Doctoral Dissertation and Research Experience

1) Defining the association between gut microbiome metabolism of dietary trimethylamines and the development of atherosclerosis (01/2011 – 05/2014)

Mentors: Stanley L. Hazen, M.D.,Ph.D., Joseph DiDonato, Ph.D., Robert Koeth, M.D.,Ph.D.

Institution: Department of Cellular and Molecular Medicine, Lerner Research Institute, Cleveland Clinic

Study: During my three and a half-year tenure in Dr. Hazen's laboratory, I supported and executed experiments on multiple ongoing projects characterizing the link between dietary lipid, the gut microbiome and atherosclerosis. The Hazen laboratory had demonstrated both phosphatidylcholine and L-carnitine are metabolized to an intermediate gaseous compound, trimethylamine (TMA) by the gut microbiome. TMA is further oxidized by liver flavin monooxygenases to yield trimethylamine N-oxide (TMAO). This terminal product is an independent predictor of major adverse cardiovascular events and has been shown to promote cholesterol accumulation and enhance platelet hyperactivity and thrombosis risk. My work centered on trimethylamine-containing metabolites related to L-carnitine, namely Gammabutyrobetaine (ɣBB). We discovered ɣBB is a gut-microbiome dependent product of L-carnitine and is also converted to TMA in a gut-microbiome-dependent manner. Gut microbial composition and functional metabolic studies reveal that distinct taxa in separate areas of the gut are associated with the production of ɣBB or TMA/TMAO from dietary L-carnitine. Moreover, chronic dietary exposure to L-carnitine or ɣBB promotes development of functionally distinct microbial communities optimized for the metabolism of L-carnitine or ɣBB, thereby linking lipid intake with a pro-atherogenic microbiome.

Techniques: Early in my training, I spent time breeding and genotyping the large mouse colony. I would also maintain ongoing large-scale mouse feeding studies and perform subsequent procedures like cardiac punctures, oral gavage, peritoneal lavage for macrophages, saphenous vein bleeding, and complete tissue harvest. I eventually learned various procedures associated with tissue analysis from these *in vivo* studies: sectioning, Oil Red O staining, and quantifying aortic lesions; RNA extraction from sample followed by RT-qPCR; plasma analysis with stable isotope dilution LC/MS/MS; triglyceride and cholesterol counts in the liver via GC/MS and spectrophotometer, respectively. I participated in the organic synthesis of deuterated trimethylamine-containing compounds for murine and human clinical studies. As I became more proficient in these techniques, I was given more responsibility and designed and executed these experiments from beginning to end.

Accomplishments: This opportunity provided me with exceptional mentorship from MD/PhD students and staff scientists alike and a new understanding of the critical and exciting potential for scientific discovery in medicine. Importantly, I was able to make productive contributions to several ongoing projects, as reflected by peer-reviewed co-authorships in *Cell Metabolism, Cell,* and *Nature Medicine*.

1. Huang Y, DiDonato JA, Levison BS, Schmitt D, Li L, Wu Y, Buffa JA, Kim T, Gerstenecker GS, Gu X, Kadiyala CS, Wang Z, **Culley MK**, Hazen JE, Didonato AJ, Fu X, Berisha SZ, Peng D, Nguyen TT, Liang S, Chuang CC, Cho L, Plow EF, Fox PL, Gogonea V, Tang WH, Parks JS, Fisher EA, Smith JD, Hazen SL. An abundant dysfunctional apolipoprotein A1 in human atheroma. *Nat Med* **20**, 193-203 (2014)
2. Koeth RA, Levison BS, **Culley MK**, Buffa JA, Wang Z, Gregory JC, Org E, Wu Y, Li L, Smith JD, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. γButyrobetaine is a proatherogenic intermediate in gut microbial metabolism of *L*-carnitine to TMAO. *Cell Metab*. **20**, 799-812 (2014).
3. Wang Z, Roberts AB, Buffa JA, Levison BS, Zhu W, Org E, Gu X, Huang Y, Zamanian-Daryoush M, **Culley MK**, DiDonato AJ, Fu X, Hazen JE, Krajcik D, Didonato JA, Lusis AJ, Hazen SL. Non-lethal inhibition of gut microbioal trimethylamine production for the treatment of atherosclerosis. *Cell* **163**, 1585-95 (2015).

2) Caspase-11 activation by lipopolysaccharide contributes to the inflammatory response in hepatocytes (06/2014 – 08/2014)

Mentors: Timothy R. Billiar, M.D., Melanie J. Scott, M.D.,Ph.D.

