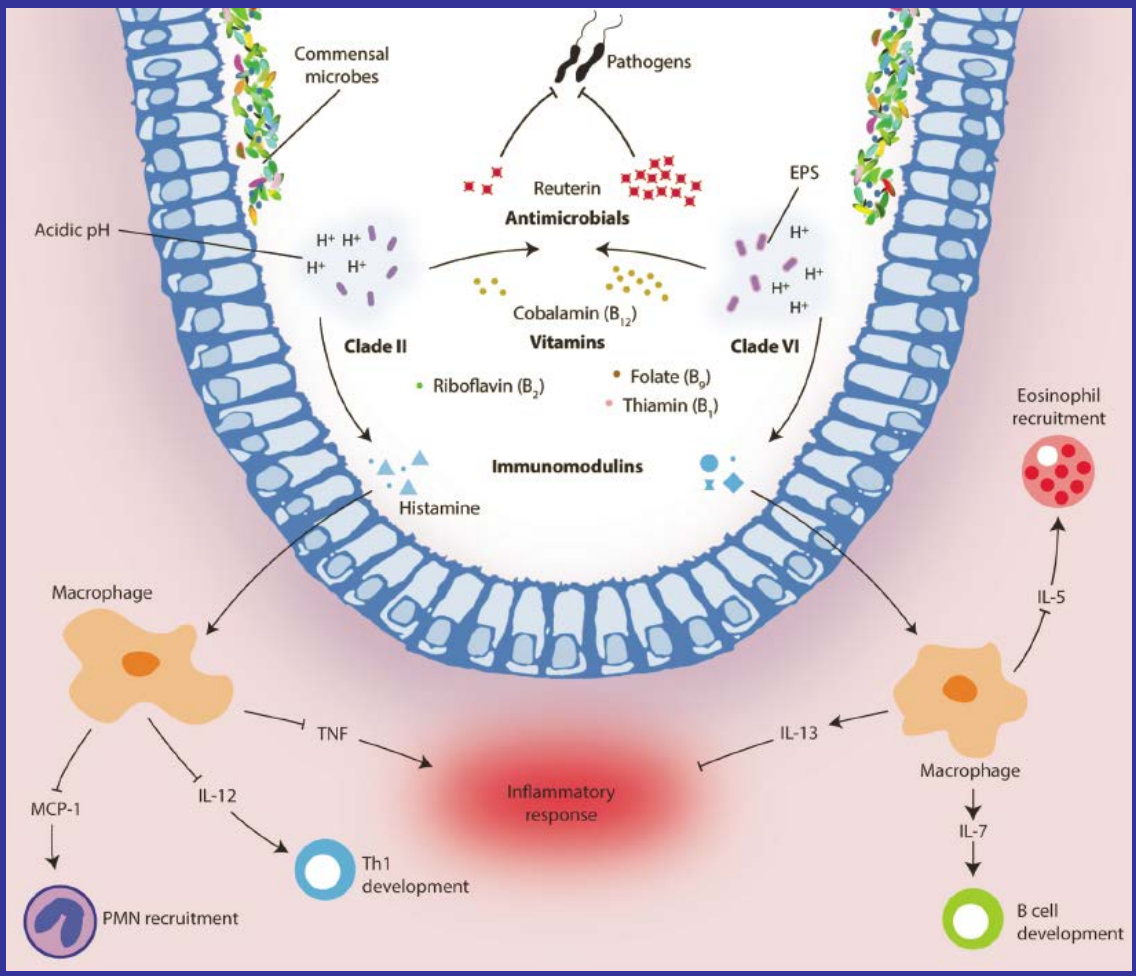
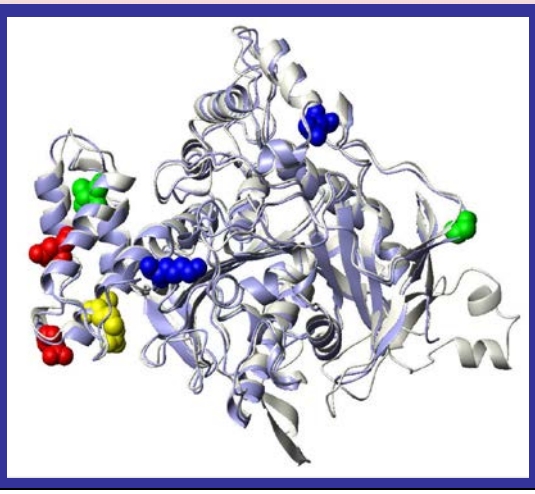
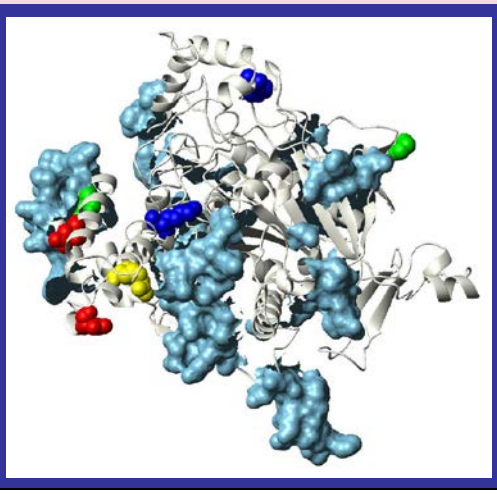
Texas Medical Center Digestive Diseases Center
5th Annual *"Frontiers in Digestive Diseases"* Symposium:
The Gut Microbiome in Health & Disease





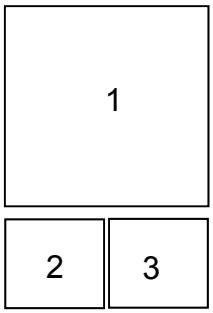


Saturday, February 8, 2014
MD Anderson – Onstead Auditorium
Houston, Texas

Texas Medical Center Digestive Diseases Center presents the 5th Annual
Frontiers in Digestive Diseases Symposium: The Gut Microbiome in Health & Disease

Saturday, February 8, 2013
MD Anderson - Mitchell Research Building
Onstead Auditorium
6767 Bertner, Houston, Texas

On the cover:



(1) P-40. Jennifer Spinler. Functional illustration of human-derived Lactobacillus reuteri ecotypes. The vertebrate gut symbiont, L. reuteri, has diversified into separate host-based clades, and human-derived strains from clades II and VI have evolved into functionally distinct subspecies ecotypes. These differences affect their functional repertoires and probiotic applications. Human-derived L. reuteri strains each differentially promote acidic environments, produce the antimicrobial reuterin, synthesize essential vitamins, and generate immunomodulatory compounds that effect immune signaling in the host. The differences associated with each ecotype are illustrated here.

(2) P-34. Numan Oezguen. Ribbon representation of the structural alignment of human neuroligin 3 (NLGN3) model (in gray) and human NLGN4-X linked crystal structure (in light blue, PDB code 2xb6). The NLGN3 model is based on rat NLGN1 (PDB code 3vkf). Highlighted in spheres are autism spectrum disorders (ASD) associated mutation sites. In yellow is the R451C NLGN3 site and in red, blue, and green are other mutation sites reported for NLGN4-X linked.

(3) P-34. Numan Oezguen. Human NLGN3 model is shown in ribbon representation. ASD associated mutations in NLGN3 and NLGN4-X linked are shown in spheres. Additionally, NLGN3 predicted protein-protein interface patches are highlighted.

Texas Medical Center Digestive Diseases Center presents the 5th Annual
***Frontiers in Digestive Diseases Symposium*: The Gut Microbiome in Health & Disease**

A G E N D A
Saturday, February 8, 2014
MD Anderson - Mitchell Research Building
Onstead Auditorium
6767 Bertner
Houston, Texas

7:45 am ***Coffee and Continental Breakfast***

Session I. Theme: Gut Microbiome: Basic Systems Biology

8:15- 8:45 am ***Welcome.*** Hashem B. El-Serag, MD, MPH, Co-Director, TMC Digestive Diseases Center; Professor and Chair; Michael E. DeBakey Veterans Affairs Medical Center and Baylor Clinic

Moderator: James Versalovic, MD, PhD, Professor, BCM

8:45 – 9:25 am ***"Neonatal Gut: Early Development of Gut Microbiome"***
Phillip I. Tarr, MD, Professor, Washington University in St. Louis

9:25 – 10:05 am ***"Gut Microbiome and Impact of Diet"***
Frederic D. Bushman, PhD, Professor, University of Pennsylvania

10:05 – 10:35 am ***"Metagenome to Metabonome"***
Tor C. Savidge, PhD, Associate Professor, Texas Children's Hospital, BCM

10:35 – 11:00 am ***Morning Coffee Break***

Session II. Theme: Gut Microbiome and Intestinal Disease Phenotypes

Moderator: Joe Petrosino, PhD, Associate Professor, BCM

11:00 – 11:40 am ***"Inflammatory Bowel Disease and Fecal Microbiome Therapy"***
Richard Kellermayer, MD, Associate Professor, Texas Children's Hospital, BCM

11:40 – 12:10 pm ***"IBS and Abdominal Pain: The Brain-Gut Axis"***
Emeran Mayer, MD, Professor, University of California Los Angeles

12:10 – 1:45 pm ***Lunch & Poster Viewing/Judging***

1:45 – 2:15 pm **"*Genotoxic E. coli in Macaques with Colitis-Associated Cancer and a BCM Germ-Free Mouse Facility Project Update"***
Alton Swennes, DVM, Clinical Veterinarian, BCM

2:15 pm **Discussion and Closing Remarks**
Mary K. Estes, PhD, Director, TMC Digestive Diseases Center; Professor, BCM

L I S T O F A B S T R A C T S

21.	David Y. Graham, MD DDC Clinical Research Core Director Professor, Medicine-GI BAYLOR	Triple Bacteroides fecal replacement therapy for of relapsing Clostridium difficile diarrhea (fecal transplantation sans feces).
22.	Diane Hutchinson Graduate Student BAYLOR	Norwalk virus infection and the gut microbiome
23.	Joseph M. Hyser, PhD ☆ Assistant Professor BAYLOR	Activation of Store-operated and Voltage-activated Calcium Channels by Rotavirus NSP4-mediated Release of ER Calcium Stores
24.	Baijun Kou Research Associate BAYLOR	Characterization of cross-reactive norovirus-specific monoclonal antibodies
25.	Lenard Lichtenberger, PhD DDC Associate Director Director Integrative Biology Core UTHealth	NSAID injury to the small intestine is dependent upon bile and is associated with overgrowth of enterococci
26.	Yuying Liu, PhD ☆ Assistant Professor UTHealth	Lactobacillus reuteri DSM 17938 prolongs the survival of Treg-deficient Scurfy mice
27.	Janielle P Maynard Research Assistant BAYLOR	Dysregulation of purinergic signaling in Hepatocellular Carcinoma
28.	Sharada Mokkapati, PhD Postdoctoral Associate MDACC	Liver Cancer In Mice With Wnt Pathway Activation in Unique Fetal Liver Progenitors.
29.	Yuko Mori-Akiyama, MD ☆ Assistant Professor BAYLOR	SOX9 and NFY converge on the promoter regions of cell cycle regulatory genes
30.	Kosuke Murakami, PhD Postdoctoral Associate BAYLOR	Screening of candidate proteins for norovirus co-receptors
31.	Dorottya Nagy-Szakal MD Postdoctoral Associate BAYLOR	Microbiome and host factors communicate protection against acute murine colitis following omega-6 fatty acid induced transient pediatric obesity
32.	Theresa Nguyen, BS Medical Student BAYLOR	The length of newly diagnosed Barrett's esophagus has been decreasing over time
33.	Theresa Nguyen, BS Medical Student BAYLOR	Statin use may decrease the risk of Barrett's esophagus: A case-control study of US veterans
34.	Numan Oezguen, Dr. rer. nat. Instructor BAYLOR	Gastrointestinal disease in neuroligin-3 linked autism is hardwired in enteric neurons
35.	Lin Qu, PhD Postdoctoral Associate BAYLOR	NoroGLuc: A cell-based human norovirus protease reporter system
36.	Kevin Riehle, MS Lead Bioinformatics Programmer BAYLOR	16S rRNA and Whole Metagenome Shotgun Analysis using the Genboree Workbench
37.	Kapil Saxena Medical Student BAYLOR	Human Intestinal Enteroid Cultures: a New Functional Model of Human Rotavirus Infection

38.	Jeremy Schaefer, PhD ☆ Assistant Professor, UTHealth - Dentistry	MicroRNAs in Crohn's disease and ulcerative colitis
39.	Amrita Sontakke, MSc Research Assistant I BAYLOR	Lactobacillus reuteri supplementation is a viable option for preventative treatment of *Clostridium difficile*-associated diarrhea
40.	Jennifer K. Spinler, PhD Instructor BAYLOR	From prediction to function using evolutionary genomics: Human-specific ecotypes of Lactobacillus reuteri have diverse probiotic functions
41.	Yuxiang Sun, MD, PhD Assistant Professor BAYLOR	Ablation of ghrelin receptor attenuates high fructose corn syrup (HFCS)-induced adipose inflammation and insulin resistance
42.	Bryan Tackett Graduate Student BAYLOR	Early activation of P2Y2 purinergic signaling is essential for efficient hepatocyte proliferation in response to partial hepatectomy
43.	Clavia Ruth Wooton-Kee, PhD ☆☆ Postdoctoral Associate, BAYLOR	Alterations in hepatic nuclear receptor function in a Wilson's disease mouse model and implications for Wilson's disease patients
44.	Irving J. Zamora, MD Postdoctoral Research Fellow BAYLOR.	Low Abdominal NIRS Values and Elevated Serum Intestinal Fatty Acid-Binding Protein Predict Necrotizing Enterocolitis in a Premature Piglet Model

☆ Denotes past DDC Pilot/Feasibility awardees

☆☆ Denotes 2014 DDC Pilot/Feasibility awardees

Oral Administration of Pulmonary Surfactant Protein-A Reduces Pathology in an Experimental Model of Necrotizing Enterocolitis

Hector D. Quintanilla, MD, Yuying Liu, PhD, Nicole Y. Fatheree BS, Constance L. Atkins, BS, Syed S. Hashmi, PhD, Joanna Floros, PhD, Francis X. McCormack, MD, Jon M. Rhoads, MD and Joseph L. Alcorn, PhD

OBJECTIVES: Necrotizing enterocolitis (NEC) frequently results in significant gastrointestinal tract morbidity and mortality in premature infants. Others reported that mice deficient in pulmonary surfactant protein-A (SP-A) born and raised in a nonhygienic environment succumb to a pathology resembling NEC, and enteral administration of purified SP-A significantly reduced mortality. We hypothesized that oral administration of purified SP-A can ameliorate pathology in an experimental model of neonatal NEC.

