drives Gastric Tuft Cell Hyperplasia

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Background and Aims: Tuft cells are rare chemosensory epithelial cells that play key roles in mucosal epithelial homeostasis, immune response, and tumorigenesis. While tuft cells have been widely studied in the intestine and airway, their role in the stomach remains poorly characterized. We previously found that administration of a high-fat diet (HFD) to wildtype C57BL/6J mice damages the gastric epithelium and elicits robust inflammation. We hypothesize that long-term HFD feeding results in increased IL-13 production by immune cells, which promotes tuft cell hyperplasia and contributes to the development of gastric metaplasia, a known pre-cancerous lesion. **Methods:** C57BL/6J mice were fed a control or HFD for 26 weeks. Gastric tissue was collected for histological and immunofluorescent analyses. **Results:** HFD-fed mice showed pronounced gastric epithelial remodeling compared with control-fed mice, including tuft cell hyperplasia and gastric metaplasia. In the intestine, tuft cells have been recently clustered into two classes based on transcriptomic profiles, with type 1 tuft cells exhibiting a neuronal associated gene signature and type 2 tuft cells being enriched in immune response related genes. In the stomach, we found that HFD-induced injury led to a significant increase in neuronal-associated type 1 tuft cells and immune-associated type 2 tuft cells. Tuft cells, normally confined to the neck of the gastric gland in control-fed mice, redistributed throughout the gland in HFD mice. We observed an increased prevalence of tuft cells at the base and middle of the gland in mice fed an HFD. Spatial distribution analysis demonstrated that expansion of neuronal-associated type 1 tuft cells was localized to the base of the glands, while immune-associated type 2 tuft cells expanded throughout the gland. Gastric tuft cells also underwent distinct morphological changes, including changes in size and shape. Immunostaining for immune cells and for IL-13 demonstrated a significant increase in immune cell derived IL-13 in HFD. IL-13 is known to drive tuft cell expansion in other organs and is likely a key driver of the tuft cell hyperplasia that we observed in our HFD gastric injury model. **Conclusion:** Our data demonstrates that tuft cells are expanded during HFD induced gastric injury. Collectively, these data suggest that chronic dietary injury impacts tuft cell number and structure, with implications for gastric malignancy.

Session 2: Host-Microbe Interactions, Barrier Defense and Surveillance

Abstract 006

Abiotrophia defectiva Degrades Mucins in the Oral Cavity

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Background: The oral cavity harbors a diverse microbial community that is continually exposed to mucin-rich saliva. Salivary mucins contribute to lubrication, microbial clearance, and protection of the oral epithelium. In the gastrointestinal tract, many bacteria degrade mucin glycans and use the released sugars as nutrients. Mucin-degrading bacteria may not consume all liberated sugars, allowing these nutrients to support neighboring microbes through metabolic cross-feeding. Thus, mucin degradation could influence the composition, spatial organization, and activity of oral microbial communities. However, the bacteria responsible for mucin degradation in the oral cavity remain poorly characterized. We therefore sought to identify and characterize oral mucin-degrading microbes. **Methods & Results:** To assess the mucin-degrading capacity of the oral microbiome, we queried the Integrated Microbiome Genome Database, which contains >100,000 bacterial genomes. Interestingly, we found that the oral commensal bacterium *Abiotrophia defectiva* had multiple enzymes for removing mucin glycans. We found that all the *A. defectiva* genomes had the enzymes to remove sialic acid (EC 3.2.1.18), fucose (EC 3.2.1.51), GlcNAc (EC 3.2.1.52), GalNAc (EC 3.2.1.97), and Galactose (EC 3.2.1.22). However, it did not have enzymes to remove sulfate (EC 3.1.6.14). To explore the ability of *A. defectiva* to consume mucus in vitro, we grew *A. defectiva* ATCC 49176 in a chemically defined medium, ZMB1, with intact human salivary mucus or varying concentrations of mucin oligosaccharides. As expected, we found that *A. defectiva* had robust growth with glucose, and it was also able to grow with human salivary mucus. It also could use galactose, GlcNAc, GalNAc, and mannose to support its growth. Interestingly, it did not use fucose or sialic acid. Since *A. defectiva* had the enzymes to remove fucose and sialic but did not consume them, we next explored the cross-feeding of *A. defectiva* with oral *Streptococcus* and *Fusobacterium* species as well as the effects of their secreted products on oral cells. **Conclusions:** We identified *A. defectiva* as a mucin-degrading microbe that is capable of changing the dynamics of the oral microbiome, potentially influencing overall oral health.

Abstract 007

Examining the Therapeutic Potential of IL-22-induced Adherent Mucin Production in *Clostridioides difficile* Infection

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Background: *Clostridioides difficile* (*C. difficile*) is a toxin-producing enteric pathogen that causes severe diarrhea and inflammation. Intestinal adherent mucins are known to play a major role in epithelial protection and wound repair, but their levels during *C. difficile* infection are unclear. We aimed to characterize the levels of adherent mucins during *C. difficile* infection and identify the pathways that regulate these mucins. **Methods:** Antibiotic-treated mice were gavaged with PBS or *C. difficile* and monitored for 10 days. RNA sequencing was used to identify differentially expressed genes at days 3, 4, 5, 8, and 10 post-infection. Human and murine colonic cells and organoids were treated with IL-22 to determine the effects of IL-22 on adherent mucin expression *in vitro*. MNK3 (ILC3) cells and peritoneal macrophages were treated with *C. difficile* toxins, and IL-22 or IL-1 β levels were measured via ELISA. To evaluate the therapeutic potential of IL-22, antibiotic-treated mice were gavaged with PBS, *C. difficile*, or *C. difficile* and engineered *Limosilactobacillus reuteri* designed to release IL-22. Immunostaining and histological analysis were observed at day 3 post-infection in all mouse models. **Results:** Mice infected with *C. difficile* exhibited significant weight loss at day 3 post-infection. Also at day 3 post-infection, infected mice showed increased colonic MUC13 gene and protein levels compared to controls. MUC13 is known to be under the control of IL-22. Mirroring this, we also observed increased IL-22 expression in infected mouse colon. When treated with IL-22, colonic cells and organoids exhibited increased MUC13 expression. While *C. difficile* toxins did not stimulate IL-22 production in MNK3 cells, they did increase IL-1 β levels in peritoneal macrophages. Supernatant from these cells, as well as recombinant IL-1 β , stimulated IL-22 release from MNK3 cells. Mice treated with a combination of IL-22 producing *L. reuteri* and *C. difficile* exhibited less weight loss, reduced intestinal damage and inflammation, and an increased mucus barrier compared to *C.*

difficile alone mice. **Conclusion:** Together these findings suggest that *C. difficile* toxins indirectly promote IL-22 production through macrophage-derived IL-1 β signaling and that IL-22 upregulation drives an increase in colonic adherent mucins and reduces *C. difficile* associated damage. This work reveals a potential therapeutic strategy to reduce intestinal damage during *C. difficile* infection.

Abstract 008

***Clostridioides difficile* toxins inhibit the functions of colonic ATP-binding cassette transporters**

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Background: *Clostridioides difficile* (*C. difficile*) is an enteric pathogen that is known for its ability to produce toxins. *C. difficile* can produce three types of toxins: TcdA, TcdB, and binary toxin. The effects of these toxins on the body can be devastating as they can cause unremitting diarrhea, toxic megacolon, and even death. As a result, research is needed to better understand how *C. difficile* causes disease. ATP-binding cassette (ABC) transporters are membrane proteins that regulate the movement of diverse molecules across the intestinal epithelium. These transporters contribute to epithelial homeostasis by controlling the absorption and efflux of metabolites, lipids, and potentially harmful compounds. However, the effects of *C. difficile* toxins on intestinal ABC transporters remain poorly understood. This study aimed to elucidate the interaction between *C. difficile* toxins and their ability to disrupt the functions of important ATP-binding cassette transporters. **Methods and Results:** Analysis of RNA-sequencing (RNAseq) data from *C. difficile* infected mice revealed decreased expression of colonic ABCB1, ABCC2, and ABCG2 compared to vehicle control mice. Immunostaining of CDI mouse colonic tissue for ABCB1 levels confirmed decreased ABCB1 levels in CDI mice at the peak of infection compared to healthy mice. We also examined RNAseq data from mice receiving a rectal administration of TcdA or TcdB. We found that after 16 hrs of toxin exposure, the colonic cells had significantly reduced ABCB1 expression compared to mock treated mice. These data suggest that both TcdA and TcdB can reduce ABC transporters. To assess if *C. difficile* toxins could directly impact these ABC transporters, we treated human colonic T84 cells with *C. difficile* toxins TcdA, TcdB, or binary toxin and examined transcription by RNAseq. Excitingly, we found that all toxins could reduce ABCB1. To analyze the function of ABCB1, we performed a Rhodamine 123 assay in T84 cells. We found that the administration of *C. difficile* toxins significantly reduced ABCB1's ability to efflux Rhodamine 123; suggesting a loss of ABCB1 function in response to *C. difficile* toxins. **Conclusions:** These data provide the first evidence of *C. difficile* toxin-mediated inhibition of ATP-binding cassette transporters such as ABCB1. We speculate that disruption of these transporters may contribute to epithelial dysfunction and disease severity during *C. difficile* infection.

Abstract 009

Exploring Synergistic Interactions Between *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

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Background: Burn wounds are highly dynamic, nutrient-rich environments that can support complex polymicrobial communities and promote colonization by opportunistic pathogens. Despite growing appreciation for the microbiome in human health, the burn wound microbiome has been relatively understudied. As a result, the diversity of burn wound-associated communities and functional interactions between organisms remain poorly defined. We **hypothesized** that burn wounds harbor distinct microbial signatures and that microbial interactions contribute to inflammatory outcomes. **Methods & Results:** We performed next-generation sequencing on burn wound samples and identified 65 unique bacterial species, with communities ranging from 2–15 organisms per wound site. Notably, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were frequently detected. To assess whether these organisms could coexist and exhibit cooperative behaviors, we established mono- and co-cultures and quantified growth across three media conditions using CFU enumeration and imaging. Both species grew robustly in co-culture, indicating stable coexistence without competitive exclusion. Conditioned media generated from mono- and co-cultures did not enhance proliferation of either

organism, suggesting that secreted soluble factors were not sufficient to promote growth. Using Biolog Phenotypic Microarrays, we found evidence of niche partitioning, with *P. aeruginosa* preferentially utilizing arginine and lysine while *A. baumannii* consumed distinct amino acid substrates. Co-culture did not increase quorum sensing activity, and biofilm biomass in co-culture was comparable to *P. aeruginosa* mono-culture. Despite limited synergy in growth or biofilm formation, co-cultures exhibited significantly increased antibiotic resistance to kanamycin and tetracycline compared to mono-cultures. Finally, murine keratinocytes exposed to conditioned media from mono- or co-cultures demonstrated increased inflammatory cytokine production. **Conclusions:** These data show that *A. baumannii* and *P. aeruginosa* can stably coexist and that community context enhances antibiotic tolerance and inflammatory signaling even without synergistic growth or biofilm phenotypes. This highlights the importance of polymicrobial dynamics in burn wound infections and therapeutic susceptibility.

Abstract 010

Optimizing Genomic Sequencing Workflows for Pathogen Detection in Northeastern Tick Populations

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Background: Tick-borne diseases represent a growing public health concern in the Northeastern United States, underscoring the need for rapid, high-resolution surveillance methods. Traditional molecular screening approaches may be limited in their ability to detect unexpected organisms or characterize complex coinfections. This study aimed to optimize an integrated genomic workflow combining *in silico* assay design, dual RNA and DNA extractions, and complementary short- and long-read sequencing to improve pathogen detection in regional tick vectors. **Methods:** Field-collected ticks underwent optimized dual RNA and DNA extractions and were processed using short-read Illumina (Illumina Microbial Amplicon Prep) and long-read Oxford Nanopore Technologies (ONT GridION) workflows. Long-read sequence datasets were assembled *de novo* with Flye, followed by taxonomic assignment using NCBI BLAST and computational filtering to distinguish microbial sequences from host genomic background. **Results:** The analytical workflow successfully distinguished tick host sequences, including sequences assigned to *Haemaphysalis longicornis*, from microbial signatures. Filtering of long-read assemblies enriched bacterial and endosymbiotic contigs, supporting targeted microbial identification while reducing host-sequence interference. **Conclusions:** Integration of Illumina short-read sequencing with ONT long-read sequencing provided complementary resolution for tick-associated metagenomic analysis. This workflow offers a scalable approach to reducing host-sequence interference and improving microbial characterization in ticks, with potential applications in pathogen surveillance and vector-borne disease monitoring.

Abstract 011

Optimizing Physiological Monitoring for Distinguishing Exploration Medical Conditions

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Background: Clinically distinct pathological states can produce overlapping physiological manifestations, complicating diagnosis when access to comprehensive testing is limited. This challenge is amplified during long-duration exploration missions, where medical monitoring must operate under substantial resource constraints. We evaluated whether a reduced set of physiological measurements could preserve the qualitative condition-distinguishing information available across NASA's 119 Exploration Medical Conditions. **Methods:** A 119×35 condition-by-measurement matrix was constructed from published clinical and aerospace-medicine literature. In a conservative analysis, only direction-resolved physiological responses (increased, decreased, or normal) were permitted to distinguish condition pairs, while uncertain or nonspecific responses were excluded. Minimum test-cover and maximum-coverage mixed-integer linear optimization were used to identify the smallest measurement subsets preserving available pairwise distinctions, with independent combinatorial verification. **Results:** The complete 35-variable matrix supported 132 pairwise condition distinctions. Seven measurements: heart rate,

blood pressure, respiratory rate, core temperature, CO₂/ventilation, audiometry, and visual acuity, formed the minimum set preserving all 132 distinctions. A four-measurement subset preserved 118/132 distinctions (89.4%), five measurements preserved 126/132 (95.5%), and six preserved 131/132 (99.2%). **Conclusions:** Most of the qualitative condition-distinguishing information available in the full physiological matrix could be preserved using a substantially reduced measurement set. These findings provide a quantitative framework for prioritizing physiological measurements in resource-constrained exploration medicine and support future optimization efforts that incorporate richer disease phenotypes and hardware constraints.

Session 3: Immune Remodeling, Inflammation, and Therapeutic Response

Abstract 012

Alcohol Reshapes Liver Zonal Plasticity and Immune-Metabolic Reprogramming in Metabolic-Syndrome Associated Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) commonly arises in metabolic dysfunction-associated steatohepatitis (MASH), alcohol-related liver disease (ALD), and metabolic dysfunction-associated ALD (MetALD), yet how zonal metabolic programs govern tumor lineage and immune responses remains unclear.

