Investigating implications and mechanisms of diet induced obesity for multi organ function in a murine model of early sepsis
Investigating implications and mechanisms of diet induced obesity for multi organ function in a murine model of early sepsis

By Momina Khan

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Title: Investigating implications and mechanisms of diet induced obesity for multi organ function in a murine model of early sepsis

Author: Momina Khan BSc. (Hon) (McMaster University)

Supervisor: Dr. Alison Fox-Robichaud

Supervisory Committee: Dr. Patricia Liaw
Dr. Geoff Werstuck
Dr. Bernardo Trigatti

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1.0 Abstract

Given the current obesity epidemic, the prevalence of overweight and obese patients with critical illness is increasing rapidly, however how obesity shapes critical illness and immune response to infection is not entirely understood. We developed a clinically relevant murine model of obesity in the context of sepsis, and examined organ specific inflammatory responses. Male C57BL/6 mice were fed either a high fat Western Diet (WD) (Modified Breslow, 21% Butterfat and 0.15% cholesterol) or normal chow diet (NCD) for 6, 15 or 27 weeks. Sepsis was induced by cecal ligation and perforation (CLP), and six hours post-surgery, plasma and tissue samples were harvested and flash frozen in liquid nitrogen. Septic obese mice at 15 and 27 weeks had significantly (p<0.0001) lower levels of lung myeloperoxidase (26.3±3.8 U/mg tissue) compared to age matched ad libitum (44.1±2.8 U/mg tissue) and diet restricted (63.2±5.60 U/mg tissue) controls, indicative of less lung inflammation. Obese mice (4.23±0.10g) had significantly enlarged livers compared to controls (1.55±0.80g and 1.22±0.031g), with pronounced steatosis, and hepatocyte ballooning, independent of sepsis. These findings are in congruence with clinical observations that obese individuals are protected from sepsis-induced lung injury, however the mechanisms involved are not entirely clear.

We also examined effects of housing conditions on susceptibility to developing metabolic syndrome, and inflammatory response in our obesity and sepsis model. For this study, animals were fed either WD or NCD for 15 weeks and were housed in static or
ventilated cages. Unlike static cages, ventilated cages have HEPA filtered air supply
system and exhaust air ventilation, protecting the animals from air borne particles and
preserving the microbiological barrier. Therefore, ventilated cages provide a more sterile
environment compared to static cages. After 15 weeks, fecal matter was collected from
the cages and mice were subjected to sepsis using the CLP technique. Six hours post
surgery, animals were sacrificed and tissues were harvested, snap frozen and stored at
-80°C. The animals from the more sterile environment (ventilated cages) had
significantly (p<0.0001) less weight gain and did not show signs of overt hyperglycemia,
compared to mice housed in a less sterile environment (static cages). In addition, obese
mice housed in static cages had less lung injury compared to controls during early sepsis,
however this difference was not evident in mice from ventilated cages. There were also
significant differences in the fecal microbe composition, where ventilated groups had
greater Firmicutes (69% ± 0.06% for WD and 76% ± 0.03%) and less Bacteroidetes
population (15% ± 0.04% for WD and 12% ± 0.02% for NCD) compared to static groups
(Firmicutes: 42% ± 0.08% for WD and 24% ± 0.02% for NCD, Bacteroidetes: 37% ± 12%
for WD and 53% ± 29% for NCD). This study highlighted the impact of environment on
the susceptibility to developing metabolic syndrome, and the potential impact on the
associated immune responses, in our mouse models of obesity and sepsis.

Leptin is an important mediator of immune responses to infection, and the levels
are elevated during diet induced obesity in both mice and humans. We found that mice
treated with leptin one hour prior to surgery, had significantly less injury (32.62±1.6 U/mg tissue) compared to saline treated animals (46.58±3.48 U/mg tissue), as evident from lung myeloperoxidase levels and histopathology scores. In addition, proprotein convertase subtilisin/kexin type 9 (PCSK9) over expressing mice on a normal diet, had significantly greater lung injury (46.51±4.51 U/mg tissue myeloperoxidase levels) compared to knockouts (31.14±1.75 U/mg tissue), this difference was not observed in WD fed mice with differential PCKS9 expression. In conclusion, WD fed mice had significantly less lung inflammation but greater hepatic injury. Furthermore, both leptin and PCSK9 are important mediators of lung inflammation in early sepsis.
1.1 Acknowledgments

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Lastly, I would like to thank my family that provided endless support through this journey and were always a source of strength.
1.2 List of Abbreviations

Activated Protein C (APC)
Acute Lung Injury (ALI)
Acute Respiratory Distress Syndrome (ARDS)
Ad libitum (Ad lib)
Advanced Glycation End products (AGEs)
American Association of Clinical Endocrinologists (AACE)
Jun amino-terminal kinase (JNK)
Cardiovascular Disease (CVD)
Cecal Ligation and Puncture (CLP)
Cholecystokinin (CCK)
Cholesteryl Ester Transfer Protein (CETP)
Cluster of Differentiation 4 (CD4)
Cluster of Differentiation 4 (CD8)
Colon Ascendens Stent Peritonitis (CASP)
Colony Forming Units (CFU)
Coronary Heart Disease (CHD)
Colony Forming Units (CFU)
Diet Induced Obesity (DIO)
End Stage Renal Disease (ESRD)
Escherichia coli (E. coli)
European Group for the study of Insulin Resistance (EGIR)
Extracellular signal Related Kinase (ERK)
Familial Hyper Cholesterolemia (FH)
Free Fatty Acids (FFA)
Germ Free (GF)
Glucose Transport 4 (GLUT 4)
Glucose Tolerance Test (GTT)
Hematoxylin and Eosin (H&E)
Hexadecyl trimethyl ammonium bromide (HTAB)
High Fat Diet (HFD)
High Mobility Group Box-1 Protein (HMGB-1)
Hormone-Sensitive Lipase (HSL)
Hypoxia Inducible Factor-1 (HIF-1)
Inhibitor of nuclear factor kappa-B kinase (IKK)
Insulin Receptor (IR)
Insulin Receptor Substrate-1 (IRS-1)
Intensive Care Unit (ICU)
Interferon-γ (INF-γ)
Interleukin-1 (IL-1)
Interleukin-1 alpha (IL-1α)
Interleukin-1 beta (IL-1β)
Interleukin-6 (IL-6)
Interleukin-4 (IL-4)
Interleukin-10 (IL-10)
Interleukin -12 (IL-12)
International Diabetes Federation (IDF)
Intracellular Adhesion Molecule-1 (ICAM-1)
Intraperitoneal (I.P)
Intravenous (I.V)
Isolated Lymphoid Follicles (ILFs)
Keratinocyte Chemoattractant (KC)
Knock Out (KO)
Lipopolysaccharide (LPS)
Low Density Lipoprotein (LDL)
Low Density Lipoprotein Receptor (LDLR)
Macrophage Inflammatory Protein-1 (MIP-1)
Matrix Metalloproteinases (MMPs)
Methicillin Resistant S. Aureus (MRSA)
Monocyte Chemoattractant Protein-1 (MCP-1)
Myeloperoxidase (MPO)
National Cholesterol Education Program Adult Treatment Panel (NCEP/ATP)
Neuropeptide-Y 1 Receptor (NPY1R)
New Zealand Obese (NZO)
Nitric Oxide (NO)
Normal Chow Diet (NCD)
Normal Chow Diet Restricted Group (NCD DR)
Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB)
Otsuka Long Evans Tokushima Fatty (OLETF)
Over expressing Transgenic (Tg+)
Peroxisome Proliferator-Activated Receptor γ (PPARγ)
Phosphate Buffered Saline (PBS)
Plasminogen Activator (PA)
Plasminogen Activator Inhibitor-1 (PAI-1)
Plasminogen Activator Inhibitors (PAIs)
Plasma Lipid Transfer Protein (PLTP)
Polymorphonuclear Neutrophil (PMN)
Principal Coordinates Analysis (PCoA)
Proopiomelanocortin (POMC)
Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9)
Proprotein Convertase Subtilisin/Kexin type 9 Knock Out (PCSK9 KO)
Protease Activated Receptors (PARs)
Regenerating Islet-Derived 3 γ (REG3 γ)
Standard Error of the Mean (SEM)
Specific Pathogen Free (SPF)
Systemic Inflammatory Response Syndrome (SIRS)
Toll-like Receptor (TLR)
Tumor Necrosis Factor-α (TNF-α)
type-Plasminogen Activator (t-PA)
urokinase type-Plasminogen Activator (u-PA)
Western Diet (WD)
White Blood Cell (WBC)
Wistar Kyoto fatty (WDF)
Wistar-Kyoto (WKY)
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1.4 Format and organization of thesis

This thesis was prepared in the ‘sandwich format’ as outlined by the School of Graduate Studies, in the ‘Guide for Preparation of Thesis’. This thesis comprises of a general introduction, followed by three original research papers, followed by a summary of findings and future directions. Manuscript 1 is published, while manuscripts 2 and 3 are in preparation for submission.
2.0 Metabolic syndrome: obesity and type II diabetes

For tens of thousands of years, the human body has battled the agony of famine but for the last several decades it’s been fighting an onslaught of food. This radical reversal of conditions has disrupted normal physiological process, giving rise to the metabolic syndrome. Characteristics of the metabolic syndrome were first observed by a Swedish physician, Kylin, in the 1920s (Kylin 1923). Kylin identified the link between hypertension, hyperglycemia and gout (Kylin 1923). In 1947 firm connections between visceral adiposity, cardiovascular diseases and type II diabetes were established (Vague 1947). Furthermore, in 1965, Avogaro and Crepaldi grouped hypertension, hyperglycemia and obesity as part of one complex syndrome (Avogaro and Crepaldi 1965). The condition was initially referred to as ‘Syndrome X’ (Reaven 1988), ‘The Deadly Quartet’ (Kaplan 1989) and later in 1992, as the ‘The Insulin Resistance Syndrome’ (Haffner et al. 1992). Over the years several definitions of metabolic syndrome have been suggested, however the most commonly used ones are by World Health Organization (Albertini et al. 1998), the European Group for the study of Insulin Resistance (EGIR) (Balkau and Charles 1999), National Cholesterol Education Program Adult Treatment Panel (NCEP/ATP) (Detection, Evaluation, and Adults 2001), American Association of Clinical Endocrinologists (AACE) (Einhorn et al. 2003) and International Diabetes Federation (IDF) (International Diabetes Federation 2015). Although the definitions overlap, there are differences among the cut offs suggested for body weight, lipids, blood pressure and glucose levels. The current commonly used definition is the
one proposed by the IDF in April 2005 (International Diabetes Federation 2015). It was also observed that certain ethnic groups have a higher risk of type II diabetes at a much lower level of adiposity and waist circumference. Keeping these variations in sight, the IDF proposed specific criteria for certain ethnic groups (Alberti et al. 2009; International Diabetes Federation 2015).

The term metabolic syndrome refers to a series of physiological processes that have been observed to occur together, and which include chronic inflammation, central visceral adiposity, insulin resistance, high blood pressure and dyslipidemia, as depicted in illustration A ‘characteristics of metabolic syndrome’ (Alberti et al. 2009). If not corrected, these factors increase the risk of developing type II diabetes by five-fold and cardiovascular diseases by two-fold (Alberti et al. 2009). Individuals with metabolic syndrome also have two to four times increase in the risk of developing stroke and myocardial infarction, and two times greater risk of dying from these conditions (Alberti et al. 2016). Although there isn’t enough research examining the link between metabolic syndrome and risk of infection, studies do suggest that it increases the risk of post surgery infectious complications (Sahin et al. 2015). Obesity has been identified as a risk factor for surgical site, nosocomial, gum and skin infections (Huttunen and Syrjanen 2013). There is also data to suggest that obesity increases the risk of developing influenza (Louie et al. 2011; Yu et al. 2011), bacteremia (Ashley et al. 2004; Spelman et al. 2000) and sepsis (Kakarla et al. 2011).
Illustration A: Characteristics of Metabolic Syndrome

Metabolic syndrome is a cluster of conditions that include chronic inflammation, central visceral adiposity, high waist circumference, insulin resistance, high blood pressure and dyslipidemia. It increases the risk for development of type II diabetes (Wilson et al. 2005), heart disease (Wilson et al. 2005) and stroke (Gupta et al. 2010).
2.1 Insulin resistance and type II diabetes

Insulin and glucagon are hormones released by cells in the islet of Langerhans in the pancreas, and are responsible for regulating the storage and use of dietary fuel including protein, carbohydrates and lipids, in response to energy intake and expenditure (Burke, Karlstad, and Collier 2016). This is mainly achieved by maintaining the balance between gluconeogenesis and glycogen synthesis in the liver, skeletal muscle and adipose tissue. Blood glucose level is required to be constantly maintained in a narrow range that is considered optimal (70-100 mg/dL or 3.9-5.6 mmol/L) (Burke, Karlstad, and Collier 2016). Under normal conditions carbohydrate intake increases plasma glucose levels, and the release of insulin from the pancreas (Samuel and Shulman 2012). Insulin stimulates glucose uptake and glycogenesis in the skeletal muscle and liver (Samuel and Shulman 2012). In the adipose tissues, insulin stimulates lipogenesis and prevents lipolysis from occurring (Samuel and Shulman 2012). During fasting there is a reduction in circulating insulin levels, promoting hepatic glycogenolysis and lipolysis in the adipose tissue (Samuel and Shulman 2012). In type II diabetes, excess lipid accumulation prevents normal glucose uptake by the skeletal muscle in response to insulin, redirecting glucose for liver uptake (Samuel and Shulman 2012). Hepatic lipid accumulation disrupts insulin regulation of gluconeogenesis and glucose storage (Samuel and Shulman 2012). In the liver lipogenesis continues, contributing to the development of non alcoholic fatty liver disease (Samuel and Shulman 2012). Therefore,
insulin orchestrates a fine balance between glycogenesis and gluconeogenesis in response to energy intake and output. This balance is disrupted by lipid accumulation, that often occurs in conjunction with chronic inflammation, bringing forth the onset of hyperglycemia.

**2.2 Epidemiology of metabolic syndrome**

The prevalence of metabolic syndrome differs between urban and rural areas as well as gender, age and ethnicity. IDF reported that at least one quarter of the world’s adult population has metabolic syndrome (International Diabetes Federation 2015). Factors such as gender, age, socioeconomic status and ethnicity influence susceptibility to developing metabolic syndrome (Genovefa et al. 2007). Poor diet, physical inactivity, smoking and having a family history also increase the risk. Normal weight individuals have a 4.6%, overweight a 22.4% and obese individuals a 59.6% risk of developing metabolic syndrome (Park et al. 2003). Similarly, there is a 10% risk of developing metabolic syndrome for individuals in the age group of 20-29, 20% for 40-49 and 45% for 60-69 age groups (Ford et al. 2002). Gender also plays a role, where males have an 8% to 43% risk, for females the risk is between 7% to 56% (Cameron, Shaw, and Zimmet 2004). Palaniappan et al. in a study with 714 white, black and Hispanic, non diabetic participants sought to investigate the predictors of metabolic syndrome (Palaniappan et al. 2004). Fasting glucose, fasting insulin, lipids (HDL, triglycerides), blood pressure, waist size and exercise levels were all quantified (Palaniappan et al. 2004). It was determined
that waist circumference was the optimal predictor of the onset of metabolic syndrome, along with cholesterol, HDL and proinsulin levels (Palaniappan et al. 2004). Losing 5% in weight reduced systolic blood pressure as well as adipocytes expression of renin, aldosterone and angiotensinogen system (Engeli et al. 2005). Weight loss also lowered lipid levels (Van Gaal, Wauters, and De Leeuw 1997), hypertension (Whelton et al. 1998) and improved glucose tolerance (Wing et al. 1987). Thus, treatment for metabolic syndrome includes losing excess weight through a balanced diet and regular physical activity, and in some cases bariatric surgery. Medication such as statins may be used for treating dyslipidemia (Clark 2003) and metformin and thiazolidinediones (Diabetes Prevention Research Group 2005) for controlling hyperglycemia. Therefore, obesity precedes metabolic syndrome, and reducing waist circumference (Palaniappan et al. 2004) and overall body weight may be vital in preventing the occurrence of metabolic syndrome in nondiabetics.

2.3 Pathophysiology of metabolic syndrome

Obesity is associated with the increase of adipose tissue, which consists of adipocytes, stromal preadipocytes, immune cells and endothelium. In response to the nutrient level, adipose tissue may undergo adipocyte hypertrophy or hyperplasia (Halberg et al. 2008). The main cell types of adipose tissues are referred to as adipocytes which hold excess calories in the form of triglycerides (Perrini et al. 2013). Expansion of adipocytes as in the case of obesity, cause interstitial oxygen tension to
Hypoxia Inducible Factor-1 α (HIF-1α) is an oxygen sensor, which is degraded under non-hypoxic conditions (Nallamshetty et al. 2013). During hypoxia, HIF-1α stabilizes and upregulates genes with HIF response element (Nallamshetty et al. 2013). Over expression of HIF-1α in adipocytes have pro fibrotic and pro inflammatory effects (Halberg et al. 2009). The proinflammatory effects occur due to the increased c-Jun N- terminal kinase (JNK) and inhibitor of nuclear factor kappa-B kinase (IKK) expression (He et al. 2011). This activates NFκβ, and the p65 subunit binds to cyclooxygenase-2 promoter, upregulating the inflammatory gene expression (Fitzpatrick et al. 2011).