METHODS: NEC was induced in newborn Sprague-Dawley rat pups by daily formula gavage and intermittent exposure to hypoxia (FH). Purified human SP-A (5 μ g/day) was administered orally via gavage (FHS). After 4 days, surviving pups were sacrificed and NEC was assessed by histological examination of distal terminal ileal sections. Intestinal levels of inflammatory cytokines (IL-1β, IFN-γ and TNF-α were assessed by ELISA. Intestinal toll-like receptor 4 (TLR4) levels were assessed by western analysis.

RESULTS: Exposure of rat pups to hypoxia (FH) significantly increased mortality and the incidence and severity of NEC. Oral administration of SP-A (FHS) significantly reduced mortality and assessment of experimental NEC. Intestinal levels of pro-inflammatory cytokines (IL-1 β TNF-α and IFN-γ) were significantly increased in FH pups. Administration of SP-A significantly reduced IL-1 β and TNF-α levels, but had little effect on elevated levels of IFN-γ. Expression of intestinal TLR4, key in NEC pathogenesis, was significantly reduced in the FHS group as compared to the FH group.

CONCLUSIONS: In an experimental rat model of NEC, oral administration of SP-A reduces intestinal levels of pro-inflammatory cytokines and TLR4 protein, and ameliorates adverse outcomes associated with NEC.

Evaluation of P2Y2 Purinergic Receptor Function in the Pathogenesis of Sepsis.

Athis Arunachalam, Bryan Tackett and Sundararajah Thevananther

Department of Pediatrics, Baylor College of Medicine/Texas Children's Hospital

Background: Severe sepsis is a major cause of morbidity and mortality. Cellular stress triggers ATP release into extracellular milieu. Extracellular ATP, via activation of P2Y2 purinergic receptors, plays a pivotal role in inflammation and immunomodulation. However, the functional significance of P2Y2 receptor activation in the modulation of inflammation, multi organ injury, and death is not well understood. The overall goal is to test the **hypothesis** that P2Y2 purinergic receptor activation is essential for the induction of inflammatory cascades and multi-organ injury secondary to microbial infection.

Methods: Sepsis was induced by cecal ligation and puncture (CLP) in adult (12-16 weeks) wild type (WT; C57BL6/J) and P2Y2-/- (KO; C57BL6/J) mice. Tissues (liver, lungs) and blood were collected at 3,6,12, 21h and 7 days after CLP. Tissues were analyzed for leukocyte infiltration, apoptosis, and pro-inflammatory cytokine/chemokine protein and RNA (quantitative RT-PCR) expression.

Results: WT mice subjected to CLP developed symptoms of severe sepsis and were moribund (100%) between 21-48 hr. However, KO mice survived longer (70% survival at 7 days). In response to CLP, induction of serum cytokines/chemokines was significantly attenuated in KO mice (IL-6, 67%; IFN□, 91%; MCP-1, 96%; MIP-2, 75%), as compared to WT mice at 21 hr. CLP induced massive liver injury in the WT mice, with elevated serum ALT, leukocyte infiltration into the hepatic parenchyma, hepatocyte injury, and hemorrhagic necrosis at 21 hr. Liver injury was attenuated in the KO mice in response to CLP at 21 hr, with decreased induction of TNFα (58%), IL-6 (72%), IL-1β (49%) and MCP-1 (90%) mRNA expression, as compared to WT livers. Additionally, attenuation of markers of lung inflammation and kidney injury were noted in the KO mice as compared to WT.

Conclusions: P2Y2 receptor function is critical for sepsis-induced mortality in mice. Early induction of hyper-inflammatory phase is dependent on intact P2Y2 receptor expression. These findings highlight the functional significance of purinergic signaling in the pathogenesis of sepsis and eventually will lead to development of novel therapeutics.

Delayed Initiation of Enteral Formula Feeding Reduces the Incidence of Necrotizing Enterocolitis (NEC) inPpreterm Piglets

Caroline Bauchart-Thevret[1], Nada Ghoneim[1], Barbara Stoll[1], Madhulika Kulkarni[1], Berthe Oosterloo[1], Miguel Saenz de Pipaeon[1], Oluyinka Olutoye[1], Irving Zamora[1], Brian Berg[2], Anja Wittke[2], Douglas G. Burrin[1].

[1] USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas. [2] Mead Johnson Pediatric Nutrition Institute, Evansville, Indiana.

Background: Necrotizing enterocolitis (NEC) is a major complication of enteral feeding in premature infants with a high morbidity and mortality. Early enteral feeding of fortified human milk is considered optimal nutrition for the preterm infant. However, human milk is not always available, and commercial formulas are needed that mimic human milk as closely as possible. **Objective:** Our aim was to test the effects of early vs. late enteral feeding of an intact vs. partially hydrolyzed protein formula on NEC incidence in a preterm piglet model being developed to closely simulate clinical practice.

Design/Methods: Moderately preterm pigs (at 90% of gestation) were randomized to either an early (EA) or late (LA) feeding protocol. The EA and LA groups received 2 d and 5 d of total parenteral nutrition (TPN), and orogastric formula feeds (50% full intake) began on d of life 3 and 6, respectively and PN continued. Pigs in the EA and LA groups were also randomized to one of two formulas containing either intact or hydrolyzed protein. All four groups were euthanized due to NEC onset or after 5 d formula feeding. NEC severity and incidence was assessed based on macroscopic and histological scoring in the stomach, proximal jejunum, distal ileum, and colon.

Results: Twenty-eight of 40 pigs in the EA group developed NEC (70%) as compared to 9 of 22 pigs in the LA group (41%) ($p=0.033$). The mean total clinical NEC severity score was significantly greater in the early vs late group (12.50 vs. 7.27; $p=0.003$). There was no significant difference in the incidence of NEC in pigs that received hydrolyzed protein formula (67% in EA, 45% in LA) and those that received intact protein formula (77% in EA, 36% in LA).

Conclusions: Although TPN has been associated with impaired gut development, this study provides evidence that delayed initiation of enteral feeding at 5 d vs. 2 d is protective and reduced both the incidence and severity of NEC in preterm pigs. The formula containing intact or hydrolyzed protein had no effect on NEC development. Future studies will explore whether more gradual rather than an abrupt introduction of feeds impacts NEC incidence.

Impact of Parenteral Lipid Emulsions on Metabolomic Phenotype in Preterm TPN-Fed Piglets

Madhulika A. Kulkarni[2], Hester Vlaardingerbroek[4], Barbara Stoll[1], Olga Ilkayeva[5], Christopher Newgard[5], Oluyinka Olutoye[3], Johannes B. Van Goudoever[6], Douglas Burrin[1].

[1]USDA-ARS Children's Nutrition Research Center, [2]Newborn Section, [3]Department Surgery, Baylor College of Medicine, Houston, TX; [4]Erasmus MC - Sophia Children's Hospital, Rotterdam, Netherlands; [5]Duke University Medical Center, Durham, NC; [6]VU University Medical Center, Amsterdam, Netherlands.

BACKGROUND: Lipids in parenteral nutrition provide essential fatty acids and are a major source of energy for hospitalized neonates. Intralipid (IL) is the only approved lipid emulsion in the U.S, but new generation emulsions including Omegaven (OV) and SMOFlipid (SL) have been approved in Europe and are being considered by the FDA. Our studies in TPN-fed preterm piglets given IL lipid emulsion leads to hepatic cholestasis and steatosis implicating metabolic dysfunction in the liver. There are no studies describing the metabolite profile in neonates comparing IL with new generation lipid emulsions.

OBJECTIVE: Our objective was to perform metabolomic profiling of liver and muscle tissue in total parenteral nutrition (TPN) fed piglets given different lipid emulsions.

DESIGN/METHODS: Preterm pigs were assigned to 4 groups (7-14 pigs/group; equal daily macronutrient intake with 5 g/kg lipid): PN+IL, PN+OV, PN+SL and an enteral group fed milk formula (EN). These emulsions are based on soybean oil (IL), fish oil (OV), or a blend of lipids soy, fish, olive, medium chain fatty acids (SL). Cesearean-derived pigs received their treatments beginning at birth until 14 d of age. Plasma, muscle and liver tissue were collected after 14 d and subjected to analysis of fatty acid, amino acid and citric acid cycle metabolites by various HPLC and GC/MS methods.

RESULTS: We found marked increase in hepatic steatosis in IL vs EN pigs that was prevented in both new generation emulsions (OV and SL). The difference in hepatic steatosis was associated with insulin resistance in IL vs EN and SL pigs. In liver tissue, acyl-CoA species were most abundant for the dominant fatty acids in the respective lipid emulsions. Tissue free carnitine in EN pigs was 4-fold higher than TPN groups and this resulted in a reduced liver, but not muscle acyl-carnitine levels. The acyl-CoA: acyl-carnitine ratios were higher in liver than muscle tissue and also higher in all TPN groups' vs EN. Among citric acid cycle intermediates, only citrate was higher in EN vs. all TPN groups.

CONCLUSIONS: We conclude that metabolomic profiles revealed a carnitine deficiency in TPN groups that suggests impaired hepatic fatty acid oxidation. A defect in hepatic fatty acid oxidation may explain steatosis in IL pigs. The protective effect of new generation emulsions against hepatic steatosis appears to be independent of carnitine status.

Vitamin E Added to Intralipid Positively Impacts Hepatic Bile Acid and Fatty Acid Homeostasis in TPN-Fed Preterm Pigs

K. Ng[1]; B. Stoll[1]; M Sáenz de Pipaón[2], D. Burrin[1]

1. Department of Pediatrics, Division of Pediatric Gastroenterology, Hepatology, and Nutrition, and USDA Children's Nutrition Research Center, and Division of Pediatric Surgery, Baylor College of Medicine, Houston, TX, United States; Department of Neonatology, La Paz University Hospital, Madrid, Spain.

Background: Prolonged total parenteral nutrition (PN) may lead to cholestasis and parenteral nutrition associated liver disease (PNALD). The etiology of PNALD is unknown, but constituents of lipid emulsions may positively or negatively impact nuclear receptors involved in bile acid homeostasis (BAH) and steatosis. Plant phytosterols present in soybean oil-based lipid emulsions (e.g. Intralipid) have been suggested to negatively impact BAH by antagonizing the bile acid sensing farnesoid X receptor (FXR) and in turn its downstream targets. The fish oil-based lipid emulsion Omegaven, abundant in vitamin E and docosahexaenoic acid (DHA) yet devoid of phytosterols, may positively impact the nuclear receptors pregnane X (PXR) and peroxisome proliferator-activated receptor-alpha (PPARα) and their downstream targets thus protecting hepatocytes against bile acid and fatty acid homeostatic dysregulation. We investigated the serum and hepatic tissue bile acid biomarkers of liver injury, as well as target genes involved in BAH and fatty acid metabolism in TPN-fed preterm pigs given 4 different lipid emulsions.

Methods: Preterm pigs were assigned to receive 14 d of either, 1) TPN + Intralipid (100% soybean oil)(IL); 2) TPN + Intralipid + Vitamin E (ILE); 3) TPN + Omegaven (100% fish oil)(OV); or 4) TPN + Omegaven + Phytosterols (PS). The final vitamin E concentration in the ILE group equaled the concentration in Omegaven. The three principal phytosterols found in Intralipid (campesterol, β-sitosterol, & stigmasterol) were added to Omegaven in the PS group.