Methods: The study utilized complementary murine models of steatohepatitis-associated hepatocarcinogenesis. Additionally, experimental interventions included pharmacologic AhR inhibition and hepatocyte-specific β -catenin deletion to evaluate tumor burden and immune therapy response. **Results:** We show that CTNNB1-mutant MASH-HCC originates from periportal and midlobular hepatocytes through perivenous reprogramming. This transition is characterized by β -catenin activation, loss of periportal metabolic functions, and induction of the immunosuppressive IDO1-kynurenine-AhR axis. In contrast, ethanol exposure suppresses perivenous xenobiotic programs, destabilizes the β -catenin/AhR/CAR axis, and increases tumor heterogeneity by generating both progenitor/biliary- and hepatocyte-derived MetALD-HCC that remain sensitive to anti-programmed death-1 (aPD1) therapy. Furthermore, pharmacologic AhR inhibition or hepatocyte-specific β -catenin deletion reduces MASH-HCC burden and restores sensitivity to aPD1 treatment. **Conclusions:** Together, these findings identify AhR as a central mediator of β -catenin-driven tumor immunosuppression and a potential therapeutic target in CTNNB1-mutant HCC, highlighting context-dependent mechanisms of immune escape in alcohol-associated HCC.

Abstract 013

Single-Cell Profiling Reveals Previously Unrecognized Intrahepatic Neutrophil States in Cholestatic Liver Disease

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Background: Neutrophils exhibit functional plasticity during chronic inflammation, but their role in cholestatic liver disease remains undefined. In primary sclerosing cholangitis (PSC), injured cholangiocytes establish a pro-inflammatory niche that recruits neutrophils to the bile duct; however, their functional heterogeneity and contribution to disease are unclear. We hypothesized that cholestatic injury induces temporally dynamic neutrophil states that contribute to PSC pathogenesis. **Methods:** Single-cell RNA sequencing was performed on intrahepatic leukocytes from wild-type controls, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-diet model (2-days and 10-days exposure), and *Mdr2*^{-/-} mice. Neutrophils were identified using canonical markers and analyzed by subclustering and differential gene expression. Immunofluorescence (IF) was performed to validate neutrophil heterogeneity and evaluate markers. **Results:** Neutrophils constituted a distinct intrahepatic immune population across models. Initial subclustering identified inflammatory/pro-survival neutrophils (52.25%) expressing *Il1b*, *Cxcl2*, *Ptgs2*, *Tnfaip3*, and *Bcl2a1b*, and terminally mature/degranulating neutrophils (46.87%) enriched for

Retnlg, *Mmp8*, *Ngp*, *Cd177*, and *Padi4*. Higher-resolution analysis resolved stress-responsive (45.74%), tissue-adapted (36.25%), and terminally mature (14.39%) neutrophil states. Acute DDC injury enriched stress-responsive and tissue-adapted populations, whereas terminally mature neutrophils were predominantly derived from *Mdr2*^{-/-} mice (60.47%). Early (2-day) DDC neutrophils demonstrated increased *Cd74* and MHC-II-associated gene expression across all subclusters compared with 10-day DDC injury, suggesting activation of immune-interacting programs early in cholestatic injury. IF confirmed distinct CD177+ and CD177- neutrophil populations. Variable CD74 expressions within the peribiliary niche supported distinct neutrophil activation states. **Conclusions:** These findings identify previously unrecognized neutrophil states that emerge during distinct stages of cholestatic liver injury. Early *Cd74*/MHC-II-associated programs and accumulation of terminally mature CD177+ neutrophils reveal candidate immune pathways linked to disease initiation and progression. Targeting stage-specific neutrophil programs may represent a novel therapeutic strategy for PSC. **Funding:** National Institute of Health (NIH), AASLD Gupta Award in PSC, PSC Partners Seeking a Cure-Canada, and Mayo Clinic K2R Award.

Abstract 014

IRF3 Deficiency Protects from CCl₄-Induced Liver Fibrosis in Mice by Modulating Neutrophil Responses

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Background: Interferon regulator factor 3 (IRF3) executes multiple transcriptional and non-transcriptional functions in the development of metabolic liver diseases. While *Irf3*-deficient mice are protected from CCl₄-induced liver fibrosis, the specific cell types mediating its effects remain unclear. Liver fibrosis is characterized by excessive extracellular matrix accumulation and involves recruitment and activation of innate immune cells. Neutrophils play a dual role in fibrosis, promoting fibrosis through cytokines and granule proteins while also degrading the matrix. Yet regulation of neutrophils by IRF3 is largely unstudied in liver. Therefore, we investigated the role of neutrophils in liver fibrosis and assessed the contribution of IRF3 to neutrophil responses. **Methods:** C57BL/6J (WT), *Irf3*-deficient (*Irf3*^{-/-}), and mice expressing only non-transcriptional IRF3 function (*Irf3*^{S1/S1}) were exposed to CCl₄-induced liver fibrosis. To determine the functional role of neutrophils, mice were treated with anti-Ly6G antibody to deplete neutrophils. Primary neutrophils were isolated from control WT, *Irf3*^{-/-}, and *Irf3*^{S1/S1} mice to characterize their functional activities. **Results:** Liver bulk RNA-seq showed that chronic CCl₄ triggered neutrophil-associated response, which was attenuated in *Irf3*^{-/-} compared to WT mice. NET formation, assessed by citrullinated histone staining, was also reduced in *Irf3*^{-/-} livers. Interestingly, NIMP-R14 staining revealed greater hepatic neutrophil accumulation in *Irf3*^{-/-} than in both WT and *Irf3*^{S1/S1} mice, without a corresponding increase in neutrophil chemoattractants. Primary neutrophil degranulation and NETosis were induced by challenge with PMA. Despite intact ROS production, NET formation and release of granules were restricted in primary *Irf3*^{-/-} neutrophils compared to neutrophils isolated from WT and *Irf3*^{S1/S1}. Finally, neutrophil depletion in mice abolished the protective effect of *Irf3* deficiency on CCl₄-induced liver fibrosis. **Conclusions:** *Irf3*-deficiency impaired suicidal NETosis both after CCl₄ treatment *in vivo* and in stimulated primary neutrophils from *Irf3*^{-/-} mice. However, it enhanced hepatic neutrophil retention and protected against CCl₄-induced fibrosis. Importantly, normal neutrophil responses were restored in *Irf3*^{S1/S1} mice, indicating that the non-transcriptional function of IRF3 contributes to critical neutrophil NET formation, likely facilitating the progression of hepatic fibrosis.

Abstract 015

Identification & Characterization of Liver-Derived Hematopoietic Factors in Healthy and Diseased Livers

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Background: Chronic liver disease (CLD) is associated with disrupted hematopoiesis, contributing to hepatic and systemic inflammation. While the liver regulates immune function, its role in modulating hematopoiesis through liver-derived factors remains poorly understood. We hypothesize that healthy and diseased livers produce distinct hematopoietic factors that can influence bone marrow (BM) function and immune responses. This study aims to identify and characterize liver-derived hematopoietic factors and define their potential role in

liver-BM crosstalk during health and disease. **Methods:** A curated panel of hematopoietic factors was selected based on established roles in hematopoietic stem/progenitor cell maintenance, differentiation, mobilization, and immune cell development from the published literature. Image-based screening using the Human Protein Atlas (HPA) identified liver-expressed factors and their localization within hepatocytes, non-parenchymal cells, or both. Candidate factors were evaluated for differential expression across stages of CLD using publicly available Gene Expression Omnibus (GEO) datasets. **Results:** Thirty hematopoietic factors were curated and evaluated for hepatic expression. Of these, 18 hematopoietic factor probes were identified in the HPA. One factor was exclusively expressed by hepatocytes, eight by non-parenchymal cells, and nine by both cell populations. Analysis of normalized GEO expression data showed that most liver-derived hematopoietic factors remained relatively stable throughout CLD progression. In contrast, JAG1 and SPP1 were consistently and significantly upregulated in advanced stages of CLD while being expressed within the liver. These findings identify JAG1 and SPP1 as potential mediators of liver-BM communication and regulators of hematopoiesis during chronic liver injury. Ongoing studies are evaluating their hepatic expression and secretion in mouse models of liver fibrosis, alcohol-associated liver disease, and metabolic dysfunction-associated steatotic liver disease. **Conclusions:** Our findings identify a panel of liver-derived hematopoietic factors and suggest that the hepatic secretome may contribute to the regulation of BM hematopoiesis during CLD. Disease-associated upregulation of JAG1 and SPP1 highlights these factors as potential mediators of liver-BM crosstalk and systemic immune dysregulation. Further studies are underway to validate their role in altered hematopoiesis and inflammation during CLD.

Abstract 016

RNA Binding Protein, FMRP, Influences IL-6 Gene Expression and Pyroptosis in Macrophages

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Background: The RNA binding protein, Fragile X messenger ribonucleoprotein (FMRP) has roles in translation, transcription and transport of mRNA. Silencing of the gene *FMR1*, that encodes for FMRP, has been documented to cause Fragile X Syndrome (FXS). FXS is an intellectual disorder that results in inhibited neural development. Not much is known for how individuals with FXS, those lacking FMRP, react in response to infection or the role FMRP has in the immune response. **Methods and Results:** Our research explores the immune response primarily in bone marrow derived macrophages to *Listeria monocytogenes*. In response to a bacterial pathogen, macrophages lacking FMRP had reduced *IL6* gene expression and protein production. To assess if the loss of FMRP affected canonical inflammation pathways, cells were primed via LPS or Pam3CSK4 then activated. To activate the NOD2 or NLRP3 or AIM2 inflammasome pathways, macrophages were stimulated using agonists: nigericin, muramyl dipeptide (MDP) and Poly(dA:dT), respectively. Macrophages lacking FMRP treated with nigericin and MDP had significantly more cell death than with cells that produced FMRP. **Conclusions:** These results show that FMRP is involved in regulating inflammatory gene expression as seen in a lack of IL6 response during infection and may offer some protection against pyroptosis.

Abstract 017

Modulating EETs to Reprogram Glioblastoma Toward a Pro-resolution Phenotype

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Background: Glioblastoma (GBM) is characterized by an immune-rich tumor microenvironments with macrophages/microglia comprising up to 70% of the tumor mass. However, endogenous pathways that actively resolve inflammation remain poorly understood in GBM. Despite a median overall survival of only 12-15 months, therapeutic strategies targeting inflammation resolution in GBM have received little attention.

Epoxyeicosatrienoic acids (EETs) are endogenous bioactive lipid mediators with anti-inflammatory, pro-resolving, neuroprotective, and tissue-protective functions but are rapidly degraded by soluble epoxide hydrolase (sEH). EC5026 is a potent sEH inhibitor with no overt toxicity in several clinical trials. Here, we investigated whether impaired inflammation resolution contributes to GBM progression and systemic tissue wasting in mice bearing orthotopic CT2A glioblastoma tumors using EC5026. **Methods:** We utilized immunohistochemistry, and immunofluorescence of macrophage polarization markers, and fibrosis- and senescence-associated genes were assessed by qPCR and western blot analysis. **Results:** Immunohistochemistry revealed robust expression of the pro-resolving receptors LGR6, GPR37, and GPR18 throughout the GBM tumor microenvironment. Multiplex immunofluorescence demonstrated co-localization of LGR6 and GPR37 with Iba1-positive tumor-associated microglia and macrophages, while co-staining with iNOS and CD206 identified distinct pro-inflammatory and pro-resolving macrophage populations. Molecular profiling suggested reduced inflammatory signaling, decreased expression of fibrosis- and senescence-associated genes, and a shift toward a pro-resolving macrophage phenotype following EC5026 treatment. Histologically, EC5026 preserved skeletal muscle fiber cross-sectional area and reduced cachexia-associated markers in tumor-bearing mice. **Significance:** sEH inhibition reprograms the glioblastoma immune microenvironment toward a pro-resolving phenotype. **Conclusion:** Taken together, these findings suggest that failed resolution of inflammation contributes to both glioblastoma progression and GBM-induced muscle wasting. Furthermore, the localization of pro-resolving receptors to tumor-associated immune cells identifies previously unexplored resolution pathways in GBM and supports targeting the sEH/EET pathway as a strategy to enhance endogenous inflammation resolution, reprogram the tumor microenvironment, and mitigate glioblastoma-associated cachexia.

Abstract 018

***Acinetobacter calcoaceticus* Shapes Epithelial-Macrophage Signalling in Colorectal Cancer**

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Background: The gut microbiota plays a critical role in colorectal cancer (CRC), yet the functional contributions of many bacterial species enriched within colorectal tumors remain poorly understood. Recent studies have identified *Acinetobacter* species in CRC-associated microbial communities; however, their potential role in promoting tumor-associated inflammation has not been defined. **Methods & Results:** To investigate the effects of *Acinetobacter calcoaceticus* on the colonic epithelium, murine CMT-93 colonic epithelial cells were treated with cell-free *A. calcoaceticus* supernatants. Exposure significantly increased the expression of the pro-inflammatory mediators MCP-1, IL-8, and TNF, which recruit and activate immune cells, like macrophages. To determine whether *A. calcoaceticus* also impacts macrophage, primary bone marrow-derived macrophages (BMDMs) were exposed to *A. calcoaceticus* cell-free supernatants. Treatment significantly increased IL-6 production, a pro-inflammatory cytokine implicated in epithelial proliferation and colorectal tumorigenesis. Fractionation of the supernatant revealed that the >10 kDa fraction of *A. calcoaceticus* secreted compound elicited increased IL-6 production in BMDMs, suggesting that the macrophage-stimulatory activity is associated with a higher-molecular-weight compound. We next investigated whether the macrophage response could influence the colonic epithelium. Conditioned medium collected from *A. calcoaceticus*-stimulated BMDMs was applied to CMT-93 cells and resulted in increased expression of the proliferation marker *Ki67*, supporting a potential macrophage-mediated effect on epithelial proliferation. To explore the clinical relevance of these findings, we analysed the Bacteria in Cancer database. CRC tumors enriched with *Acinetobacter* exhibited elevated expression of several proliferation and cancer-associated genes. Finally, we examined the inflammatory effects of *A. calcoaceticus* *in vivo*. Antibiotic-treated mice were orally gavaged with either PBS (vehicle control) or *A. calcoaceticus*. Colonized mice exhibited increased immune cell infiltration and elevated expression of pro-inflammatory cytokines in the colon. Furthermore, in mice treated with 3% DSS to induce colitis, *A. calcoaceticus* colonization significantly exacerbated DSS-induced intestinal pathology. **Conclusion:** These findings suggest that *A. calcoaceticus* drives inflammatory and proliferative signals that could participate in CRC development.