Hormone-sensitive lipase (HSL) causes the release of fatty acids in adipose tissue (Jaworski et al. 2007). Insulin blocks HSL function, trapping fatty acids within the cell. Insulin resistance however, allows free fatty acids to enter the circulation and infiltrate organs including the liver and skeletal muscle, further contributing to insulin desensitization of these organs. Furthermore, inflammation in the adipose tissue attracts migration of macrophages and the associated release of pro-inflammatory cytokines, exacerbating the pre-existing insulin resistance. In addition, peroxisome proliferator-activated receptor γ (PPARγ) which usually activates the production of new fat cells from stem cells (Rosen et al. 1999), is blocked by Tumour Necrosis Factor α (TNF-α) (Ye 2008). Since the tissue is unable to produce more cells, the existing fat cells continuously expand until they can no longer accommodate more, and cell death occurs,
attracting more neutrophils and macrophages to clean up the debris, as depicted by illustrations B1 and B2 (Cinti et al. 2005). These immune cells further increase adipocytokines including free fatty acids (FFA), TNF-α, Interleukin 6 (IL-6), Plasminogen Activator Inhibitor 1 (PAI-1) and C-reactive protein (CRP) (Lau et al. 2005). Therefore, obesity is considered a pro-inflammatory state and is associated with enlargement of the adipose tissues that experience hypoxia, attract immune cells and thus contribute to the pre-existing inflammation.

Free fatty acids are utilized as fuel by the heart, skeletal muscle and the liver (Coppack, Jensen, and Miles 1994), and are especially important during prolonged fasting (Felber et al. 1977). However, high levels of plasma FFA decrease insulin-stimulated glucose uptake in both diabetics and non-diabetics (Boden and Chen 1995). Increase in the levels of free fatty acids and intramyocellular triglyceride negatively affect skeletal muscle insulin sensitivity and overall glucose metabolism (Yu et al. 2002). Also, intracellular lipid accumulation leads to an increase in acyl-CoA, activating serine/threonine kinases which can interact with insulin receptors (Yu et al. 2002). Insulin receptor substrate 1 (IRS-1) inhibition inversely affects glucose-transport 4 (GLUT 4) activity and glucose uptake (Yu et al. 2002). FFA are important source of energy, however, in excess contribute to development of insulin resistance.
Illustration B1: Lean Adipose Tissue

Illustration B2: Obese Adipose Tissue
Illustration B: Differences between lean and obese adipose tissue

Lean adipose tissue comprises of resident macrophages, preadipocytes and adipocytes as shown in B1. In lean adipose tissue, there is greater secretion of adiponectin. In the case of obesity as shown in B2, the adipocyte expands to accommodate increased fat content. There is greater secretion of leptin and less adiponectin. Secretion of MCP-1 and other pro-inflammatory cytokines such as IL-6, TNF-α, attract dendritic cells, T cells and M2 macrophages into the tissue, that further recruit more immune cells (McArdle et al. 2013). This positive feedback loop of inflammatory agents, creates a highly inflamed and hypoxic environment, disrupting insulin signalling leading to insulin resistance (McArdle et al. 2013).
Amongst all the pro-inflammatory cytokines IL-6 has important implications for insulin resistance. Senn et al. have reported that IL-6 can block insulin receptor (IR) signaling and insulin function in mouse hepatocytes and HepG2 cell line (Senn et al. 2002). They showed that IL-6 treatment decreased tyrosine phosphorylation of IR substrate (IRS-1) and reduced the interaction between p85 subunit of phosphatidylinositol 3-kinase and IRS-1, as well as a 75% decline in glycogen synthesis (Senn et al. 2002). Furthermore, Klover et al., have also reported that a 5 day subcutaneous infusion of IL-6 led to loss of normal murine hepatic insulin receptor signaling (Klover et al. 2003). There was a 60% reduction in insulin receptor autophosphorylation as well as reduced insulin sensitivity (Klover et al. 2003). IL-6 is an important mediator of inflammation that disrupts the insulin signalling pathway, contributing to insulin resistance.

Plasminogen activator (PA) prevents intravascular thrombosis by generating the enzyme plasmin from the inactive precursor plasminogen (Collen, Lijnen, and Plow 1986). Tissue type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA) are the two types of PAs found in mammals (Brutsaert et al. 1997). Activation of plasminogen is tightly regulated by plasminogen activator inhibitors (PAIs) (Ijnen et al. 1994), mainly plasminogen activator inhibitor-1 (PAI-1) (Sprengers and Kluft 1987). It was reported by Thögersen et al., that increased plasma levels of PAI- and tPA were indicative of the first occurrence of acute myocardial infarction in both men and
women (Thögersen et al. 1998). Elevated PAI-1 levels were also closely associated with insulin resistance. Festa et al. explored the link between levels of CRP, fibrinogen and PAI-1 levels in 1,047 non-diabetic patients, and the development of diabetes within five years (Festa et al. 2002). Elevated levels of PAI-1 coincided with adiposity (Hassanin, Elhusien, and Osman 2013) and predicted the onset of type II diabetes (Festa et al. 2002).

2.4 Animal models of obesity and type II diabetes

Animal models of obesity and type II diabetes are characterized by increased food intake, weight gain, blood insulin and glucose levels. Models of obesity and type II diabetes are divided into two groups, monogenic and polygenic. Monogenic models involve spontaneous mutations in a single gene, such as complete deletion or partial dysfunction (Lutz and Woods 2012). Among the monogenic models, the ones that involve mutations in the leptin pathway are the most commonly used and well-studied. Leptin is a hormone produced by adipocytes in parallel to the amount of stored fat. Mutations in genes for leptin or leptin receptors cause morbid obesity (Bray and York 1979). The mouse obese (ob) gene codes for leptin, and a single mutation in this gene is associated with premature termination of the leptin protein (Bray and York 1979). More specifically, there is a single autosomal recessive mutation on the leptin coding gene, where a nonsense mutation in codon 105 replaces an arginine with a stop codon, resulting in premature truncation and a non-functional protein (Friedman et al. 1991).
The phenotype of this model includes early obesity with hyperphagia, hyperinsulinemia, hyperglycemia, hyperthyroidism, infertility and a decrease in growth hormone (Bray and York 1979; Pelleymounter et al. 1995).

The mouse diabetes (db/db) gene involves a mutation in the gene for the leptin receptor, and causes leptin insensitivity due to lack of functional receptors. A single autosomal recessive mutation replaces glycine with a threonine, generating a donor splice site that introduces a novel exon, producing an abnormal long form transcript (Chen et al. 1996). The db/db model is commonly used to study type II diabetes. The main characteristics of this model include obesity, severe insulin resistance, diabetes and infertility (Chua et al. 1996). There are several rat models used to study obesity related type II diabetes as well, including the Zucker diabetic fatty (ZDF) (Peterson et al. 1990) and Goto-Kakizaki (GK) models (Kakizaki and Masaki 1975). The differences between these models include severity of obesity, islet pathology, age of onset and severity of insulin resistance and hyperglycemia (Nugent, Smith, and Jones 2008). The ZDF model is the most common rat model utilized to study type II diabetes (Norihide et al. 2013). This model involves mutations in the leptin receptor, where leptin receptors are synthesized but only few are expressed due to intracellular retention caused by the mutation (Crouse et al. 1998).

Other less common monogenic models that involve the leptin pathway include Proopiomelanocortin (POMC) knockout mouse (Challis et al. 2004) and POMC/Agouti
related protein knockout mice (Corander et al. 2011). Mutations in the genes for hormone cholecystokinin (CCK) receptors are also important monogenic models. CCK signals satiety and is produced by the gastrointestinal tract in response to interaction with food. The Otsuka Long Evans Tokushima Fatty (OLETF) is a common rat model which involves mutations in the receptor gene for CCK, leading to a mild form of obesity. There are also several other monogenic models such as melatocortin receptors 3 and 4 (Krude et al. 1998), that involve manipulation of receptors in the hypothalamus involved in satiety and energy expenditure. There are a variety of other monogenic models that are generated by targeting specific genes involved in the homeostasis of energy expenditure and food intake, and are utilized to study obesity and type II diabetes. Monogenic models allow us to examine the function of a target gene or protein, at both physiological and molecular levels. One of the limitations of monogenic models is that human diseases are generally multifactorial, therefore manipulating only one gene may result in findings not consistent with what is observed in the clinical setting.

Polygenic animal models share many similarities with human obesity and usually obesity is induced using high fat and western diets. To establish a diet induced obesity (DIO) model, both non-transgenic as well as genetically manipulated animals are utilized. Several different types of diet with varying fat content may be used, depending on the nature of the study. Short term high fat diet feeding causes insulin insensitivity and altered expression of hypothalamic hormones, leading to hyperphagia (Clegg et al. 1998).
HFD is associated with altered expression of hypothalamic peptides that regulate satiety such as Apolipoprotein A-IV, a gut-brain peptide that limits food intake and allows for weight regulation (Woods et al. 2004). Animal studies often utilize mice predisposed to obesity in DIO models such as the New Zealand obese (NZO) model. This model is genetically manipulated to increase susceptibility to adiposity and hyperglycemia, and is characterized by severe obesity and type II diabetes (Joost 2010). Mice with genetic manipulations utilized for DIO studies also include GLUT4 (Shepherd et al. 1993), beta-3 adrenergic receptor (Susulic et al. 1995) and Neuropeptide-Y 1 receptor (NPY1R) knockouts (Kushi et al. 1998).

Therefore, both monogenic and polygenic models are useful tools that have provided insight into the diabetes disease process, and have allowed for testing of potential therapeutic agents. However, since human diabetes is clinically heterogeneous, translating these findings to the clinical setting continues to be a challenge. No single animal model of type II diabetes can exhibit all the characteristics of the disease; however, they allow for us to examine selective features of the condition. In addition, there are physiological differences between human diabetes and animal models. For instance, animal models develop diabetes without the same islet pathology as humans (Nugent, Smith, and Jones 2008). Also, not all animal models develop diabetes associated complications, for instance C57BL/6 are resistant to nephropathy (Brosius et al. 2009).
2.5 Innate Immune response to foreign antigen and leukocyte recruitment

The immune system is a complex network of lymphoid organs, cells and proteins that shield the host from foreign pathogens, virus and cancer cells (Parkin and Cohen 2001). Innate immunity is the first line of defense against the entry of foreign antigens. It is a non-specific (antigen-independent) immune response that occurs rapidly, within minutes to hours, post initial insult (Basset et al. 2003). Adaptive immunity is a delayed antigen dependent immune response, that is more specific, efficient and retains immunologic memory (Basset et al. 2003; Mogensen 2009). Upon host intrusion by a foreign antigen, the innate immune system is activated rapidly due to the interaction between pathogen molecules and the immune surveillance mechanisms (Basset et al. 2003; Mogensen 2009). Pathogen-associated molecular patterns (PAMPs) are conserved molecules present on pathogen surfaces, and are essential for pathogen survival (Janeway 1989). They are recognized by host germ line encoded pattern recognition receptors (PRRs), expressed on dendritic cells, macrophages and other immune cells (Blasius et al. 2010). Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD like receptors (NLRs) and DNA receptors are all types of PRRs, that detect different types of pathogen molecules including proteins, lipids and carbohydrates (Blasius et al. 2010). When PRRs identify PAMPs, a cascade of signalling pathways are initiated that activate the production of cytokines, chemokines and cell adhesion molecules (Mogensen 2009). TLRs, the most extensively studied PRR, upon interaction with PAMPS, induce receptor
oligomerization that initiate intracellular signal transduction using several adaptor molecules, including MyD88 and Mal (Mogensen 2009). In addition, the innate immune system also utilizes complement activation and phagocytosis to inhibit the spread of the invading pathogen (Mogensen 2009). Phagocytosis involves recognition of conserved pathogen motifs by macrophages, neutrophils and complement receptors, and the rearrangement of the cytoskeleton in order to internalize the endotoxin (Aderem and Underhill 1999). The complement system is made up of plasma proteins, several of which are proteases, that aid in destroying the pathogen (Dunkelberger and Song 2009).

Recruitment of leukocytes to the site of injury is an essential step in the immune response to infection. It was first described in 1987 that exposure to TNF-α, IL-1 or E. coli, induces the activation of pro-adhesive properties on the endothelial surface (Bevilacqua and Gimbrone 1987). Leukocyte recruitment involves the interaction between adhesion molecules present on the cell surface of endothelial cells and their ligands expressed on leukocytes. The common steps of leukocyte recruitment involve tethering, rolling, adhesion, crawling and transmigration as depicted in illustration C (Kolaczkowska and Kubes 2013). Using intra vital video microscopy in rabbits, Drs. von Andian and Butcher visualized the effects of anti-Leukocyte Endothelial Cell Adhesion Molecule-1 (LECAM-1) (now termed L- Selectin) and anti-CD18 monoclonal antibody on leukocyte interaction with mesenteric venules (von Andrian et al. 1991). Anti LECAM-1 antibodies resulted in lack of leukocyte rolling, while anti CD-18 blocked firm
attachments of leukocytes to vessel wall (von Andian et al. 1991). Hence it was proposed that leukocyte recruitment and rolling required LECAM-1, which was followed by β2 integrin dependent firm attachments and adhesion (von Andian et al. 1991).
Figure C. Process of leukocyte recruitment

Leukocyte recruitment involves the interaction between adhesion molecules expressed on the endothelium and their ligands present on leukocytes. Tethering, rolling, adhesion, crawling and transmigration are the main steps of this process (Kolaczkowska and Kubes 2013). P-selectin on the endothelium binds P-selectin glycoprotein ligand-1 (PSGL-1) which causes tethering and rolling of neutrophils (Ley et al. 2007; Zarbock et al. 2011). Further binding of LFA-1 and MAC-1 to ICAM-1 and ICAM-2 on the endothelium causes firm adhesion. Neutrophils then engage in a crawling behaviour and examine the surroundings (Zhelev, Alteraifi, and Chodniewicz 2004). A chemokine gradient allows for neutrophils to move towards the sites of transmigration (Makino et al. 2005; Zhelev, Alteraifi, and Chodniewicz 2004). Transmigration at cell-cell junctions involve interaction between integrins, CAMs, platelet/endothelial cell adhesion molecule-1 (PECAM-1), CD99, junctional adhesion molecules (JAM), leukocyte-specific protein 1 (LSP1) and epithelial cell adhesion molecules (ECAM) among others (Ley et al. 2007; Petri et al. 2011; Reymond et al. 2004). Transmigration may occur through paracellular (between endothelial cells) or transcellular (through endothelial cell) means at sites with the least resistance (Burns et al. 2000).
Due to their bactericidal properties, neutrophils are usually the first leukocytes to be recruited to the site of infection. The endothelial surface changes in response to inflammatory molecules released from the interaction between tissue resident sentinel leukocytes and the pathogens (Ley et al. 2007). The surface can also be activated through the discovery of pathogen through PRRs (Ley et al. 2007). Activation of the endothelium refers to expression of adhesion molecules, P- selectin and E-selectin (Ley et al. 2007). P-selectin binds P-selectin glycoprotein ligand-1 (PSGL-1) which causes tethering of neutrophils, followed by rolling (Ley et al. 2007; Zarbock et al. 2011). Rolling involves dissociation of the P-selectin and PSGL-1 bond at one end, and formation of a new bond at the leading end (Ley et al. 2007; Zarbock et al. 2011). Neutrophils express integrins, Lymphocyte Function Associated Antigen 1 (LFA1) and Macrophage Antigen 1 (MAC1), that undergo a conformational change in order to bind to Intracellular Adhesion Molecule-1 and 2 (ICAM-1 and ICAM-2) expressed on the endothelium, and result in firm adhesions (Phillipson et al. 2006). While attached to the endothelium, neutrophils examine their surroundings using pseudopods (Zhelev, Alteraifi, and Chodniewicz 2004). They engage in crawling behaviour, releasing bonds at one end while making new ones as they move forward, ensuring attachment to the endothelium especially during conditions of sheer stress (Makino et al. 2005; Zhelev, Alteraifi, and Chodniewicz 2004). Active crawling involves the interaction between ICAM-1 and MAC-1. Chemokine gradients allow for neutrophils to move towards sites of transmigration (Makino et al. 2005; Zhelev, Alteraifi, and Chodniewicz 2004).
They transmigrate at cell-cell junctions which require integrins, CAMs, Platelet/endothelial cell adhesion molecule-1 (PECAM-1), CD99, junctional adhesion molecules (JAM), leukocyte-specific protein-1 (LSP-1) and epithelial cell adhesion molecules (ECAM) among others (Ley et al. 2007; Petri et al. 2011; Reymond et al. 2004). Movement can occur via paracellular (between endothelial cells) or transcellular (through endothelial cell) means at sites with the lowest amount of ECM molecules, reducing the resistance (Burns et al. 2000). Once inside the tissue the neutrophils follow the chemokine gradient in order to arrive at the site of infection and destroy the pathogen through phagocytosis, releasing bactericidal enzymes and neutrophil extracellular traps (Kolaczkowska and Kubes 2013).

### 2.6 Hyperglycemia and type II diabetes associated increased susceptibility to infection

Diabetes is associated with an increased risk of infections and mortality. More specifically, diabetics have an increased risk of surgical site infections, community acquired pneumonia (Saibal et al. 2012), foot infections (Hokkam 2016), malignant external otitis (Grandis, Branstetter IV, and Yu 2004), rhinocerebral mucormycosis (Kumar et al. 2013) and urinary tract infections (Boyko et al. 2002). Acute (stress) hyperglycemia is also associated with an increased risk of mortality in non-diabetic patients after stroke (Capes et al. 2001). These patients experience a longer hospital stay and greater admission rates to the intensive care unit (Umpierrez et al. 2002). Gram positive bacteria such as Methicillin- resistant *S. aureus* (MRSA) are the primary cause of
diabetes associated infections. A study from Denmark examined medical records of 30,000 people over a twelve-year time period, and noted that type I diabetics were 7.2 times, and type II diabetics 2.7 times more at risk of developing *S. aureus* blood infection outside of the hospital (Smit et al. 2016). In addition, poor management and the duration of diabetes also increased the risk.