Institution: Department of Surgery, University of Pittsburgh Medical Center

Study: Sepsis, in response to severe gram-negative bacterial infection, is the driving cause of morbidity in hospital intensive care units. Lipopolysaccharide (LPS), endotoxin from gram-negative bacteria, elicits a rapid innate immune response by stimulating the release of cytokines IL-1β and IL-18 and other inflammatory mediators from immune cells. Toll-like Receptor 4 (TLR4) mediates this response in coordination with canonical inflammasome activation of caspase-1. LPS can also activate the noncanonical inflammasome to cleave procaspase-11 to caspase-11 (caspase-4 and -5 in humans), promoting cell death in a TLR4-independent manner. The function of activated caspase-11 in non-immune cells has yet to be explored. We sought to determine the function of caspase-11 in hepatocytes stimulated with LPS by employing poly-A RNA sequencing. Preliminary data generated via RT-qPCR revealed treatment of primary C57Bl/6 hepatocytes with LPS significantly increased caspase-11 mRNA expression. TLR4-/- did not demonstrate increased caspase-11

expression with LPS treatment, illustrating caspase-11 mRNA upregulation may TLR4-dependent in hepatocytes. Primary hepatocytes from C57Bl/6, TLR4-/- and Caspase-11-/- mice were treated with LPS and collected for poly-A RNA sequencing. Results from the CuffDiff biostatistical program revealed over 3000 genes of interest. Preliminary review of RNA sequencing demonstrated a large increase in proinflammatory chemokines, cytokines, and receptors with LPS stimulation in hepatocytes from C57Bl/6 or Caspase11-/- mice with differential expression and fold variations dependent upon genotype. Results confirming TLR4-dependent activation of caspase-11 by LPS in hepatocytes improve our understanding of LPS recognition, uptake, and clearance by the liver and highlight a role for non-immune cells in the innate immune response to severe gram negative bacterial infection. Marked fold changes in specific proinflammatory transcripts could provide new targets for blocking inflammation associated with sepsis.
Techniques: I spent my short time in the Billiar laboratory focused on optimizing an RNA extraction and purification protocol. I worked closely with a collaborating laboratory to better understand poly-A RNA sequencing and perform initial bioinformatics analysis on the produced data set.

3) mRNA stability of Fbxo3 is marginally increased by LPS treatment of MLE cells (06/2015 – 08/2015)
Mentors: Rama K. Mallampalli, M.D., Yutong Zhao, M.D., Ph.D., Jing Zhao, M.D., Ph.D.
Institution: Department of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine
Study: A key feature of severe bacterial infections is TRAF-mediated release of large amounts of cytokines, which in turn leads to severe inflammation and tissue injury. Tumor necrosis factor receptor-associated factors (TRAFs) are a group of adaptor proteins that transduce immune signals to control aberrant cytokine release and inflammation. This is a critical feature of acute lung injury. Specific recognition of TRAFs by Fbxl2, a pan-reactive F box subunit in the Skp-Cullin1-F box (SCF) superfamily of ubiquitin E3 ligases, has been shown to decrease TRAF stability. Fbxl2 itself is targeted for ubiquitination and degradation by a pro-inflammatory F box protein, Fbxo3, which leads to stabilization of TRAF and an increase in cytokine-driven inflammation. However, the mechanism of regulation of the Fbxo3 gene remains unknown. We hypothesized LPS treatment of lung epithelial cells would increase the transcription of Fbxo3. Conversely, there was no increase in Fbxo3 protein when MLE cells were stimulated with LPS at variable times and doses. Full promoter activity did not increase from baseline when stimulated with LPS. More experimentation is required to complete the story of pathological posttranscriptional regulation of Fbxo3 expression.
Techniques: I cultured and treated MLE cells and collected them for mRNA and protein quantification by RT-qPCR and immunoblotting. I also worked to create plasmids with different fragments of Fbxo3 promoter sequence and transcriptional activity was measured via luciferase assay.
Accomplishments: I was able to contribute both experimentally and conceptually to a side project while working with Drs. Yutong and Jing Zhoa. I also drafted a significant portion of the manuscript.

1. Nan L, Wei J, Jacko AM, **Culley MK**, Zhao J, Natarajan V, Ma H, Zhao Y. Cross-talk between lysophosphatidic acid receptor 1 and tropomyosin receptor kinase A promotes lung epithelial cell migration. *Biochim Biophys Acta* **1863**, 229-35 (2016).

These two distinctive rotations (**2**, **3**) allowed me to train in diverse environments and learn new experimental techniques while also reinforcing that my genuine scientific interest is in vascular biology. Taken together, these experiences provided an excellent introduction to medical science and will serve as a strong foundation in starting graduate school. I completed my third and final research rotation with Dr. Stephen Chan (07-2016 – 09 2016) during which time I began to generate the preliminary data present in this proposal. I will stay in Dr. Chan's laboratory for my dissertation and thus my fellowship training. All of these research experiences build upon one another, ultimately propelling me in my training to become a physician scientist. I have learned how to formulate hypotheses, execute experiments, and formulate my findings into manuscripts and presentations. The skills I have developed, guided by professional and individualized mentorship, will allow me to be successful during my graduate education and in future research endeavors.