Abstract 019

Efflux Transporters Are Elevated and Microbially Regulated in Head and Neck Cancer

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Background: Head and neck squamous cell carcinoma (HNSCC) accounts for approximately 4% of cancer diagnoses and was projected to cause 16,680 US deaths in 2025. Major risk factors include tobacco and alcohol use and high-risk human papillomavirus (HPV) infection. Although chemotherapy remains important for HNSCC treatment, its effectiveness can be limited by efflux transporters that remove drugs from cancer cells. Currently, the levels of drug efflux transporters in HNSCC remain poorly characterized. We aimed to examine the levels of efflux transporters in HNSCC and identify if oral microbes were capable of impacting efflux activity. **Methods & Results:** We analyzed gene expression data from The Cancer Genome Atlas (TCGA) to determine the expression of drug efflux transporters in HNSCC. We found that drug efflux transporters ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, and ABCG1 were increased in the HNSCC tumors compared to control tissues. We chose to focus on ABCC2, which encodes the drug efflux transporter MRP2, since it is known to transport several chemotherapy drugs out of cancer cells. We found that ABCC2 expression was elevated in tumors from patients with stage I-IV cancer, across all tumor grades (1-3), in both male and female patients, and across multiple racial groups, including Caucasian, African American, and Asian patients. Interestingly, ABCC2 expression was significantly higher in HPV-negative tumors, but not in HPV-positive tumors. To determine the bacteria found most commonly found in HNSCC, we examined the Bacteria in Cancer (BIC) Database. We found *Prevotella* was the top genera in HNSCC tumors, followed by *Fusobacterium* and *Streptococcus*. To determine if these bacteria affect MRP2 activity, we cultured multiple *Prevotella* and *Streptococcus* strains in a defined bacterial medium and applied cell-free bacterial supernatant to human CAL-27 tongue squamous cell carcinoma cells. MRP2 activity was measured using the fluorescent dye 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA), which is exported from cells by MRP2. We found that secreted compound from *P. intermedia* and *P. melaninogenica*, but not *S. oralis*, *S. salivarius*, *S. sanguinis*, *S. parasanguinis* or *S. gallyticus* altered CDCFDA efflux in CAL-27 cells. **Conclusions.** These findings suggest that bacteria in HNSCC could impact drug efflux activity.

Session 4: Development, Matrix Biology, and Fibrotic Remodeling

Abstract 020

Hepatocyte HIF Signaling is Dispensable for β -catenin-dependent Liver Zonation

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Background: The adult liver is divided into three zones along the porto-central axis, where hepatocytes located in each zone play distinguished metabolic functions to maximize functional efficiency. Zone 1 hepatocytes near the portal vein, where blood flows into the liver, are exposed to the highest oxygen concentration, whereas Zone 3 hepatocytes near the central vein, where blood leaves the liver, are exposed to the lowest oxygen concentration. We have previously shown WNT/ β -catenin signaling is a main regulator of Zone 3 identity. What remains unclear is the upstream regulator of Zone 3 WNT activity. Since liver metabolic functions are dictated by the environmental factors where they reside, we hypothesized that hypoxia-inducible factor (HIF) signaling in hepatocytes driven by relative hypoxia regulates Zone 3 identity. **Methods:** The transcriptional activity of HIF requires both HIF α (either 1 α or 2 α) and HIF1 β subunits to dimerize. To test our hypothesis, we generated transgenic mouse models to delete *Hif1a* and *Epas1* (HIF2 α) (HC-Hif1a-Hif2a-DKO) or *Arnt* (HIF1 β) (HC-Hif1b-KO) from hepatocytes. Mice were intraperitoneally injected with AAV8-TBG-eGFP (control) or AAV8-TBG-Cre at 8 weeks of age, and livers were resected after 14 days post-injection. Liver tissues were stained with H&E and Sirius Red to assess liver morphology and fibrosis, respectively. Immunohistochemistry (IHC) was performed to stain for glutamine synthetase (GS) and CYP2E1 as zone 3 markers and CYP2F2 as a zone 1 marker, to determine the changes in liver zonation. **Results:** All control, HC-Hif1a-Hif2a-DKO, and HC-Hif1b-KO mice did not show difference in liver weight-to-body weight ratio after *Hif1a/Epas1* or *Arnt* deletion compared

to control mice. We also did not see any morphological differences in livers from three mouse groups, as well as no changes in the degree of fibrosis. There was no difference in serum AST, ALT, total bilirubin, and alkaline phosphatase to suggest hepatocyte injury or cholestasis. We also did not see differences in serum glucose, triglyceride, and cholesterol level. More importantly, we did not see any difference in the liver zonation in all three mouse livers based on IHC of GS and CYP2E1 (Zone 3 markers) and CYP2F2 (Zone 1 marker) based on %area quantifications. **Conclusion:** Overall, these data demonstrated that HIF signaling in adult hepatocytes does not have a direct effect on liver homeostasis or liver zonation. Our data is meaningful in that it addresses the long-standing question in the field of whether relative hypoxia has a direct effect on β -catenin activity in hepatocytes to regulate liver zonation.

Abstract 021

Investigating the Mechanisms of Retinoic Acid Signaling in Liver and Hepatic Stellate Cell Development

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Background: Retinoic acid (RA) signaling is key to several developmental and pathologic processes, acting as a mediator of target gene expression. Hepatic stellate cells (HSCs) are the primary storage site for retinoids. In mice, loss of HSC retinoid storage leads to increased RA signaling in the liver and decreased hepatocellular carcinoma (HCC) incidence (Shirakami Y, 2012, *Carcinogenesis*, 33:268-274). We hypothesize that RAR signaling is essential for normal liver development and for reducing a proliferative phenotype that is a frequent hallmark of precancerous cells. **Methods:** This project is investigating the RA signaling pathway in larval zebrafish by pharmacologically modulating RA activity in the period when HSCs are entering the liver (56-80 hours post fertilization (hpf)). We treat larval zebrafish with RA pathway agonists and inhibitors, then evaluate HSCs and liver phenotype by confocal imaging and gene expression by in situ stains and qPCR. The resulting proliferative state and gene expression expose the involvement of the targeted RA components in late-stage liver development. **Results:** Treatment of larval zebrafish with the retinoic acid receptor- α (RAR α) agonist AM580 decreases the number of HSCs in the larval liver at (hpf), while treatment with the retinoid X receptor (RXR) agonist methoprene acid (MA) increases HSC number (Yin C, Evason KJ, 2012, *Hepatology*, 56:1958–70). I confirmed that MA increases HSC number at 80 hpf and extended these findings, showing that liver volume was not affected by MA at 80 hpf but was increased at 144 hpf. Treatment with DEAB, a drug which inhibits ALDH from synthesizing RA, results in a higher number of HSCs at 80 hpf. Dissimilar to the effect of MA on liver volume, DEAB treatment did not significantly impact liver size. Together, these findings suggest that HSC and hepatocyte proliferation may not be controlled by the same RA factors, but still the modulation of RA signaling results in abnormal HSC and liver development. **Conclusions:** During early embryonic stages of development, RA plays an important role in embryo patterning, hepatic fate specification, and liver budding. Despite the mature liver being the primary site of retinoid storage, hepatic RA signaling mechanisms are poorly understood after the liver bud formation stage. This project aims to fill that gap by modulating RA signaling during the developmental stage during which HSCs are entering the liver.

Abstract 022

Senescence in Healthy Lung Aging Contributes to Changes in the Extracellular Matrix Proteome

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Background: Aging is characterized by several hallmarks, including senescence, implicated in chronic lung diseases. Previous studies have focused on cellular outcomes of senescence, however, effects on the extracellular matrix (ECM) altered upon lung aging remain elusive. We aim to decipher changes in the ECM proteome in healthy aging lung and investigate whether trigger- and cell-specific senescence is sufficient to drive ECM changes. **Methods:** Twenty healthy human donor lung tissues (4mo-95yrs) were obtained for label-free

liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis completed in the Schilling Lab. Precision cut lung slices (PCLS), an *ex vivo* model for multicellular interactions and ECM, were prepared from human lung tissue into 300um thick and 1cm diameter slices. Samples were treated with bleomycin (15ug/mL) to induce senescence. **Results:** 5,138 protein groups were identified and quantified with at least two unique peptides in human lung tissue aging cohort. In old (>65years) vs young adult (< 50 years) lung tissues, 740 protein groups reached significance with 508 up-regulated, and 232 down-regulated. Statistical analysis revealed significant changes in abundance and expression of proteins, with the largest group representing ECM proteins, identified through the MatrisomeDB. To further investigate whether these changes are mediated by cellular senescence, we analyzed senescent human PCLS confirmed by assessing p21/p16 mRNA and protein levels, and b-galactosidase staining. 2,899 protein groups were similarly identified in the senescent human PCLS cohort, with 61 key proteins significantly altered with aging and senescence. Nineteen ECM proteins, including secreted factor S100A9 and matrix metalloproteinases (MMP9, MMP2) regulated across age and senescence were termed Senotype A (senescence-type) “ECM-based Healthy Aging Senotype”. Twenty-six ECM proteins, including nidogens (NID1), collagens (COL1A1, COL3A1), and laminins (LAMA3, LAMB2) regulated only with senescence-induction were termed Senotype B “ECM-based Fibrosis Senotype”. **Conclusion:** The lung ECM is significantly altered over the healthy lifespan. Autocrine and paracrine signaling of senescent cells contributing to ECM alterations. Two ECM-based lung senotypes were identified for healthy and diseased senescence-driven dysregulation. **Acknowledgements:** NIA U54 AG075931 (Koenigshoff) and U54 AG075932 (Schilling)

Abstract 023

The Hippo Pathway Component AMOTL2 Impacts Lung Epithelial Cell Reprogramming in Pulmonary Fibrosis.

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Background: Idiopathic pulmonary fibrosis (IPF) is a severe and usually fatal lung disease characterized by increased ECM deposition, stiffening of lung tissue, and destruction of alveoli. IPF is thought to be in part, driven by the failure of appropriate differentiation of alveolar stem cells (primarily alveolar type 2 – AT2) into the gas-exchanging alveolar type 1 (AT1). Single-cell RNA sequencing has identified an aberrant AT2 cell phenotype in IPF, referred to as basaloid cells. A similar aberrant cell type has been found in murine models of pulmonary fibrosis, alveolar differentiation intermediate (ADI) cells. We and others show that the Hippo effector Yes-associated protein 1 (YAP) is upregulated in AT2 cells in IPF and promotes ADI formation; here we examine its regulator AMOTL2. We hypothesize that AMOTL2 downregulation in AT2 cells leads to increased nuclearization of YAP where it upregulates genes related to reprogramming of AT2 cells. **Materials and Methods:** We examined localization of AMOTL2 in human lung tissue from healthy and IPF donors (n=10/group -IPF vs control) via single-cell RNA Seq and immunofluorescent (IF) staining of AMOTL2 alongside specific AT2 cell-markers. We knocked out AMOTL2 in cultured primary mouse AT2 cells from *Amotl2* fl/fl mice using Adeno-cre *ex vivo* and analyzed up and downregulated genes and pathways in addition to their ADI phenotype using bulk RNASeq (n=4). AT2 specific AMOTL2 knockout mice were treated with bleomycin to induce lung fibrosis and were compared to controls in bodyweight, lung function and histologically. **Results:** We found that AMOTL2 is downregulated in AT2 cells in IPF, and its loss in primary mouse AT2 cells *ex vivo*, induces transcription of pro-fibrotic and altered epithelial identity pathways. The AMOTL2 knockout cells also show upregulation of ADI-associated genes such as Keratin 8 and Claudin 4, indicating loss of normal AT2 cell reprogramming. Our *in vivo* experiment with bleomycin-induced lung injury in mice with AT2 cell-specific loss of AMOTL2 showed that our knockout mice lose less bodyweight following bleomycin, but they exhibit increased lung stiffness compared to control. These data indicate worsened fibrotic mechanical remodeling despite a potential attenuated initial immune-related systemic effect from the bleomycin treatment. **Conclusion:** Loss of AMOTL2 contributes to AT2 cell reprogramming and thus might contribute to the development and progression of pulmonary fibrosis.

Abstract 024

A Novel, Preclinical Model Recapitulates Neonatal Marfan Syndrome and Uncovers Potential Mechanisms Underlying Disease Severity

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Background: Neonatal Marfan syndrome (nMFS) is a severe, early-onset form of Marfan syndrome, a connective tissue disorder caused by mutations within exons 23-32 of fibrillin-1 (*FBN1*). Patients are born with cardiovascular, skeletal and ocular dysfunction, with 95% dying within 1-2 years. Cardiovascular complications include congestive heart failure, valve dysfunction and aortic aneurysms with risk of dissection. Currently, there is no cure for nMFS, and the molecular mechanisms driving its early-onset and severe progression remain poorly understood, in part due to a lack of suitable pre-clinical models. **Methods:** Based on clinical findings of two nMFS patients with deletion of *FBN1* exon 30, we generated a conditional *Fbn1* exon 30 allele that was ubiquitously deleted via *Cmv-Cre*. Exon 30 deletion and nMFS phenotype were assessed by qPCR, survival analysis, and histology. RNAscope and immunofluorescence were performed on postnatal day 14 (PND14) heart sections to investigate underlying mechanisms, and bulk RNA-seq of PND0 hearts was used to identify potential molecular targets. **Results:** *Fbn1* exon 30 deleted heterozygous mice (ex30^{del/+}) phenocopy nMFS characteristics as early as PND0, with 98% dying within 24 days. qPCR validated a 50% reduction in *Fbn1* exon 30 in ex30^{del/+} hearts. Hart's staining revealed increased elastic fiber breakage, enlarged wall thickness and dissections in ex30^{del/+} aortas. Ex30^{del/+} hearts have enlarged aortic and mitral valves with increased proteoglycan content and disorganized collagen. *Fbn1* RNAscope revealed significantly increased *Fbn1* RNA staining in the aortic and mitral valves, left ventricular compact myocardium and aorta. However, immunofluorescence staining revealed decreased fibrillin-1 protein. Bulk RNA-seq and Ingenuity Pathway Analysis uncovered genes and upstream pathways involved in the manifestation of nMFS, such as *Tnc*, *Mmp9*, *Mmp12* and TGF- β . **Conclusion:** We successfully generated a preclinical model that phenocopies nMFS patient characteristics that has identified potential genes involved in its disease pathology. Future studies will test molecular targets to uncover therapeutic interventions.