Muller et al. in a 12 month prospective cohort study compared the risk of infections in 705 adults with type I diabetes, 6712 adults with type II diabetes and 18,911 control patients (Muller et al. 2005). The risk of lower respiratory tract, urinary tract, skin and mucous membrane infections were found to be greater for both type I and type II diabetics compared to controls (Muller et al. 2005). Shah et al. have also shown using a retrospective cohort study comparing 513,749 diabetic individuals from Ontario, and an equal number of non-diabetic controls, that diabetes increased the risk for developing infections and death (Shah and Hux 2003). According to the study, 46% of diabetics had at least one hospitalization or case of infectious disease claimed by physician, compared to 38% in the non-diabetic group and similarly there was an increase in mortality rates for diabetics as well (Shah and Hux 2003). Diabetics are at a greater risk of mortality regardless of population and type of infection (Bertoni, Saydah, and Brancati 2001; Liu 2013; Yende et al. 2010). Insulin therapy utilized to normalize glucose levels and maintain levels at or below 110 mg/dl, has been reported to reduce mortality rates in critically ill patients in the intensive care unit (Van den Berghe et al. 2001). Therefore, it
has been known for several decades that diabetes increases the risk of developing infections and is also associated with diminished ability to fight the infection.

2.7 Mechanisms for diabetes associated increased risk of infection

Several studies have investigated the mechanisms involved in diabetes associated increased risk and reduced ability to combat infectious diseases. The main reason for the increased prevalence of infectious diseases in diabetics is defects in the immune system. Neutrophils are immune cells that migrate to the site of infection, and eliminate pathogens as explained previously. Using Polymorphonuclear (PMN) neutrophils from 61 diabetic patients without infections, it was found that expression of adhesion molecules, chemotaxis, phagocytosis and bactericidal activity were all significantly reduced in diabetic patients, increasing the risk of infection and inhibiting the ability to combat foreign pathogens (Delamaire et al. 1997). Collison et al. reported that advanced glycation end products (AGEs) in diabetics inhibited trans-endothelial migration and reduced bactericidal capacity in neutrophils in-vivo (Collison et al. 2002). Yano et al. investigated the effects of insulin on neutrophil function during a *S. aureus* infection in db/db and diet induced diabetic mice (Yano et al. 2012). When these mice were treated with insulin, there was a reversal in the decreased phagocytosis and bactericidal function of neutrophils (Yano et al. 2012).

Hyperglycemia also increases glycosylation of proteins such as immunoglobulins, which are important for opsonisation. Glycosylation is a process that adds glycans
(sugars) to proteins and mediates protein function. In rats, hyperglycemia causes glycosylation of immunoglobulins rendering them inactive, increasing susceptibility to infection (Black et al. 1990). Furthermore, it has also been reported that increasing glycation reduces IL-10 production by myeloid cells, as well as Interferon-γ (IFN-γ) and TNF-α by T cells (Price et al. 2010). In addition, glucose interaction with the third component of complement C3 prevents attachment of C3 to microbial surface for opsonisation (Hostetter 1990). Pathogens such as Candida albicans have a glucose inducible protein that have structural and functional similarities to mammalian complement receptor, allowing for adhering and inhibition of phagocytosis (Hostetter 1990). In another study it was shown that high levels of glucose inhibit glucose-6-phosphate dehydrogenase, an enzyme that protects from oxidative stress, and predisposition to cell death (Zhang et al. 2000). Therefore, diabetes increases the risk of infections and morbidity due to nephropathy, impaired defense mechanisms such as reduction in the production of interleukins, phagocytosis and chemotaxis of neutrophils, as well as glycosylation of proteins essential for the immune response.

2.8 Microflora and susceptibility to disease

Trillions of bacteria thrive in the human gut and are important factors in health and disease (Devaraj, Hemarajata, and Versalovic 2013). Recent studies have begun to explore the role of microbiota in the onset of metabolic syndrome and type II diabetes. Larsen et al. found that type II diabetics have changes in composition of intestinal
microbiome (Larsen et al. 2010). They examined 36 adult males, of which 18 had type II diabetes (Larsen et al. 2010). Based on the analysis of the fecal bacteria composition, the phylum *Firmicutes* and class *Clostridia* were significantly reduced, and *Betaproteobacteria* was more abundant in diabetics compared to controls (Larsen et al. 2010). Turnbaugh et al. examined gut microbiome in both obese mice as well as obese humans (Turnbaugh et al. 2006). In both cases, obesity was associated with an increased ratio of *Firmicutes* to *Bacteroidetes* (Turnbaugh et al. 2006). Also, in comparison to “lean microbiota”, germ free mice when colonized with the obese microbiota, had an increase in body fat (Turnbaugh et al. 2006). Furthermore, microbiome harvested from Peyer’s patches of DIO mice, there were significantly less inhabiting Lactobacillus, specifically *Lactobacillus reuteri*, while it was the most abundant strain in the controls (Sun et al. 2015). Therefore, obesity is associated with occurrence of greater numbers of pro-inflammatory strains of bacteria, resistant to oxidative stress (Sun et al. 2015).

The gastrointestinal (GI) microbiome also has a complex relationship with the immune system. Germ free mice have defects in antibody production (Lamousé-Smith, Tzeng, and Starnbach 2011) and isolated lymphoid follicles (ILFs) (Bouskra et al. 2008) compared to animals from specific pathogen free (SPF) environments. The GI microbiome also influences the expression of pathogen recognition receptors and have changes in production of antimicrobial peptides (Round and Mazmanian 2009). For instance, gram negative *Bacteroides thetaiotaomicron* but not gram-positive
*Bifidobacterium longum* is associated with the expression of ‘regenerating islet-derived 3 γ’ (REG3-γ), an antimicrobial peptide. REG3-γ specifically targets gram positive bacteria, therefore it can be speculated that gut bacteria direct immune function in a way that allows them to thrive (Round and Mazmanian 2009). Therefore, the gut microbiome plays an important role in shaping host immune system and therefore influence susceptibility to diseases including metabolic syndrome and type II diabetes.

### 2.9 Pathophysiology of sepsis

Sepsis is a condition characterized by severe organ deterioration due to impaired host physiological response to infection (Singer et al. 2016). In the clinical setting, severe sepsis associated organ dysfunction has a Sequential Organ Failure Assessment (SOFA) score of 2 points or greater, which is associated with a 10% mortality rate (Singer et al. 2016). Septic shock is defined as having severe circulatory, cellular and metabolic dysfunction, with mortality rates of 40% or greater (Singer et al. 2016). Sepsis patients are likely to have poor outcomes if they have at least two of the following clinical criteria; respiratory rate of 22/min or higher, systolic blood pressure of 100mm Hg, or less and altered mental state (Singer et al. 2016). Severe sepsis and septic shock are not only associated with high rates of mortality but claim tremendous amount of health care resources in forms of therapeutic interventions. In 2013, it was reported that treatment costs of sepsis were the most expensive, with aggregate hospital cost at $23,663 million in the United States (Tario and Moore 2016).
It is observed that sepsis is associated with an initial pro-inflammatory state that involves over production of pro-inflammatory cytokines, fever, tachycardia and tachypnea (Vachharajani 2008). This is followed by an immuno-suppressed state with lymphopenia and loss of immune function (Vachharajani 2008). Upon first encounter with inflammatory stimuli (pathogen), the immune system reacts by releasing cytokines and activates the complement and coagulation cascades (Vachharajani 2008). Following this hyper-inflammatory response, the immune system stimulates the synthesis of anti-inflammatory agents to limit the inflammation and prevent collateral damage to tissues. If the “pro and anti-inflammatory” agents achieve a balance, there is little to no tissue damage. However if this balance is not achieved, organ damage and even death may occur (Vachharajani 2008).

The pathophysiology of sepsis involves both extrinsic and intrinsic mediators (Jean-Baptiste 2007). The extrinsic mediators include endotoxins, and other protein or nucleic particles from viruses, fungi or bacteria. They activate macrophages that release various pro-inflammatory agents, such as TNF-α, Interleukin-1 (IL-1), IL-6, high mobility group box-1 protein (HMGB-1), PAF, nitric oxide (NO), and eicosanoids (Shapiro et al. 1993; Wang et al. 1999). IL-6 is the main inducer of acute phase protein production, synthesized by the liver in response to inflammation or tissue injury (Pannen and Robotham et al. 1995). The pro-inflammatory agents released by the host immune cells are considered the intrinsic mediators of sepsis.
Inflammation is closely associated with activation of the coagulation cascade, which can further amplify the generation of pro-inflammatory cytokines in a positive feedback loop (Levi 2010). Pro-inflammatory agents such as IL-6 can activate the coagulation system through expression of tissue factor on mononuclear and endothelial cells (Levi 2010). In addition, pro-inflammatory cytokines also indirectly hinder the activity of endothelial bound anticoagulant systems including protein C and antithrombin (Levi 2010). Components of the coagulation cascade such as tissue factor VIIa complex, factor Xa and thrombin, in turn increase production of pro-inflammatory cytokines (Levi 2010).

It has been reported that blood coagulation in vitro leads to an increase in IL-1 (Mileno et al. 1995) and IL-8 (Johnson et al. 1998). Endothelial cells can also be activated by factor Xa, thrombin and fibrin, promoting IL-6 and IL-8 production (Sower et al. 1995; van der Poll et al. 2001). In humans, administration of recombinant factor VIIa induced an increased in IL-6 and IL-8 levels in the plasma (de Jonge et al. 2003). One of the mechanism through which components of the coagulation system influence inflammatory processes is through binding protease activated receptors (PARs), found on endothelial cells, platelets, fibroblasts and smooth muscle cells (Coughlin et al. 2000). The ligands for PAR 1, 3 and 4 are thrombin, while PAR 1 is activated by tissue factor VIIa complex and Xa (Camerer et al. 2000; Slofstra et al. 2007; Sevastos et al. 2007). Activated coagulation factors cleave PARs, causing exposure of a neo aminoterminus, activating the same receptor and thus initiating signaling (Camerer et al. 2000; Slofstra
et al. 2007; Sevastos et al. 2007). Together, inflammation and coagulation during sepsis contribute significantly to dysfunction in the microvascular circulation (Levi et al. 2010).

The main role of the microcirculation in vascular beds is to provide tissues with oxygenated blood for normal functioning. Pathogenic factors produced during sepsis affect almost every cellular component of the microcirculation including the endothelial cells, smooth muscle cells, leukocytes, and erythrocytes. These cells are unable to perform normal regulatory functions because of disturbed signal transduction pathways, loss of electrophysiological communication and smooth muscle control, all important for maintaining normal microcirculation (Ince 2005). Aside from antimicrobial therapy, sepsis is initially managed with fluid resuscitation, vasopressors and inotropes to regulate blood pressure to prevent organ dysfunction. Early resuscitation has been suggested as one of the best strategies to combat sepsis associated hypovolemic shock. There are two main types of fluids utilized, colloid and crystalloid. Colloid fluids include hypooncotic (gelatins and 4-5% albumin) and hyperoncotic solutions (dextrans, hydroxyethyl starches and 20-25% albumin), while crystalloids include isotonic and hypertonic solutions that fall into nonbuffered and buffered categories (Annane et al. 2013). There is ongoing debate over which type of fluid (crystalloid versus colloid) administration has the most optimal outcome (Rivers et al. 2001). Nevertheless, the key for sepsis treatment is improving microcirculation and allowing for optimal tissue perfusion to prevent organ dysfunction.
2.10 Animal models of sepsis

Mice are the most commonly used animals in sepsis models, mostly due to short reproductive cycles, feasibility, and inexpensive housing and maintenance costs. Genetic manipulation also allows targeting and exploring specific genes of interest. The most simple and common model of sepsis is the endotoxemia model, which involves administration of cell wall components of gram negative bacteria, either intraperitoneally (I.P.) or intravenously (I.V). Lipopolysaccharide (LPS), a component of gram negative bacterial cell wall, induce the pathogenesis of gram negative sepsis through release of pro-inflammatory cytokines (Parker and Watkins 2001). LPS causes these responses mainly through its interaction with Toll-like receptor (TLR) 4, present on cells such as macrophages and dendritic cells. Intravenous infusion with a live organism is also an important infection model, as it closely mimics sepsis as seen in meningococcemia and other cases of gram-negative bacteremia (Fink and Heard 1990). There are a lot of variations in this model, mainly due to differences in the types of organism used, duration and dosage. The most common bacterial species used in animal models is *Escherichia coli* (*E. coli*). Animals are usually administered intravenously with live *E.coli* (e.g. 6x10⁹ colony forming units/ml/kg) over a period of 15 minutes or longer (Lagoa et al. 2004). In some cases, the animal is injected with fluids post infusion (Lagoa et al. 2004). *E.coli* infusion is associated with a decrease in arterial pressure, cardiac index, blood flow, oxygenation, increase in lactate levels and a disruption of normal flow
in the systemic and mesenteric beds (Lagoa et al. 2004). The type of response generated depends heavily on the species used. For instance, in a porcine model of sepsis, gram positive and gram negative bacteria, induced very different responses (Dehring et al. 1983). While S. aureus induced non-significant changes, E.coli and Pseudomonas aeruginosa led to shock and failure of the respiratory system (Dehring et al. 1983). In addition, with S. aureus, a substantially higher pathogen burden is required to induce disease in animal models. In humans however, S. aureus is pathogenic and associated with bacteremia, sepsis, pneumonia and other infectious diseases (Kim, Missiakas, and Schneewind 2014).

One of the limitations of the endotoxemia model of sepsis is that some animals including mice, dogs and baboons are less sensitive to endotoxins. Therefore, to induce any type of response in mice that mimics human sepsis, a significantly larger dose needs to be administered. This dose is usually 1000 000 time greater than the dose (2-4ng/kg) used to induce modest symptoms in human volunteers (Barber et al. 2016). Administering large doses may cause toxic effects, not present in endotoxin sensitive humans (Piper et al. 1996). In addition, endotoxins are released by gram negative bacteria, however mortality rates of sepsis are similar in both gram positive and negative infections in humans (Opal and Cohen 1999). The limitation of intravenous infusion of bacteria is that it differs from clinical sepsis, due to lack of an infectious focus and overloads the system with a large bacterial load at one time point, as opposed to slow
persistent release of bacteria as seen in humans (Fink and Heard 1990). In addition, different strains of bacteria, duration of infusion as well as specie specific response all cause variability (Piper et al. 1996). Therefore, the endotoxemia model in some aspect lacks clinical relevance.

The cecal ligation and puncture (CLP) model is considered the gold standard animal model of sepsis and functions as a model of intra-abdominal sepsis, the second most common cause of sepsis in humans (Vincent et al. 2009). In this model, polymicrobial infection and bacteremia is introduced by ligating the cecum distal to the ileocecal valve, and the antimesenteric cecal surface is punctured with a needle between the ligation and cecal end (Wichterman, Baue, and Chaudry 1980). A small amount of fecal content is extruded to allow a constant flow of fecal matter post surgery, and the cecum is returned to the peritoneal cavity (Wichterman, Baue, and Chaudry 1980). Much like intra-abdominal sepsis in humans, this model consists of an infectious focus that allows consistent flow of pathogens into the host, with eventual entry of the pathogen into the blood stream (Wichterman, Baue, and Chaudry 1980). Blood and peritoneal cultures contain different strains of bacteria, including *Escherichia coli* and *Streptococcus bovis*, among others (Wichterman, Baue, and Chaudry 1980). Unlike endotoxin models, the CLP model replicates important features of bacterial peritonitis and polymicrobial infection as seen in the clinical settings, including the initial hyperdynamic phase followed by the late hypodynamic stage. The hyperdynamic phase
is usually associated with increased blood flow and hyperglycemia whereas the hyperdynamic state involves decreased blood flow, hypoglycemia and hyperinsulinemia. Other similarities include an initial increase in the concentration of TNF-α, IL-6, Keratinocyte Chemoattractant (KC)/ human growth regulated oncogene (GRO), Macrophage Inflammatory Protein (MIP-1) and Monocyte Chemoattractant Protein-1 (MCP-1), in addition to neutropenia and lymphopenia (Remick et al. 2000). Inflammation of tissue, bacteremia and the presence of bacteria in the blood stream, all provide the model with clinical relevance (Dejager et al. 2011). Some of the limitations of the CLP model include the inability to control the intensity of the septic insult and bacterial dissemination that occurs through the perforated cecum into the peritoneal cavity.

The Colon Ascendens Stent Peritonitis (CASP) model, is a different model of polymicrobial sepsis that is highly reproducible and simulates abdominal sepsis as seen in humans (Traeger et al. 2010). A small stent is inserted into the ascending colon that acts as a source of constant bacterial flow into the peritoneal cavity, inducing peritonitis and systemic bacteremia (Traeger et al. 2010). The severity of sepsis can be controlled by manipulating the diameter of the stent. An 18G diameter of the stent leads to a 50% mortality, whereas 16G leads to a 70% mortality rate (Traeger et al. 2010). TNF-α, IL-1β, IL-6, Interleukin 10 (IL-10) are all elevated both locally and systemically in plasma (Traeger et al. 2010). High concentrations of bacteria are present in the peritoneal fluid,
blood, lungs, spleen and liver (Traeger et al. 2010). This model activates the innate immune system through TLR2, TLR4 and activation of MyD88. Mice administered with anti TLR4/MD2 had reduced mortality rates in CASP model (Daubeuf et al. 2007). The pathogenesis of this model involves identification of bacterial products, followed by an inflammatory response and activation of the complement system (Entleutner et al. 2006; Yuan et al. 2011). The limitations of both CASP and CLP models are the anatomical variations among the animals such as the size of the cecum, the amount of fecal content in the cecum as well as leaking of the fecal matter from the puncture or stent (Schabbauer 2012).