Results: Serum levels of direct bilirubin, ALT, GGT, triglyceride, LDL and hepatic triglyceride content were significantly lower ($P<0.05$) in the ILE, OV, and PS compared to IL pigs. CYP7A1 (bile acid synthesis) expression was lower ($P<0.05$) in the ILE, OV, and PS groups vs. IL. CYP3A29 and MRP2 expression (bile acid oxidation and canalicular bilirubin export, respectively) were higher in ILE, OV, and PS groups vs. IL. OSTα (bile acid efflux transporter) expression was lower ($P<0.05$) in the ILE, OV, and PS groups vs. IL. BSEP (canalicular bile acid export) was slightly lower in the ILE, OV, and PS pigs vs. IL. CPT1A and CYP2E1 (mitochondrial and microsomal fatty acid oxidation) were higher in ILE, OV, and PS vs. IL pigs. Addition of phytosterols to Omegaven did not induce evidence of liver injury. The findings suggest that supplemental vitamin E and DHA are associated with up-regulated expression of PXR and PPARα downstream target genes involved in bile acid and fatty acid metabolism andoxidation. These changes resulted in decreased bile acid synthesis and increased bile acid breakdown and canalicular bilirubin export; this triggered a compensatory down-regulation of the alternative bile acid export pathway (OSTα). Increased mitochondrial and microsomal fatty acid oxidation protects against hepatic triglyceride accumulation and steatosis.

Conclusions: Vitamin E and DHA found in greater quantities in fish-oil vs. soybean oil-based lipid emulsions may provide hepatocyte protection via activation of bile acid as well as fatty acid metabolic and oxidative pathways. Importantly, the supplemental vitamin E in the ILE group may have prevented the detrimental effects of abundant phytosterols.

Interaction of BMP, Apelin and PTHrP pathways in pancreatic duct ligation-induced chronic pancreatitis

Y. Cao,[1,2] C. Rastellini,[2] S. Han,[2] V. Bhatia,[2] M. Falzon,[2] G. Greeley, Jr [2] and T.C. Ko[1,2]
[1]Dept. of Surgery, UTHSC-Houston; [2]UTMB at Galveston, TX

Background: Our laboratories have shown that pancreatic bone morphogenetic protein (BMP), apelin and parathyroid hormone-related protein (PTHrP) signaling pathways are up-regulated by cerulein-induced chronic pancreatitis (CP) in mice. Furthermore, BMP and apelin are anti-fibrogenic, while PTHrP is pro-fibrogenic. It is unclear whether these pathways interact in CP.

Objective: To examine potential interaction of BMP, apelin and PTHrP in pancreatic duct ligation (PDL)-induced CP model.

Methods: CP was induced by PDL in adult male C57/BL6 mice (n=5). The ligated and non-ligated lobes (control) of the pancreas were harvested for histology, protein and mRNA analyses. For *in vitro* experiment, cultured mouse PSCs were treated with BMP2 (50 ng/ml) or PTHrP (10^{-7} M). The effect of apelin gene knockout (APKO) on pancreatic PTHrP levels was examined.

Results: In the ligated lobes, PDL-induced CP results in inflammation, acinar degeneration and fibrosis, while the control lobes appear histological normal. Fibronectin protein levels are elevated in the ligated lobes (control 0.27±0.03 vs ligated 1.73±0.30). Pancreatic mRNA levels of BMP2 and apelin are elevated in the ligated lobes (7- and 4 to 10-fold vs control respectively, $p<0.05$). *In vitro*, BMP2 induces apelin mRNA expression (vehicle 1.1±0.01 vs BMP2 1.4±0.10), whereas PTHrP inhibits apelin mRNA expression (vehicle 1.0±0.01 vs PTHrP 0.28±0.03). Pancreatic PTHrP mRNA levels are elevated 8-fold in APKO mice compared to wildtype.

Discussion: PDL-induced changes in BMP2 and apelin levels replicate those measured in cerulein-induced CP. BMP2 and PTHrP influence apelin expression implying that BMP2 and PTHrP regulate apelin signaling during CP. Results from APKO imply a feedback axis between apelin and PTHrP.

Conclusion: BMP and apelin interact in CP that may form an endogenous network protecting the pancreas during CP. PTHrP may suppress BMP's and apelin's protective effects.

Regulation of Gremlin and Apelin Levels by Ethanol via a PTHrP-Dependent Pathway in Pancreatic Acinar Cells

V. Bhatia, Y. Cao, T.C. Ko, G. Greeley, Jr, M. Falzon

Objective: To define the effect of ethanol-induced parathyroid hormone-related protein (PTHrP) signaling on the bone morphogenetic protein (BMP) and apelin signaling pathways.

Background: Our laboratories have shown that ethanol increases PTHrP levels in acinar cells, and that the ethanol-mediated increase in cytokine release is suppressed by the PTHrP signaling antagonist PTHrP (7-34). Apelin protects against cerulein-induced pancreatic damage *in vivo;* the BMP antagonist gremlin exerts an opposite effect. BMP-2 regulates apelin levels.

Methods: Mouse acinar cells were treated with PTHrP (1-36) (10^{-7}) for 15-60 min. Acinar cells deficient in PTHrP were isolated from mice with conditional knockout of the *Pthrp* gene in acinar cells (CKO-PTHrP) and from littermate controls. Cells were treated with ethanol (50 mM) for 15-120 min. Apelin and gremlin mRNA levels were measured.

Results: PTHrP (1-36) reduced apelin mRNA levels (~3-fold) and increased gremlin mRNA levels (~2.5-fold). Apelin levels in CKO-PTHrP acinar cells were 8-fold higher than those in controls. Ethanol treatment caused a 3-fold increase in apelin levels in control mice. This effect on apelin was attenuated in CKO-PTHrP cells. Conversely, gremlin levels were ~2.5-fold lower in CKO-PTHrP cells. Treatment with ethanol caused a 3-fold increase in gremlin levels in control cells, but was ineffective in CKO-PTHrP cells.

Discussion: Previous findings show that pancreatic insult enhances PTHrP signaling, leading to an inflammatory and fibrotic response. Results presented here indicate that increased PTHrP signaling may suppress the BMP and apelin signaling pathways, endogenous pathways which are normally protective.

Conclusion: The BMP and apelin pathways form an endogenous protective network. Inactivation of this protective network by PTHrP prevents the reestablishing of homeostasis following injury, thereby increasing the susceptibility to developing chronic pancreatitis.

Targeting TGF-β regulated telomerase: a novel therapeutic approach for liver and gastrointestinal cancers

Jian Chen[1], Jiun-Sheng Chen[1], Zhixing Yao[1], Wilma Jogunoori[2], Bibhuti Mishra[2], Lopa Mishra[1]

[1]Department of Gastroenterology, Hepatology & Nutrition, University of Texas MD Anderson Cancer Center
[2]Institute of Clinical Research, Veterans Affairs Medical Center, Washington DC

Background: A hereditary human cancer stem cell syndrome, Beckwith-Wiedemann syndrome (BWS) is currently linked to deregulated imprinting at chromosome 11p15, IGF2, CDKN1C and others, and develops multiple cancers, including liver and gastrointestinal cancers. Yet, causal molecular defects and genetic models of this overgrowth syndrome have remained elusive to date in the majority of cases. CCCTC-Binding Factor CTCF is a highly conserved zinc finger protein that has diverse regulatory functions, including transcriptional activation/repression/imprinting of molecules such as *TERT, IGF-2* and *c-Myc*. Recent studies demonstrate a high frequency of *TERT* promoter mutations in early stage of multiple cancers, suggesting that these promoter mutations may function as driver events that contribute to oncogenesis through TERT deregulation. However, telomerase remains a challenge to target effectively. We have recently discovered identified a mouse model for BWS with loss of TGF-β signaling with multiple liver and GI cancers. CTCF expression is markedly decreased, with concomitantly high TERT levels in these cancers.

Hypothesize: TGF-β/β2SP/Smad3/ signaling regulates CTCF and *TERT* regulation. Rescuing TGF-β signaling may successfully reduce tumor burden with inhibition of telomerase.

Materials & Methods: Generation of *β2SP+/-/Smad3+/-* mice and genotype analysis. Database of Genomics (COSMIC) and transcriptomics (TCGA) were analyzed. BWS tumors and mice liver, pancreas and stomach tissue specimens were immunostained with anti-TERT antibody. ChIP assays were to demonstrate the recruitment of β2SP/Smad3/CTCF at the promoter of TERT.

Results: (1) *β2SP+/-/Smad3+/-* heterozygote mice spontaneously develop visceromegaly, multiple cancers, phenocopy a hereditary human cancer stem cell syndrome BWS. (2) Genomics and transcriptomics analyses revealed aberrant TGF-β signaling in *β2SP+/-/Smad3+/-* mice and human BWS. (3) Increased TERT expression in *β2SP+/-/Smad3+/-* mice is similar to that observed in human BWS. (4) CTCF levels are markedly decreased in *β2SP+/-/Smad3+/-* mice tissues. (5) CTCF interacts with β2SP/Smad3 in cell nucleus in a TGF-β-dependent manner. (6) TGF-β promotes the complex of β2SP/Smad3/CTCF at *TERT* promoter region.

Conclusions: Loss of TGF-β signaling pathway leads to dysfunctional chromosome modulation through *TERT* deregulation. Smad3/β2SP/CTCF complex regulates *TERT* transcriptional activity. Our results provide a better mechanistic understanding of this dysfunctional stem cells cancer syndrome. Importantly, this study may lead to identify new treatment strategies and functional markers for the early detecting lethal cancers.

TGF-β Regulated E3 Ligases are Novel Therapeutic Targets for Primary Liver Cancer

Jian Chen[1], Jiun-Sheng Chen[1], YoungJin Gi[1], Lior H Katz[1], Ji-Hyun Shin[1], Liem Phan[1], Wilma Jogunoori[2], Vivek Shukla[3], Bibhuti Mishra[2], Shulin Li[1], Milind Javle[1], Mien-Chie Hung[1], Lopa Mishra[1]

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Background: Hepatocellular Cancer (HCC) is lethal and difficult to treat due to late diagnosis, few viable targeted therapeutics and unclear molecular profiling of each stage of tumor development, from cirrhosis to nodule formation to carcinoma. A large number of studies have demonstrated that the TGF-β pathway plays a fundamental role in the biology of the GI tract, and as such, several components of the pathway are commonly targeted in various GI cancers. TGF-β signaling is regulated by the ubiquitin–proteasome pathway, in which E3 ubiquitin ligases recognize and target proteins for degradation by the proteasome. Numerous E3 ubiquitin ligases have been identified as negative regulators of different components of the TGF-β pathway. More recently, Praja and Keap1 have been identified as E3 ligases that ubiquitinates β2-spectrin (β2SP) in a TGF-β-dependent manner. Accordingly, Praja E3 ligase activity regulates TGF-β signaling by controlling β2SP abundance through ubiquitin-mediated degradation.

Therefore, we **hypothesis** is that Specific E3 ligases, which disrupt TGF-β/β2SP/Smad3 tumor suppressor pathway lead to uncontrolled activation of chromatin and tumor formation. Therefore, small molecule inhibitors that specifically target these E3 ligases could present a novel therapeutic approach for liver cancer.

Materials & Methods: Broad genome datasets including 110 cases of HCC patients were retrieved from The Cancer Genome Atlas (TCGA) and analyzed. 23 HCC and normal liver tissue specimens were immunostained with anti-Praja1 antibody. A small chemical compound library was screened for E3 ligase inhibitors targeting Praja1.

Results: (1) Broad genome analysis reveals that E3 ubiquitin ligases regulating the TGF-β pathway are altered in GI cancers mainly by increase in mRNA and that they negatively correlate with patient survival. (2) Praja1 expression is dramatically raised in human HCCs with loss of TGF-β signaling. (3) Praja1 inhibits TGF-β/Smad3 tumor suppressor function and increases c-Myc activities. (4) Inhibition of Praja1 leads to apoptosis and suppresses its oncogenic activities. (5) Small chemical inhibitors suppress Praja1 activities and restores TGF-β tumor suppressor function in HCC cells. (6) Small chemical inhibitors induce apoptosis and inhibit HCC cell growth and tumorigenesis.

Conclusions: E3 ubiquitin ligases that modulate the TGF-β pathway are frequently increased in HCC. Praja1 presents a therapeutic target- it disrupts the TGF-β tumor suppressor pathway, and its overexpression promotes cancer cell survival and proliferation. Small molecule inhibitors such as triterpenoids that specifically target Praja1 could be very useful in HCC therapy, through targeting c-Myc, restoring TGF-β tumor suppressor function. This study may lead to new therapeutics targeting this lethal cancer and potentially a Phase I clinical trial in HCC.

The Pathophysiology of Lipid Metabolism During Rotavirus Infection

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Rotavirus, the causative agent of viral gastroenteritis in children and young animals, requires the formation of lipid droplets for assembly of viroplasms that are sites of genome replication and particle assembly. However, the precise mechanism of rotavirus-induced formation of lipid droplets and whether these lipid droplets play other roles during infection remain unknown. Lipid droplets are composed of a core of neutral lipids, triglycerides and sterol esters, surrounded by a phospholipid monolayer containing lipid droplet-specific perilipin proteins. A major function of lipid droplets is to store neutral lipids that can be processed by fatty acid-oxidation to produce ATP.