Abstract 025

A Patient-specific ADAMTSL2 Variant Causes Geleophysic Dysplasia Connective Tissue Changes in a Preclinical Mouse Model

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Background: Geleophysic dysplasia (GD) is caused by recessive mutations in ADAMTSL2 (a disintegrin and metalloprotease with thrombospondin type I motifs-2; GD1), and is characterized by severe short stature and other skeletal abnormalities, characteristic facial features, thick skin, and hyper-muscular build. Progressive heart valve disease and narrowing of the large airways results in approximately 33% mortality before the age of 5 years. Despite high childhood mortality and significant morbidity, no disease-modifying treatments exist for GD. **Methods:** To model disease progression and enable efficacy testing of mechanism-based therapeutic approaches, a mouse model for severe GD1 was generated by introducing the patient-specific ADAMTSL2 c.499G>A (p.D167N) mutation into the mouse *Adamtsl2* locus. X-ray, histology and antibody staining were undertaken to determine changes in extracellular matrix and molecular signaling. **Results:** Homozygous *Adamtsl2*^{D167N/D167N} (D167N) mice had reduced postnatal survival and short stature. Radiographs demonstrated significantly shortened hind limb and forelimb bones with delayed mineralization and abnormally shaped vertebrae. Histologic investigation revealed a shortened growth plate, suggesting abnormalities in chondrogenesis. Cardiac histomorphometry revealed dysplastic aortic heart valves, consistent with progressive heart valve disease observed in patients with GD1. In the lungs, bronchial obstruction was observed, as

previously reported for global *Adams12* knockout mice, likely resulting in occlusion of the affected airways. TGF β signaling was increased in D167N mice as compared to control. **Conclusion:** D167N mice recapitulate key clinical manifestations of patients with GD1 and can be utilized further to determine the molecular mechanisms of GD1 towards therapeutic intervention.

Session 5: Microbial Metabolism, GI Homeostasis, and Inflammatory Disease

Abstract 026

Hydroxyphenyllactic Acid Promotes Intestinal Homeostasis Through Antioxidant, Anti-inflammatory, and Barrier-Protective Effects

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Background: *Lactobacillus* species are known to support intestinal development and homeostasis early in life. These effects are partly mediated by metabolites produced from dietary substrates. In a previous study, we identified that hydroxyphenyllactic acid (HPLA), a tyrosine-derived metabolite, was robustly produced by multiple *Lactobacillus* species in response to human milk, but not infant formula. Although HPLA has demonstrated to have free-radical-scavenging activity *in vitro*, its other potential functions, particularly in the gut, remain uncharacterized. Here, we investigated the effects of HPLA on intestinal oxidative stress, inflammation, and barrier function. **Methods:** Antioxidant activity of HPLA was assessed in fetal intestinal epithelial cells (CCD-841CoN) using H₂DCFDA to measure intracellular reactive oxygen species (ROS) induced by H₂O₂. Anti-inflammatory effects were evaluated in CCD-841CoN cells stimulated with lipopolysaccharide and flagellin, followed by RNA-seq analysis. Barrier function was tested in human colonic monolayers using lucifer yellow permeability assays and RT-qPCR analysis of tight junction gene expression. *In vivo*, the effects of orally administered HPLA (20 mg/kg) were examined in adult C57BL/6 mice with 7-day dextran sulfate sodium (DSS)-induced colitis; inflammation was assessed by H&E histology and colonic cytokine expression by RT-qPCR. **Results:** *In vitro*, HPLA significantly reduced H₂O₂-induced ROS accumulation in fetal intestinal cells. Transcriptomic analysis revealed that HPLA attenuated activation of key inflammatory pathways, including TNF- α , IFN- α/γ , and IL-6/JAK/STAT3 signaling. HPLA also enhanced epithelial barrier integrity, evidenced by reduced monolayer permeability and increased expression of tight junctional genes *Cldn1*, *Cldn3*, and *Ocln*. In the DSS colitis model, HPLA administration upregulated the anti-inflammatory cytokine *Il10*, although no differences were noted for the pro-inflammatory genes *Kc*, *Tnfa*, or *Ifng*. HPLA treatment also rescued DSS-induced suppression of *Cldn3* and increased expression of *Cldn1* and *Cldn4* relative to the control and DSS vehicle group, respectively. **Conclusions:** HPLA is a diet-dependent microbial metabolite that mitigates oxidative stress, inflammation, and barrier dysfunction, providing a potential mechanistic link between human milk-microbe interactions and intestinal homeostasis.

Abstract 027

Diverse Microbes From the Human Gut Microbiome Secrete Extracellular Purines

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Background: Extracellular purines, including ATP and ADP, serve as important signaling molecules to maintain homeostasis. Several studies have identified that microbial and mammalian cells release purines into their environment. While purinergic signaling, via specific receptors, have been well characterized in immune and neural responses, less is known within the gastrointestinal tract especially about which microbiome communities are capable of secreting extracellular purines. To address this, we sought to identify human gut microbes capable of ATP production. **Methods & Results:** We examined >100,000 annotated genomes using the Integrated

Microbiome Genome Database, as well as the enzymes involved in ATP production and clearance in bacteria. Genomes were sorted based on those identified as part of the human microbiome using the IBD TAMMA database. Interestingly, we found that >70% of the human gut microbiota had the enzymes involved in ATP and ADP production, suggesting that ATP/ADP production is conserved amongst bacterial taxa. To assess ATP production, we grew a representative cohort of bacteria and fungi in a chemically defined medium for 6 and 24 hrs and performed a luciferin-based ATP assay on microbial supernatant. In general, we found that ATP concentrations peaked at 6 hours and were sustained at the 24-hour time point. We identified that *Streptococcus mitis*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* generated similar levels of ATP, while *Streptococcus pyogenes* secreted the highest amount (~500nm) of ATP. We also found that fungal strains *Candida glabrata* and *C. tropicalis* produced ~150 nM ATP in supernatant. We did not observe striking differences of ATP production between commensal and pathogenic microbes. To assess whether microbial ATP could activate host purinergic receptors, we performed agonist assays on cells expressing the calcium indicator GCaMP6s and observed increased intracellular calcium signaling from bacteria supernatant containing ATP. **Conclusions:** Collectively, our findings demonstrate that several microbes are capable of secreting exogenous ATP, specifically Gram-positive and fungal species, and that this ATP can activate host purinergic signaling. This work reveals unique patterns of exogenous ATP production among diverse bacterial groups.

Abstract 028

***Limosilactobacillus reuteri* Creates Novel Microbially Conjugated Bile Acids Which Impacts Bile Acid Signaling**

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Background: Multiple microbes in the gastrointestinal tract can transform primary bile acids into secondary bile acids and other derivatives. Microbes can also conjugate bile acids with diverse amino acids and bioactive amines, but the responsible organisms and physiological consequences of this conjugation remain poorly understood. We investigated bile acid conjugation by *Limosilactobacillus reuteri* ATCC 6475 and the functional effects of its conjugates. **Methods and Results:** Using targeted LC-MS/MS, we found that *L. reuteri* conjugated bile acids to several amino acids, including aspartate, glutamine, glutamate, histidine, lysine, and threonine. The abundance of individual conjugates varied according to the bile acid substrate, with UDCA yielding the greatest levels of aspartate- and glutamate-conjugated products and CDCA producing the highest levels of histidine conjugation. To investigate possible receptor-level effects, we modeled interactions between the identified conjugates and FXR or TGR5. Surprisingly, we found that many of the identified *L. reuteri* conjugated CDCAs bound the receptors more strongly than unconjugated CDCA. However, predicted receptor-bound poses of amino acid-conjugated CDCA exhibited high RMSD values relative to unconjugated CDCA. These data suggest that although *L. reuteri* conjugated bile acid have a predicted stronger affinity, their orientation and/or conformation in the FXR and TGR5 binding pockets are substantially altered, and these compounds may not activate the receptors. Consistent with these predictions, experiments in transfected HEK293FT cells showed that *L. reuteri*-derived CDCA conjugates reduced FXR and TGR5 activation relative to unconjugated CDCA. FXR competition assays further suggested that this reduction may be attributable, at least in part, to competitive antagonism, as conjugated bile acids diminished activation elicited by the potent FXR agonist XL335. RNA sequencing of human colonic epithelial cells revealed that *L. reuteri*-conjugated CDCA produced a distinct transcriptional response compared with unconjugated CDCA. Microbial conjugation attenuated CDCA-induced activation of the unfolded protein response, reactive oxygen species signaling, and xenobiotic metabolism pathways. **Conclusions:** These findings demonstrate that a single microbe can produce a diverse range of amino acid-conjugated bile acids and that these microbial metabolites can modulate bile acid receptor signaling and epithelial responses.

Abstract 029

Patients With Ulcerative Colitis Have Alterations in Purinergic Receptors Expression and Function

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Background: Purinergic receptor activation is known to play an important role in immune responses to intestinal inflammation and infection. However, less is known about the role of intestinal epithelial purinergic signaling during inflammation. As inflammation impacts epithelial function, we hypothesized that active inflammation in ulcerative colitis alters intestinal purinergic receptors expression and signaling. **Methods:** To assess the distribution of the 19 purinergic receptors in the human gut epithelium and immune cells, we queried single-cell RNA sequencing data from the Human Protein Atlas. Using publicly available RNA seq data from individuals with ulcerative colitis and non-IBD controls, we assessed alterations in purinergic receptors across histological severity scores (GSE109142). We also analyzed RNA seq data from human intestinal organoids to identify potential inflammatory cytokines responsible for altered purinergic receptor expression (GSE289072). To evaluate functional P2 receptor signaling, we used human intestinal organoids expressing the calcium indicator GCaMP6s to measure epithelial calcium responses as a read out of receptor activation. **Results:** Single-cell analysis revealed limited P1 receptor expression in the intestinal epithelium, with greater expression across immune cell populations. In contrast, P2RY1, P2RY2, and P2RX4 were broadly expressed across epithelial cell types in the healthy small and large intestine. Analysis of ulcerative colitis transcriptomic data revealed significant reductions in epithelial-associated P2RY1, P2RY2, and P2RX4 expression with increasing inflammation, while P1 receptor expression remained unchanged. Several immune-associated purinergic receptors were increased in ulcerative colitis patients. Exposure to pro-inflammatory cytokines decreased P2Y1 and P2Y2 expression in ulcerative colitis organoids. Functionally, inhibition or knockout of P2Y1 significantly reduced epithelial calcium signaling in human intestinal organoids, consistent with impaired purinergic communication. P2Y1 inhibition, but not P2Y2 inhibition, delayed wound healing in epithelial scratch assays, suggesting P2Y1 signaling contributes to repair. **Conclusions:** These findings reveal an imbalance in epithelial and immune cell purinergic signaling during ulcerative colitis. This highlights distinct roles for purinergic receptors in intestinal epithelial function and repair.

Abstract 030

Green Tea Kombucha Protects Against Murine Colitis by Averting Oxidative Colon Damage

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Background: Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) associated with intestinal inflammation, oxidative stress, intestinal barrier disruption, and increased risk of colorectal cancer. Although 5-aminosalicylic acid (5-ASA) preparations are commonly used in the management of IBD, their use may be limited by cost and adverse effects, including nausea and vomiting. These limitations highlight the need for safer and more affordable therapeutic alternatives. Kombucha, a fermented tea beverage, possesses considerable antioxidant properties and may therefore have therapeutic potential in oxidative stress-associated disorders. We hypothesized that green tea Kombucha (GTK) could attenuate colonic injury by mitigating oxidative stress. **Methods:** The antioxidant capacity of GTK was initially evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay. Colitis was subsequently induced in male Wistar rats by a single intrarectal administration of 500 μ L of 4% (v/v) acetic acid. Following induction, rats received daily oral administration of water, Pentasa mesalazine (30 mg/kg), or GTK at doses of 100 and 500 mg/kg for seven days. Animals were euthanized on day eight, the colons were excised, weighed, and their weight-to-length ratios determined. Colonic homogenates were also analyzed for the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Histopathological changes were evaluated following hematoxylin and eosin staining. **Results:** GTK demonstrated significant antioxidant activity, as evidenced by its ability to scavenge DPPH free radicals. Intrarectal administration of acetic acid produced marked colonic injury, characterized histologically by crypt loss, oedema, and pronounced leukocyte infiltration into the lamina propria. Treatment with GTK attenuated these

inflammatory and morphological alterations, with effects comparable to those observed following mesalazine treatment. The increased colon weight-to-length ratio associated with acetic acid-induced colitis was also reduced following GTK administration. In addition, GTK reversed the colitis-associated reduction in colonic CAT activity, suggesting an improvement in antioxidant defense. **Conclusion:** These findings suggest that GTK ameliorates murine ulcerative colitis by enhancing antioxidant defense and reducing colon inflammation and tissue injury. GTK may therefore represent a promising natural therapeutic candidate for the management of UC.

Abstract 031

Reduced Myosin 5b Expression is Associated with Increased Epithelial Proliferation in Ulcerative Colitis and Colorectal Cancer

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Background: Inflammatory Bowel Disease (IBD) is a chronic condition that causes extensive intestinal inflammation and is currently without a cure. IBD is divided into two subtypes: Crohn's disease and ulcerative colitis. Ulcerative colitis is characterized by persistent inflammation and ulcers in the large intestine (colon). Individuals with IBD, including those with ulcerative colitis, have an increased risk of developing colorectal cancer (CRC) due to the presence of widespread inflammation. Myosin 5b is a motor protein that is highly expressed throughout the colonic epithelium and mutations in Myosin 5b have recently been identified in IBD cohorts.

Objective: We aimed to investigate alterations in Myosin 5b expression during colonic inflammation in ulcerative colitis patients and colorectal cancer. **Methods & Results:** We analyzed human bulk and single cell RNA sequencing datasets to identify the distribution of Myosin 5b expression in human tissue and the colon. We found that Myosin 5b is broadly expressed across all major colonic epithelial cells and is uniformly expressed throughout the different anatomical regions of the human colon. We also observed that Myosin 5b expression was markedly decreased in both inflamed ulcerative colitis tissue and in CRC. Immunofluorescent staining and imaging of human colonic tissue confirmed decreased levels of Myosin 5b in ulcerative colitis samples, in dysplasia and in CRC tissue. In a tamoxifen inducible-intestinal specific Myosin 5b knockout mouse model we found that loss of Myosin 5b resulted in increased colonic proliferation *in vivo* and *in vitro* using colonic organoids. We identified that tissue from ulcerative colitis patients and CRC samples, which had low levels of Myosin 5b compared to healthy tissue, also exhibited increased proliferation. **Conclusions:** Collectively, these findings identify decreased Myosin 5b expression in active colonic inflammation in individuals with ulcerative colitis and in CRC. We also demonstrate that loss of Myosin 5b in the colonic epithelium is associated with a significant increase in proliferation within colonic crypts both *in vivo* and *in vitro*. These data suggest that decreased Myosin 5b may be a feature of colonic inflammation and could increase proliferation thereby impacting pathological states.