A model of sepsis where the dose of the insult as well as the pathogen type can be closely controlled is the fibrin clot model (Ahrenholz and Simmons 1980). A specific strain and colony of bacteria are implanted in a fibrin clot (Ahrenholz and Simmons 1980). The fibrin allows for a gradual systemic release of the entrapped bacteria, developing a chronic intraperitoneal abscess (Ahrenholz and Simmons 1980). This model was first introduced in canines, where fibrin clot infected with E. coli was implanted intraperitoneally in the 10 unsedated dogs, and cardiac function was examined (Natanson et al. 1986). The animals had a decrease in systolic ventricular performance and ventricular dilation (Natanson et al. 1986). This model is easily reproducible and has many similarities to clinical sepsis, including the hyperdynamic cardiovascular state and high rates of mortality (Natanson et al. 1986). The pneumonia model of sepsis involves
intratracheal inoculation of *Klebsiella pneumoniae* in mice (Sordi et al. 2013). 6, 24 and 48 hours post inoculation, there is an increase of cells in the bronchoalveolar lavage, presence of leukopenia, lung pathology, hypotension, hyper responsiveness and bacteria in heart and spleen homogenates (Sordi et al. 2013). The pneumonia model of sepsis is a clinically relevant model that causes systemic inflammatory response along with dysfunction of the vital organs (Sordi et al. 2013).

Small and large animal models have allowed for extensive study of sepsis pathogenesis. However, most models have failed to predict the outcome of human clinical trials for several reasons. In the clinical setting, many patients require mechanical ventilation, all receive antibiotics and resuscitation fluids. Vasopressors to support hemodynamic instability and nutritional supplements among other interventions may be required. However, majority of animal models do not receive any such treatments. In addition, sepsis is most frequently seen in older individuals in the presence of comorbid conditions such as diabetes, however, sepsis is usually exclusively studied in young animals without any comorbid complications. Also, every sepsis model has its limitation in the ability to mimic the pathogenesis of human sepsis correctly. Majority of the animal models lack clinical relevance and are not completely reliable in developing therapeutic treatments, that will serve to be effective in the clinical setting. Therefore, any findings from animal models should not be extrapolated directly to humans and an effort to create clinically relevant models of sepsis are needed.
2.11 Link between obesity and critical illness

Reports from animal studies suggest that Western Diet (WD) associated obesity have negative implications for sepsis outcomes. The WD constitutes of excessive consumption of dairy, refined sugars, fatty meat and salt (Cordain et al. 2005). Rivera et al. investigated the impact of WD on hepatic inflammation in C57 mice with sepsis. They discovered that feeding these mice WD for three weeks, increased the adhesion of leukocytes in the sinusoids and hepatic venules by 8 fold, within the first six hours (Rivera et al. 2010). They also reported an increase in expression of TNF-α, MCP-1 and Intracellular Adhesion Molecule 1 (ICAM-1) (Rivera et al. 2010; Weaver et al. 2004). The majority of current animal studies report that WD associated obesity exacerbates pro-inflammatory responses during sepsis in animal models (Rivera et al. 2010). The limitations of these current models as previously discussed, include utilizing young mice in the absence of any comorbid conditions, when in the clinical setting sepsis mostly occurs in older individuals (Starr and Saito 2014) with comorbid condition (Ashley et al. 2004; Kakarla et al. 2011; Spelman et al. 2000).

Clinical studies report mixed findings with regards to the implications of obesity for outcomes of critical illness, where some report an increase in mortality risk (Spagnolo et al. 2010), others suggest beneficial effects of obesity. A study by Pickkers et al. examined the link between BMI and outcomes of critical illness. They conducted an observational cohort study with 154,308 ICU patients from Dutch urban and non-urban
teaching hospitals, and discovered an inverse relationship between obesity and hospital mortality, with obese patients (BMI 30-39.9kg/m²) having the lowest risk of mortality (Pickkers et al. 2013). Similarly, Wacharasint et al. reported from a retrospective analysis comparing outcomes of septic shock patients based on BMI, that obese patients had the lowest 28 day mortality and less incidence of lung and fungal infections (Wacharasint et al. 2013). They attributed these benefits to the fact that obese patients received less fluids, vasopressin and norepinephrine (Wacharasint et al. 2013). A more recent study by Prescott et al. determined 1 year mortality rates among other factors in patients with severe sepsis (Prescott et al. 2014). There were 1,404 patients with severe sepsis, of which 42.5% were normal weight, 33.7% overweight and 23.8% were obese. They concluded that obesity was associated with improved survival rates (Prescott et al. 2014). This finding contradicts preclinical work, which suggests that obesity enhances the risk of infection and mortality rates. One of the reasons for this contradiction may be lack of clinical relevance among animal models. The majority of preclinical studies utilize young mice, and induce sepsis using methods that do not simulate characteristics of clinical sepsis. This leads to a disconnect between findings in animal models and clinical observations.

2.12 Obesity and sepsis induced changes in immune cell function

Ongoing obesity usually involves infiltration of macrophages/monocytes into the liver and visceral fat tissue and increase the expression of cytokines and chemokines
(Schenk, Saberi, and Olefsky 2008; Fantuzzi 2005; Hillenbrand et al. 2010). As discussed above, obesity is considered a chronic state of inflammation with an increase in the levels CRP, IL-6, TNF-α and PAI-1 (Lau et al. 2005). Studies have shown that morbidly obese patients have reduced adiponectin levels and elevated MCP-1, PAI-1, IL-1α, IL-6 and IL-10 levels (Lau et al. 2005).

2.13 Leptin and obesity

As previously discussed, leptin is a hormone produced by adipocytes and is widely known to play a key role in maintaining the balance between the storage and use of energy and relaying information regarding nutritional status to other physiological systems (Friedman 2010). It is coded by the “ob” gene and a mutation in the gene (such as in the case of ob/ob mice), leads to leptin deficiency. From murine models of diet induced obesity, it is known that consuming a high fat diet causes an increase in circulating leptin levels that directly correlate with the amount of adipose tissue (Krempler et al. 1998; Frederich et al. 1995). In addition, leptin levels plummet after weight loss in both humans and mice (Maffei, Halaas and Pratley 1995). Leptin has both short and long-term effects on the regulation of energy levels. It can also influence plasma glucose levels, plasma amino acids, CCK and other hormones. Ob/ob mice have low body temperature, energy expenditure, and infertility, all of which can be corrected by leptin replacement (Chehab, Lim, and Lu 1996). Over the year studies have elucidated the effects of leptin on the immune system. It has been observed that starvation is
associated with poor immune status and that infectious diseases are the common cause of death in malnourished individuals (Shears 1991). Lord et al. reported that leptin differentially regulates the proliferation of naïve and memory T cells and increases Th1 and suppresses Th2 cytokine responses (Lord et al. 1998). Furthermore, leptin receptors are found on a variety of immune cells such as macrophages, supporting the role of leptin in directly mediating immune responses (Lee et al. 1999).

2.14 Leptin in context of infectious diseases

Studies investigating the effects of leptin in the context of sepsis report mixed findings, and there is debate over the pro and anti-inflammatory capacities of this hormone. Shapiro et al., using two models of sepsis, cecal ligation and puncture and endotoxemia, reported pathogenic role of leptin where this protein and soluble leptin receptor were elevated in septic mice in both models (Shapiro et al. 2010). The administration of exogenous leptin increased rates of mortality, adhesion and coagulation molecules and loss of endothelial integrity, which were not observed in long form leptin receptor deficient mice. Faggioni et al. on the other hand showed that ob/ob mice challenged with LPS had greater mortality rates, and lower IL-10 and IL-1 receptor antagonist levels. Treatment with leptin reduced LPS associated mortality rates in these mice (Faggioni et al. 1999). In context of TNF associated pathology, ob/ob mice administered with exogenous leptin had less TNF associated injury, suggesting that leptin may prevent host injury by inhibiting pro-inflammatory responses (Takahashi,
Waelput, and Guisez 1999). Interestingly, leptin also played a role in preventing lung injury in different animal models. Dong et al. reported that LPS or oleic acid induced lung injury and was associated with greater mortality rates in mice. These effects were reversed by exogenous administration of leptin, through down-regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 and ERK pathways.

Furthermore, some clinical studies also report positive association between the levels of leptin and survival in septic patients, with average leptin levels three times greater in survivors than non-survivors of acute sepsis (Bornstein et al. 1998). Therefore, current literature suggests that leptin may be an important component of host defense against infection and may protect against development of lung injury in the context of infection.

2.15 Proprotein Convertase Subtilisin/Kexin Type 9

It was first discovered in 2003 that mutations in proprotein convertase subtilisin/kexin type 9 (PCSK9) in familial hypercholesterolemia (FH) are associated with variation in Low Density Lipoprotein (LDL) metabolism (Abifadel et al. 2003). Abifadel et al. reported that autosomal dominant hypercholesterolemia was associated with a decrease in low density lipoprotein receptor, and therefore was a risk factor for coronary heart disease (Abifadel et al. 2003). Maxwell et al. were among the first to elucidate the mechanism involved in PCKS9 inhibition of Low Density Lipoprotein Receptor (LDLR) (Maxwell, Fisher, and Breslow 2005). Using LDLR knockout mice
infected with PCSK9 adenovirus, they demonstrated that these mice had no change in plasma cholesterol levels compared to mice infected with control adenovirus (Maxwell, Fisher, and Breslow 2005). Furthermore, they also detected a complete lack of LDLR protein in mice over expressing PCSK9 (Maxwell, Fisher, and Breslow 2005). More recent studies reported that PCSK9 increase LDL – C by reducing the available hepatic LDLR, through binding and targeting LDLR for lysosomal degradation (Lambert et al. 2012). Low expression of PCSK9 allows for recycling of LDLR to the surface and binding and degradation of LDL-C, reducing cholesterol levels (Lambert et al. 2012). PCKS9 expression can be reduced or inhibited completely through monoclonal antibodies, silencing RNA expression and vaccinations. There have been 20 short term clinical trials investigating the clinical potential of PSCK9 inhibition with monoclonal antibodies (Stoekenbroek, Kastelein, and Huijgen 2015). These studies reported PSCK9 as an important therapeutic agent for lowering cholesterol and would help patients that are unable to benefit from current available treatments. (Stoekenbroek et al. 205; Navarese et al. 2015; Zhang et al. 2015)

2.16 PCSK and sepsis

During sepsis, pathogens that initiate the cytokine storm contain lipids including LPS and lipoteichoic acid (Walley et al. 2014). Clearance of these lipids from the circulation reduces the magnitude of the inflammatory response and improve chances of survival. There are distinct similarities between the mechanisms associated with clearance of
cholesterol and lipid pathogens. Lipid pathogens usually trigger an immune response by binding TLRs. Proteins that bind these pathogen lipids include Ligand Binding Proteins, structurally analogous to lipid transfer proteins, such as Plasma Lipid Transfer Protein (PLTP) and Cholesteryl ester transfer protein (CETP). Therefore lipid transfer proteins can also bind pathogen lipids and incorporate them into lipoprotein particles (Azzam and Fessler 2012; Gautier and Lagrost 2011). Thus, it was proposed by Walley et al., that PCSK9 may also affect clearance of lipid pathogens (Walley et al. 2014). It was reported using human liver cells, that PCSK9 was in fact involved in inhibiting LPS uptake. Furthermore, PSCK9 knock out mice, administered with LPS, had a decrease in inflammatory cytokine production (Walley et al. 2014). These beneficial effects were absent in LDLR knock out mice. In the clinical settings, septic shock patients with loss of function PCSK9, had a decrease in inflammatory cytokines and improved survival rates (Walley et al. 2014). These findings were not present in humans with a particular LDLR variant resistant to PCSK9 (Walley et al. 2014). Similarly, studies have reported the statin therapy lowered pro-inflammatory cytokine levels in patients with bacterial infections (Novack et al. 2009), and prior stain use reduced mortality rates in patients with community acquired pneumonia (Mortensen et al. 2005) and severe sepsis (Mansur et al. 2015). Therefore, PCSK9 plays an important role in lipid clearance in sepsis, mediated through LDLR (Walley et al. 2014).
A more recent study by Boyd et al., has shown that PCSK9 levels significantly increase in sepsis. In a single-center observational cohort, with 200 sepsis patients, plasma PCSK9 and lipid levels were measured (Boyd et al. 2016). PCSK9 concentrations above 250 ng/ml were associated with impaired endotoxin clearance in human hepatocytes (Boyd et al. 2016). PCSK9 concentration between 250 and 500ng/ml were associated with respiratory and cardiovascular failure, while patients with concentrations in the range of 220-380 ng/ml did not develop organ failure (Boyd et al. 2016). Therefore, at higher concentrations PCSK9 is associated with organ failure, and therefore is a potential therapeutic target for sepsis (Boyd et al. 2016)

3.0 Hypothesis

Obesity is associated with reduced lung injury during early sepsis, as indicated by lung myeloperoxidase (MPO) and histopathology scores. These responses may be dependent on gut microbiome and mediated though multifactorial processes that involve leptin and differential PCSK9 expression.

4.0 Aims

1. Develop a clinically relevant murine model of diet induced obesity and examine organ specific inflammatory responses.

2. Investigate if housing conditions of mice influence susceptibility to developing hyperglycemia and examine effects on immune responses during early sepsis.
Investigate the mechanisms through which lung inflammation is potentially mediated in our murine model of obesity and early sepsis.

**5.0 Methods:**

**Experimental Animals:**

C57BL/6 mice (Taconic) were fed western high fat diet (modified choline deficient diet, Dyets Inc.) for 15 weeks and induced with sepsis. Two groups of controls were utilized, one had *ad libitum* access to normal chow diet (NCD), while the other was 30% diet restricted and referred to as the ‘diet restricted group’ (NCD DR). Diet restriction was introduced gradually after approximately 2 months of age as previously done by Bonkowski et al. (Bonkowski et al. 2006). Approximately 5-6 mice were used for all experiments. All animal protocols were approved by the Animal Research Ethics Board at McMaster University and in accordance with the Canadian Council of Animal Care regulations.

**Leptin Experiments**

Mice (Taconic) were housed in static cages for 10-12 weeks. One hour prior to surgery, mice were injected with 0.1 mg/kg recombinant leptin protein (R&D), or the equivalent volume of saline. Mice were induced with sepsis using the CLP technique, and sham operated mice were used as controls. 6-hour post-surgery; tissues were harvested, snap frozen in liquid nitrogen and stored at -80°C.

**Sepsis Model: Cecal Ligation and Puncture**
Polymicrobial sepsis was induced using CLP. Isoflurane anaesthetized mice were weighed and subcutaneously injected with analgesic and 2mL Ringer’s Lactate. A catheter was inserted into the right jugular vein, secured with 4-0 sutures and tunneled to the back of the neck. The cecum was exposed through a one centimeter wide incision, one centimetre of the cecum distal to the ileal cecal junction, was ligated and punctured once with an 18G needle. Sham surgery only involved catheterization of the right jugular vein, followed by incision to the abdominal muscle, however, no ligation or puncturing of the cecum was involved. Animals received 1ml Ringer’s Lactate post-surgery. Six hours post-surgery; animals were sacrificed and plasma as well as tissue samples were collected, snap frozen in liquid nitrogen and stored at -80°C.

**Lung Myeloperoxidase activity (MPO) Assay**

Lung tissue samples were collected, washed in PBS, snap frozen in liquid nitrogen and stored at -80°C. Samples were homogenized for 30 seconds in 1mL PBS and centrifuged at 10,000 rpm for 10 minutes. The pellet was re-suspended in 1 mL HTAB, homogenized for 30 seconds and centrifuged at 30000 rpm for 15 minutes. 7μl of each sample were added in triplicate to a 96 well plate. 50 μl of 0.021% H₂O₂ solution was added to a cocktail mix of distilled water, potassium phosphate buffer and O-dianiside. 200 μl of this solution was then added to each well. Changes in absorbance were measured at 450 nm by a spectrophotometer for 90 seconds. Results are represented in units of MPO activity per gram of tissue.
Glucose Tolerance Test (GTT)

Mice were fasted for 6 hours and administered with an intraperitoneal (I.P) injection of 2g/kg of 20% sucrose solution. Blood glucose levels were measured at 15 minute intervals for the first hour, followed by a final measurement at the 2-hour time point.

Histopathology

Tissues from animals were placed in cassettes, and stored in 10% neutral buffered formalin. Tissues were then processed and embedded in paraffin. 5µm thick section were stained with hematoxylin and eosin to visualize tissue morphology. Stained organs were visualized under 200x magnification. Scoring was performed by two blinded histopathology experts. The severity of inflammatory cell infiltration, congestion, steatosis as well as over all microvascular damage were used for scoring (ranging from 0-3, where 0 represented no organ pathology and 3 rereferred to severe damage).

Statistical Analysis:

Data are expressed as mean and SEM. All data were analyzed using ANOVA (with Bonferroni correction of 0.05) and student t test. A p value of less than 0.05 was considered significant.
6.0 Manuscript 1: Inflammation, oxidative stress and multi-organ function during early sepsis in a diet induced murine model of obesity

Forward:
This manuscript was published in Bio Med International (2014). Momina Khan, Amanda Patrick and Alison E. Fox-Robichaud are the authors. The corresponding author is Alison E. Fox-Robichaud. The experiments were conducted by Momina Khan with assistance from Amanda Patrick. The experiments were designed by Momina Khan and Alison E. Fox-Robichaud. The manuscript was written by Momina Khan and edited by Alison E. Fox-Robichaud. This manuscript is reproduced with permission from the publisher.
6.1 Abstract

**Rationale:** Sepsis, the systemic response to infection, is a global health issue and the most common cause of mortality in the Intensive Care Unit. **Objective:** In context of the current obesity epidemic, the aim of this study was to investigate the impact of a high fat Western diet (WD) induced obesity on outcomes associated with early sepsis.