To evaluate lipid droplet formation, lipid droplets and rotavirus antigens were detected in rotavirus-infected cells. Rotavirus infection induces lipid droplets with different localization and lipid source. Early in infection, lipid droplets form from triglycerides that preexist in the infected cell and colocalize with viroplasms. From 2-3 hours post infection, nascent lipid droplets form from fatty acids taken up by the infected cell and do not colocalize with viroplasms. The triglycerides in both lipid droplets undergo lipolysis as shown by pulse-chase experiments with a fluorescently-labeled fatty acid.

To determine whether the rotavirus-induced lipid droplets are an energy source required for rotavirus replication, the yield of rotavirus was evaluated in the presence or absence of lipolysis or fatty acid-oxidation inhibitors. The yield of virus was significantly reduced (94% or 97%) in the presence of the general lipase inhibitor, E600, or in the presence of etomoxir, a specific inhibitor of CPT-1, respectively. Additionally, the level of ATP was significantly reduced in infected cells by inhibiting fatty acid-oxidation. In contrast to other viruses, such as hepatitis C and dengue virus, the lipid droplets induced by rotavirus were not utilized by fatty acid-oxidation to provide an energy source for virus replication.

The importance of a lipid droplet-specific protein, PLIN3, was assessed in the mouse model of rotavirus infection. PLIN3 knockout mice shed 40% less rotavirus than wild-type mice. Furthermore, PLIN3-positive large vacuoles were observed in enterocytes of rotavirus-infected mouse pups. These results suggest a pathophysiological role for lipid metabolism in rotavirus infection.

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Human Norovirus Infection Elicits Cross-Reactive Surrogate Neutralizing Antibodies

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Noroviruses (NoVs) are the leading cause of epidemic nonbacterial gastroenteritis. Our understanding of the determinants of protective immunity to NoV is incomplete. Serum antibody that inhibits virus binding in vitro to the histo-blood group (HBGA) family of host glycans, the putative NoV attachment factors, is the only known correlate of protection from NoV gastroenteritis. Antigenic drift in the capsid protein of human NoVs is well-documented, but the genotype-specificity of HBGA-blocking antibody is not known. In this study, we investigated the breadth of the blocking antibody response elicited by NoV infection.

The study population consisted of healthy adults who participated in a previously conducted experimental challenge study with Norwalk virus (genotype GI.1). Serum was collected before (-3 to 0 days) and 7, 14, 28, and 180 days after infection. A modified ELISA to measure blocking antibody in serum was developed and carried out against NoV virus-like particles representing homotypic and heterotypic NoVs. NoV virus-like particles (VLPs) structurally and antigenically recapitulate native antigen and were produced in a baculovirus expression system for the capsid proteins of selected NoVs representing genotypes GI.1, GI.4, GI.7, and GII.4. All persons infected with Norwalk virus developed an increase in blocking antibody titer to Norwalk VLPs (n=18). Furthermore, 22-39% of infected persons had a serological rise (≥4-fold rise between pre-infection and peak blocking titers) to at least one heterotypic NoV VLP. In contrast, no seroresponse to any antigen occurred among uninfected or placebo recipients.

There is no antiviral therapeutic for the human NoVs, underscoring the need for a vaccine. In the absence of cell culture or small animal replication models, blocking antibody is considered a surrogate neutralizing antibody and serves as an endpoint in the evaluation of norovirus vaccine candidates. However, the substantial genetic diversity and co-circulation of distinct NoV genotypes pose challenges to the development of a broadly effective vaccine. Our results show that infection with a human NoV can elicit blocking antibodies with cross-genotype specificity, suggesting that a broadly effective vaccine may be feasible.

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Microfluidic isolation of bacteria from diverse specimens for metagenomics analysis

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Metagenomic analysis of the human microbiome has revealed a multiplicity of microorganisms and their previously unsuspected involvement in human health. The prevailing metagenomic paradigm attempts to decipher host-microbe interactions by analyzing massive quantities of genetic sequence data *in silico*. An important limitation of this strategy, especially in low bacterial biomass samples, is that host nucleic acids in the specimen interfere with the interpretation of DNA sequence data. To address this limitation our multidisciplinary team of bioengineers, clinicians and biologists is developing a microfluidic apparatus to isolate and concentrate bacterial cells prior to extracting nucleic acids. We showed that gram-positive and gram-negative bacteria could be isolated from small volumes of resuspended stool, saliva and skin specimens with >75% efficiency at a processing rate of 100 µL/min, and could be released from the apparatus in a new fluid medium. Comparative metagenomic analyses between input samples and system isolates were carried out using 16S ribosomal subunit RNA genes and revealed that isolates represent the diversity of microorganisms present in the original specimen. Whole genome sequencing of processed skin specimens demonstrated enrich of microbial DNA against the background of host DNA. These data demonstrate feasibility for applying this approach to enhance metagenomic analyses by depleting host DNA and enriching low-level bacteria. The microfluidic approach requires little sample preparation and exploits the intrinsic biophysical properties of viable microorganisms to accomplish isolation, thereby obviating the need for expensive biochemical labels and bioengineered tags. Upon completion of this project we expect that this technology will enhance microbiome research as a preparative and potentially analytical tool to rapidly purify and profile bacteria present in clinical specimens. This project may have further applications in screening transfusion products and diagnosing bloodstream infections.

Microbiome and metabolomic determinants of necrotizing enterocolitis in the preterm pig

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Background: Using our preterm piglet model of necrotizing enterocolitis (NEC), we have observed that a formula diet enriched in maltodextrins versus lactose as the carbohydrate source produces a striking increase in NEC incidence (91% versus 27%, respectively). These studies and others in human infants illustrate that diet-dependent increases in NEC and exacerbation of clinical symptoms correlate with nutrient malabsorption and bacterial dysbiosis within the gastrointestinal tract (GIT). Furthermore, clinical NEC does not develop *in utero* or in germ-free animal models in response to formula feeding, highlighting the critical role of the microbiome and metabolism in this disease process**. Objective:** Determine the pattern and metabolic products of the gut microbiome in preterm pigs fed different dietary carbohydrate compositions and whether this correlates with NEC incidence. **Methods:** Preterm pigs are delivered by caesarian section at ~103 days of gestation (100% gestation = 115 days) and 4-6 hours after birth surgically implanted with a jugular vein catheter for the administration of total parenteral nutrition (TPN) and an orogastric tube for enteral formula feeding. Pigs are randomly assigned to one of four treatment groups (n= 10-20/group). After delivery, a cohort of pigs (**Group 1**) are immediately sacrificed to provide a baseline measurement of the "naïve" microbiome and metabolome. The remaining pigs receive TPN for 2 days and are subsequently randomized to receive 1 of 3 formulas. These formulas differ only in carbohydrate composition, containing either Lactose, Corn Syrup Solids (CSS), or a 1:1 mixture of Lactose:CSS (Mix) (**Groups 2-4**). Enteral feedings are introduced gradually and administered every 3 hours for 5 days or until the development and advancement of NEC requires euthanasia. Clinical and histological scoring of each region of the GIT is performed in conjunction with a morphometric analysis to quantify the development and severity of NEC. Tissue and luminal contents are collected from the stomach, distal ileum and colon for analysis of the microbiome by 16S rRNA sequencing. Colonic contents and plasma are additionally prepared for analysis by LC/GC mass spectrometry to determine the metabolomic profile. **Results:** Based on the average clinical and histological scores in each group, the NEC incidence rate was 12% in the Lactose group (n=17), 35% in the CSS group (n=23) and 40% in the Mix group (n=10). A significant decrease in crypt depth in the colon was observed in the CSS and Mix groups versus the Lactose group, suggesting that Lactose may protect against or reduce the severity of disease developing in the large intestine. Metabolomic analysis revealed a decrease in luminal 2,3-butanediol with a matching increase in the plasma levels in the CSS versus the Lactose groups. While histidine→histamine metabolism was favored in the CSS group, metabolism in the colon of the Lactose group favored an alternative pathway of histidine metabolism with increases in the peak intensity for the compounds trans-urocanate and imidazole propionate. Anti-inflammatory endocannabinoid-like compounds increased in the luminal contents with increasing concentrations of Lactose. **Conclusions:** These data suggest that dietary carbohydrates influence inflammation locally within the GIT as well as systemically and therefore the development and severity of NEC. Based on the changes in the profile of metabolites and recent investigations, we hypothesize that the microbiome analysis may reveal dysbiosis within the Proteobacteria and Firmicutes phyla.

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Human Organoid and Jejunal Enteroid Cultures as a Functional Model of Human Small Intestine to Study Infection with Human Enteric Microbes

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A significant limitation in translational research is the absence of reliable pre-clinical models that mimic relevant human physiology and disease pathology. This is particularly true in the field of human enteric diseases, which are most commonly caused by gastrointestinal (GI) microbes that are difficult to culture in cancer-derived cell lines. In many cases, the cell type that supports replication of human GI viruses is not known. The goal of the current study was to establish 3D small intestinal epithelial cultures, called organoids or enteroids, and validate them as an appropriate pre-clinical model of clinically important enteric infections. We have successfully cultured human intestinal organoids (HIOs) from approved stem cell lines as well as human enteroids from jejunal tissues (jHIEs) obtained from patients undergoing bariatric surgery. Both HIOs and jHIEs have been maintained in culture for greater than 5 months, and can be frozen and recultured. The enteroids, self-organized into villus-crypt structures in culture, contain multiple intestinal cell types, including enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. We confirmed the expression of a number of ion channels, which may contribute to the diarrhea observed in enteric infections. We tested methods (immunofluorescence, RT-PCR, plaque assay) to detect cultivation of enteric viruses in both HIOs and jHIEs. Lab strains and fecal samples of rotaviruses, and picornaviruses have been tested and they replicate well either in organoids or enteroids or both. This work demonstrates that HIOs and jHIEs offer a promising new model to study enteric viruses and they may be useful to study a variety of gastrointestinal-microbe interactions.

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Attenuation of Colonic Inflammation by Probiotic *Lactobacillus Reuteri* via Histamine Production

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Supplementation with probiotic *Lactobacillus reuteri* strains that naturally colonize the gut of mammals has been effective at ameliorating intestinal inflammation in rodent colitis models, but the underlying mechanisms are unknown. Pangenomic studies showed that *L. reuteri* strains with anti-inflammatory properties contain a complete *hdc* gene cluster which is responsible for synthesis and secretion of histamine, indicating a potential role for histamine in alleviation of inflammation. *L. reuteri* 6475 which contains an intact *hdc* gene cluster was found to suppress TNF production in activated THP-1 cells through the production of histamine and activation of histamine receptor 2 (H2R). Targeted mutagenesis of the *hdc* genes resulted in diminished anti-TNF activity and loss of histamine production, indicating the anti-TNF activity of histamine *in vitro*. Using a trinitrobenzene sulfonic acid-induced mouse model of colitis, *L. reuteri* 6475 administration protected eight-week female BALB/c mice against colitis, as indicated by significantly decreased weight loss, colonic damage graded by the Wallace score and serum amyloid A protein concentrations compared to media control. Positron emission tomography (PET) imaging also showed that *L. reuteri* 6475 significantly reduced the uptake of [18F]fluorodeoxyglucose ([18F]FDG) in the colon, indicating attenuation of colonic inflammation by *L. reuteri.* Further experiments showed that the *hdcA* mutant of *L. reuteri* 6475 which failed to produce histamine showed diminished ability to attenuate colitis. Moreover, H2R was detected in the mouse colon by immunohistochemistry and blocking H2R with its specific antagonist ranitidine diminished the anti-inflammatory ability of *L. reuteri* 6475. In addition, feeding mice with a histidine-free diet diminished *L. reuteri*'s ability to attenuate colitis. These combined investigations indicate that *L. reuteri* 6475 attenuates experimental colitis *via* histamine production, and provide important insights into understanding the molecular mechanisms underlying probiotic immunomodulation.

Triple *Bacteroides* fecal replacement therapy for of relapsing *Clostridium difficile* diarrhea (fecal transplantation sans feces).

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Purpose: In 1989 Tvede reported 5 subjects with chronic C. difficile (C. diff) diarrhea (Lancet 1989;1:1156), Following 5 days of vancomycin to suppress C diff, each received a mixture of 10 aerobic and anaerobic bacteria, including Bacteroides ovatus, Bacteroides vulgatus, and Bacteroides thetaiotaomicron. Fecal Bacteroides were absent during the patients' illness. Of the instilled bacteria, only the Bacteroides sp. were present in all cases after recovery.

Methods: Based on Tvede's experience, we cultivated B. ovatus, B. vulgatus, and B. thetaiotaomicron strains in vitro to prepare fresh inocula containing a mixture of 109 cells of each strain resuspended in 200 mL of preservative-free saline (with 1% human albumin) for infusion into the proximal intestine of patients with chronic or recurrent C. diff diarrhea.