Session 6: Endothelial Immunobiology, Vascular Remodeling, and Repair

Abstract 032

A Novel Role for PECAM in the Regulation of Leukocyte Transendothelial Migration

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Background: Mechanical traction between platelet endothelial cell adhesion (PECAM) on a transmigrating leukocyte and PECAM at the endothelial cell border initiates leukocyte diapedesis in areas of inflammation. The PECAM-initiated signaling pathway mediates transendothelial migration (TEM) by promoting the movement of a storage membrane compartment, the lateral border recycling compartment (LBRC), to the site of TEM. Previously, we demonstrated that kinesin light chain 1, variant 1 (KLC1c) regulates TEM by controlling the targeted delivery of the LBRC to the TEM site. The goal of this study was to identify and characterize KLC1c-interacting proteins in endothelial cells and to elucidate the molecular pathways through which KLC1c moves the LBRC and facilitates TEM. **Methods:** We performed an unbiased proteomics screen using FLAGAPEX2-

tagged KLC1c expressed in endothelial cells to label neighboring proteins. Biotinylated proteins were affinity-purified and analyzed via mass spectrometry. In parallel, we conducted mass spectrometry on proteins co-immunoprecipitated with KLC1c. We utilized high-performance computing and AlphaFold-Multimer to model potential protein interactions between KLC1c and candidate binding partners. **Results:** Across both datasets, we identified a convergent set of top candidate interactors, many of which are associated with cytoskeletal dynamics, membrane trafficking, and scaffolding – hallmarks of KLC1 function. PECAM was the only transmembrane protein identified. AlphaFold Multimer modeling revealed PECAM as a direct binding partner for KLC1c. Notably, the PECAM ITIM1 motif (previously shown to be essential for TEM) docked onto the 6TPR domain on KLC1c, a region typically associated with cargo and adaptor binding. Co-immunoprecipitation experiments in endothelial cells expressing Flag-tagged KLC1c confirmed this interaction. **Conclusions:** Our data suggest that PECAM is not only involved in the initiation of TEM but also in the final stages of recruitment of the LBRC. To test this, we are synthesizing novel peptides to disrupt PECAM:KLC1c interactions. We are using RFdiffusion to generate 3D peptide backbones, followed by ProteinMPNN to optimize the final amino acid sequences. If successful, these could serve as potent and selective anti-inflammatory therapies.

Abstract 033

Endothelial Senescence in Skin Wound Repair

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Background: Angiogenesis is required for organ regeneration and repair from injury. Cellular senescence, an irreversible cell-cycle arrest, is involved in age-related diseases, including cancer. However, cellular senescence is also required for tissue regeneration and repair; it has been reported that depletion of senescent cells inhibits wound repair. **Methods and Results:** The role of endothelial cell (EC) senescence in angiogenesis and wound repair has not been examined before. When we create a dorsal wound in *C57BL/6* mice, the wound closes and blood vessel density increases 7 days after wounding. Expression of the senescent marker p16 is increased in ECs during wound healing in a mouse skin wound model. Bulk RNA-seq analysis of wounded skin reveals an increase in senescence genes during wound repair. Wound closure is significantly delayed, and blood vessel formation is inhibited in *p16-3MR* mice, in which p16⁺ senescent cells are depleted after ganciclovir treatment. Consistently, p16 knocked down ECs isolated from human subcutaneous tissues suppress vascular formation in the human EC organoid system. **Conclusion:** These results suggest that EC senescence is necessary for angiogenesis during wound repair.

Abstract 034

Lymphatic YAP1 in hypoxia-induced vascular remodeling

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Background: Pulmonary hypertension (PH) is a life-threatening pulmonary vascular disease characterized by sustained elevation of pulmonary arterial (PA) pressure. Remodeling of distal pulmonary arterioles (PAs) is a key feature of PH and involves accumulation of PA smooth muscle cells (PASMCs) to PAs. Lymphatic endothelial cells (LECs) contribute to cardiovascular and lung diseases associated with PH. **Methods and Results:** The role of lymphatics in PH pathology has not been examined before. Depletion of lymphatics accelerates hypoxia-induced PA remodeling and right ventricular (RV) hypertrophy. Expression of Hippo signaling transducer, Yes associated protein (YAP1) increases in hypoxia-treated mouse lung LECs. Hypoxia induced PA remodeling and RV hypertrophy are enhanced in the *Prox1-Cre^{ERT2}-Yap1^{fl/fl}* mouse lungs, in which expression of APLN that inhibits PA remodeling in LECs decreases. Conditioned medium collected from hypoxia-treated LECs or LECs treated with YAP activator suppresses DNA synthesis of SMCs; in contrast, an APLN receptor inhibitor inhibits the effects. **Conclusion:** These results suggest that lymphatic YAP1 signaling is necessary for suppressing hypoxia-induced distal PA remodeling.

Abstract 035

Biglycan in Lung Vascular Regeneration

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Background: Impaired lung regeneration and repair contribute to the pathogenesis of chronic lung diseases. Angiogenesis plays a crucial role in organ regeneration and repair from injury. Understanding the mechanism of angiogenesis during regenerative lung growth is required for the development of more efficient strategies for chronic lung diseases. We have reported that endothelial cell (EC) proliferation during regenerative lung growth after unilateral pneumonectomy (PNX) is more prominent at the peripheral region compared to proximal region.

Methods and Results: ECM components in the post-PNX mouse lungs are spatially different between peripheral vs. proximal regions. Transcriptomic analysis reveals that the expression of extracellular matrix molecule biglycan is higher in the proliferating EC populations of the post-PNX lungs compared to sham operated mouse lungs. Biglycan knockdown inhibits EC DNA synthesis and angiogenic factor expression. **Conclusion:** Biglycan may be one of the therapeutic targets to stimulate lung vascular regeneration. **Funding:** This project is supported by NIH R21AG084736, R01HL139638, R01HL178475, and AHA 967800.

Abstract 036

HMGB1/Caspase-8/RIP3 Axis Mediates Mitochondrial Apoptosis in Septic Cardiac Microvascular Endothelial Injury

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Background: Sepsis-induced cardiac microcirculation disturbance significantly causes cardiac dysfunction and increased mortality. Cardiac microvascular endothelial cells (CMECs) closely interact with cardiomyocytes, and endothelial injury is a critical link in sepsis-induced cardiac injury. HMGB1 and caspase-3 are elevated in sepsis, potentially inducing mitochondrial dysfunction and subsequent apoptosis signaling, but the specific mechanism remains unclear in CMECs. **Materials and Methods:** A mouse sepsis model was established via intraperitoneal LPS injection. Endothelial-specific HMGB1 knockout mice were used for in vivo assessment. CMECs were isolated and cultured for in vitro experiments. HMGB1, RIP3, and caspase-8 were detected by Western blot and immunofluorescence. Microvessel density, caspase-3 activity, and cardiac function were evaluated by CD31 staining, activity assay, and echocardiography, respectively. Mitochondrial ROS and fragmentation were assessed via MitoSOX and live-cell labeling. Apoptosis was evaluated by TUNEL and cleaved caspase-3/PARP detection. **Results:** Sepsis significantly increased HMGB1 and RIP3 in CMECs, decreased microvessel density, and worsened cardiac function. At the gene level, HMGB1 modulated RIP3 splicing, reducing the splice variant RIP3 γ and relatively increasing full-length RIP3. At the protein level, HMGB1 down-regulated caspase-8, attenuating RIP3 post-translational cleavage and promoting its mitochondrial accumulation. This mitochondrial anchoring of RIP3 activated mitochondrial apoptosis, driving excessive fission, ROS production, and caspase-3-dependent apoptosis. Endothelial HMGB1 knockout alleviated these pathological changes. **Conclusions:** This study reveals a novel dual regulatory mechanism in septic cardiac microcirculatory dysfunction. At the gene level, HMGB1 modulates RIP3 splicing to reduce RIP3 γ . At the protein level, HMGB1 down-regulates caspase-8 to attenuate RIP3 cleavage. This dual regulation promotes RIP3 mitochondrial anchoring, driving excessive fission and ROS production, ultimately activating caspase-3-dependent apoptosis in CMECs and leading to cardiac dysfunction.

Abstract 037

ADAMTS6 Is Necessary for Heart Valve Stratification and Myocardium Development

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Background: Congenital heart defects (CHDs) are the leading cause of child morbidity and mortality. ADAMTS6 is a protease known to regulate the extracellular matrix (ECM) and is necessary for heart development as patient variants lead to a myriad of CHDs. Additionally, *Adamts6*-deficient embryos have CHDs, including myxomatous heart valves and cardiomyopathy, and die at birth. However, the role of *Adamts6* in distinct cardiac lineages remains unknown. **Methods:** *Adamts6* was conditionally inactivated in multiple cell lineages to isolate defects without the comorbidities witnessed in global *Adamts6* deletion. Since premature endocardial cushions become mature heart valves through epithelial to mesenchymal transition, *Adamts6* was conditionally inactivated in *Cdh5*Cre-positive endothelial cells to ascertain valve development. Additionally, *Adamts6* was conditionally inactivated in *Myh6*Cre-positive cardiomyocytes to ascertain its role in myocardium development. Histology and immunofluorescence staining were performed to evaluate the morphology and composition of valves and muscle. **Results:** *Adamts6*^{fl/fl}; *Cdh5*Cre mice are viable but showed significant pulmonary and aortic valve enlargement persisting into adulthood as compared to littermate controls. Histology revealed increased proteoglycan content and disorganized collagen (hallmarks of myxomatous valves). Immunofluorescence staining revealed an accumulation of fibrillin-1, a substrate of ADAMTS6. A decrease in collagen 1 and tropoelastin (components of ECM) immunofluorescence staining was also witnessed. Similarly, *Adamts6*^{fl/fl}; *Myh6*Cre mice are viable but showed a significant increase in trabecular myocardium and overall ventricular width. **Conclusion:** These findings establish that ADAMTS6 plays a unique role in distinct cell lineages of the hearts and is thus essential for ECM organization and long-term integrity of heart function. Future studies will uncover specific substrates and molecular mechanisms in heart valves and myocardium via proteomics.

Abstract 038

Disrupting Angiofibrotic Retinal Signaling Through Nanoparticle-Based NADPH Oxidase Inhibition

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Background: Angiofibrotic retinal diseases, including neovascular age-related macular degeneration (nAMD), remain a leading cause of vision loss worldwide. Despite first-line intravitreal anti-VEGF therapy, up to 56% of patients develop subretinal fibrosis and irreversible vision loss within five years, warranting research into alternative therapies. NADPH oxidases are major generators of reactive oxygen species (ROS) and can modulate angiogenic and fibrotic signaling pathways, including endothelial-to-mesenchymal transition (EndMT). This study evaluated the efficacy of a non-selective NADPH oxidase inhibitor; VAS2870. Due to its hydrophobicity, acetalated dextran (AcDex) nanoparticles (NPs) were synthesized to improve the bioavailability for intravitreal delivery. **Methods:** VAS2870 was loaded into AcDex NPs using a single emulsion method. Primary human retinal endothelial cells (HRECs) were treated with free VAS2870 (8 μ M), VAS2870-loaded AcDex NPs (equivalent concentration), and either pro-fibrotic TGF- β 2 (10 ng/ml) or pro-angiogenic VEGF (10 ng/ml). Angiogenic responses were assessed by tube formation over 16 hours (n=6), with cell viability confirmed with calcein AM. Expression of EndMT markers (α -SMA, vimentin) and the endothelial marker VE-cadherin was quantified by immunofluorescence following 72-hour treatment (n=3). Cell migration was assessed using a scratch wound assay over 24 hours (n=6). Mitochondrial ROS levels were measured using MitoSOX over 24 and 72 hours (n=3). **Results:** VAS2870 was successfully encapsulated into AcDex NPs with an average size of ~200 nm. Free VAS2870 and VAS2870-loaded AcDex NPs significantly reduced tube formation and cell migration compared with untreated, VEGF- and TGF- β 2-treated HRECs. TGF- β 2 induced a mesenchymal phenotype characterized by upregulated α -SMA and vimentin and downregulated VE-cadherin expression. VAS2870 attenuated pro-fibrotic marker expression and restored endothelial marker levels, consistent with

inhibition of TGF- β 2-mediated EndMT. Notably, VAS2870 was associated with an increase in mitochondrial ROS generation at 24 and 72 hours. **Conclusions:** Non-selective NADPH oxidase inhibition with free and NP-encapsulated VAS2870 demonstrates anti-angiogenic and anti-fibrotic activity in retinal endothelial cells, supporting further *in vivo* evaluation as a novel therapy for nAMD. The redox crosstalk between NADPH oxidases and mitochondrial ROS remains complex and requires further mechanistic investigation.

Abstract 039

Monomethyl Fumarate Inhibits Angiogenesis and Fibrosis in Neovascular Age-related Macular Degeneration Models

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Background: Neovascular age-related macular degeneration (nAMD) is a sight-threatening disease, characterized by abnormal blood vessel growth (choroidal neovascularization) and fibrosis. These pathological changes occur through a process involving endothelial cell migration and endothelial-mesenchymal transition (EndMT). We previously showed that dimethyl fumarate (DMFu), an FDA-approved drug for multiple sclerosis, has anti-inflammatory and anti-angiogenic activity in the retina. We explored whether monomethyl fumarate (MMF), the active metabolite of DMFu, could suppress angiogenesis and fibrosis in primary human retinal endothelial cells (HRECs) and mouse choroidal explants, offering a novel therapeutic approach for nAMD.

Methods: HRECs were treated with 100 μ M MMF or DMSO (vehicle control) in the presence of transforming growth factor- β 2 (TGF- β 2, 10 ng/mL; pro-fibrotic) or vascular endothelial growth factor (VEGF, 10 ng/mL; pro-angiogenic) to model nAMD pathology. Angiogenic and fibrotic responses were assessed using scratch wound migration (n=6), tube formation (n=4), and cytotoxicity assays (n=6). Expression of EndMT markers, α -smooth muscle actin (α -SMA) and vimentin, was evaluated by immunofluorescence (n=3). In an *ex vivo* mouse choroidal sprouting model (n=9), choroidal explants isolated from C57BL/6 mice (4-5 weeks old) were cultured, treated with VEGF and/or MMF. Sprouting area was quantified to assess anti-angiogenic capacity. **Results:** MMF was tested from concentrations 50 μ M to 200 μ M, at 100 μ M showed minimal cytotoxicity ($\leq 15\%$). MMF significantly reduced TGF- β 2-induced cell migration, blocking wound closure up to 14 hours (TGF- β 2 vs. TGF- β 2 + MMF: $p < 0.0001$). MMF also suppressed VEGF-induced tube formation ($p < 0.05$). Furthermore, MMF reduced TGF- β 2-induced upregulation of EndMT markers α -SMA and vimentin. The anti-angiogenic potential was further demonstrated using the mouse choroidal sprouting assay, VEGF increased sprouting response compared to the control. MMF significantly suppressed VEGF-induced sprouting compared to vehicle control (< 0.05). **Conclusion:** MMF demonstrates anti-angiogenic and anti-fibrotic efficacy positioning it as a promising therapeutic agent for nAMD. However, MMF undergoes rapid clearance and poor bioavailability. Future studies will investigate *in vivo* efficacy and explore nanoparticle-based drug delivery systems to enhance bioavailability and achieve sustained release.