**Methods:** Male C57BL/6 mice were fed either a high fat (Modified Breslow, 21% Butterfat and 0.15% cholesterol) WD or normal chow diet (NCD) for 6, 15 or 27 weeks. Sepsis was induced by cecal ligation and perforation and six hours post-surgery, plasma and tissue samples were collected and snap frozen. **Findings:** Septic obese mice at 15 and 27 weeks had significantly (p<0.0001) lower levels of lung myeloperoxidase (26.3±3.80 U/mg tissue) compared to age matched ad lib (44.1±2.86 U/mg tissue) and diet restricted (63.2±5.60 U/mg tissue) controls, indicative of less lung inflammation. Low levels of lung inflammation were not associated with changes in hepatic cytokines and oxidative stress levels. Obese mice had significantly enlarged livers compared to controls (4.23±0.107g compared to 1.55±0.798g and 1.22±0.0314g). Histological examination of the liver demonstrated that WD fed mice suffered from greater hepatic inflammation with pronounced fat infiltration, steatosis, and hepatocyte ballooning, independent of sepsis. **Conclusions:** These data agree with recent clinical observations that obese individuals are protected from sepsis-induced lung injury. Damage to the hepatic microcirculation from the WD may be responsible for less lung injury due to trapping of neutrophils in the liver preventing their influx downstream to the lungs.
key words: sepsis, inflammation, cytokines, glutathione, obesity
6.2 Introduction

Given the current obesity epidemic, the ratio of overweight and obese patients with critical illness are increasing rapidly however how obesity shapes critical illness and immune response to infection is understudied. Prolonged nutrient surplus causes macrophages to accumulate in the liver and intramuscular visceral fat, triggering synthesis of pro-inflammatory mediators such as Interleukin (IL-6), Tumor Necrosis Factor alpha (TNF-α), Macrophage chemoattractant protein-1 (MCP-1), Matrix Metalloproteinases (MMPs) and fatty acid binding protein (Fantuzzi 2005), that contribute to the development of cardiovascular diseases and metabolic syndrome.

The link between metabolic disorders and inflammation was first discovered in 1993 by Hotamisligil et al., where they reported adipocytes to continuously express TNF-α and that enhanced TNF-α levels in obese mice could be reversed with weight loss (Hotamisligil, Shargill, and Spiegelman 1993). From clinical studies it is known that morbidly obese patients have reduced adiponectin levels and elevated Plasminogen Activator Inhibitor 1 (PAI-1), Interleukin-1α (IL-1α), IL-6 and TNF-α levels (Lundgren et al. 1996; Mohamed-Ali et al. 1997; Yudkin et al. 1999).

Since obesity alone is considered a pro-inflammatory state, exposure to an external inflammatory stimulus, such as bacterial infection, is assumed to further exacerbate the inflammatory response with worse outcomes for obese compared to normal weight (BMI<25Kg/m²) patients. Sepsis is a critical illness characterized by systemic
inflammatory response of host immune system to infection. Stressors such as burns, injuries or infections induce Systemic Inflammatory Response Syndrome (SIRS) that further develops into sepsis. Sepsis is characterized by widespread inflammation with an increase in body temperature, white blood cell and heart and respiratory rates. When the condition progresses to septic shock, the patients stop responding to treatment, develop hypo-perfusion and multi-organ dysfunction and mortality rates at this stage are as high as 60% (Annane et al. 2003). Therefore, sepsis has the highest rates of mortality in the ICU and thus is a huge burden on the health care system.

Several studies have investigated implications of obesity for outcomes of critical illness, however not only is there lack of consistency between results from different studies, but majority of the studies utilize transgenic obese mice (ob/ob) that do not mimic human obesity and non-clinically relevant models of sepsis. Therefore, systemic and local implications of obesity in the context of sepsis are unclear.

This study investigates the link between obesity and early sepsis, primarily focusing on hepatic and pulmonary inflammation, oxidative stress and subsequent organ dysfunction in a clinically relevant murine model of diet induced obesity.

6.3 Methods:

Experimental Animals:

C57BL/6 mice (3-5 weeks old) were ordered from Taconic Farms. Animals were housed in a facility with regulated temperature and a 12 hour light-dark cycle. They
were fed a high fat western diet (WD) (from Dyets Inc., 'Modified choline deficient diet', catalogue #510151) for 6, 15 or 27 weeks and allowed water ad libitum (ad lib), prior to being induced with sepsis. There were two sets of age matched controls; both fed normal chow diet (NCD). One group had normal access to food (ad libitum) whereas the other group were 30% diet restricted. Diet restriction was introduced gradually after approximately 2.5 months of age as previously done by Bonkowski et al. (Bonkowski et al. 2006). Approximately 5-6 mice were used for most experiments. All animal protocols were approved by the Animal Research Ethics Board at McMaster University and in accordance with the Canadian Council of Animal Care regulations.

**Sepsis Model: Cecal Ligation and Puncture**

Polymicrobial sepsis was induced using cecal ligation and puncture (CLP). Isoflurane anaesthetized mice were weighed and injected with analgesic and 2mL Ringer’s Lactate subcutaneously. A catheter was inserted into the right jugular vein and secured with 4-0 sutures, followed by a one centimeter wide incision to the abdominal muscle along the midline. The cecum was exposed, one centimeter ligated and punctured once with an 18G needle. The animal was then administered with 500 µl of Ringer’s Lactate through the catheter. The catheter was tunnelled to the back and sutured in place for future fluid administration. Mice received 1mL Ringer’s Lactate through an intravenous catheter post-surgery. After six hours; mice were sacrificed and plasma as well as tissue samples were collected and snap frozen in liquid nitrogen. Sham
surgery only involved catheterization of the right jugular vein followed by incision to the abdominal muscle however no ligation or puncturing of the cecum was involved.

**White Blood Cell Count (WBC)**

White blood cell count was utilized to quantify the number of leukocytes in blood samples obtained from all experimental groups. 25 μl of blood was mixed with 30% acetic acid and crystal violet solution. White blood cells were counted using a hemocytometer and expressed as cells/L.

**Lung Myeloperoxidase activity (MPO) Assay**

Lung samples were snap frozen in liquid nitrogen and stored at ‐80⁰C. For the assay, samples were homogenized for 30 seconds and centrifuged at 10,000 rpm for 10 minutes. The pellet was re-suspended in HTAB, homogenized again for 30 seconds and centrifuged at 30000 rpm for 15 minutes. 7μl of the sample was added in triplicate to a 96 well plate. 50 μl of 0.021% H₂O₂ solution was added to a cocktail mix of distilled water, potassium phosphate buffer and O-dianiside and added to each well. Changes in absorbance were measured at 450 nm by a spectrophotometer for 90 seconds. Results are represented in units of MPO activity per gram of tissue.

**Glucose Tolerance Test (GTT)**

Mice were fasted for 6 hours and administered with an intraperitoneal injection of 2g/kg of 20% glucose solution. Blood glucose levels were measured from the tail vein at 15 minute intervals for the first hour followed by a final measurement at the 2 hour
time point using a standard glucometer.

**Insulin Tolerance Test (ITT):**

Random-fed mice were administered with 0.75U/kg of insulin intraperitoneally. Glucose levels were measured from the tail vein before the injection as well as at 15, 30, 60 and 120 minute time points using a standard glucometer.

**Glutathione Assay:**

Glutathione assay was performed using kits purchased from Cayman, and the protocols provided were followed.

**Cytokine Assay:**

Cytokine levels were measured with Bioplex assay kits purchased from Bio-Rad and protocols provided were followed.

**Statistical Analysis:**

Data are expressed as mean and SEM. All data was analyzed using ANOVA (with Bonferroni correction of 0.05) and student t test. A p value of less than 0.05 was considered significant.

**6.4 Results:**

**6.4.1 WD feeding induce weight gain and changes in response to glucose and insulin challenges**

Mice fed WD developed obesity by gaining significantly more weight (50.8g±1.05 at 27 weeks) compared to age matched controls (39.6±1.18g) as shown in figure 1. Due
to the increase in weight, there were also changes in response to glucose and insulin challenges. WD fed mice at 15 weeks had significantly higher glucose levels post a glucose challenge compared to both control groups as seen in figure 3, suggesting glucose intolerance. After 27 weeks however, normal chow diet fed ad lib (NCD AL) control group also showed the same response to both glucose and insulin challenges as WD fed obese mice, as seen in figure 4 and 5. Normal chow diet restricted (NCD DR) control group however, continuously had significantly lower glucose levels compared to the other two groups. Therefore, as expected increase in body mass was strongly associated with glucose intolerance and symptoms of metabolic syndrome not only in the WD fed obese mice but also in the ad lib controls after 27 weeks on WD.

6.4.2 Obese septic mice had significantly lower lung inflammation compared to age matched controls

Lung MPO levels were quantified for all groups as a measure of airway inflammation. As anticipated MPO levels were the lowest in naive mice and highest during sepsis for all diet and age groups. Obese septic mice at 6 weeks did not have a significant difference in MPO levels (46.9±2.20 U/mg tissue) compared to controls (51.2±3.38 U/mg tissue) as seen in figure 6. However obese mice at 15 weeks had significantly lower lung MPO (26.3±3.80 U/mg tissue) compared to NCD AL (44.1±2.86 U/mg tissue, p<0.01) and NCD DR controls (63.2±5.60 U/mg tissue, p<0.0001). Similar results were found at 27 weeks where obese septic mice again had significantly (p<0.01)
lower MPO levels (28.3±5.08 U/mg tissue) compared to NCD AL (47.5±2.70 U/mg tissue) and NCD DR (43.9±3.29 U/mg tissue) as illustrated in figures 7 and 8. Therefore WD induced obesity is associated with low MPO levels and thus less airway inflammation during early sepsis.

### 6.4.3 Low MPO levels were not associated with changes in hepatic oxidative stress and inflammation in obese septic mice

Hepatic glutathione was quantified as a marker of oxidative stress. Obese mice at 15 weeks had no significant difference in terms of glutathione levels in naive (153 ±18.5 µM) and sham (144 ±7.32 µM) compared to septic (119 ±7.66 µM) mice, as shown in figure 9. However obese mice at 27 weeks had significantly greater (p<0.0001) glutathione concentration in naive (241 ±8.93 µM) and shams (184 ±11.0 µM) compared to septic mice (127 ±10.3 µM), as shown in figure 10. In terms of controls, in both NCD AL and NCD DR groups at both 15 and 27 weeks, shams (220 ±22.0 µM, 212 ±16.5 µM) and naive (219 ±19.0 µM, 230 ±20.0 µM) mice had significantly (p<0.05) greater glutathione levels compared to septic mice (129 ±11.3 µM, 134 ±12.3 µM). There was no significant difference in terms of hepatic glutathione levels between obese septic mice compared to normal weight or diet restricted septic mice at any age.

Oxidative stress and synthesis of glutathione is closely linked with cytokine expression. Therefore, hepatic Interleukin-1beta (IL-1β), IL-6, TNF-α and MCP-1 levels were quantified in a different set of mice as illustrated in figures 11-14. IL-1β, IL-6 and
MCP-1 levels were all up-regulated in sepsis in both obese mice and NCD AL controls, as seen in figure 11, 12 and 14. However there were no significant differences in hepatic pro-inflammatory cytokine levels in obese compared to control septic mice.

6.4.4 Obesity was associated with changes in hepatic structure with significant steatosis, hepatocyte ballooning and neutrophil infiltration

An increase in body mass was also reflected in age dependent changes in hepatic structure and morphology. Mice fed WD had progressively larger livers the longer they were on the diet as shown in figure 2, where obese mice at 27 weeks had significantly larger liver (4.23 ± 1.07g) compared to obese mice at 6 (1.86 ±0.0820g) and 15 (3.40 ±0.257g) weeks. The controls also followed the same trend however, had significantly smaller livers compared to WD fed groups.

Liver samples were stained with hematoxylin and eosin (H&E) to score damage to the hepatic microvasculature. As shown in Figure 15A, WD fed mice had greater damage to the liver with pronounced lipid deposition between and inside the hepatocytes, as well as hepatocyte ballooning and neutrophil infiltration independent of surgery. Both groups of controls had very few or no lipid deposits in or around the hepatocytes, as shown in figure 16A and 17A.

6.4.5 Obesity is not associated with low white blood cell counts during sepsis

As shown in figure 18, naive mice had greater white blood cell (wbc) counts compared to septic and sham mice in all diet groups. There were no differences in terms
of wbc counts in obese septic mice compared to control septic mice at 6 weeks 
((2.32±0.283 vs. 2.19±0.490) x10⁹ cell/L), 15 weeks ((1.87±0.275 vs. 2.25±0.271 and 
1.51±0.212) x10⁹ cell/L) or at 27 weeks ((2.03±0.235 vs. 1.35±0.127 and 1.74±0.330) x10⁹ 
cells/L). These results suggest that obesity is not associated with lower wbc counts 
during infection in our model.

6.5 Discussion

Our study highlights that obesity may reduce incidence of lung inflammation during 
sepsis and is not associated with changes in hepatic pro-inflammation and oxidative stress levels (Prabhakar et al. 2016). Based on current literature it is unclear how obesity affects critical illness, with mixed reports from standpoints of clinical and animal studies. Some clinical studies suggest that obesity predisposes to as much as 50% increase in 
mortality risk and that obese individuals do not recuperate from critical illness as well as normal weight patients (Nasraway et al. 2006). However, others report obesity to have no effect on outcomes of critical illness and to even be protective (Davos et al. 2003). In terms of animal studies, the majority indicate that obesity has negative implications for sepsis outcomes. Rivera et al. reported WD feeding increases adhesion of leukocytes in the sinusoids and hepatic venules by 8 fold within the first six hours of sepsis (Rivera et al. 2010). Changes in cytokine production and increase in expression of TNF-α, MCP-1 and Intracellular Adhesion Molecule 1 (ICAM-1) have also been reported (Rivera et al. 2010).
The most important finding of our study was that obesity protected from lung injury. Acute lung injury is the leading cause of death in sepsis (Varisco 2011) and requires several different types of therapeutic interventions, contributing to the high cost of care associated with the condition (Rossi et al. 2006). Based on our findings obese septic mice had significantly less airway inflammation and neutrophil sequestration therefore, protecting these mice from lung injury. These results are supported by Wacharasint et al. that conducted a retrospective analysis of body mass and sepsis outcomes (Wacharasint et al. 2013). They discovered that obese patients had the lowest 28 day mortality rates and both obese (BMI>30kg/m²) and overweight (25<BMI<30kg/m²) patients had significantly (p=0.03) less lung infection compared to controls (BMI <25 kg/m²) (Wacharasint et al. 2013). The mechanism behind these finding is not clear but is thought to be linked to obese patients receiving less norepinephrine, vasopressin and fluid on day 1 compared to controls (Wacharasint et al. 2013). Similarly, further experimentation is required to elucidate the mechanism behind obese mice protected from lung injury during sepsis in this study.

The second important finding of this study was that reduced lung injury in obese mice was not due to down-regulation of hepatic pro-inflammatory and oxidative stress mediators. Lung and liver functions are very closely linked and hepatic dysfunction directly affects pulmonary function through cytokine production and clearance (Spagnolo et al. 2010). However, we found no changes in hepatic pro-inflammatory
cytokine levels to account for reduced lung injury. Obesity is usually associated with overload of pro-inflammatory cytokines including IL-6, TNF alpha and MCP-1, MMPs and fatty acid binding protein produced by visceral fat, macrophages and adipocytes (Fantuzzi 2005b). Obesity is also associated with an increase in oxidative stress and usually a reduction in expression of glutathione (Sastre et al. 1989). Sastre et al. reported that in mice fed high fat diet, glutathione levels in hepatocytes were 45% lower and oxidized glutathione levels were 54% lower compared to controls (Sastre et al. 1989). NADPH oxidase catalyzes conversion of oxygen to free radical species that further activates the pro-inflammatory transcription factor NF-κB. Dandona et al. also reported that in obese individuals a 48 hour fast led to a 50% reduction in the levels of reactive oxygen species and the expression of NADPH oxidase was also reduced (Dandona et al. 2001). In this study obesity was not associated with significant differences in pro-inflammatory cytokine and glutathione levels compared to controls in naive or septic mice.

Obesity associated damage to the hepatic microcirculation may account for less neutrophil infiltration into lung tissues during sepsis. Based on Hematoxylin and Eosin stains obese mice had significant steatosis, hepatocyte ballooning and neutrophil infiltration. Thus, there is a possibility that severe damage to the hepatic microcirculation may trap neutrophils preventing their movement into tissues situated
downstream such as the lungs. Further investigation is required to elucidate the mechanism behind the low levels of lung inflammation associated with obesity.

6.6 Conclusion

In summary, from this study we were reported that WD induces obesity and reduces glucose tolerance at 15 and 27 weeks. In context of sepsis, obesity is associated with significantly lower airway inflammation and lung injury. Reduced lung injury in these mice is not associated with changes in levels of hepatic pro-inflammatory cytokines or antioxidants. Obesity induced damage to hepatic microvasculature however may sequester neutrophils and prevent infiltration into the lungs, however further research is required to elucidate the specific mechanisms involved.
6.7 Figures

Figure 1: Average weight of each diet group at their respective end points:
Data is presented as the mean (SE) (n=5/group). #p<0.0001 WD compared to NCD AL and NCD DR. ^p<0.0001 NCD AL compared to NCD DR
Figure 2: Glucose tolerance test at 15 weeks: Results are expressed as changes in glucose levels after an I.P challenge of 2g/kg glucose:

Data is presented as the mean (SE) (n=5 for NCD AL and NCD DR and n= 15 for WD).