Results: Results: A man with chronic co-morbidities was admitted because of C. diff diarrhea that began while receiving augmentin. Stools were C. diff toxin positive by PCR and enteric pathogen negative. He received flagyl 500 mg TID for 14 days; stools normalized for 5 days. He returned after about 2 weeks because of recurrent diarrhea and confusion. Diarrhea continued until day 11 but recurred on day 17 with at least 4 watery BM and crampy abdominal pain. Vancomycin 125 mg q6h was begun with minimal effect. After 7 days he received a Bacteroides transplant of 200 mL of freshly prepared triple species Bacteroides infused into the proximal intestine via an ultraslim endoscope. He improved rapidly and within 24 hours was clinically well. He was discharged after 12 days with one formed BM every other day and has remained without diarrhea for more than 3 months. Assessment of changes in the stool microbiota was done by 16S rDNA deep sequencing using DNA isolated from samples collected on the day of treatment (before treatment), one to ten days post-treatment, and from a final sample collected at a 40 day follow-up visit. Treatment with the triple species Bacteroides infusion resulted in marked changes in the microbial communities found in the stool of the subject during the first 10d of treatment. By day 40, a dramatic rise in Bacteroides was observed. Studies are underway to determine whether these are the transplanted species.

Conclusion: Relapsing chronic C. diff was successfully treated with Bacteroides replacement therapy which avoided the additional risks of using human feces. In contrast to fecal transplants, only three bacterial strains were required for successful amelioration of disease. Additional clinical studies await receipt of an IND. Although additional experience is needed, it appears that Tvede was correct and fecal transplant does not require human feces.

Norwalk virus infection and the gut microbiome

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The intestinal microbiome has recently been shown to play a role in the pathogenesis of viral enteric infections by enhancing viral infectivity. Norovirus (NoV) pathogenesis is not fully understood, and the effect of the gut microbiota in the context of NoV infection has not been described thoroughly. To assess the interaction between NoV, the intestinal microbiota, and the human host, we used fecal samples collected from the Norwalk virus (NV) challenge study carried out at Baylor College of Medicine. The study population consisted of 57 individuals who participated in an experimental challenge with NV. Longitudinal fecal samples were collected prior to, during, and subsequent to infection. NV infection was defined as the excretion of virus in stool or a ≥4-fold increase in serum titer of antibody to virus-like particles by total immunoglobulin ELISA. Fecal samples were collected from all subjects at 6 timepoints (days -7, 2, 4, 8, 21, and 56) to establish a baseline for the microbiome and to assess changes in the gut microbiota related to NV infection. Of the 55 subjects included in our study, 35 were uninfected (9 non-secretors and 6 placebo) while 20 subjects were infected. Bacterial gDNA was extracted from 328 samples, and the V4 hypervariable region of the 16S rDNA was amplified and sequenced on the Illumina MiSeq platform. The resulting sequencing data was analyzed using QIIME. During analysis, several parameters were applied to stratify the samples including: secretor status, infection status, symptoms (vomiting and/or diarrhea), antibody response, and shedding duration. In addition, we looked at each timepoint individually due to the inherent similarity of a person's microbiome over time. Quantitative beta-diversity (between sample diversity) Principal Coordinate Analysis (PCoA) was performed separately for each stratification. PCoA demonstrated no differences in microbiome composition according to secretor status. Additionally, pre-challenge microbiome composition did not affect susceptibility to NV infection, and infection did not induce changes to the structure of the microbiome. Following infection, presence of symptoms did not alter microbiome composition as determined by PCoA. When comparing infected individuals, the microbiome of long shedders was more similar to the pre-challenge composition than that of short shedders, indicating that the microbiome of short shedders changed over the course of infection. These results indicate that NV infection does not alter the composition of the fecal gut microbiome except for those individuals who block the viral replication (short shedders) faster.

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Activation of Store-operated and Voltage-activated Calcium Channels by Rotavirus NSP4-mediated Release of ER Calcium Stores

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Rotavirus (RV) is the leading cause of viral childhood gastroenteritis and despite implementation of two licensed vaccines causes >500,000 deaths worldwide. Our studies use a combination of *in vitro*, *in vivo* and the newly developed intestinal enteroid model systems to understand the molecular mechanisms that underlie RV pathophysiology. A hallmark of RV infection is disruption of Ca^{2+} homeostasis, including increased permeability of the endoplasmic reticulum (ER) to Ca^{2+} and activation of plasma membrane (PM) Ca^{2+} entry channels, resulting in elevated cytoplasmic Ca^{2+} ($[Ca^{2+}]c$), which is essential for RV replication and thought to contribute to secretory diarrhea. We showed RV nonstructural protein 4 (NSP4) is a viral pore-forming protein (viroporin) in the ER, which causes the elevated $[Ca^{2+}]c$, but how viroporin activity caused ER Ca^{2+} permeability and PM Ca^{2+} channel activation remained uncharacterized. We hypothesized that NSP4 forms a Ca^{2+} channel to release luminal ER Ca^{2+} and ER Ca^{2+} depletion activates store-operated Ca^{2+} entry (SOCE) through the PM.

Electrophysiological studies demonstrated that purified NSP4 has intrinsic ion channel activity when reconstituted into synthetic lipid bilayers or liposomes and conducts Ca^{2+}. Further, recombinant NSP4 had channel activity in native ER membranes by patch clamp of the outer nuclear envelope, which is contiguous with the ER, and increased channel activity correlated with the elevation in $[Ca^{2+}]c$. To evaluate effects of NSP4 on SOCE, we generated a cell line stably expressing YFP-tagged stromal interaction molecule 1 (YFP-STIM1), an ER transmembrane sensor of luminal Ca^{2+} that activates SOCE upon ER Ca^{2+} store release. We used these cells as a biosensor for SOCE activation. We found that RV infection or recombinant NSP4 expression activated YFP-STIM1, which triggered Ca^{2+} entry through Orai1 Ca^{2+} channels. By contrast, a NSP4 viroporin mutant failed to activate YFP-STIM1, did not induce Ca^{2+} entry through the PM, and did not increase $[Ca^{2+}]c$.

In addition to SOCE inhibitors, we also found that L-type voltage-activated Ca^{2+} channel (VACC) blockers significantly reduced RV replication. Using qRT-PCR and immunofluorescence, we discovered that human enterocyte cell lines and human intestinal enteroids all express the $Ca_v1.3$ VACC. Specific VACC blockers inhibited both NSP4-induced Ca^{2+} entry and RV replication, suggesting $Ca_v1.3$ is important for RV-induced Ca^{2+} uptake.

Our studies show that RV encodes an ER viroporin with Ca^{2+} channel activity. Releasing ER Ca^{2+} activates STIM1, triggering Ca^{2+} entry through host Ca^{2+} channels in the PM. These are the first electrophysiology studies on a Ca^{2+} viroporin and demonstrate NSP4 viroporin activity globally disrupts Ca^{2+} homeostasis by activating both SOCE and VACC to acquire the Ca^{2+} needed to support RV replication. Therefore, drugs targeting cellular Ca^{2+} channels, such as $Ca_v1.3$, should be studied as potential antiviral drugs to reduce RV diarrheal disease.

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Characterization of cross-reactive norovirus-specific monoclonal antibodies

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Noroviruses (NoVs) are the most common cause of outbreaks of acute gastroenteritis. Broadly reactive diagnostic assays are essential for rapid detection of NoV infections. We previously generated a panel of broadly reactive monoclonal antibodies (MAbs). We first characterized MAb reactivity with NoV virus-like particles (VLPs) from 15 different genotypes (GI=5, GII=10) coated on a microtiter plate (direct enzyme immunoassay [dEIA]) and by Western blot. The MAbs were classified as genotype-specific, genogroup-specific or broadly reactive, and some recognized overlapping epitopes by competition EIA. We next applied surface plasmon resonance (SPR) using a Biacore system to measure MAb dissociation constants (Kd) as a surrogate for binding affinity. The MAbs were immobilized on a chip and a panel of NoV VLPs were flowed past the immobilized MAbs. A Kd could not be measured by SPR for some MAbs because of a lack of interaction with the VLPs. To further assess this lack of MAb-VLP interaction, the MAbs were evaluated for their ability to capture NoV VLPs in a sandwich EIA. Those MAbs for which a Kd could not be measured also failed to capture the NoV VLPs in the sandwich EIA; in contrast, those with a measurable Kd gave a positive signal in the sandwich EIA. Kd values were in the nanomolar range for most NoV genotypes tested, although measured values were higher for NoV VLPs representing GI.7, GII.6 and GII.7 genotypes. Although some MAb pairs recognized overlapping epitopes in the P domain, they were discordant in their ability to capture VLPs in the sandwich EIA. Thus, broadly cross-reactive epitopes in the P domain may be partially masked on intact particles, adversely affecting the ability to target these epitopes in diagnostic assays. Nevertheless, several MAbs that recognize intact VLPs were identified for further evaluation in the development of different diagnostic immunoassay formats.

NSAID injury to the small intestine is dependent upon bile and is associated with overgrowth of enterococci

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Background: The use of indomethacin (INDO) and related nonsteroidal anti-inflammatory drugs (NSAIDs) to treat pain/inflammation is limited due to the drugs' GI toxicity, an action not completely understood. Lower gut NSAID injury in rodents is known to be dependent on the presence of bile. INDO has been reported to increase the proportion of *Enterococcus faecalis* in the gut microbiome (*Appl Environ Microbiol* 72:6707-15, 2006). *E. faecalis* is a normal gut commensal, but also a source of nosocomial infections and sepsis.

Objective: To determine the relationship between NSAID-induced intestinal injury and (1) the small bowel presence of *Enterococcus* spp., (2) the dissemination of the bacteria to other organs and (3) the contribution of bile to these pathological responses.

Methods: Male SD rats received either a sham operation (SO) or a bile duct ligation (BDL) a day before they were administered daily injections of saline or INDO (7.5 mg/kg) for 2 days, and euthanized 24 hr after the last dose. Biopsies of the distal small intestine, liver, and kidney were collected sterilely, homogenized and plated on Enterococcosel agar (EA - selective for enterococci) medium. The presence of *E. faecalis* was confirmed by colony hybridization with an *ace* gene probe under high stringency. The luminal flush was assayed for bile acid, to verify the BDL. Fecal hemoglobin (Hb) and hematocrit (Hct) were measured to assess intestinal bleeding.

Results: Total luminal bile acid was reduced to near zero in all BDL rats. SO-rats treated with INDO experienced a significant increase in fecal Hb and reduction in Hct ($p<0.05$), whereas BDL-rats treated with INDO had values comparable to SO/saline and BDL/saline rats, indicating that BDL attenuated INDO-induced intestinal injury. 10^3-10^4 CFUs of enterococcal colonies were derived from the distal intestine of SO/saline rats. There was a significant increase in the SO/INDO rats (10^6-10^7 CFUs; $p<0.003$) and an absence of enterococcal colonies in BDL/saline rats (0 CFUs; $p<0.01$), whereas the BDL/INDO rats had levels similar to the SO/saline rats (10^3-10^4 CFUs). In both the liver and kidney, enterococcal colonies were not detected in SO/saline rats (0 CFUs), but were detectable in the SO/INDO rats (10^2-10^3 CFUs), indicating bacterial dissemination.

Conclusion: Bile plays an important role in NSAID-induced gut injury and correlates with an increase of *Enterococcus* spp. in the intestine and bacterial dissemination. The importance of NSAIDs in the increase of *E. faecalis* in the small bowel has yet to be ascertained. It may be a compensatory response to gut injury or part of the pathogenic process itself.

Lactobacillus reuteri DSM 17938 prolongs the survival of Treg-deficient Scurfy mice

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Background: Foxp3$^+$ regulatory T (Treg) cells modulate the function of multiple immune cell types. Loss of Tregs causes lethal, CD4$^+$ T-cell-dependent multi-organ autoimmune disease in both mice and humans. Anti-inflammatory effects of probiotic *Lactobacillus reuteri* DSM17938 (LR17938) have been shown in our animal models of LPS-induced intestinal inflammation and necrotizing enterocolitis (NEC). We showed LR17938 reduces ileal cytokines via a TLR-pathway and differentially modulates of T effector and Treg cell populations in the intestine. We hypothesized that the beneficial effects of LR17938 in inflammatory diseases may be partially independent of Tregs. Therefore, in this study, we used Scurfy (sf) mice, which have a deletion in the forkhead domain of Foxp3, fail to develop thymic-derived Foxp3$^+$Tregs, and die of a lymphoproliferative syndrome with multi-organ inflammation at 16-26 days of age.

Aims: To determine if LR17938 improves survival and changes immunological function in sf mice.

Methods: Scurfy mice were obtained by breeding pairs of Foxp3sf heterozygous female and C57BL/6J normal male (Jackson Labs). Because the Foxp3 gene is on the X chromosome, 25% of the offspring will be hemizygous males with the mutation (sf). Male sf mice generally develop the scurfy phenotype (scaly, crusted skin on the tail, poor growth) on day of life (DOL) 13-15. Mice were administrated LR17938 (10^7 CFU/day) by gavage starting on DOL17, daily for 3 weeks (survival study) or for 7 days (immunological measurements) to compare sf with wild-type (WT) mice. Lymphocytes isolated from terminal ileum, mesenteric lymph node (MLN), and spleen were labeled for CD4, CD8, CD44, CD45RB, CD11c, CD103, CD11b, and intracellular Foxp3 and were then analyzed by flow cytometry.