Session 7: Tissue Stress, Fibrotic Remodeling, and Neural Injury

Abstract 040

Targeting De Novo Pyrimidine Synthesis via CAD Inhibition Suppresses Hepatic Stellate Cell Activation and Liver Fibrosis

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Background: Chronic liver injury leads to fibrosis and cirrhosis, a leading cause of death worldwide, and is driven by hepatic stellate cell (HSC) activation, characterized by proliferation and collagen deposition. HSC activation, when stimulated by platelet-derived growth factor (PDGF), has been shown to involve increased glycolysis, but the involvement of other metabolic pathways remains elusive. Pyrimidine metabolism from glutamine (de novo) or recycled nucleic bases (salvage) (Thymine, Cytosine, and Uracil) generates nitrogenous

bases required for DNA/RNA synthesis. How de novo drives HSC activation and subsequent liver fibrosis remains unknown, which is the focus of the study. **Methods:** We pharmacologically inhibited the Carbamoyl-phosphate synthetase 2, Aspartate transcarbamoylase, and Dihydroorotase complex (CAD) in primary human HSCs *in vitro* and patient-derived precision-cut liver slices *ex vivo*. **Results:** *In vitro*, PDGF increased HSC proliferation compared to vehicle as measured by uptake of 5-Ethynyl-2'-deoxyuridine, which was abrogated by sparfosic acid, a CAD inhibitor. *Ex vivo*, PDGF increased collagen deposition compared to vehicle in human PCLS, which was attenuated by sparfosic acid. Collagen deposition was assessed by total collagen staining via picrosirius red and collagen 1 expression via western blot. **Conclusion:** Collectively, these findings demonstrate that pyrimidine from de novo synthesis promotes HSC proliferation and fibrosis, presenting a novel therapeutic target.

Abstract 041

WNK1 Inhibition Reduces H3K9ac Expression in Hepatic Stellate Cells and Precision Cut Liver Slices

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Background: Liver fibrosis is a leading cause of mortality worldwide, with no known cures. Fibrosis arises when chronic liver injury activates hepatic stellate cells (HSCs), signaled by growth factors such as platelet derived growth factor (PDGF). HSC activation increases proliferation, migration, and collagen secretion. To meet their increased energy demands activated HSCs upregulate glycolysis via Lysine-Deficient Kinase 1 (WNK1) signaling. This increases histone 3 lysine 9 acetylation (H3K9ac), which drives pro-fibrogenic gene expression. Whether WNK1 and H3K9ac interact directly, and the mechanism of possible interactions, is unknown. Decreased H3K9ac in response to WNK1 inhibition with WNK-IN-11 would provide evidence of a functional relationship between the two. **Objective:** The aim of this study is to determine a possible link between H3K9ac and WNK1 in HSC activation and liver fibrosis, specifically whether WNK1 inhibition with WNK-IN-11 leads to decreased H3K9ac expression in PDGF-activated HSCs and PDGF-fibrosis induced Precision Cut Liver Slices (PCLS). **Methods:** HSCs were treated for 4 hours in 10 μ M WNK-IN-11 in serum starved media, and 2 hours in 20 ng/ml PDGF. PCLS were treated for 120 hours under the same concentrations. Cells or PCLS were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, and blocked with either 5% BSA or 10% FBS in PBS for 1 hour. Samples were incubated with primary antibodies overnight at 4° C, followed by fluorophore-conjugated secondary antibodies and DAPI for nuclear staining. Staining was performed for H3K9ac, pWNK1, and Desmin, an intermediate filament protein which marks HSCs in tissue stains. **Results:** Pharmacological inhibition of WNK1 attenuated expression of H3K9ac in response to PDGF treatment. Compared to the DMSO controls, WNK-IN-11 treatment resulted in a reduced fold change in the intensity of H3K9ac expression in isolated HSCs as well as in Desmin-positive cells in PCLS. **Conclusions:** We found inhibition of WNK-1 with WNK-IN-11 attenuated expression of H3K9ac in response to PDGF treatment in both *in vitro* and *ex vivo* models. How H3K9ac and WNK1 interact is still unclear and further research is needed.

Abstract 042

Long-term deficits to memory and motor coordination ability are sustained in a murine model of pediatric Type C HE after liver injury resolution.

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Introduction: Chronic liver disease (CLD) in the pediatric population is a growing problem worldwide with an estimated 3-5% of all CLD cases globally occurring in children under 18. Stemming from both lifestyle and genetic causes, CLD complications such as hepatic encephalopathy (HE) in children pose a greater risk to quality of life due to the vulnerable state of development the body, and most importantly the brain, is at. Case studies have identified a potential association between experiencing an HE event during neurodevelopment and long-term deficits to learning and memory, cognitive, and neuromuscular abilities. Little is understood of the unique pathology of HE in pediatric cases and the current study aimed to investigate these long-term neurologic changes in a novel murine model of Type C HE in juveniles. **Methods:** Mice were given 0.2 mL/kg CCl₄ via intraperitoneal

injection for 8 weeks beginning at PND14. Neuromuscular and motor coordination, learning and memory, and neuropsychiatric assessments were performed prior to tissue collection and biochemical assessments. A second cohort was allowed 8 weeks to recover from CCl₄ treatment, then followed the same behavioral assessment paradigm. Liver function was assessed via serum chemistries, Sirius Red and H&E staining, and blood ammonia levels. Neuroinflammation was assessed via immunohistochemistry for markers of glial cell activation. Morphological assessments to determine glial cell immune subtype were performed in ImageJ. **Results:** Neuromuscular ability and motor coordination in mice in recovery from CCl₄-induced Type C HE improved significantly but still showed a significant delay in ability compared to peers receiving only vehicle. This same trend was observed when assessing long-term memory ability, but interestingly no long-term changes in anxiety or depression-like behaviors were observed. **Conclusions:** Despite allowing the liver to make a full recovery from CLD, the consequences of experiencing HE during crucial points in neurodevelopment are long-lasting and potentially irreversible. Key pathways such as synaptogenesis, myelination, and synaptic pruning may be impacted during HE pathology and could be responsible for the long-term cognitive changes observed.

Abstract 043

From Breath to Call: Neuromodulatory Role of IL-6 in Neonatal Brainstem function. Ultrasonic Vocalization in Neonates

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Background: Neonatal sepsis is the leading cause of morbidity and mortality in newborns. IL-6 is emerging as a cytokine with neuroprotective or detrimental effects and has been shown to regulate neurodevelopmental processes. Our previous work demonstrates that IL-6 plays a key role modulating neonatal immune activation and brainstem neuroinflammation, which disrupts breathing. The brainstem circuits controlling autonomic breathing overlap with those that generate vocalizations, transforming respiratory rhythms into patterned calls. Ultrasonic vocalizations (USV) are a key communicative behavior in mice that provide sensitive readout for respiratory-vocalization circuit integrity. Our study sought to determine how IL-6 loss alters neonatal brainstem circuitry, using USVs as an integrative measure of respiratory-vocalization network function. **Methods:** *Il6^{tm1Kopf}/J* (IL6^{KO}) and IL6^{+/+} mice were utilized throughout postnatal day (PD) 5 to 7. At PD5 they were *i.p* administered with either saline or LPS. Following 3, 24, 48 and 72 hours USVs were recorded for 5 min in a soundproof chamber using a condenser microphone connected to Ultrasound Gate hardware. All analysis was performed using the VocalMat add on MATLAB and R Studio for the data analysis. **Results:** To evaluate the neuromodulatory effects of IL6 in neonatal brainstem circuits, we first determined whether IL-6 ablation alters USVs. Baseline recordings revealed that IL6^{KO} pups exhibited a significant reduction in call number and mean bandwidth compared to IL6^{+/+} at PD7 and PD8. To further characterize these differences, we mapped syllables features using a diffusion map, which revealed genotype-dependent shifts in syllables centroid distances, highlighting the impact of IL6 loss on vocalization structure. Neonatal immune activation by LPS resulted in a significant reduction calls in acute phase (3h) in both IL6^{+/+} and IL6^{KO}. However, during the early-adaptive phase (24 h), this effect was reversed exclusively in IL-6^{+/+} mice, which displayed an increased number of calls that persisted throughout the adaptive response at 72 h. Consistent with these findings, syllables centroid distances demonstrated that LPS induced marked changes in call patterns in IL-6^{+/+} pups, whereas IL-6 ablation prevented these adaptive shifts at 24 h. At 48 h post-LPS, the vocalization repertoire in IL-6^{+/+} mice became more similar between LPS-treated and control groups, suggesting convergence of call patterns during adaptation. In contrast, IL-6^{KO} pups that received LPS maintained a distinct syllable repertoire throughout the adaptive phase. Analysis of established calls categories showed positive association with flat and short calls in IL6^{KO} regardless of the immune activation with LPS. **Conclusions:** Our findings highlight IL-6 as a key neuromodulator of neonatal breathing-vocalization circuits, shaping adaptive vocal behavior in response to immune activation. **Acknowledgements:** This work was supported by: NIH/NHLBI R01HL132355 for JJO and FIU Start-up for JO and MA.

Abstract 044

Micro- and Nanoplastics Exposure Exacerbates Alzheimer's Disease Pathology in APP/PSEN1 Mice

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Background: Alzheimer's disease and related dementias (AD/ADRD) are among the most prevalent cognitive disorders, affecting more than 6 million Americans over the age of 65. While the exact cause remains unknown, risk factors include genetics, lifestyle, and environmental toxins. In recent years, micro- and nanoplastics (MNPs) (1 μm – 5 mm and $\leq 1 \mu\text{m}$ in size, respectively) have emerged as pervasive environmental pollutants. Studies have shown that MNPs can accumulate in peripheral tissues throughout the body of both humans and animals, raising concerns about their potential health impacts, as evidence suggests they may induce inflammation, oxidative stress, and disruption of metabolic and endocrine functions. Furthermore, MNPs have been demonstrated to cross the blood-brain barrier and accumulate throughout the brain, resulting in alterations in cognitive behavior and immune markers consistent with neurological disease. In line with this, we have previously demonstrated that exposure to MNPs impairs cognitive function and increases inflammation in both young and aged mice, as well as in mice carrying the APOE4 allele, the strongest genetic risk factor for sporadic Alzheimer's disease, in a sex-dependent manner. **Methods and Results:** In the present study, we investigated the effects of chronic MNPs exposure in a transgenic mouse model of Alzheimer's disease pathology (APP/PSEN1). Following chronic 3-month exposure to pristine polystyrene MNPs, APP/PSEN1 mice exhibited a significant increase in amyloid- β plaque burden. This was accompanied by exacerbated cognitive impairment, reduced synaptic integrity, impaired lysosomal function, and increased microglial abundance. **Conclusions:** These results collectively suggest that MNPs exposure may accelerate key neuropathological processes associated with Alzheimer's disease progression.

Abstract 045

Unravelling the Effects of Blue Light Exposure on Human Choroidal Melanocytes

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Background: Chronic exposure to environmental blue light, that can reach the retina, retinal pigment epithelium and choroid, may play key roles in both the physiological development and photooxidative decline of ocular tissue. A similar mechanism has been identified in skin melanocytes where blue light can impact melanogenesis via light-sensitive pathways involving Opsin 3 (OPN3; a non-visual opsin). This study explores the possibility that human choroidal melanocytes (HCMs) may exhibit blue-light sensitivity through the expression of non-visual opsins. **Methods:** Human donor eyes provided by the Lions NSW Eye Bank, with human research ethics approval and consent, and without obvious ocular pathology were dissected and imaged. Primary HCMs were isolated from donor eyes (n=4; 63-77 years), grown to 80% confluence, with passage (P) 2 to P7 cells used for experiments. HCMs seeded at 50,000 cells/well were exposed to 1.3mW/cm² 460nm blue light for 24 hours; HCMs not exposed to blue light served as controls. Following this, RNA was extracted and purified, and bulk RNA sequencing performed (Novogene, Singapore) for blue-light and control samples. Differentially expressed genes (Log2FC > |1|, FDR < 0.05) were identified and gene set enrichment analysis of hallmark gene sets, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG), was performed to identify enriched signaling pathways. HCMs were also immunolabelled for melanogenesis and oxidative stress-related proteins including TYR, TRYP1, DCT, MC1R, OPN3 and HMOX1, visualized using fluorescence and confocal microscopy. **Results:** Bulk RNA sequencing of HCMs with and without blue light identified 84 differentially expressed genes (34 upregulated, 50 downregulated). Highly upregulated genes included *HMOX1*, *NQO1* and *SLC7A11*. Highly downregulated genes included *DCT*, *CXCL13*, and *ADAMTS15*. Key positively enriched pathways included 'glutathione metabolism' and 'oxidative stress'; negatively enriched pathways included 'melanogenesis' and 'ferroptosis'. Immunolabelling of HCMs confirmed the expression of melanocyte markers including TYR, TYRP1 and DCT, and OPN3. **Conclusion:** HCMs responded to low energy blue light exposure via regulation of oxidative

stress, pigmentation, and ferroptosis gene transcription. HCMs expressed OPN3, a blue-light-sensitive opsin previously identified in skin melanocytes, suggesting a potential blue-light response mechanism in the choroid.

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Session 8: Cancer Genomics, Metabolic Adaptation, and Systemic Effects

Abstract 046

Regulation of Lipogenic Programs in the Meibomian Gland by MYC and Sterol Regulatory Element-Binding Protein 1 (SREBP1)

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Background: Meibomian glands (MGs) are sebaceous glands of the eyelid which form the lipid layer of the tear film. Sebaceous carcinoma (SebCA) may arise following unchecked meibocyte proliferation. SebCA is a rare but aggressive cancer in which gain-of-function mutations and copy number variations at the *MYC* locus have been demonstrated. *MYC* upregulation activates SREBP1 and downstream metabolic pathways to promote tumor growth and invasion. We aimed to characterize the effects of *MYC* modulation on SREBP1 and lipogenesis and to define the impact of local fatty acid synthase (FASN) inhibition on *MYC*-mediated meibum alterations.