#p<0.0001 WD compared to NCD AL and NCD DR. *p<0.01 for WD compared to NCD AL.
^p<0.000 and @p<0.001 NCD AL compared to NCD DR.
Figure 3: Glucose tolerance test at 27 weeks: Results are expressed as changes in glucose levels after an I.P challenge of 2g/kg glucose:

Data is presented as the mean (SE) (n=5 for NCD AL and NCD DR and n= 15 for WD). #p<0.001 for WD compared to NCD AL and NCD DR. Φ p<0.05 for WD compared to NCD AL. @p<0.001, ^p<0.0001, Δp<0.05 NCD AL compared to NCD DR.
Figure 4: Insulin tolerance test at 27 weeks: Results are expressed as changes in glucose levels after an I.P challenge of 0.75U/kg insulin:

Data is presented as the mean (SE) (n=5/group). #p<0.0001 for WD compared to NCD AL and NCD DR. Φp<0.05 for WD compared to NCD AL. Yp<0.001 for WD compared to NCD DR. Δp<0.05 NCD AL compared to NCD DR
Figure 5: Lung myeloperoxidase levels after 6 weeks:
Data is presented as the mean (SE) (n=5/group). δ p<0.0001 for WD CLP compared to WD sham and WD naive. α p<0.001 for NCD AL CLP compared to NCD naive and shams.
Figure 6: Lung myeloperoxidase levels after 15 weeks:
Data is presented as the mean (SE) (n=5/group). δ p<0.05 for WD CLP compared to WD sham and WD naive. α p<0.001 for NCD AL CLP compared to NCD AL naive and shams. β p<0.0001 for NCD DR CLP compared to NCD DR sham and naive. *p<0.01 for WD CLP compared to NCD AL CLP. #p<0.0001 for WD CLP compared to NCD DR CLP.
**Figure 7: Lung myeloperoxidase levels after 27 weeks:**

Data is presented as the mean (SE) (n=6/group). \( \delta p<0.05 \) for WD CLP compared to WD sham and \( (p<0.0001) \) WD naive. \( \alpha p<0.0001 \) for NCD AL CLP compared to NCD AL naive and \( (p<0.01) \) shams. \( \beta p<0.0001 \) for NCD DR CLP compared to NCD DR naive. *\( p<0.001 \) for WD CLP compared to NCD AL CLP. #\( p<0.01 \) for WD CLP compared to NCD DR CLP.
Figure 8: Hepatic glutathione levels after 15 weeks:
Data is presented as the mean (SE) (n=4 for naïve and n=5 for sham and CLP groups). α p<0.05 for NCD AL CLP compared to NCD AL sham and naive. β p<0.0001 for NCD DR CLP compared to NCD DR sham and naive. #p<0.05 for NCD AL sham compared to WD sham. @ p<0.05 for NCD DR sham compared to WD sham.
Figure 9: Hepatic glutathione Levels after 27 weeks:
Data is presented as the mean (SE) (n=5 for CLP and n=4 for sham and naive). δ p<0.0001 for HFD CLP vs. HFD naive. α p<0.01 for NCD AL CLP compared to NCD AL sham and naive. β p<0.01 for NCD DR CLP compared to NCD DR sham and naive.
Figure 10: Hepatic IL-1β Levels after 27 weeks:
Data is presented as the mean (SE) (n=6/group). δ p<0.01 for WD CLP compared to WD sham and (p<0.05) WD naïve. α p<0.001 for NCD AL CLP compared to NCD AL naïve and (p<0.0001) shams.
Figure 11: Hepatic IL-6 Levels after 27 weeks:
Data is presented as the mean (SE) (n=6/group). δ p<0.01 for WD CLP compared to WD sham and (p<0.05) WD naïve.
Figure 12: Hepatic TNF-α levels after 27 weeks:
Data is presented as the mean (SE) (n=6/group). * p<0.05 for NCD AL naïve compared to WD naïve group. A p<0.01 for NCD AL naïve compared to NCD AL sham.
Figure 13: Hepatic MCP-1 levels after 27 weeks:
Data is presented as the mean (SE) (n=6/group). δ p<0.0001 for WD CLP compared to WD sham and WD naive. α p<0.0001 for NCD AL CLP compared to NCD AL naive and sham.
Figure 14: Liver weights of each diet group at their respective end points:
Data is presented as the mean (SE) (n=15/group). #p<0.0001 for WD compared to NCD AL and NCD DR. *p<0.01 for WD compared to NCD AL at 6 weeks.
Figure 15: WD group at 27 weeks:
A) induced with sepsis B) Sham group C) Naive group. H and E staining of the liver x100 magnification.
Figure 16: NCD AL control group at 27 weeks:
A) induced with sepsis B) Sham group C) Naive group. H and E staining of the liver x100 magnification.
Fig 17: NCD DR control group at 27 weeks:
A) induced with sepsis B) Sham group C) Naive group. H and E staining of the liver x100 magnification.
Figure 18: White Blood Cell Counts for all diet groups at their respective end points:
Data is presented as the mean (SE) (n=5/group). * p<0.05 for NCD AL Naive vs. NCD AL CLP at 6 weeks. # p<0.0001 for NCD DR naive vs. NCD DR CLP at 15 weeks. α p<0.01 for NCD AL naive vs. NCD AL CLP at 27 weeks. β p<0.05 for NCD DR Naive vs. NCD DR CLP at 27 weeks.
6.8 References


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Sciences 45(2): 183–87.


7.0 Manuscript 2: Effects of environment and housing conditions on shaping immune response to sepsis

Forward:

This manuscript is in preparation. The authors are Momina Khan, Ji Zhou, Dhruva J. Dwivedi, Laura Rossi, Mikaela Eng, Michael Surette, Patricia C. Liaw and Alison E. Fox-Robichaud. The corresponding author is Alison E. Fox-Robichaud. The animal experiments were completed by Momina Khan. Mikaela and Laura Rossi performed the Bioilluminescence assays on the fecal samples. Dhruva Dwivedi and Ji Zhou performed the histopathology scoring of lung and liver samples. The studies were designed by Momina Khan and Alison E. Fox-Robichaud. Dr. Mike Surette and Dr. Patricia Liaw provided feedback on the experimental design and results. The manuscript was written by Momina Khan with significant input from Alison E. Fox-Robichaud.
7.1 Abstract

Rationale: In the context of disease and drug discovery, it has been rare for findings from animal studies to be extrapolated to the clinical setting. Often, influence of external and internal factors on the animal are not taken into consideration, leading to disparity in reports. Objectives: The primary objective of this study was to examine potential effects of housing conditions on susceptibility to developing metabolic syndrome, and inflammatory response to early sepsis in a diet induced obesity murine model. Methods: For the experiments two types of cages were utilized, static and ventilated. Compared to static cages, ventilated cages have HEPA filtered air supply system and exhaust air ventilation, protecting the animals from air borne particles and preserving the microbiological barrier. Animals were housed in either static or ventilated cages, and obesity was induced in C57BL/6 mice by feeding a high fat diet (modified choline deficient diet) for 15 weeks. Age matched controls were fed a 30% restricted normal chow diet (18% protein, Tekland). After 15 weeks on the respective diets, fecal matter was collected from the cages and animals were induced with sepsis. Six hours post-surgery animals were sacrificed and tissues and plasma harvested, snap frozen in liquid nitrogen and stored at -80°C. Data was analyzed using ANOVA (with Bonferroni correction of 0.05) and student t test. A p value of less than 0.05 was considered significant. Findings: Animals housed in more sterile environment (ventilated cages), had significantly (p<0.0001) less weight gain and lower glucose levels in response to a glucose challenge, compared to the less clean environment (static cages). In addition,
there were no differences in lung MPO levels between obese (32.7±10.3 U/mg tissue) and control (28.1±3.2 U/mg tissue) groups. However, in the static cages, the WD group had significantly less (p<0.0001) lung injury as indicated by MPO (29.1 ±9.1 U/mg tissue), and histopathology scores (1.2±0.2) compared to controls NCD DR (53.0±3.5 MPO U/mg tissue and histopathology score of 2.25±0.3). A beta diversity test was performed to assess potential differences in fecal microorganism composition between the two cages, by generating a distance/dissimilarity matrix. There were significant differences in fecal microbe composition; ventilated groups had greater Firmicutes (69% ± 0.06% for WD and 76% ± 0.03% for NCD) population compared to static groups (42% ± 0.08% for WD and 24% ± 0.02% for NCD), and less Bacteroidetes population (15% ± 0.04% for WD and 12% ± 0.02% for NCD) compared to ventilated groups (37% ± 12% for WD and 53% ± 29% for NCD). **Conclusion:** In conclusion, the environment and gut microbiome have substantial implications for susceptibility to metabolic syndrome and immune responses associated with early sepsis.
7.2 Introduction

Animal models provide foundations for validifying clinical correlations as well as testing and developing innovative treatments. Most animal models are designed carefully to include all parameters of the condition being researched. However often, there are certain external factors that are not taken into consideration. These factors may have important implications that can completely alter the associated responses.

Gut microbiome is an important mediator of immune responses, and variations in which can have important implications for disease outcome. The role of microflora is so important that it has been suggested to be classified as a hidden organ (O’Hara and Shanahan 2006). The human bowel has greater than 400 anaerobic species with concentrations of $10^{11}$ to $10^{12}$ CFU/g (Moore and Holdeman 1974). The combined genome of the gut microflora has approximately 100 times more genes than the human genome (Savage 1977). Intestinal microbiota are mostly anaerobic commensal bacteria that are not harmful to the host and aid in digestion.

Susceptibility to developing obesity and phenotypes of metabolic syndrome are directly influenced by gut microbiome. Bäckhed et al. demonstrated that introducing germ free (GF) C57BL/6 mice with microbiota from conventionally raised mice, led to a 60% increase in fat content and development of insulin resistance in two weeks (Bäckhed et al. 2004). Transferring microbiota harvested from conventionally raised, ob/ob mice into germ free mice transmitted obese traits to the recipient (Turnbaugh et
al. 2006). Similarly, microbiome harvested from Diet Induced Obese (DIO), and transferred to germ free mice, led to an increase in fat deposition (Turnbaugh et al. 2016). It is suggested that the “obese microbiome” can harvest more energy and has greater abundance of genes that code for enzymes involved in digestion of dietary polysaccharides. Obese microbiome also absorbs monosaccharides from the gut, leading to hepatic lipogenesis (Bäckhed et al. 2004).

Gut microflora is an important modulator of immune responses to infection. Intestinal flora mediates cytokine (IL-1, IL-4, TNF and IL-12) production from bone marrow and spleen derived macrophages, differently, suggesting an important role in Th1 and Th2 responses (Nicaise 1999). Similarly, peritoneal macrophages and Kupffer cells from Germ Free (GF) mice secrete less cytokines compared to controls (Nicaise et al. 1993). And LPS treatment increased serum amyloid and acute phase proteins in both conventional and GF mice, however the response was diminished significantly in GF mice (Ikeda et al. 1999). In addition, the serum concentrations of TNF, IL-1 and IL-6 were also significantly lower in GM treated mice (Ikeda et al. 1999).

We previously established a diet induced obese model where obese animals were protected from lung injury. While establishing our model in a new facility, we only had access to ventilated cages as opposed to our original model where only static cages were used. Compared to static cages, ventilated cages have HEPA filtered air supply system and exhaust air ventilation, protecting the animals from air borne particles and
preserving the microbiological barrier. Our new cohort, now housed in ventilated cages had very different weight and glycemic profiles and did not show the same lung inflammatory response. To replicate our original model at the new facility, we changed the housing conditions back to static cages with a new cohort of mice. We investigated if changing the housing conditions will have any effects on weight gain, glycemic state and inflammatory response as well as gut microbiome. We hypothesized that microbiome composition will be altered between the two types of cages, and mice housed in static cages and fed WD, will have increased weight gain, glucose intolerance and there will be significant differences in lung inflammation compared to NCD group.

7.3 Methods

Experimental Animals:

C57BL/6 mice (Taconic) were housed in ventilated or static cages and fed western high fat diet (modified choline deficient diet from Dyets Inc.) for 15 weeks and induced with sepsis. 30% diet restricted mice, referred to as normal chow (18% protein, Tekland) diet restricted group (NCD DR), were used as age matched controls. Diet restriction was introduced gradually after approximately 2 months of age as previously done by Bonkowski et al. (Bonkowski et al. 2006). Approximately 5-6 mice were used for all experiments. All animal protocols were approved by the Animal Research Ethics Board at McMaster University and in accordance with the Canadian Council of Animal Care regulations.
Sepsis Model: Cecal Ligation and Puncture

Polymicrobial sepsis was induced using CLP. Isoflurane anaesthetized mice were weighed and subcutaneously injected with analgesic and 2mL Ringer’s Lactate. A catheter was inserted into the right jugular vein, secured with 4-0 sutures and tunnelled to the back of the neck. The cecum was exposed through a one centimeter wide incision, one centimetre of the cecum distal to the ileal cecal junction, was ligated and punctured once with an 18G needle. Sham surgery only involved catheterization of the right jugular vein, followed by incision to the abdominal muscle, however, no ligation or puncturing of the cecum were involved. Mice received 1mL Ringer’s Lactate through an intravenous catheter post-surgery. Six hours post-surgery; animals were sacrificed and plasma as well as tissue samples were collected, snap frozen in liquid nitrogen and stored at -80°C.

Lung Myeloperoxidase (MPO) Assay

Lung tissue samples were collected, washed in PBS, snap frozen in liquid nitrogen and stored at -80°C. Samples were homogenized for 30 seconds in 1mL PBS and centrifuged at 10,000 rpm for 10 minutes. The pellet was re-suspended in 1 mL HTAB, homogenized for 30 seconds and centrifuged at 30000 rpm for 15 minutes. 7μl of each sample were added in triplicate to a 96 well plate. 50 μl of 0.021% H₂O₂ solution was added to a cocktail mix of distilled water, potassium phosphate buffer and O-dianiside. 200 μl of this solution was then added to each well. Changes in absorbance were
measured at 450 nm by a spectrophotometer for 90 seconds. Results are presented in units of MPO activity per gram of tissue.

**Glucose Tolerance Test (GTT)**

Mice were fasted for 6 hours and administered with an intraperitoneal (I.P) injection of 2g/kg of 20% sucrose solution. Blood glucose levels were measured at 15 minute intervals for the first hour, followed by a final measurement at the 2-hour time point.

**Bioilluminescence Assay**

Genomic DNA was extracted from 2 mouse fecal pellets as described in Whelan *et al.* with some modifications (Whelan et al. 2014). Briefly samples were transferred to screw cap tubes containing 2.8mm ceramic beads, 0.1mm glass beads, GES and sodium phosphate buffer. Samples were bead beat and centrifuged and the supernatant was further processed using the MagMAX Express 96-Deep Well Magnetic Particle Processor from Applied Biosystems with the multi sample kit (Life Technologies#4413022). Purified DNA was used to amplify the variable region 3 of the 16S rRNA gene with PCR using Illumina adapted primers as described in Whelan *et al.* Resulting PCR products were normalized using the SequalPrep normalization kit (ThermoFisher#A1051001) and sequenced with the Illumina MiSeq platform. Resulting sequences were run through an in house bioinformatic pipeline as described in Whelan *et al* (Whelan et al. 2014). All further analysis was done using QIIME.
**Histopathology**

Tissues from animals were placed in cassettes, and stored in 10% neutral buffered formalin. Tissues were then processed and embedded in paraffin. 5µm thick sections were stained with hematoxylin and eosin to visualize tissue morphology. Stained organs were visualized under 200x magnification. Scoring was performed by two blinded histopathology experts. The severity of inflammatory cell infiltration, congestion, steatosis as well as overall microvascular damage were used for scoring (ranging from 0-3, where 0 represented no organ pathology and 3 referred to severe damage).

**Statistical Analysis:**

Data are expressed as mean and SEM. All data were analyzed using ANOVA (with Bonferroni correction of 0.05) and student t test. A p value of less than 0.05 was considered significant.

**7.4 Results**

**7.4.1 WD feeding induced weight gain but no major changes in glycemic state observed when housing conditions were changed**

Figure 1A and 1B displays images of the two different types of cages utilized in this study. 1A displays static cages with no mechanical air filtration, while 1B shows ventilated cages that provide HEPA filtered air ventilation. Figure 2 displays weight gain of mice fed different diets over the course of 15 weeks, housed in either static or ventilated cages. As shown in figure 2, WD fed mice in both static and ventilated cages had significant weight gain, however mice in ventilated cages were significantly smaller.
(p<0.0001). Similarly, there were differences in the glycemic states of these mice. In the ventilated cages, as shown in figure 3, even though there were significant differences between WD and NCD DR mice at 15 and 30 minute time points, unlike the static cages, glucose levels remained below 11mmol/L at all times.

7.4.2 Variation in lung injury observed in association with difference in environmental conditions

Lung MPO levels were quantified for all groups as a measure of airway inflammation. As anticipated MPO levels were the lowest in naive mice and highest during sepsis for both diet groups. We previously found that mice housed in static cages and fed WD for 15 weeks, when induced with sepsis had significantly (p<0.0001) lower lung MPO levels (26.3±3.8 U/mg tissue), compared to age matched diet restricted controls (63.2±5.6 U/mg tissue) (Khan, Patrick, and Fox-Robichaud 2014). However, as shown in figure 4, this response was not observed in mice housed in ventilated cages and we did not detect a significant difference in lung MPO levels between WD (32.7±10.3 U/mg tissue) and NCD DR (28.1±3.2 U/mg tissue) groups. However, when the housing were changed back to static cages with a new cohort of mice, similar pattern of lung injury (WD (29.1 ±9.1 U/mg tissue) and NCD DR (53.0±3.5 U/mg tissue)) was observed as our published model.
7.4.3 Variation in pulmonary damage as indicated by H&E staining and histopathology scores

Using H&E stained lung sections, it was observed that NCD CLP static group (5C) had greater damage as shown by congestion, leukocyte infiltration in vessel walls, and intra-alveolar migration compared to the WD CLP static group (5A). The level of histopathological damage was similar between WD and NCD CLP vent groups (5B and 5D). Sham groups had less damage compared to CLP groups but there was some degree of inflammation observed (5E-H). These reports were further supported by histopathology scoring of the H&E stained lung sections, as shown in figure 6. Based on lung histopathology scores, NCD CLP static group (2.25±0.3) had a significantly (p<0.0001) higher score compared to the WD CLP static group (1.2±0.3). Mice housed in the ventilated cages had no significant differences between the lung histopathology scores.