Results: Feeding LR17938 significantly increased the survival rate of sf mice ($p<0.001$). Eighty-five % of sf mice after feeding LR17938 had a greater 2-fold increase in life span, but with consistent presence of sf clinical signs especially dermatitis and impaired growth (weight) in surviving sf mice compared to normal WT mice ($p<0.001$). The absence of Foxp3 expression on sf lymphocytes was not affected by feeding LR17938. In the ileum, MLN and spleen of the poorly growing (untreated) sf mice, we observed increases in the percentages of CD4$^+$T cells ($p<0.01$) and T effector cells (CD44$^+$CD45Rb^{lo}) ($p<0.001$). Feeding LR17938 to sf mice reversed these changes ($p<0.01$). We also observed that the % of "tolerogenic" CD103$^+$CD11c$^+$ dendritic cells (DCs) was decreased but % of CD11b$^+$CD11c$^+$ "inflammatory" DCs was increased in the intestine of sf compared to WT; but the ratio could be reversed by LR17938 feeding. In cultured splenocytes, the percentages of proinflammatory IL17A$^+$CD4$^+$ T cells were decreased and anti-inflammatory IL10$^+$CD4$^+$T cells were increased in LR17938-fed sf compared to sf without LR.

Conclusions: Major anti-inflammatory effects of probiotic LR17938 can be observed even in the absence Foxp3$^+$Tregs. LR17938 may be beneficial for human autoimmune and inflammatory diseases.

Dysregulation of purinergic signaling in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the third most lethal cancer worldwide, but molecular mechanisms of its pathogenesis are not well understood. Recent studies suggest that extracellular ATP-mediated activation of P2Y2 purinergic receptor induces hepatocyte proliferation in response to partial hepatectomy and ATP treatment alone was sufficient to induce hepatocyte proliferation *in vitro*. The purpose of this study was to characterize extracellular nucleotide effects on HCC cell proliferation and to examine the role of P2 purinergic signaling in the pathogenesis of HCC in patients and Mst1/2$^{-/-}$, a mouse model of HCC.

Hypothesis: *Dysregulation of purinergic signaling facilitates aberrant cell proliferation underlying hepatocellular carcinogenesis.*

Methods. HCC human-derived Huh7 cells, maintained in serum free media for 24h, were treated with ATPγS, or ADP (100μM) for different time intervals. SP600125 pretreatment was used to inhibit c-Jun N-terminal Kinase (JNK) signaling. Western blotting, qRT-PCR and 5-Bromo-2'-deoxy-uridine (BrdU) incorporation analysis were done. Mst1/2$^{-/-}$ and WT mouse livers (1, 3, & 6 months) and HCC patient livers (n=27) were analyzed by qRT-PCR for all 15 P2 purinergic receptor isoforms.

Results. Extracellular nucleotide treatment alone was sufficient to induce cell cycle progression in Huh7 cells, evidenced by increased BrdU incorporation and increased cyclin D3, E, and A mRNA and protein expression. We observed downregulation of cyclin D1 mRNA, however, as previously reported in a subset of HCC with high tumor grade. JNK inhibition attenuated nucleotide-induced cyclin D3, E and A protein expression, but enhanced downregulation of cyclin D1. Mst1/2$^{-/-}$ mouse tumors (at 3-6 months) exhibit dysregulated expression of multiple P2 purinergic receptor isoforms as compared to WT. In HCC patients, multiple P2 purinergic receptor isoforms were elevated ≥2-fold in liver tumors as compared to uninvolved areas in up to 52% of patients. P2 purinergic receptor upregulation was more prevalent among HCC patients infected with hepatitis C virus (HCV) (75%) as compared to non-viral groups (20%) identifying a unique subset of viral-induced HCC overexpressing P2 receptors.

Conclusions. Our results suggest that extracellular nucleotides are potent mitogens in Huh7 cells, inducing downregulation of cyclin D1 and upregulation of cyclin E, which are associated with poor prognosis in HCC patients. Our analysis of HCC patient and Mst1/2$^{-/-}$ mice livers has uncovered a likely role for purinergic signaling in the pathogenesis of HCC, highlighting P2 purinergic receptors as potential biomarkers and novel therapeutic targets for HCC.

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Liver Cancer In Mice With Wnt Pathway Activation in Unique Fetal Liver Progenitors.

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Hepatocellular cancer is the most common primary liver malignancy. Gene expression studies suggest that they can also arise from fetal progenitor cells or their adult progenitor progeny. We identified a unique population of fetal liver progenitors using a BAC transgenic mouse. *Cited1-GFP-CreER^{TM}*-expressing cells (*Cited1-GFP*$^{+}$) represent 4.0% of liver cells at E11.5, express markers characteristic of fetal hepatoblasts, and give rise to adult hepatocytes, cholangiocytes and SOX9^{+} periductal cells. Since the Wnt signaling pathway is critical in regulating progenitor/stem cell self-renewal and has been implicated in the etiology of cancers of rapidly self-renewing tissues, we hypothesized that its activation in *Cited1-GFP*$^{+}$ progenitors would result in HCC. We somatically and mosaically stabilized β-catenin in the *Cited1*-GFP^{+} cells, we generated tumor watch mice, *Cited1-GFP-CreER^{TM}*; *Ctnnb1*$^{ex3(fl)}$. By 26 weeks of age, >90% of *Cited1-GFP-CreER^{TM}*; *Ctnnb1*$^{ex3(fl)}$ mice developed HCC and ocassionally lung metastases. HCCs were histologically like human tumors, showed activation of Wnt, Ras/Raf/MAPK and PI3K/AKT/mTOR pathways, and expressed stem/hepatoblast markers. These data demonstrate that Wnt pathway activation is sufficient for malignant transformation of these progenitors and provide experimental support for a fetal/adult progenitor origin of some human HCCs. This model will be a valuable tool for understanding the cellular etiology and biology of HCCs and for preclinical studies.

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SOX9 and NF-Y converge on the promoter regions of cell cycle regulatory genes

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The transcription factor SOX9 plays critical roles on cell lineage specification by directly regulating target genes in various tissues. Aberrant SOX9 expression is observed in various cancers and SOX9 has also been shown to regulate cellular proliferation; however, the mechanism underlying this regulation has not been well studied. Here, we report a novel mechanism of SOX9-mediated regulation of cell division. Our genome-wide analysis of SOX9 binding to the chromatin of HT-29 human colorectal cancer cells revealed that SOX9 binds to same site of the nuclear factor Y (NF-Y), a histone-like CCAAT-binding trimer. The NF-Y complex plays a central role to support proliferation by regulating the basal transcription of regulatory genes responsible for cell cycle progression particularly in the G2/M checkpoint. In addition to the SOX9 consensus binding sequences, a motif analysis revealed a novel SOX9-binding motif (CCAAT) on the cell cycle genes that are also regulated by NF-Y, including *CCNB1*, *CCNB2*, *CDK1*, and *Topo IIa*. The peaks of SOX9 chromatin affinity on the genome overlapped with NF-Y binding to regulatory sequences of genes involved in cell cycle regulation. Interestingly, lowering the levels of SOX9 resulted in increased affinity of NF-Y proteins to its target genes. *SOX9* knockdown resulted in elevated cellular proliferation by an accumulation in the G2/M phase and increased expression of genes involved in the G2/M transition in human colorectal cancer cell lines. Collectively, our results suggest that SOX9 interferes with NF-Y binding to its target genes and that SOX9 suppresses cell proliferation via inhibition of NF-Y mediated activation of cell-cycle regulatory genes. Therefore, SOX9 levels are critical for the control of cellular proliferation and differentiation.

Screening of candidate proteins for norovirus co-receptores

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Human norovirus (HuNoVs) are a major cause of acute non-bacterial gastroenteritis; however, the mechanism of cell binding is not clear. Histo-blood group antigens (HBGAs) have been implicated in the initial binding of NoVs to cells. To determine the involvement of HuNoVs and HBGAs in cell binding, we previously investigated the localization of HuNoV virus-like particles (VLPs) and HBGAs in a human intestinal cell line, Caco-2, by immunofluorescence microscopy. Following incubation of Caco-2 cells with GII.6 VLPs, we found that VLP-cell binding depended on the state of cell differentiation, but not on the presence of type-H1, -H2 and -Le^b HBGAs. This result suggested that VLPs utilize molecule(s) other than HBGAs during binding to cells.

To explore molecules involved in HuNoV-cell binding, we screened highly expressing proteins in differentiated cells by 2D-differential gel electrophoresis (2D-DIGE). Following comparison of 2D-spot patterns of undifferentiated cells with differentiated cells, 15 spots, whose intensities increased more than 3.5-fold, were identified by MS analysis. Virus overlay binding protein assay (VOBPA)/proteomic analysis was performed to screen from another viewpoint. The VOBPA using VLPs was performed as previously reported. Concurrent with VOBPA, the same samples were subjected to another SDS-PAGE/Sypro Ruby staining, and stained-bands corresponding to VOBPA-bands were analyzed by MALDI-TOF/MS. In the VOBPA/proteomic analysis, VLPs detected 4 bands and 7-17 proteins were identified from these bands. From these experiments, we obtained candidate proteins that are being pursued as possible molecules for co-receptors for norovirus binding.

Our group has developed a plasmid-based NoV reverse genetic systems (NoV-RGS) that can produce viral particles containing genomic RNA with inserted GFP gene. We are preparing the evaluation assay to estimate candidate proteins for co-receptor function by using this system. However, production of viral particles from NoV-RGS is still not efficiently, therefore, we are optimizing parameters to increase production efficient for further analysis.

Microbiome and host factors communicate protection against acute murine colitis following omega-6 fatty acid induced transient pediatric obesity

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Background: Dietary influences may affect microbiota composition and host immune responses at the intestinal surface. These nutritionally induced biologic component changes may modulate propensity towards inflammatory bowel diseases (IBD), including Crohn disease (CD) and ulcerative colitis (UC). IBD present most commonly in young adults, indicating childhood as a potentially vulnerable developmental period for disease pathogenesis. Dietary omega-6 fatty acids (ω-6) have been associated with UC in prospective human epidemiologic studies. However, the critical developmental period, when ω-6 consumption may induce UC is not known. We examined the prolonged effects of transient high ω-6 diet during pediatric development in a murine colitis model.

Methods: C57Bl/6J mice received high (40% caloric content [cc]), or control (12% cc) ω-6 diet from postnatal day 30 (P30) to P80, then reversed to control diet for 40 days (P120). Body composition was examined by qMRI. Colitis was induced by dextran sulfate sodium (DSS). The severity of colitis was assessed by weight loss and histological scoring. Selective microbiome effects on DSS colitis were studied following fecal transplantation into germ-free mice. The mucosal microbiome was interrogated by next-generation pyrosequencing of the bacterial *16S rRNA* gene. Microarrays to study DNA methylation and gene expression from colonic mucosa were utilized. T cell populations in mesenteric lymph nodes (MLNs) and spleen were analyzed by flow cytometry. Plasma of 12 treatment naïve (TN) UC, 11 TN CD and 10 controls were used for quantification of circulating CXCL13 levels by ELISA.

Results: Mice transiently became obese on high ω-6 diet. Surprisingly, those were protected against DSS colitis. Protection against colitis was fat type and dietary reversal dependent. Germ-free mice receiving cecal content from the transiently obese mice were protected against colitis. No significant colonic mucosal DNA methylation or gene expression changes were detected. In the meantime, the number of CD4+ cells was decreased in both the spleens and MLNs of the high ω-6 reversed mice. MLN Cxcr5+/CD4+ cells, specifically, were decreased following transient obesity. Anti-Cxcl13 (the ligand of Cxcr5) antibody treatment decreased DSS induced histological colitis severity. Elevated CXCL13 concentrations (CD: 1.8-fold, p=0.0077; UC: 1.9-fold, p=0.056) were found in the serum of human pediatric IBD patients.

Summary: Loss of ω-6 diet induced pediatric obesity protected against acute colitis in mice. This phenotype was communicated by prolonged microbiome changes and associated with immune organ composition modification. The Cxcr5-Cxcl13 pathway was indicated to be an important host factor in modulating DSS colitis severity following the dietary reversal. Our human serologic observations supported the translational relevance of our findings.

The length of newly diagnosed Barrett's esophagus has been decreasing over time

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Objectives: Incidence rates of esophageal adenocarcinoma (EAC) have been increasing in the United States, however the rate of increase has slowed during the past few years (Hur et al, 2013). Longer segments of Barrett's esophagus (BE) has been implicated as a risk factor for dysplasia and EAC. Few studies have examined the temporal trends of length in newly diagnosed BE and arrived at conflicting results. The aim of this study was to identify whether there has been a change over time in the length of BE at the time of diagnosis.

Methods: This is a retrospective, single-VA center, observational study of newly diagnosed BE between 2008 and 2013. We reviewed previous records in the VA Computerized Patient Record System, verified the date of BE diagnosis, and excluded patients who were diagnosed with BE before the study period. All cases were defined by the presence of endoscopically visible BE and histologic confirmation of intestinalized columnar epithelium with goblet cells. Using the Prague C and M criteria, BE length was defined as the maximum (M) extent of the endoscopically visualized BE segment. We examined temporal changes in 1-year intervals (except for 2012 was combined with 2013) in the length of BE at the time of diagnosis.