Methods: Human meibocytes (hMGECs) and a primary human SebCA cell line were treated with *MYC* and fatty acid desaturase inhibitors with and without oleic acid (OA) or vehicle control (DMSO). Proliferation, apoptosis, differentiation, invasion, and SREBP1 binding activity were assessed. Adult wildtype (wt) and tamoxifen (4-OHT)-inducible *MYC*-overexpressing transgenic (TG) C57B6 mice were administered 4-OHT followed by topical application of a FASN inhibitor (C75) alone, or with OA, or DMSO. Tear Film Break-Up Times (TBUTs) were obtained before and after 4-OHT administration and following topical intervention. Eyelids were harvested and subject to assessments of morphology, differentiation, proliferation, apoptosis, and lipogenic transcript expression. **Results:** *MYC* inhibition suppressed SREBP1 expression and binding activity and impaired proliferation, clonogenicity, and invasiveness while promoting lipogenic stress and apoptosis in both cell lines. The addition of OA partially rescued this phenotype. FASN inhibition *in vivo* accelerated TBUTs, increased apoptosis, and attenuated *MYC* expression, proliferation, and lipid droplet formation in all mice. Lipogenic transcripts were upregulated in TG mice relative to wt littermates, and FASN inhibition downregulated these genes relative to DMSO controls. This phenotype was partially rescued by concurrent OA administration.

Conclusions: *MYC* inhibition impairs SREBP1 activity and downstream lipogenic programs, leading to decreased proliferation and increased apoptosis in meibocytes. Topical FASN inhibition phenocopies key aspects of *MYC* inhibition, including tear film instability and altered lipid droplet formation. OA achieves partial lipotoxic rescue. SebCA's reliance on *MYC*-driven lipogenic programming unveils a metabolic vulnerability and represents a potential therapeutic target.

Abstract 047

Genomic Complexity and Survival Differences Between Ewing Sarcoma and Desmoplastic Small Round Cell Tumor

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Background: Ewing sarcoma and desmoplastic small round cell tumor (DSRCT) are aggressive round cell sarcomas driven by distinct oncogenic fusions, yet differences in their broader genomic architecture and clinical behavior remain incompletely characterized. We investigated whether DSRCT and Ewing sarcoma differ in chromosomal genomic complexity and whether genomic alteration burden is associated with survival. **Methods:** We performed a secondary analysis of the MSK 2023 cBioPortal cohort comprising 290 tumor samples from 277 patients. To avoid non-independence from repeated sampling, one tumor sample per patient was retained. One discordant Ewing sarcoma of soft tissue case was excluded, yielding 276 patients: 172 with Ewing sarcoma and 104 with DSRCT. Fraction genome altered (FGA) was compared between diagnoses using the Mann–Whitney U test. Overall survival (OS) and progression-free survival (PFS) were evaluated using Kaplan–Meier analysis and log-rank testing. Cox proportional-hazards models assessed FGA as a continuous predictor of survival. **Results:** DSRCT showed significantly greater chromosomal alteration burden than Ewing sarcoma, with median FGA of 0.129 versus 0.066, respectively ($p < 0.001$). Survival was markedly inferior in DSRCT: 60-month OS was 16.1% versus 54.4% in Ewing sarcoma, and 60-month PFS was 12.8% versus 36.3% (both log-rank $p < 0.001$). Across the cohort, each 0.10 increase in FGA was associated with worse OS (HR 1.28, 95% CI 1.16–1.42; $p < 0.001$) and PFS (HR 1.19, 95% CI 1.09–1.30; $p < 0.001$). **Conclusions:** DSRCT demonstrates substantially greater chromosomal genomic complexity and poorer survival than Ewing sarcoma. Higher FGA was independently associated with adverse outcomes, supporting chromosomal alteration burden as a potentially informative marker of aggressive sarcoma biology.

Abstract 048

Fibroblast Subtype Composition at Diagnosis Improves Progression Risk Estimates in Non-Muscle-Invasive Bladder Cancer

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Background: About 75% of bladder cancers are non-muscle-invasive at diagnosis, and the central clinical problem is predicting which will progress. Guideline risk groups that guide the choice between intravesical BCG and cystectomy are built from tumor features alone. Cancer-associated fibroblasts (CAFs) are heterogeneous, but whether CAF subtype composition adds prognostic information in non-muscle-invasive disease has not been tested against a progression endpoint. **Methods:** We integrated five published single-cell RNA sequencing datasets into an atlas of 315,628 cells from 34 treatment-naïve tumor samples (26 patients) and resolved 5,950 fibroblasts into four subtypes, separating fibroblasts from smooth muscle cells and pericytes. We characterized each subtype by matrix gene expression and compared subtype shares between tumor and normal bladder by spatial transcriptomics (four tumor sections, one normal section). We estimated subtype fractions in bulk RNA sequencing from an independent cohort of 530 treatment-naïve patients (UROMOL) by Bayesian deconvolution. We applied log-ratio transforms for Cox modeling and measured added value for progression-free survival as the change in Harrell's C-index, tested by likelihood ratio. **Results:** Two subtypes had opposite associations with progression. The dominant collagen and extracellular matrix producer was associated with higher risk. This subtype expressed ACTA2 (alpha-SMA) in 67.9% of cells, yet ACTA2 separated smooth muscle cells more strongly than it separated this subtype (detection difference 0.700 against 0.467). The same subtype made up 2.7% of fibroblasts in normal bladder against 24.7 to 67.6% across tumor sections. A second subtype was associated with lower risk. Adding this protective subtype to a clinical model of age, sex, smoking and clinical risk score raised the C-index from 0.772 to 0.796 (likelihood ratio $p = 4.4 \times 10^{-4}$, 1 degree of freedom). Adding total stromal cell abundance instead raised it to 0.774 ($p = 0.151$). **Conclusions:** Fibroblast subtype composition adds prognostic information to clinical risk factors in non-muscle-invasive bladder cancer, and in this model it outperforms total stromal abundance. The adverse subtype is enriched in tumor relative to normal bladder, and alpha-SMA alone does not distinguish it from smooth muscle. Subtype fractions can be estimated from bulk sequencing of diagnostic tissue, without an additional biopsy.

Abstract 049

Endometrial Cancer Exploits Myosin 5B to Support Nutrient Uptake and Metabolism

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Background: Uterine corpus endometrial carcinoma (UCEC) is the most common gynecologic malignancy in the United States, with approximately 68,000 new cases and 14,000 deaths annually. Although most UCEC cases are diagnosed early, advanced tumors have high rates of recurrence and limited treatment options. Cancer cells reprogram their metabolism and increase nutrient acquisition to support continued growth. Myosin 5b (MYO5B) is an actin-based molecular motor that transports cargo, including nutrient transporters, to the apical membrane of epithelial cells. Our group has previously demonstrated that MYO5B is critical for trafficking nutrient transporters in the intestine, but its role in other tissues and tumors, specifically in UCEC, is unknown. We hypothesized that UCEC cells exploit MYO5B-dependent trafficking to increase glucose transporter localization, nutrient uptake, and tumor metabolism. **Methods & Results:** Analysis of UCEC tumor biopsies and control tissue revealed that MYO5B was significantly upregulated at both the mRNA and protein levels across all stages of UCEC. Immunostaining confirmed increased apical MYO5B localization in UCEC tumors compared with adjacent normal tissue. Unfortunately, elevated MYO5B expression was associated with reduced patient survival. Increased MYO5B expression correlated with elevated levels of the glucose transporters GLUT1 and GLUT3. Metabolic pathway analysis identified significant enrichment of metabolic pathways in UCEC, and expression of key metabolic genes positively correlated with MYO5B. To identify the specific role MYO5B plays in UCEC, we generated MYO5B-deficient HEC1A uterine cancer cells. We found that the MYO5B-deficient cancer cells had reduced metabolism and lowered glucose uptake compared to parental cells. RNA-sequencing further revealed large-scale shifts in gene expression in MYO5B-deficient cells. MYO5B-deficient cells had reduced expression of amino acid transporters and genes involved in lipid synthesis and fatty-acid metabolism. We also observed decreased levels of several mitochondrial genes, and by Resipher, we found that MYO5B-deficient cells had decreased OCR. Finally, we found MYO5B-deficient cells had reduced proliferation and migration compared to the parental HEC-1A cell line. **Conclusions:** These findings suggest that MYO5B regulates nutrient transporter trafficking and metabolism in UCEC. As a result, targeting MYO5B could be a promising therapeutic avenue for UCEC.

Abstract 050

Long-Term Effects of Chemotherapy in Mouse Survivorship Model

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Background: Survivors of adolescent and young adult (AYA; age 15-39 years at diagnosis) cancers are a growing population with the potential to live for many decades after treatment completion. However, they often face long-term treatment-related toxicities that can affect the immune system and reproductive and cognitive health for decades after therapy. Although cisplatin, cyclophosphamide, and doxorubicin remain the first line of cancer treatments, the cellular mechanisms driving these late survivorship effects are not well defined. The goal of this study is to characterize chemotherapy-induced late effects across hematopoietic/immune, reproductive, and nervous-system tissues through longitudinal phenotyping and multi-organ single-cell transcriptomic profiling in a mouse survivorship model. **Methods:** Male C57BL/6J wild-type mice (2 months old) were treated with two weekly doses of PBS, cisplatin (4 mg/kg), cyclophosphamide (50 mg/kg), or doxorubicin (2 mg/kg) and monitored periodically after chemotherapy administration. Peripheral blood was analyzed by flow cytometry at baseline and at serial post-treatment time points. Behavioral assessments were conducted during late survivorship. **Results:** Post-chemotherapy analyses showed durable remodeling of peripheral T-cell populations, particularly in doxorubicin-treated mice. Compared with controls, chemotherapy-treated mice had reduced CD44⁺CD62L⁺

naïve T cells and increased CD44⁺CD62L⁺ central-memory-like T cells, indicating persistent adaptive immune reprogramming beyond acute cytotoxic depletion and without acute pathology. This trend persisted after several months post treatments, suggesting that, after chemotherapy, persistent T-cell dysregulation may promote chronic inflammation, and potentially contribute to reproductive dysfunction. **Conclusion:** This work provides new insights on chemotherapy-related late effects and might shed new light on potential strategies to restore immune, reproductive, and cognitive health in cancer survivors. This preliminary work could help with finding treatments and interventions aimed at improving long-term quality of life for AYA cancer survivors.

Abstract 051

Modulating Epoxy-Eicosanoids to Attenuate Glioblastoma-Induced Sarcopenia

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Background: Glioblastoma (GBM) is the most aggressive primary brain tumor in adults resulting in poor clinical outcomes. GBM can drive systemic complications such as cachexia which is characterized by progressive skeletal muscle wasting (sarcopenia). Sarcopenia predicts poor prognosis in GBM patients. Epoxyeicosatrienoic acids (EETs) are bioactive lipid mediators with anti-inflammatory, vasoprotective, and neuroprotective functions. Soluble epoxide hydrolase (sEH) degrades EETs into less active metabolites, diminishing their protective effects. We hypothesized that GBM-derived factors induce cachectic changes in skeletal muscle cells through disruption of sEH/EET signaling and that restoration of EET activity either by supplementing 14,15-EET or by treatment by sEH inhibition via EC5026 would protect against GBM-induced muscle wasting. **Methods:** C2C12 myoblasts were differentiated into myotubes and exposed to CT2A GBM cells to establish an in vitro cachexia model. Oil Red O staining quantified intramyocellular lipid droplet accumulation as an indicator of metabolic dysfunction and myosteatosis. **Results:** Exposure to CT2A-conditioned media reduced myotube size and density, consistent with a GBM-induced cachectic phenotype. In contrast, treatment with 14,15-EET or EC5026 partially preserved myotube thickness and abundance, suggesting protection against GBM-induced muscle degeneration. Consistent with these morphological findings, our results demonstrated increased expression of the pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α , elevated levels of the atrophy markers MuRF1 and MAFbx, and increased *Ephx2* (sEH gene) expression in myotubes exposed to CT2A-conditioned media. Conversely, treatment with 14,15-EET or EC5026 suppressed inflammatory signaling, reduced expression of atrophy-associated genes and proteins, preserved MyHC, MyoD, and Pax7 expression, and decreased lipid accumulation within myotubes. Together, these molecular and phenotypic changes demonstrate a protective role for EET/sEH inhibition in mitigating GBM-induced cachectic remodeling of skeletal muscle. **Significance:** EETs supplementation and/or sEH inhibition may preserve musculoskeletal health, thereby improving the quality of life and survival of glioblastoma patients. **Conclusions:** Enhancement of EET signaling via EETs and/or sEH inhibition protect from GBM-induced muscle wasting, identifying the sEH/EET axis as a potential therapeutic target to prevent GBM-induced cachexia and sarcopenia.

Session 9: Breast Cancer Inflammation, Stroma, and Therapeutic Resistance

Abstract 052

Immune Infiltration in Breast Cancers With Germline Alterations in DNA Repair Genes: Effects of Age and Ancestry.

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Background: Approximately 10% of breast cancers (BCs) harbor germline alterations in homologous recombination deficiency/DNA damage response (gHRD/DDR) genes. *BRCA1/2* alterations have been associated with increased immune infiltration, but data on other HRD/DDR genes are limited. Here, we sought to define associations between gHRD/DDR genotype and tumor infiltrating lymphocytes (TILs) extent and determine whether age and ancestry modify these associations. **Methods:** We analyzed 204 BCs harboring germline pathogenic/likely pathogenic variants (gP/LPV) across 20 HRD/DDR genes with paired tumor/normal targeted sequencing and genetic ancestry. TILs were assessed by the International TILs Working Group criteria. Allelic status was assessed using FACETS. Mutational signatures were inferred with SigMA. **Results:** The HRD/DDR genes most frequently affected by gP/LPV were *BRCA2* (n=50), *BRCA1* (n=37), *CHEK2* (n=32), *ATM* (n=20), and *FANCA* (n=15). Genetic ancestry was predominantly European (n=105) and Ashkenazi Jewish (ASJ; n=33), followed by African and Asian (n=17, each). ASJ patients were enriched for g*BRCA1/2* variants ($P=0.01$). g*BRCA1* patients were younger at diagnosis than other gHRD/DDR carriers ($P<0.01$), and their BCs showed higher TILs, followed by g*BRCA2*, g*ATM*, and g*CHEK2*. Most g*BRCA1* (76%) and g*BRCA2* (72%) BCs had a dominant HRD signature (SBS3), whereas clock/aging was predominant in g*ATM* (62%) and g*CHEK2* (60%) BCs. While TILs declined with age overall, gene-specific analyses showed that this association was restricted to g*ATM* ($\rho=-0.59$; $P=0.006$) and g*CHEK2* ($\rho=-0.54$; $P=0.001$) BCs, and was absent in g*BRCA1*, g*BRCA2* or g*FANCA* BCs. The age-related TIL decline was confined to non-SBS3 BCs ($\rho=-0.57$; $P=0.001$), even within g*BRCA1/2* BCs ($P=0.034$). TILs were higher in ASJ and African BCs vs European BCs (both, $P<0.01$), which remained significant after adjusting for gHRD/DDR gene, age, and ER/HER2 status ($P=0.006$). **Conclusions:** Immune infiltration in gHRD/DDR BCs varies by genotype, ancestry and age. Age-related TIL decline was restricted to *ATM*- and *CHEK2*-associated BCs, and more broadly to tumors lacking an HRD signature, even within g*BRCA1/2* cases. ASJ ancestry was independently associated with higher TILs beyond other relevant covariates. These findings suggest that inherited DNA repair defects and their downstream mutational processes, together with age and ancestry, shape the tumor microenvironment and may inform therapeutic stratification.