7.4.4 Difference in microflora between the static and ventilated conditions

We performed an analysis of the bacterial colonies present in fecal matter of mice housed in static and ventilated cages. Differences in gut microbiome composition were assessed by Principal Coordinates Analysis (PCoA), a measure of dissimilarity based on evolutionary relatedness. A beta diversity test was performed to assess potential differences between the two groups by generating a distance/dissimilarity matrix. Figure 7 displays data from the beta diversity distance matrix in a 2 dimensional plot. The PCoA plot showed significant differences in the composition of gut microbial communities.
between static and ventilated groups, after 15 weeks on diet. PC1 or first principal coordinate, representing response to type of cage, explains 50.9% of the inter sample variance. Thus, there is clear segregation between static and ventilated groups, suggesting that the type of cage is a significant force in shaping gut microbiome composition.

Differences in diet had some effects on gut microbial biodiversity of mice housed in the same type of cage, as shown in figure 8. This suggests that the type of diet (WD vs. NCD) contributed to some degree of variance, however the differences were not significant.

Based on analysis of microbial taxa distributions at the phylum level, we found that mice housed in ventilated groups had a greater percentage of Firmicutes (69% ± 0.06% for WD and 76% ± 0.03%) compared to the static groups (42% ± 0.08% for WD and 24% ± 0.02%), as shown in figure 9. Ventilated groups also had Verrucomicrobia which were not present in the static group. The static groups had greater numbers of Bacteroidetes (37% ± 12% for WD and 53% ± 29% for NCD), compared to the ventilated groups (15% ± 0.04% for WD and 12% ± 0.02% for NCD). Static groups also had Tenericutes and Actinobacteria, which were completely absent in the ventilated groups (Figure 9).
We also found differences between the microbial compositions at the genus level (figure 10). We found that fecal matter from static cages contained certain families that were completely absent in the ventilated group, including *Desulfovibrionaceae*, *Bifidobacterium*, *tm7*, *Helicobacteraceae*, and *Riknellaceae*. Ventilated cages had a slightly greater abundance of *Verrucomicrobiaceae* (5.8±1.7% for WD and 8.7±1.6%) compared to static cages (3.7%±3.7% for WD and 0% for NCD), however the difference was no significant.

Figure 11 displays the different strains of bacteria found in static and ventilated cages, labelled as pathogenic, commensal or both. This figure conveys that overall there were more pathogenic and commensal strains present in static cages compared to ventilated ones. Furthermore, there were no significant differences in terms of diversity between WD and control groups of the same cage type.

### 7.5 Discussion

Animal models have been imperative tools in understand disease pathogenesis and the effectiveness of therapeutic interventions. It is however essential to consider specific environmental conditions when designing these models, as external factors may influence susceptibility to disease and the associated immune responses. We previously established a clinically relevant murine model of obesity and sepsis (Khan, Patrick, and Fox-Robichaud 2014). We found that mice fed a WD for 15 weeks developed obesity,
hyperglycemia and had significantly less inflammation, as indicated by lower lung MPO levels compared to controls (Khan, Patrick, and Fox-Robichaud 2014). The conditions of our original model included mice being housed in static cages for the duration of the study.

While re-establishing our model at a new animal facility, animals were housed in ventilated cages. We noticed a different pattern of weight gain and glycemic states in mice fed WD for 15 weeks (figure 2 and 3). More specifically these animals gained significantly less weight (figure 2) and did not show a drastic response when challenged with an intraperitoneal glucose bolus (figure 3). Furthermore, the control group had low levels of lung inflammation in response to early sepsis (figure 4). We then investigated if changing the cages back to static ones (as our original model), would have any effects on weight gain and the inflammatory responses. A new cohort of mice were housed in static cages for 15 weeks, and fed either a WD or NCD. We observed that the animals in static cages gained more weight (figure 2), and developed hyperglycemia (figure 3) compared to mice in ventilated cages. In addition, in static cages mice on NCD had greater lung injury compared to the NCD group in ventilated cages, as indicated by lung MPO levels (figure 4) and histopathological scores (figure 5 and 6). The primary difference between the two groups were the types of cages the animals were housed in, therefore that led us to examine any potential differences in the fecal microflora.
It has been known for decades that intestinal microflora plays an important role in the pathophysiology of metabolic syndrome. The primary function of microbiota of the large intestine is to break down starch and dietary fiber, that remain unprocessed by enzymes of the small intestines. The main products released from processing of fiber are short chain fatty acids, acetate, propionate and butyrate (Macfarlane and Gibson 1997). The composition of the microbiome may be altered in response to the diet being consumed, in order to improve processing of simple sugar for efficient energy harvest (Turnbaugh et al. 2006).

In our study, we assessed and compared the compositions of fecal microbiota collected from mice housed in static and ventilated cages. We gathered from fecal microbial beta diversity analysis, that there were significant differences in the compositions of gut microbiome between the static and ventilated groups (figure 7). More specifically, there was a greater abundance in *Bacteroidetes* compared to *Firmicutes* in the static group (with overt obesity and hyperglycemia), while the opposite was true for the ventilated groups (figures 9 and 10). Differences in the type of diet alone did not induce significant changes in the compositions of the gut microbiome (figure 8).

Our findings are supported by a large study conducted by Schwiertz, where it was reported that the ratio of *Bacteroidetes* to *Firmicutes* increased in overweight and obese subjects after controlling for confounding factors (Schwiertz et al. 2010). Duncan et al.,
reported that in human fecal samples from obese, non obese and obese on weight loss diets, there were no differences detected in the abundance of *Bacteroidetes* (Duncan et al. 2008). However, there was a reduction in Firmicutes in obese subjects on weight loss diets (Duncan et al. 2008).

There are also some studies that contradict our findings. For instance, Turnbaugh et al., reported that ratio of *Firmicutes* (*Mollicutes*) to *Bacteroidetes* increases with a HFD, and this ratio is reversed when the animals are switched to a low fat or low carbohydrate diet (Turnbaugh et al. 2006). Ley at al., also reported that the abundance of *Bacteroidetes* to *Firmicutes* is decreased in obese humans, and can be reversed with weight loss and a low calorie diet (Ley et al. 2006). The limitations of these studies include small sample size, and not controlling for confounding factors such as physical activity and daily energy intake, which may have important implications for shaping gut microbiome (Schwiertz et al. 2010).

Both *Firmicutes* and *Bacteroidetes* phyla are responsible for fermentation of carbohydrates, producing short chain fatty acids (SCFAs), CO₂ and H₂ (den Besten et al. 2013). *Bacteroidetes* mainly produces acetate and propionate, while Firmicutes produces butyrate (Ismail et al. 2011). Butyrate has been shown to increase insulin sensitivity in mice (Hartstra et al. 2014), and has anti inflammatory action (Brahe, Astrup, and Larsen 2013). In addition, studies have shown that Butyrate can protect against diet induced obesity (Lin et al. 2012) and increases leptin gene expression (Harris
et al. 2012). A greater number of Firmicutes were found in the ventilated (WD) groups, which potentially explain the low weight gain and subdued inflammatory response, compared to static cages (WD group) with greater Bacteroidetes: Firmicutes ratio.

Furthermore, WD changes gut microbiome by decreasing gut barrier protecting bacteria, leading to higher gut permeability, increasing pathogens that release LPS and thus high level of inflammation that may lead to the development of metabolic syndrome (Cani et al. 2007; C. Zhang et al. 2009). Changes in microbiome are detected by PPRs which can recognize structural components of microbes (such as LPS, lipoproteins and peptidoglycans), and regulate inflammatory responses. PRRs are also involved in development of insulin resistance and other metabolic conditions. (Cani et al. 2007). In our study Firmicutes (gram positive) was associated with reduced weight gain. While Bacteroidetes (gram negative) was associated with greater weight gain, and high levels of lung inflammation in the NCD group. These findings are supported by studies which shown that LPS from gram negative gut bacteria are associated with low grade inflammation and insulin resistance (Wellen and Hotamisligil 2005). Furthermore, Cani et al., reported that increasing gram positive bacteria, negatively correlated with endotoxemia and improved glucose tolerance and decreased levels of plasma and adipose tissue proinflammatory cytokines (Cani et al. 2007).

In addition, there were no significant differences in terms of the diversity of strains between WD and NCD housed in the same type of cages (figure 8). There were
similar strains of pathogenic and commensal bacteria found in both diet groups (figure 11), although the abundance differed. The abundance of strains also varied significantly between static and ventilated cages (figures 9 and 10). Static cages had greater diversity and some strains found in fecal matter from static cages, were completely absent in the ventilated group including, Desulfovibrionaceae, Bifidobacterium, tm7, Helicobacteraceae, Riknellaceae and in general there was less diversity in the phylum of Bacteroidetes. It has been reported that sulfate reducing bacteria such as Desulfovibrionaceae increase with a high fat diet and contribute to the development of metabolic syndrome (Zhang-Sun et al. 2015). Ventilated cages had a slightly greater abundance of Verrucomicrobiaceae, which have been reported to be associated with weight loss in mice (Liou et al. 2013).

7.6 Conclusion

In conclusion, our study highlights the important implications of environmental conditions of animal models and variations in gut microbiome composition, on the development of metabolic syndrome and inflammatory responses. We found that variations in the types of cages, lead to a shift in the ratio of specific bacteria strains potentially altering immune responses. In this study, mice housed in static cages developed obesity and hyperglycemia, and had a greater ratio of Bacteroidetes to Firmicutes. While mice in ventilated cages had a greater abundance of Firmicutes, and did not develop overt obesity or glucose insensitivity and the NCD group had subdued
lung inflammatory response during early sepsis. Limitations of this study include assessment of microbial composition as a snap shot at the end of 15 weeks, as opposed to a more detailed trajectory at several time points. That may have provided more detail and enhanced our understanding of the effects of gut microbiota on the development of metabolic syndrome.
7.7 Figures

Figure 1A

Figure 1B

Figure 1: Images of static and ventilated cages:

1A Static cages were placed on a rack and air was filtered through the lid. Mice were housed in static cages for 15 weeks and fed either a WD or NCD. 1B Ventilated cages were plugged into a rack that provided HEPA filtered air and protected the animals from any airborne particles. Mice were housed in ventilated cages for 15 weeks and fed either a WD or NCD.
Figure 2: Average body weight of each diet group at 15 weeks housed in static and ventilated cages:
4-5 weeks old mice were housed in either ventilated or static cages and weight gain was monitored. Data is presented as the mean (SE) (n=5/group). ^p<0.0001 WD 15 week ventilated compared to WD 15 week static. * p<0.0001 WD compared to NCD DR in each group.
Figure 3: Glucose tolerance test at 15 weeks comparing WD and NCD DR groups housed in static and ventilated cages:
4-5 weeks old mice were housed in either ventilated or static cages and glucose tolerance was assessed by an intraperitoneal glucose challenge. Results are expressed as changes in glucose levels after a 2g/kg I.P challenge. Data is presented as the mean (SE) (n=10 for ventilated, n=6 for static) *p<0.05 WD compared to NCD DR. ^p<0.0001 for static WD compared to vent WD groups.
Figure 4: Lung MPO levels of mice housed in ventilated and static cages after 15 weeks on diet:

WD and NCD fed mice housed in either ventilated or static cages were subjected to CLP or sham surgeries. Lung injury was quantified by using MPO as a surrogate marker of inflammation. Data is presented as the mean (SE) (n=5-6/ group) * p<0.01 for CLP compared to sham. ^p<0.0001 for static WD compared to static NCD CLP. # p<0.0001 for NCD CLP static compared to NCD CLP Vent.
Figure 5: H&E stained images of lung samples from WD and NCD fed mice housed in static and ventilated cages:
Tissues were harvested six hours post surgery and stored in buffered formalin. Lung tissues were processed, embedded in paraffin wax and 5 μm thick sections were stained with hematoxylin and eosin for visualizing overall morphology. Photomicrographs of stained tissues were examined under 200x magnification (25μm). Figures A-D represent CLP groups, E-F represent sham groups. A. WD CLP Static, B. WD CLP Vent, C. NCD CLP Static, D. NCD CLP Vent, E. WD Sham Static, F. WD Sham Vent, G. NCD Sham Static and H. NCD Sham Vent. Data is presented as the mean (SE) (n=4-5/ group).
Figure 6: Histopathology scores of lung samples from mice housed in ventilated and static cages after 15 weeks on diet:

WD and NCD fed mice housed in either ventilated or static cages were subjected to CLP or sham surgeries. Harvested lung tissues were scored for injury by two blinded researchers with expertise in pathology. Tissues were scored for inflammatory cell infiltrate, interstitial edema, cell necrosis, vascular congestion and assigned a score from 0-3. Healthy sham animals were assigned a score of 0. Data is presented as the mean (SE) (n=4-5/group). *p<0.05 for static NCD CLP compared to static WD CLP. ^p<0.0001 for static NCD CLP compared to static NCD Sham.
Figure 7: Fecal microbial beta diversity analysis of static and ventilated conditions: Fecal samples were collected from mice housed in static and ventilated cages. Bioilluminescence assay were conducted to determine diversity between microbial compositions of the different groups. The pink dots represent static and the green represent ventilated groups. Data is shown in percentages (n=3/group).
**Figure 8: Fecal microbial beta diversity analysis of mice fed WD or NCD:**
Fecal samples were collected from mice housed in static and ventilated cages and fed WD or NCD. Bioilluminescence assay were conducted to determine diversity between microbial compositions of the different groups (WD and NCD). The pink dots represent WD and the green represent NCD. Data is shown in percentages (n=3/group).
Figure 9: Fecal microbial taxa distribution (phylum) of mice housed in static and ventilated conditions:

Fecal samples were collected from mice housed in static and ventilated cages. Bioilluminencescence assays were conducted to determine diversity at the phylum level between microbial compositions of the different groups. Figure 9A represents static WD, 9B static NCD, 9C vent WD and 9D vent NCD. Data is shown in percentages (n=3/group).
10A. Static WD

10B. Static NCD

10C. Vent WD

10D. Vent NCD
Figure 10: Fecal microbial taxa distribution (genus) of mice housed in static and ventilated conditions:

Fecal samples were collected from mice housed in static and ventilated cages. Bioilluminescence assays were conducted to determine diversity at the genus level between microbial compositions of the different groups. Figure 10A represents static WD, 10B static NCD, 10C vent WD and 10D vent NCD. Data is shown in percentages (n=3/group).
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**Figure 11: Comparison of pathogenic versus probiotic strains of microbes (phyla; class; order; family):**

Fecal samples were collected from mice housed in static and ventilated cages. Bioilluminiscence assays were conducted to determine microbial composition between different groups (n=3/group). The microbes were further divided based on their function as pathogenic, commensal or potential for both (Clavel et al. 2014; Zhang-Sun et al. 2015; Jandhyala et al. 2015; Pruneau et al. 2014; Jakobsson et al. 2015; Soo et al. 2014)
7.8 References


8.0 Manuscript 3: Investigating potential mechanisms protecting mice on western diet from lung inflammation during sepsis

Foreword:

This manuscript is in preparation. The authors are Momina Khan, Dhruva J. Dwivedi, Ji Zhou, Annik Prat, Nabil G. Seidah, Patricia C. Liaw and Alison E. Fox-Robichaud. The corresponding author is Alison E. Fox-Robichaud. All animal experiments were completed by Momina Khan. Dhruva Dwivedi and Ji Zhou performed the histopathology scoring. PCSK9 mice were provided by Dr. Nabil Seidah and animal breeding was conducted by Dhruva Dwivedi with the assistance of Peter Grin. The studies were designed by Momina Khan and Alison E. Fox-Robichaud. Dr. Patricia Liaw provided feedback on the experimental design and the results. The manuscript was written by Momina Khan with significant input from Alison E. Fox-Robichaud.
8.1 Abstract

Rationale: Obesity increases the risk of sepsis, but how obesity shapes the immune responses to infection is unknown. In congruence with findings from clinical studies, we previously demonstrated that Western diet fed obese mice have reduced lung inflammation during early sepsis. **Objective:** In this study, we further explored potential mechanisms to explain this finding. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) is a protein involved in cholesterol homeostasis and is implicated in sepsis survival. Leptin is a hormone produced by adipocytes that regulates energy homeostasis, and the levels are elevated with obesity and sepsis. We hypothesized that either PCSK9 and/or leptin contributed to the obesity-associated lung protection in early sepsis. **Methods:** PCSK9 knock out, PCSK9 overexpressing and wild type mice on a C57/BL6 background were fed either a high fat Western diet (WD), or a normal chow diet (NCD) for 15 weeks (n=5/group). Sepsis was induced by cecal ligation and puncture (CLP). Tissues were harvested six hours post surgery. For the leptin studies mice were housed in static cages for 10-12 weeks prior to sepsis surgery. Mice were injected with recombinant leptin protein (1mg/kg) one hour prior to CLP, then re-anesthetized and tissues and plasma harvested at 6 hours. All mice were resuscitated with 2ml of lactated Ringers SQ pre surgery, and 1ml IV post surgery. Lung injury was assessed by myeloperoxidase (MPO in U/mg of tissue) assay of lung tissues and histopathology scores. Data are expressed as mean ± SEM and analyzed using ANOVA or t-test.