Results: There were 212 patients with documented BE who were first diagnosed between February 15, 2008 and July 15, 2013. The mean BE length was 2.7 ± 2.9 cm in 2008 and 2.2 ± 2.9 cm in 2012-2013. The proportion of patients diagnosed with BE ≥ 3 cm per year declined during the study period, while the proportion of patients with BE ≥ 1 and < 3 cm increased, and those with BE < 1 cm remained stable (Figure 1). For example, during 2008, 33% of BE patients had BE ≥ 3 cm compared with 21% in 2012-2013. The ratio of BE < 3 cm to BE ≥ 3 cm was 2.1 in 2008 but increased to 3.8 in 2012-2013.

Most (97.2%) of the patients were male with no differences between BE < 3 cm and BE ≥ 3 cm (p= 0.34 by chi-square). Approximately 85.4% of all BE patients were Caucasian, and 14.2% were African-American with no significant yearly differences (p=0.33 by chi-square). The mean age at the time of BE diagnosis was 61.5 years (SD 7.4 years) and did not change significantly during the study period (p=0.39 by ANOVA).

Conclusion: The mean length of newly diagnosed BE has decreased during the study period as a result of a decline in BE ≥ 3 cm; these findings, if confirmed in other studies, may explain the recent slow increase in EAC.

Statin use may decrease the risk of Barrett's esophagus: A case-control study of US veterans

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Objectives: Statins have been associated with a reduced risk of esophageal adenocarcinoma, however their possible effect on the risk of developing Barrett's esophagus (BE) is unknown. This study evaluated the association between statin use and the risk of BE.

Methods: We conducted a case-control study among eligible patients scheduled for elective esophagogastroduodenoscopy (EGD) and a sample of patients eligible for screening colonoscopy recruited from primary care clinics at a single VA center. We compared 303 patients with definitive BE with two separate frequency matched control groups: 303 patients from the primary care group ("primary care controls") and 606 patients from the elective EGD group ("endoscopy controls") with no endoscopic or histopathologic BE. Index date was the earliest BE diagnosis date for cases, and the study EGD date for controls. Use of statins and other lipid lowering medications was ascertained by reviewing filled prescriptions in electronic pharmacy records during a 10-year period before index date, and use before the index date was compared between cases and controls. We calculated odds ratios (OR) and 95% confidence intervals (95% CI) using multivariable logistic regression models adjusting for age, sex, race, GERD symptoms, *H. pylori* infection, and waist-to-hip ratio (WHR).

Results: Most in the cohort were men (97.7%) and white (75.5%). Dispensed prescriptions for statins were identified in 64.6% of subjects, most of whom (94.3%) used simvastatin. The proportion of BE cases (57.4%) with filled statin prescriptions was significantly less than in endoscopy controls (64.9%; p=0.029) and primary care controls (71.3%; p<0.001). Most of the difference was related to a higher proportion of controls having statin prescriptions that were first filled earlier than 5 years before the index date than cases (p=0.001) and prescriptions that lasted for 3-10 years (33.9% vs. 28.1%; p=0.011). Longer durations of statin prescriptions were filled by the combined groups of control subjects than BE cases; mean duration (28.6 months vs. 22.1 months, p=0.001). In multivariable analysis, statin use significantly reduced the risk of BE (adjusted OR, 0.70; 95% CI, 0.52-0.93) compared with the combined control groups. The risk of BE is particularly lower with statin use among patients who were obese (OR, 0.49; 95% CI, 0.31-0.78), or had a high WHR (OR, 0.65; 95% CI, 0.48-0.89). Importantly, we found no significant association between BE and non-statin lipid lowering medications (p=0.452). Most study subjects (91.3%) reported the VA as the primary source for most medications, and 84.6% reported receiving all prescriptions from the VA pharmacy with no significant differences between cases and controls (p=0.618).

Conclusion: Statin use may decrease the risk for BE, especially among patients who are obese or have a high WHR.

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Gastrointestinal disease in neuroligin-3 linked autism is hardwired in enteric neurons

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Autism spectrum disorders (ASD) are neurodevelopmental diseases characterized by impaired social interaction, repetitive and/or restricted interests. Gastrointestinal (GI) disease is a major co-morbidity in ASD patients, and altered brain-gut function is generally assumed to be the primary etiological cause. Mutations in the neuroligin family of synaptic adhesion molecules are widely implicated in ASD. Whether such mutations also affect enteric nervous system function is not known. Here we report that GI disease in patients with a neuroligin-3 (NL3) R451C missense mutation is extended to $NL3^{R451C}$ mice, which display impaired social interaction and risperidone-reversible heightened aggression. Neuroligin-3 was identified in myenteric neurons, albeit at significantly reduced levels in $NL3^{R451C}$ mice without evidence of neuronal loss. As reported for CNS, the R451C mutation targets GABAeric signalling and altered colonic motility via $GABA_A$ receptors. Further clinical relevance was demonstrated by more frequent defecation in $NL3^{R451C}$ mice and by structural mapping of the R451C amino acid substitution to a conserved domain targeted by other ASD-associated neuroligin mutations. Structural analysis and comparison of human, rat and mouse crystal structures and homology models of NL3, show R451C located on a helix close to predicted protein-protein interface patches. This indicates conformational changes by R451C that affect protein binding. Our findings identify a novel parallel role for brain-gut communications in human disease, and implicate altered enteric synaptic function as being strongly associated with GI dysfunction in ASD.

NoroGLuc: a cell-based human norovirus protease reporter system

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Human noroviruses, a group of positive strand RNA viruses in the *Caliciviridae* family, are the leading cause of epidemic viral gastroenteritis worldwide. Our understanding of their replication and pathogenesis is limited by the inability to propagate these viruses in cell culture. Although genomic RNA isolated from Norwalk virus (NV), the prototype of human noroviruses, is able to replicate in several cell lines, a reporter system for monitoring viral RNA replication is lacking. Here we report a cell-based human norovirus protease reporter system in which the NV protein p41 is fused with *Gaussia* luciferase (NoroGLuc) through a cleavage site for the viral protease (Pro). The fusion of GLuc with membrane-associated NV protein p41 not only retains GLuc within the cells, but also renders the intracellular GLuc enzyme inactive. *Trans* cleavage of NoroGLuc by NV Pro or Pro precursors results in release and secretion of an active GLuc into the cell culture medium as a readout. Using this system, we first demonstrated that the ORF1 region of NV, which encodes the entire nonstructural polyprotein including Pro, and its processing intermediates p22VpgProPol and p22VPgPro have the most potent protease activities. We further showed that transfection of NV stool RNA resulted in measurable increase of secreted GLuc, validating that this system can detect viral RNA replication. Although designed for NV (genogroup GI.1), this system is also suitable for viral proteases of noroviruses in other genogroups. We thus have developed a cell-based, wide-spectrum human norovirus protease reporter system that can serve as a platform for detection of viral replication, identification of host factors that regulate viral replication, and validation of antiviral drugs.

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16S rRNA and Whole Metagenome Shotgun Analysis using the Genboree Workbench

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The decreasing cost, accessibility, and increasing volume of sequencing data provides unprecedented opportunities for scientific discovery. However, next-generation sequence data sets pose non-trivial data management and analysis challenges to individual investigators. There exists a need for an infrastructure that provides leading "omics" analysis tools without the overhead, cost, and learning curve required to develop and manage such a system. We have integrated a suite of analytical tools and pipelines on a web-based platform, the Genboree Workbench (http://genboree.org), to bring large data management and analysis capabilities to the individual investigator. The Genboree Workbench constitutes a cloud-based platform for collaborative sequence-centric research for web-based data analysis.

To address the needs of the metagenomics community, we have developed, integrated, and combined leading open-source tools for researchers to analyze their data without requiring programming or scripting of the tools themselves. Using a web browser, investigators can upload and analyze either 16S rRNA or whole metagenome shotgun (WMS) sequencing data. The Genboree Workbench contains tools for analyzing 16S rRNA data to explore diversity (alpha and beta diversity), taxonomic abundances, and perform analyses using machine learning methods (feature selection, classification, etc.).

We are currently developing and integrating into the Genboree Workbench a pipeline to support WMS data and analysis. The computational and hardware requirements necessary for processing WMS sequence data is dramatically higher than with 16S rRNA sequence data, which can pose significant challenges. Additionally, there is a scarcity of polished, peer-reviewed tools available for WMS experiments as compared to the availability of 16S rRNA toolsets. We have crafted a novel WMS pipeline using open-source tools that allow users to explore taxonomic abundances, produce phylogenetic trees, normalize sequence data, assemble metagenomes (contigs), predict genes, map predicted genes onto the KEGG database and derive functional annotations (orthologous genes, modules, and pathways).

Human Intestinal Enteroid Cultures: a New Functional Model of Human Rotavirus Infection

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A significant limitation in translational research is the absence of reliable pre-clinical models that mimic human physiology and disease pathology. This is particularly true in the field of human enteric diseases, which are commonly caused by gastrointestinal (GI) microbes that are difficult to culture in cancer-derived cell lines and do not infect mice. The goal of our study was to characterize new 3D human small intestinal epithelial (HIE) cultures (enteroids) as pre-clinical models of human rotaviruses (HRVs), which still kill nearly 500,000 children annually despite two licensed vaccines. We established HIEs using advancements in stem cell biology pioneered by the Clevers Lab at Hubrecht Institute in Utrecht, the Netherlands. HIEs were established from duodenal and ileal biopsy specimens and jejunal tissue from bariatric surgery patients. The HIEs self-organize into villus-crypt structures, which contain all intestinal cell types normally present in the intestinal epithelium (enterocytes, goblet cells, enteroendocrine cells, and Paneth cells). Cultures have been maintained for over 1 year and can be frozen and recultured.

RVs generally exhibit host range restriction in replication. Thus, HRVs do not replicate in mice whereas mouse RVs and some other animal RVs ([rhesus] RRV) exhibit a broader host range and do replicate in mice. To address whether RVs exhibit host range restriction in HIEs, enteroids were inoculated with either HRV or RRV. We measured the (1) amount of viral RNA using qRT-PCR and (2) number of infected cells using flow cytometry, immunofluorescence, and confocal microscopy. HRVs infected more cells within HIEs (50%) than did RRV (13%). This contrasts to replication in human colonic tumor-derived cell lines (e.g., Caco-2 or HT-29 cells) in which animal RV strains grow significantly better than HRV strains. *In vivo,* RV replication occurs in the most differentiated epithelial cells of the intestinal villi. Differentiation of cells within HIEs is driven by withdrawal of growth factors. We determined whether the state of differentiation affected the numbers of cells susceptible to infection. RV-infected cells increased from 30% to 50% when HIE differentiation was increased from 3 to 4 days, demonstrating that the status of differentiation influences the number of cells that are susceptible to HRV. Infection with RRV also increased in more differentiated HIEs from 8% to a maximum of 13% infected cells. Using electron microscopy and immunofluorescence, we have validated that RV-infected HIEs show classical features of RV-infected cells, including presence of virus factories (viroplasms) and induction of lipid droplets.

Having a robust, reproducible *ex vivo* system for HRV replication allows us to address new questions about human virus-host interactions such as what is the innate immune response to these viruses. A number of studies have examined innate responses using murine models and cultured cells and shown that results with homologous virus-host infections differ from those with heterologous virus-host infections. Moreover, data from limited studies of cytokines detected in children contradict results from the murine model. Thus, we are beginning to investigate the innate immune response to infection in the HIEs. Prior studies demonstrated that RRV and HRV infection elicits IL-8 secretion in HT-29 cells but RRV does not in Caco-2 cells. Our current results indicate RRV infection of HIEs does not induce upregulation of IL-8 as detected by qRT-PCR. However, the HIEs do produce IL-8 in response to treatment with TNF-α, a known inducer of IL-8. RNA-seq will be performed on infected enteroids to better elucidate the mechanisms by which the intestinal epithelium responds to HRV infection. These preliminary findings of host range restriction and differences in epithelial response to infection present HIEs as a new model to study HRV infection.

MicroRNAs in Crohn's disease and ulcerative colitis

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The inflammatory bowel diseases (IBD), Crohn's Disease (CD) and ulcerative colitis (UC) are chronic inflammatory conditions characterized by an inability to regulate the immune response in the gastrointestinal tract. Cytokines (IL-17A), cytokine receptors (IL-23 receptor), and pattern recognition receptors (CARD15/NOD2) are dysregulated or mutated in patients and mouse models of IBD. However, less is known about the expression of microRNAs (miRNA) in IBD. Dysregulated expression of miRNAs, both within diseased tissue and other body fluids, has been linked to a number of diseases. In an effort to develop effective non-invasive techniques for identifying colitis, we have screened a panel of miRNAs in colonic tissue, blood, and saliva samples from CD and UC patients. In these studies, we identified 4 miRNAs that were dysregulated in pooled colon samples from CD patients (miR-31, miR-101, miR-146a, and miR-375) and UC patients (miR-19a, miR-21, miR-31, and miR-101). Six and nine miRNAs were dysregulated in pooled blood samples from CD and UC patients, respectively, while one and four miRNAs were dysregulated in pooled saliva specimens. Examination of paired healthy and diseased colon biopsies from IBD patients revealed a strong correlation of elevated miRNA expression in a subset of patients. Further, we found that a miRNA target gene with immunomodulatory properties, Rc3h1, was decreased in a subset of the IBD colon specimens. These studies may have significance for developing diagnostics and immunotherapies to control intestinal inflammation.