Abstract 053

Induction of Tertiary Lymphoid Structures by Immune Checkpoint Therapy in a Murine Model of Triple-Negative Breast Cancer

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Background: Though immune checkpoint therapy (ICT) is approved for the treatment of triple-negative breast cancer (TNBC), response rates are low and non-durable. There is thus an urgent need for improved therapeutic biomarkers and deeper understanding of TNBC antitumor immune responses to tailor improved immunotherapies. Tertiary lymphoid structures (TLSs) are intra-tumoral lymphoid tissues composed of B cells and T cells that are associated with improved survival and response to ICT. Despite these associations, it is unclear what impact ICT has on these structures and whether they mediate ICT efficacy. We sought to examine if ICT may promote formation of TLSs in TNBC. **Methods:** BALB/c mice were orthotopically implanted with T11-APOBEC murine TNBC tumor cells to generate TNBC tumors. This model genocopies clinical TNBC and is dependent upon B cells and CD4⁺ T cells for response to ICT. Mice were then treated with ICT (anti-PD-1 and anti-CTLA-4 antibodies) twice weekly. TLSs were identified by immunofluorescent staining for B and T cells on tumor sections. Spatial transcriptomics was used to further characterize gene expression profiles of TLSs in murine and human TNBC tumors. **Results:** We show that ICT significantly upregulated TLS formation in the periphery of T11-APOBEC tumors, whereas tumors treated with vehicle control show little to no TLS formation ($p = 0.0286$). Using spatial transcriptomics, we show that these TLSs are identified as a spatially and transcriptionally distinct niche in an unbiased manner. TLSs in this model are significantly enriched for four widely used transcriptional signatures of human cancer-associated TLSs, and they also significantly upregulate pathways associated with B cell activation, humoral immunity, and germinal center activity. We then further show that a signature derived from these ICT-induced TLSs are focally enriched in a publicly available cohort of human TNBC samples, suggesting the formation of similar structures in TNBC patients. **Conclusions:** These data

suggest that ICT promotes the formation of TLS in TNBC, which may mediate enhanced anti-tumor adaptive immune responses. This model also provides a platform to further investigate mechanisms of formation and function of these structures in the context of ICT, which will inform predictive biomarkers and improved immunotherapy approaches for this aggressive disease.

Abstract 054

Development of Breast-adipose Co-culture Model to Decipher the Impact of Lipid Heterogeneity on Triple-negative Breast Cancer

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Background: Lipid composition has been associated with increased chemoresistance and mortality in triple-negative breast cancer (TNBC), a highly aggressive breast cancer subtype accounting for 10-15% of all breast cancer cases. Fatty acids in the tumor microenvironment (TME) promote TNBC progression by driving metabolic inflammation. Despite this, the unique impact of TNBC-associated lipids as a subsequent energy source is not fully understood. Deciphering the lipid heterogeneity in the TME will provide insight into TNBC progression to ultimately unveil novel therapeutic targets. Prior works have identified a heterogeneous distribution of lipids between primary TNBC tumors and matched breast tissue. Specifically, the lipid species linoleic acid (LA) and oleic acid (OA) were upregulated in TNBC tumors but not elevated in hormone positive breast tumors. **Methods and Results:** We hypothesized that the distinct TNBC lipid composition enriched in LA and OA fuels TNBC progression. We developed a model of the TNBC TME that is enriched for LA and OA through the co-culture of adipogenic differentiated breast adipose-derived stem cells (BrASC) and TNBC spheroids to determine the impact of the TME lipid composition on TNBC. We constructed an adipocyte-TNBC co-culture model by treating BrASC with fatty acids distinct to TNBC during differentiation to adipogenic lineage. Fatty acid production and uptake were confirmed with quantitative BODIPY fluorescence microscopy and qRT-PCR. We also showed that fatty acids increased the total triglyceride profile in differentiated BrASC. Through quantitative fluorescence microscopy, we determined that up to 200 μ M of lipid can be added to the system while retaining cell viability. Further, BrASC incorporated exogenous lipids during adipogenic differentiation, and RNA sequencing data revealed differential gene expression in LA and OA when compared to controls, including upregulation of carbohydrate and lipid metabolism pathways. Further, fluorescence microscopy demonstrated lipid transfer occurred during the co-culture of BrASC with TNBC. **Conclusions:** Using this co-culture model, future studies are poised to evaluate the impact of an altered TME lipid profile on TNBC progression.

Abstract 055

Eicosanoid Regulation of Cachexia in Triple-negative Breast Cancer Mice

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Background: Breast cancer is the most common cancer diagnosed among women. Triple-negative breast cancer (TNBC) is an aggressive malignancy associated with cachexia in up to 60% of patients. Chronic inflammation, metabolic dysregulation, and muscle loss (sarcopenia) are hallmarks of cachexia, yet no treatments exist. Breast cancer sarcopenia is associated with poor overall survival and impaired quality of life. Pro-inflammatory cytokines initiate and accelerate cachexia. Epoxyeicosatrienoic acids (EETs) derived from arachidonic acid are anti-inflammatory lipid mediators that activate anti-inflammatory cytokine programs. However, EETs are rapidly metabolized by soluble epoxide hydrolase (sEH) into their corresponding diols, which

are less biologically active. EC5026 is a potent sEH inhibitor with no reported drug-related adverse events in clinical trials. We investigated whether pharmacologic sEH inhibition with EC5026 could mitigate functional and metabolic impairments in a 4T1 TNBC-induced cachexia model. **Methods:** We utilized a metastatic, orthotopic model of TNBC leading to skeletal muscle degeneration. Functional performance was assessed using forelimb grip strength and metabolic treadmill. **Results:** Control 4T1 mice developed progressive exercise intolerance as demonstrated by reduced time to fatigue, run distance, and running speed compared to non-tumor bearing (NTB) mice. In contrast, EC5026 treatment significantly preserved exercise performance by increasing time to fatigue, average speed, and peak speed. Forelimb grip strength and altered RER patterns were maintained in EC5026-treated mice. EC5026 significantly reduced tumor burden relative to control 4T1 mice via modulation of cachexia- and inflammation-associated molecular pathways, including MAFbx, MuRF1, IL-6, and TNF- α . Thus, EC5026 restores muscle mass and function in a murine TNBC model. **Significance:** sEH inhibition may be a novel host-directed therapeutic approach to preserve musculoskeletal health, thereby improving the quality of life and survival of breast cancer patients. **Conclusion:** These findings demonstrate that sEH inhibition with EC5026 attenuates functional/metabolic decline in TNBC-bearing mice. Since sEH inhibitors (sEHi) have potential for treating multiple inflammatory diseases, our studies provide a basis for clinical translation of sEHi to prevent and/or reverse cachexia in breast cancer patients.

Abstract 056

Defining a Calcification-Associated Stromal Niche for Biomarker-Guided Bone Metastasis Prevention in Hormone Receptor-Positive Breast Cancer

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Background: Bone is the most frequent site of distant recurrence in receptor-positive (ER+) breast cancer and remains a leading cause of patient morbidity and mortality. While adjuvant bisphosphonates reduce bone recurrence and improve survival in selected postmenopausal patients, understanding the biomarkers and drivers associated with breast tumor bone tropism is critical to optimizing bisphosphonate clinical use. Hydroxyapatite (HA)-containing breast microcalcifications are associated with significantly increased odds ratio of bone recurrence, suggesting that they may represent biologically active stromal niches associated with bone tropism rather than passive radiographic findings. **Methods:** Thus, to model the calcified breast stromal niche, we utilized immunophenotypically validated donor-derived breast adipose stromal/stem cells (brASCs) induced *in vitro* to undergo osteogenic differentiation. **Results:** We observed donor-dependent heterogeneity in brASC capacity to generate HA-rich microcalcifications by alizarin red staining. HA producing donors (HA+) and HA non-producing (HA-) donors clustered into distinct transcriptomic profiles upon RNA sequencing. Specifically, HA+ brASCs exhibited increased gene expression of C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 12 (CXCL12), chemokines implicated in osteolysis, breast cancer stemness, and metastatic dissemination. Compared to HA- conditioned media, HA+ conditioned media applied to ER+ cell line MCF7 significantly upregulated proliferation and gene expression of CXCL12, Trefoil Factor 1 (TFF1), and progesterone receptor (PGR), mediators of estrogen receptor-mediated signaling and biomarkers associated with bone tropism. Finally, we found that continuous treatment with the bisphosphonate zoledronic acid during brASC osteogenic differentiation prevented HA deposition, demonstrating the potential for bisphosphonates to directly interfere with stromal calcification in the breast. **Conclusions:** Future studies will define calcification associated stromal biomarkers in archived ER+ breast tumors, determine whether calcified stromal niches promote aggressive phenotypes and bone tropism, and test whether bisphosphonates disrupt this crosstalk. This work utilizes donor-derived brASCs as an innovative platform to model the calcified breast niche and establishes a biological framework for developing stromal biomarkers to guide precision use of adjuvant bisphosphonate therapy in ER+ breast cancer.

Abstract 057

Role of Liver Kinase B1 and Its Downstream Kinases as Regulators of Estrogen Receptor Signaling in Estrogen Receptor-positive Breast Cancer

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Background: Estrogen receptor positive (ER+) breast cancer accounts for ~70% of breast cancers, relying on estrogen-ER binding for growth and progression. Although endocrine therapy is the mainstay treatment, de novo or acquired resistance in ~40% of patients, concomitant with loss of estrogen (E2) sensitivity, remains a challenge. ER interacts with kinase signaling pathways regulating cell survival, growth, and endocrine resistance. While pathways such as AKT-mTOR and MAPK are well studied, others remain underexplored, and therapy resistance remains an issue. Liver Kinase B1 (LKB1), a highly conserved tumor suppressor, functions as a co-activator of ER-dependent transcription; however, it doesn't directly phosphorylate ER. Paradoxically, LKB1 deletion in ER+ breast cancer cells promotes resistance to endocrine therapies, suggesting that the ER-LKB1 interaction may be mediated through downstream kinase signaling pathways. LKB1 phosphorylates 14 downstream adenosine monophosphate-related kinases (ARKs), regulating cellular transcription, metabolism, and growth. Although evidence suggests crosstalk between LKB1-ARKs and ER signaling pathways, a complete understanding of the LKB1-ARKs axis and ER regulation is lacking. It is hypothesized that *"LKB1 regulates ER-dependent transcription through downstream ARKs, influencing endocrine response. Further, identification of ARKs regulating ER signaling will provide novel candidate biomarkers and therapeutic targets in ER+ breast cancer"*. **Methods and Results:** To investigate this hypothesis, CRISPR-mediated LKB1 knockdown (KD) in ER+ MCF7 cells, followed by RNA sequencing, revealed broad transcriptional reprogramming, including altered ARKs expression, significantly reduced ER expression, and downregulation of hallmark estrogen-response pathways. To define the E2 response, LKB1-KD cells were evaluated under estrogen-deprived conditions with or without E2. Compared to the control, LKB1-KD cells showed significantly reduced ER and ER-dependent gene expression and substantial ARK rewiring, including increased NUA2 and MARK1 expression under both conditions. To determine a functional role of ARKs, ER+ cells were treated with ARK inhibitors and assessed for cell density. Results demonstrated that in estrogen-deprived media, SIK inhibition reduced cell density in MCF7 cells, an effect attenuated by E2. **Conclusion:** Together, these findings implicate a potential crosstalk between the LKB1-ARK axis and ER signaling through AMPK-related kinases.

Abstract 058

Structure-Guided Docking of WEA001, a Goldilocks ER α Antagonist for Endocrine-Resistant Breast Cancer

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Background: Endocrine resistance in estrogen receptor alpha (ER α)-positive breast cancer is associated with persistent receptor signaling and ligand-binding-domain mutations, including L536S and Y537S, for which no current antagonist reliably restores receptor inactivation. WEA001 was independently designed as a Goldilocks ER α antagonist candidate that combines an estradiol-like core with a structured, helix 12 (H12)-directed tail. We hypothesized that WEA001 would preferentially generate favorable docking poses in wild-type and resistance-associated ER α conformations while failing to yield a comparably stable pose in the agonist-state estradiol-bound model. **Methods:** Estradiol-bound 1A52, 4-hydroxytamoxifen-bound 3ERT, fulvestrant-bound L536S 6SBO, and Y537S 6CHZ ER α structures were prepared in Schrödinger Maestro. Comparative analysis evaluated the Glu353, His524, and Arg394 region, H12 positioning, and pocket-water dependence. WEA001 was constructed, prepared with LigPrep, and docked into prepared receptor grids using Glide Extra Precision (XP). Top-ranked poses were evaluated by GlideScore and residue-level interactions across receptor states. **Results:** Estradiol 1A52 provided the agonist benchmark, with short Glu353 and His524 contacts and a closed H12 state. Tamoxifen 3ERT showed a weakened, water-sensitive anchor network and a partially shifted H12 conformation,

whereas fulvestrant-bound L536S 6SBO showed tail-driven H12 disruption. WEA001 generated favorable XP poses in L536S 6SBO (best GlideScore -7.076 kcal/mol), 3ERT (-5.341 kcal/mol), and Y537S 6CHZ (-5.092 kcal/mol). In L536S 6SBO, the WEA001 tail packed near the H12-mouth region, including Cys530, Val533, Val534, and the L536S site. No comparably stable WEA001 pose emerged in agonist-state 1A52 under the tested docking conditions. **Conclusions:** These findings support the antagonist-design hypothesis and computationally prioritize WEA001 for evaluation in wild-type and resistance-associated ER α models, particularly L536S. This structure-guided workflow offers a testable scaffold-optimization strategy for endocrine-resistant, ER-positive breast cancer, a population with limited options after progressing on fulvestrant or CDK4/6 inhibitors. Next steps include native-ligand redocking to benchmark GlideScore, plus biochemical binding, transcriptional activity, receptor degradation, and cell-based resistance studies to define WEA001's therapeutic potential.

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