Lactobacillus reuteri supplementation is a viable option for preventative treatment of *Clostridium difficile*-associated diarrhea

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The most prolific cause of bacterial-induced diarrhea in the U.S. is infection by *Clostridium difficile.* Up to one million cases are reported annually at a cost >$3.5 billion, with rates in some hospitals approaching 40%. In the last 15 years, the incidence of *C. difficile* infection (CDI) has more than doubled. Despite a known inverse correlation between the protective gut microbiota and the development of symptoms in infected patients, there is still a major gap in our understanding of how host bacteria protect against this pathogen. Vertical transmission of *Lactobacillus* spp. from mother to child at birth is important in establishing a healthy microbiome during the first years of life. Nourishment and secondary inoculation of the infant GI microbiota from breast milk provides another maternal source of microbes rich in lactic acid bacteria. Metagenomic studies have indicated >10-fold difference in the abundance of *Lactobacillus* in the GI tract of healthy preadolescent children compared to healthy adults. Incidence of CDI increases with age and is rare in children even in the presence of this pathogen. Our metagenomic data analysis of stool specimens from adults with recurrent CDI showed the relative abundance of *Lactobacillus* is significantly decreased. We propose that lactobacilli isolated from the infant GI tract represent candidate probiotic strains that naturally interfere with *C. difficile* cytotoxicity, and repopulation of known lacto-deficient GI microbiomes with such strains may serve as an efficient means to protect against recurrent CDI. Our central hypothesis is that the Achilles heel of *Clostridium difficile* pathogenesis is its susceptibility to secreted antimicrobials by the host microbiota. The rationale for this hypothesis is based on (1) the established concept that *C. difficile* pathogenesis occurs when the normal gut microbiota is disrupted by antibiotic use, and (2) restoration of an intact intestinal microbiota by transplantation represents the most successful clinical practice to date to prevent severe, recurrent CDI. We screened probiotic *Lactobacillus* ssp. already shown to be safe in humans, and identified *L. reuteri* as an intrinsically antibiotic-resistant strain which possessed greater cytotoxicity than currently FDA-approved vancomycin and fidaxomicin. *L. reuteri* was also shown to be resistant to three antibiotics typically used to treat CDI: vancomycin, metronidazole, and fidaxomicin. We have confirmed reuterin production by *L. reuteri* is required for *C. difficile* growth inhibition using targeted mutagenesis. Finally, we demonstrated *C. difficile* killing in the gut lumen of mice following oral synbiotic *L. reuteri* and glycerol administration, allowing development of a prototypic therapeutic concept targeting microbial infection. In keeping with our long-term goal of understanding microbiota-protective mechanisms in gut inflammation, our project aims to characterize this previously unappreciated antimicrobial mechanism, and to exploit this novel finding to develop prototypic therapeutic concepts for CDI. The impact of achieving this outcome would be to provide a novel adjunct treatment for a global epidemic that is becoming one of the major public health threats of the 21st century.

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From prediction to function using evolutionary genomics: Human-specific ecotypes of *Lactobacillus reuteri* have diverse probiotic functions

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Background: The vertebrate gut symbiont *Lactobacillus reuteri* has diversified into separate clades reflecting host origin. Its strains show evidence of host adaptation, but how host-microbe co-evolution influences microbial-derived effects on hosts is poorly understood. Emphasizing human-derived strains of *L. reuteri*, we combined comparative genomic analyses with functional assays to examine variations in host interaction among genetically distinct ecotypes.

Results: Within clade II or VI, the genomes of human-derived *L. reuteri* strains are highly conserved in gene content and at the nucleotide level. Between clades, they share only 70-90% of total gene content, indicating differences in functional capacity. Human-associated lineages are defined by genes related to bacteriophages, arginine catabolism, vitamin biosynthesis, antimicrobial production, and immunomodulation. Differential production of folate, reuterin, and histamine were demonstrated in 23 strains belonging to clades II and VI. These strains also differed with respect to human cytokine production (TNF, MCP-1, IL-1β, IL-5, IL-7, IL-12, and IL-13) by myeloid cells. Microarray analysis revealed global regulation of genes within the reuterin, vitamin B_{12}, folate, and arginine catabolism gene clusters by the AraC family transcriptional regulator, PocR.

Conclusions: Human-derived strains within *L. reuteri* clades II and VI are genetically distinct and these differences affect their functional repertoires and probiotic applications. These findings highlight the biological impact of microbe:host co-evolution and illustrate the functional significance of subspecies differences in the human microbiome. Consideration of host origin and functional differences at the subspecies level may have major impacts on probiotic strain selection and considerations of microbial ecology in mammalian species.

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Ablation of ghrelin receptor attenuates high fructose corn syrup (HFCS)-induced adipose inflammation and insulin resistance

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Adipose inflammation and insulin resistance play causal roles in type 2 diabetes. High fructose corn syrup (HFCS) is the most-used sweetener in the United States, substantially replacing table sugar. Some studies have suggested that HFCS consumption correlates with obesity and insulin resistance, while others are in disagreement. Due to conflicting and insufficient scientific evidence, HFCS continues to be used as the primary sweetener in our food supplies.

In this study, we compared the metabolic effects of mice fed regular diet, high fat diet, or regular diet supplemented with 8% HFCS in drinking water (to mimic soft drinks). As expected, high fat diet-fed mice consumed the most calories, and showed the highest weight gain and fat deposition. Surprisingly, HFCS-fed mice exhibited most severe insulin resistance, which was disproportionate to calorie intake and body fat content. Adipose tissue macrophages (ATMs) play an important role in the pathogenesis of obesity and insulin resistance. Here we show that similar to high fat diet; HFCS triggered a robust increase of both pro-inflammatory ATMs ($F4/80^+CD11c^+$) and anti-inflammatory ATMs ($F4/80^+CD11c^-$) in visceral fat. Remarkably, however, the anti-inflammatory ATMs were much less abundant in HFCS-fed mice than in high fat-fed mice.

Ghrelin is an orexigenic hormone, promoting adiposity and insulin resistance. We found that deletion of ghrelin receptor growth hormone secretagogue receptor (GHS-R) ameliorates HFCS-induced adipose inflammation, insulin resistance and liver steatosis.

Thus, HFCS consumption has detrimental effect on metabolism beyond the calories associated with it, and GHS-R antagonists may represent novel therapeutic option for insulin resistance.

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Early activation of P2Y2 purinergic signaling is essential for efficient hepatocyte proliferation in response to partial hepatectomy.

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Background & Aims. Partial hepatectomy (PH) induces hepatocyte proliferation via step-wise induction of immediate early genes, reorganization of extracellular matrix, and cytokine and growth factor-mediated signaling early on during liver regeneration. However, the identity of initial trigger(s) of liver regeneration has remained elusive. ATP is released into the extracellular milieu within minutes of 70% PH. We hypothesized that extracellular ATP, via activation of its cognate cell-surface P2Y2 purinergic receptors, might play a key role in the induction of early events critical for hepatocyte proliferation in regenerating livers.

Methods. Wild type (WT) and P2Y2 purinergic receptor knockout (P2Y2-/-) mice were subjected to 70% PH and liver tissues were analyzed for efficiency of hepatocyte priming and proliferation. Influence of extracellular ATP and P2Y2 puringeric receptor signaling on hepatocyte proliferation was evaluated *in vitro*.

Results. Our findings suggest that hepatocyte proliferation in response to 70% PH was impaired in P2Y2-/- mice. Early activation of p42/44 MAPK (ERK, 5 min), early growth response-1 (Egr-1) and activator protein-1 (AP-1) DNA-binding activity (30 min) were attenuated in the remnant livers of P2Y2-/-. Correspondingly, Egr-1 and AP-1 target gene and a key mediator of extracellular matrix remodeling, matrix metalloprotese-9 (MMP-9) protein induction and HGFα/c-Met signaling were attenuated in P2Y2-/-. Extracellular ATP alone, via the activation of P2Y2 purinergic receptors, was sufficient to activate ERK/Egr-1 and proliferation of primary mouse hepatocytes *in vitro*.

Conclusions. Extracellular ATP-mediated rapid activation of P2Y2 purinergic receptors plays a key role in the initiation of hepatocyte proliferation in response to PH in mice.

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Alterations in hepatic nuclear receptor function in a Wilson's disease mouse model and implications for Wilson's disease patients

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Wilson's disease is an autosomal recessive disease that results in hepatic copper accumulation due to mutations in the Cu-transporting P-type ATPase (ATP7b) and is a chronic liver disorder that is associated with a variety of symptoms including steatosis, cholestasis, cirrhosis, and liver failure. The Atp7b$^{-/-}$ mouse has increased hepatic copper levels similar to those of Wilson's disease patients, as well as increased nuclear copper concentrations. Microarray analysis in 6-week old Atp7b$^{-/-}$ mice showed altered expression of several nuclear receptor metabolic target genes, which correlated with elevated nuclear copper levels. Previous studies demonstrated that copper has a high affinity for the estrogen receptor DNA binding domain (ER-DBD) and disrupts ER binding to ER-response elements. Our hypothesis is that elevated hepatic copper concentrations results in decreased nuclear receptor regulation of metabolic target gene expression. Results: Hepatic nuclear receptor target gene mRNA expression was decreased at 6, 12, and 20 weeks of age in the Atp7b$^{-/-}$ mice: FXR (Bsep and SHP, 40 and 20%), HNF4α (Ntcp, 20%), LRH-1 (Cyp8b1, 40%; Abcg8, 20%), and TR (Spot14 and G6Pase, 40 – 20 %). ChIP analysis confirmed decreased binding of nuclear receptors at 20 weeks of age in the Atp7b$^{-/-}$ mice: FXR (Bsep and SHP promoters), HNF4a (Cyp7a1, Cyp8b1, and apoCIII promoters) and LRH-1 (Cyp8b1 promoter). We extended our studies into adult human specimens obtained from the National Disease Research Interchange (NDRI). Hepatic copper concentration and mRNA expression was measured in the control (patients with no known liver disease) and Wilson's disease samples. Only one of the Wilson's disease samples had hepatic copper concentrations within the Wilson's disease diagnostic criteria (451.06 μg/g liver dry weight) versus 19 and 26 μg/g liver dry weight of the other Wilson's disease samples; the average copper concentration of the control samples was 18.6 μg/g liver dry weight. Relative to the control and Wilson's disease samples with normal hepatic copper levels, the Wilson's disease sample with elevated hepatic copper levels had decreased mRNA expression of BSEP (73%), SHP (75%), and SPOT14 (50%) whereas MRP2 and NTCP were unchanged. CYP7A1 mRNA expression was elevated (2.5-fold increase) relative to patients with normal copper levels, which may reflect loss of FXR-mediated repression of CYP7A1 expression. As expected, all Wilson's disease samples had increased mRNA expression of the fibrogenic markers collagen 1A1, COL1A1 (3-fold increase relative to controls) and tissue inhibitors of metalloproteinases, TIMP (3.8-fold increase relative to controls). Taken together, our studies provide new insight into the effect of excessive hepatic copper on nuclear receptor function in both the Atp7b$^{-/-}$ mouse and patients with Wilson's disease. Our future studies will include patients with cholestatic retention of copper, as well as determine therapeutic strategies for alleviating nuclear receptor disruption in models of excessive hepatic copper levels.

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Low Abdominal NIRS Values and Elevated Serum Intestinal Fatty Acid-Binding Protein Predict Necrotizing Enterocolitis in a Premature Piglet Model

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Purpose: To identify early markers of necrotizing enterocolitis (NEC), we hypothesized that continuous abdominal near-infrared spectroscopy (A-NIRS) measurement of splanchnic tissue oxygen saturation and serum intestinal fatty-acid binding protein (*s*I-FABP) can detect NEC prior to onset of clinical symptoms.

Methods: Premature piglets received parenteral nutrition for 48-hours after delivery, followed by enteral feeds every three hours until death or euthanasia at 96-hours. Continuous A-NIRS, oxygen saturation, and heart rate were measured while monitoring for clinical signs of NEC. Blood samples obtained at 6-hour intervals were used to determine *s*I-FABP levels by ELISA. Presence of NEC was assessed by a validated clinical score and confirmed by histology. Data were analyzed using Student's *t*-test and receiver operating characteristic curves.

Results: Of 43 piglets, 49% developed NEC and 51% were No-NEC littermate controls. A-NIRS values within the first 3-hours of life were lower in the NEC group (71±4.4%) compared to littermate controls (79±1.9%; p=0.003) and remained lower throughout the study. A-NIRS $\leq$75% predicted NEC with 94% sensitivity and 94% specificity. Mean *s*I-FABP was higher in animals that developed NEC (0.66±0.62ng/ml) compared to littermate controls (0.09±0.05ng/mL; p<0.001). In the NEC group sI-FABP increased from 0.04±0.06ng/mL on parenteral nutrition to (0.7±0.76ng/mL; p<0.001) after feeds. *s*I-FABP levels increased in parallel with disease progression and a value $\geq$0.25ng/mL identified animals with NEC (71% sensitivity and 95% specificity).

Conclusions: In premature piglets, low A-NIRS in the early neonatal period predicts NEC and *s*I-FABP increases with disease progression. These modalities may help identify neonates with NEC prior to clinical manifestations of disease.

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