**Findings:** Septic PCSK9 over expressing mice fed NCD had greater lung MPO levels
(46.5±4.5 U/mg tissue) compared to PCSK9 deficient mice (31.1±1.7 U/mg tissue) on NCD (p<0.01). In mice fed the WD for 15 weeks, the protection from the loss of PCSK9 was not evident, however the injury was reduced compared to NCD groups. Septic PCSK9 deficient (14.4±1.4 U/mg tissue), wildtype (17.6±0.9 U/mg tissue), and overexpressing (17.9± 1.0 U/mg tissue) mice on WD had no significant differences in lung MPO levels. This correlated with histopathology scores for PCSK9 deficient (0.7±0.2), wildtype (1.1±0.2), and PCSK9 overexpressing (1.3±0.7) septic mice. We found that leptin treated septic mice had lower lung MPO (32.6±1.6 U/mg tissue) levels, compared to saline treated septic mice (46.6±3.5 U/mg tissue) (p<0.01). Sham operated mice had significantly lower MPO levels (12.7±1.7 U/mg tissue for leptin and 11.8±1.2 U/mg tissue for saline) compared to septic counterparts. **Conclusion:** Our data suggested that both lack of PCSK9 and increases in leptin contributed to the lung protection in early sepsis. However, when exposed to a WD, the potential benefits of PCSK9 deficiency to further reduce lung injury were no longer evident. These findings have implications for potential therapeutic strategies to reduce sepsis induced lung injury.
8.2 Introduction

Sepsis is a critical condition that is the most common cause of death in non-coronary Intensive Care Units, and is associated with high cost of care. It is characterized by dysregulated systemic response to infection that leads to organ dysfunction (Gotts and Matthay 2016). The endothelium is a layer of squamous cells that line the interior surface of vessels, and regulate the influx of nutrients, signalling molecules and cells across the cell wall and into tissues, as well as vasomotor tone (Aird 2003). Systemic inflammation induces vasodilation, leukocyte adhesion, coagulation and loss of integrity of the endothelium, causing fluid to leak out of the vascular bed and into the interstitial spaces (Aird 2003). In addition, normal flow in the microvascular beds is obstructed by microthrombi and plugs of immune cells, leading to hypoxia (Koh et al. 2010). Further loss of normal organ functioning occurs due to tissue factor expression, fibrin and blunted anticoagulant processes that establish disseminated intravascular coagulation (DIC) (Levi 2010). Loss of endothelial integrity and normal function create a hypoxic environment where tissues resort to using anaerobic metabolism, leading to lactic acidosis and death (Bellomo and Ronco 1999).

The lungs are among the first organ system to be implicated during the progression of systemic inflammation. Lung injury can occur within 90 minutes of the onset of systemic inflammatory response syndrome, the first stage in sepsis disease continuum (Bhatia and Moochhala 2004). Loss of alveolar epithelial barrier function and
lung capillaries being highly permeable, allow for influx of edema into the interstitial and alveolar spaces (Matthay, Ware, and Zimmerman 2012). This leads to arterial hypoxemia, impaired gas exchange and loss of normal functioning of the lungs (Matthay, Ware, and Zimmerman 2012). In the clinical setting, oxygen delivery is usually enhanced through fluid resuscitation and mechanical ventilation. However, despite interventions lung injury associated with sepsis remains an important concern for intensivists.

In the context of the recent obesity epidemic, the number of obese patients with sepsis are on the rise. There are several mixed reports with regards to the implication of obesity for sepsis outcomes, where some reports suggest an increase in mortality risk (Spagnolo et al. 2010), others suggest the contrary. Pickkers et al. found in an observational cohort study with 154,308 ICU patients from Dutch urban and non-urban teaching hospitals, that there is an inverse relationship between obesity and hospital mortality (Pickkers et al. 2013). In their study, obese patients (BMI 30-39.9kg/m²) had the lowest risk of mortality (Pickkers et al. 2013). Furthermore, Wacharasint et al. reported from a retrospective analysis of septic shock patients that obese patients had the lowest mortality rates and less incidence of lung and fungal infections (Wacharasint et al. 2013). They suggested the reason to be use of less fluid, vasopressin and norepinephrine administration in these patients (Wacharasint et al. 2013).

We previously established a murine model of sepsis in diet induced obese mice, and we reported that obese animals had significantly less lung injury compared to age
matched controls during early sepsis. We were then interested in examining Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) and leptin as potential mediators that may be involved in the protective responses associated with obesity, observed in our obese model.

PCSK9 is a protein involved in cholesterol homeostasis, first characterized in 2003 (Seidah et al. 2003). It functions by binding hepatic low density lipoprotein receptors (LDLR) and targets them for lysosomal degradation, reducing the number of LDLR available for LDL clearance (Naureckiene et al. 2003). The important role of PCSK9 in the modulation of the innate immune response, and implications for outcomes of septic shock have recently come to light. Walley et al., were the first to show that reduced PCSK9 levels, increase the availability of LDLR, which are involved in pathogen lipid clearance (Walley et al. 2014). In the clinical setting, septic shock patients with PCSK9 loss of function mutations, had improved survival rates (Walley et al. 2014). It has also been reported that young PCKS9 knockout animals have reduced lung injury compared to wildtype and transgenic animals (Dwivedi et al. 2016). Findings from both human and animal models suggest the important role of PCSK9 in lung injury and immune response to sepsis (Walley et al. 2014).

Leptin is a hormone produced by adipocytes that relays information to the central nervous system regarding energy reserves in the body and thus regulates energy homeostasis (Friedman 2004). Over the past few decades studies have elucidated the
important role of leptin in immune response to infection. Low concentration of leptin as seen in cases of malnutrition, increases susceptibility of developing infections and is associated with immunosuppression (Lord et al. 1998). Leptin levels are directly proportion to the mass of adipose tissue, and therefore endogenous leptin levels are elevated in the case of obesity in humans (Wong et al. 2004).

For this study, we investigated the potential implications of PCSK9 expression and leptin on lung injury in the context of sepsis. We investigated the effects of differential PCSK9 expression on inflammation during early sepsis in a murine model of diet induced obesity. We also examined the effects of exogenous leptin administration on inflammatory responses during early sepsis. Based on previous literature, we hypothesized that lack of PCSK9 expression and leptin administration will be associated with reduced inflammation and lung injury associated with sepsis.

8.3 Methods

Experimental Animals:

Mice with differential PCSK9 expression were acquired from Dr. Nabil Seidah and Dr. Annik Prat at McGill University. PCSK9 knockout, transgenic and wild type animals with a C57B/6 background were fed either a high fat western diet (modified choline deficient diet from Dyets Inc.) or a normal chow diet (18% protein, Tekland) for 15 weeks. The normal chow diet group was diet restricted (30%) to prevent excessive weight gain (Bonkowski et al. 2006). For the leptin experiments, mice were housed in
static cages for 10-12 weeks. These animals were then treated (I.P) with leptin (1mg/kg) one hour pre-surgery, and lung Myeloperoxidase (MPO) and histopathology were quantified six hours post sepsis surgery. Approximately 5-6 mice were used for all experiments. All mice were housed in static cages with a 12 hour light/dark cycle and *ad lib* access to water. All protocols were approved by the Animal Research Ethics Board at McMaster University and in accordance with the Canadian Council of Animal Care regulations.

**Sepsis Model: Cecal Ligation and Puncture**

Polymicrobial sepsis was induced using CLP. Isoflurane anaesthetized mice were weighed and subcutaneously injected with analgesic and 2mL Ringer’s Lactate. A catheter was inserted into the right jugular vein, secured with 4-0 sutures and tunnelled to the back of the neck. The cecum was exposed through a one centimeter wide incision, one centimetre of the cecum distal to the ileal cecal junction, was ligated and punctured once with an 18G needle. Sham surgery only involved catheterization of the right jugular vein, followed by incision to the abdominal muscle, however, no ligation or puncturing of the cecum were involved. Animals received 1mL Ringer’s Lactate through an intravenous catheter post-surgery. Six hours post-surgery; animals were sacrificed and plasma as well as tissue samples were collected, snap frozen in liquid nitrogen, and stored at -80°C.
**Glucose Tolerance Test (GTT)**

Mice were fasted for 6 hours and administered with an intraperitoneal (I.P) injection of 2g/kg of 20% sucrose solution. Blood glucose levels were measured at 15 minute intervals for the first hour, followed by a final measurement at the 2-hour time point.

**Lung Myeloperoxidase (MPO) Assay**

Lung tissue samples were collected, washed in PBS, snap frozen in liquid nitrogen and stored at -80°C. Samples were homogenized for 30 seconds in 1mL PBS and centrifuged at 10,000 rpm for 10 minutes. The pellet was re-suspended in 1 mL HTAB, homogenized for 30 seconds and centrifuged at 30000 rpm for 15 minutes. 7μl of each sample were added in triplicate to a 96 well plate. 50 μl of 0.021% H₂O₂ solution was added to a cocktail mix of distilled water, potassium phosphate buffer and O-dianiside. 200 μl of this solution was then added to each well. Changes in absorbance were measured at 450 nm by a spectrophotometer for 90 seconds. Results are presented in units of MPO activity per gram of tissue.

**Histopathology**

Tissues from animals were placed in cassettes, and stored in 10% neutral buffered formalin. Tissues were then processed and embedded in paraffin. 5μm thick section were stained with hematoxylin and eosin to visualize tissue morphology. Stained organs were visualized under 200x magnification. Scoring was performed by two blinded
histopathology experts. The severity of inflammatory cell infiltration, congestion, steatosis as well as over all microvascular damage were used for scoring (ranging from 0-3, where 0 represented no organ pathology and 3 referred to severe damage).

**Statistical Analysis:**

Data are expressed as mean and SEM. All data were analyzed using ANOVA (with Bonferroni correction of 0.05) and student t test. A p value of less than 0.05 was considered significant.

**8.4 Results**

**8.4.1 WD was associated with increase in weight gain and glucose response to interperitoneally glucose challenge**

As shown in figure 1, there were no difference in terms of weight gain between knockout (40.77g ± 1.21g), wildtype (42.55g ± 2.13g) and transgenic (45.17g ± 0.96g) mice on the high fat diet. There were also no differences between the weights of knockout (23.93g ± 0.30g), wildtype (23.96g ± 0.59g) and transgenic (23.19g ± 0.70g) on the normal restricted diet. Animals in the WD group were significantly (p<0.0001) larger than the NCD (diet restricted) group. In terms of glucose levels, as shown in figure 2, mice on the WD had significantly greater glucose level in response to an intraperitoneal glucose challenge, compared to the NCD group. Interestingly, obese mice over expressing PCKS9 (Tg+) had significantly greater glucose levels at the 30 minute (17.1±1.03mmol/L) and 60 minute (18.38±0.45mmol/L) time points, compared to knockout (14.06±0.81mmol/L and
10.68±1.06 mmol/L) and wildtype (12.7±0.50 mmol/L and 12.1±0.60 mmol/L) at 30 and 60 minutes respectively. Knockout, transgenic and wildtype mice in the NCD group did not show any significant differences in glucose response when administered with a glucose challenge, as shown in figure 2.

8.4.2 PCSK9 over expression increased lung injury in mice on normal diet, however no effects were observed in the WD group

PCSK9 over expressing mice, fed the normal diet, had significantly greater lung MPO levels (46.51±4.51 U/mg tissue) compared to PCSK9 knockout mice (31.14±1.75 U/mg tissue) on NCD, as shown in figure 3. There were no significant differences in MPO levels between PCSK9 over expressing (46.51±4.51 U/mg tissue) and wildtype (41.24±2.29 U/mg tissue) mice on NCD. Knockout (14.40±1.41 U/mg tissue), wildtype (17.59±0.90 U/mg tissue) and over expressing (17.86±0.98 U/mg tissue) mice on WD had significantly lower MPO levels compared to their respective PCSK9 expression groups on NCD.

From the H & E stained images and histopathology scoring it was evident that PCSK9 over expressing mice on NCD had more lung damage, as indicated by cell infiltration, structural damage and damage to alveolar walls, as shown in figure 4 and 5 (F). These changes in lung morphology were not as evident in knockouts (5D). There were no significant differences between sham operated animals, as shown in figures 5 (G-L).
8.4.3 Differential PCKS9 expression had no effects on WD associated hepatic injury

Histopathology scores and H&E staining revealed that WD fed septic mice had greater hepatic damage, specifically in terms of steatosis (figures 6 and 7A-C), compared to mice on the normal diet (figures 7D-F). Hepatic steatosis was also evident in sham operated animals on WD (figures 7G-I) compared to NCD (figures 7J-L), regardless of PCSK9 expression levels.

8.4.4 Intraperitoneal administration of leptin one hour prior to surgery decreased lung injury, compared to saline administered controls during early sepsis

As shown in figure 8, leptin administered mice had significantly lower lung MPO (32.62±1.62 U/mg tissue) levels compared to saline treated mice during early sepsis (46.58±3.48 U/mg tissue). Sham operated mice had significantly lower MPO levels (12.67±1.73 U/mg tissue for leptin and 11.79±1.16 U/mg tissue for saline) compared to the septic counterparts. Based on histology scoring, mice treated with leptin had less pulmonary damage during sepsis, as shown in figure 9. There was less red blood cell accumulation, interstitial inflammation and congestion in the alveoli, compared to saline treated septic mice (as shown in figure 10 A-D). There were no significant differences observed between sham operated animals from the two treatment groups (figures 10C
8.4.5 Leptin treatment had no effects on hepatic injury during early sepsis

As shown in figure 11, based on histopathology scoring, leptin and saline administered septic mice had no significant differences in the hepatic microvasculature. H&E images from the two groups show some degree of inflammation and congestion as shown in figures 12A and B. Both sham operated groups had no significant differences compared to each other (figures 12C and D).

8.5 Discussion

In sepsis, the lungs are the most common origin of infection and are also among the first organs to fail. We previously designed a clinically relevant murine model of diet induced obesity, and determined that obese septic mice had less lung injury compared to controls (Khan et al. 2014). For the current study, we examined PCSK9 and leptin as potential factors associated with reduced lung injury in our murine model of obesity and early sepsis.

PCSK9 is an important mediator of LDLR receptor expression on hepatic cell surface. Gain of function mutations in the PCSK9 gene have been reported to cause dominant hypercholesterolemia (Abifadel et al. 2003). Elevated PCSK9 levels increase LDL in the body by reducing the density of LDLR, thereby increasing the risk for cardiovascular conditions (Abifadel et al. 2003). Recent studies have elucidated the importance of PCSK9, as a mediator of infection and inflammation (Dwivedi et al. 2016).
Pathogen cell walls are composed of lipids such as LPS and lipoteichoic acid that trigger innate inflammatory responses. Lipid transfer proteins can bind pathogen lipids and incorporate them into lipoprotein particles which are excreted by the liver (Azzam and Fessler 2012; Gautier and Lagrost 2011). Recent studies have demonstrated that pathogen clearance can be modulated by reducing PCSK9 expression, allowing for an increase in LDLR turnover and greater pathogen clearance (Walley et al. 2015). Thus, we wanted to determine if PCSK9 was an important regulator of lung inflammation in our obesity model.

In this study PCSK9 knockout, wildtype and over expressing mice were fed a western diet or normal chow (restricted) diet for 15 weeks. Regular weight gain and glycemic states were monitored for all groups. As hypothesized, mice on WD gained significantly more weight compared to age matched controls on NCD (figure 1). Similarly, WD fed animals had greater blood glucose levels in response to an intraperitoneal glucose challenge (figure 2). The highlight of our study was that PCSK9 over expressing NCD fed animals had significantly greater lung injury compared to PCSK9 knockout and wildtype animals on NCD. This finding is supported by a recent study by Dwivedi et al., that over expression of PCSK9 increases bacterial dissemination, inflammation and organ pathology in a murine model of sepsis (Dwivedi et al. 2016). Walley et al., also reported that inhibition of PCSK9 expression was associated with improved survival rates in mice (Walley et al. 2014). The mechanisms associated with differential effects of PCSK9 on
organ function are not entirely clear. Nonetheless, hepatic LDLR and adipose VLDLR are regulated by PCSK9 inversely, and both these receptors can remove LPS, reducing the systemic load (Topchiy et al. 2015).

An intriguing finding from this study was that regardless of PCSK9 expression levels, there was less lung injury in mice on WD as indicated by lung MPO levels (figure 3) and histopathology scores, compared to controls (figures 4 and 5). These finding have important implications for anti-PCSK9 treatments administered to obese sepsis patients in the clinical setting. Based on our findings, obesity was associated with diminished lung injury during sepsis and thus administering anti-PCSK9 medication may not improve the condition any further.

In terms of hepatic injury, all mice on the WD had significantly greater hepatic histopathology scores compared to the NCD groups (figure 6). There was significantly greater steatosis, hepatocyte ballooning and inflammation compared to NCD fed animals (figure 7). Therefore, obese mice have significantly less lung and significantly greater liver injury when induced with sepsis in our model. Due to severe damage to the hepatic vasculature and microcirculatory bed, immune cells may be trapped and unable to migrate down-stream to the lungs. This inverse relationship between damage to the lungs and liver needs to be further examined using intravital imaging to elucidate the movement of cells through hepatic microcirculation.

Leptin is derived from the Greek word leptos which means ‘thin’, and was first
discovered by Jeffery Friedman and Douglas Coleman (Friedman et al. 1991). Receptors for leptin are present on an array of immune cells including CD4, CD8, B cells as well as monocytes (Papathanassoglou et al. 2006) and neutrophils (Bruno et al. 2005). Leptin regulates proliferation of naïve and memory T cells, and increases Th1 immune responses and supresses Th2 responses (Lord et al. 1998). It directly influences neuroendocrine function, T cell signaling (Papathanassoglou et al. 2006), cytokine synthesis as well as the activation of immune cells, wound healing and angiogenesis (Fantuzzi and Faggioni 2000).

Leptin is an important modulator of immune function and is directly proportional to the amount of adipose tissue. Hence, leptin levels are elevated during diet induced obesity (Ahren and Scheurink 1998). It has been previously established that in a long term high fat diet model (12 weeks) of (polymicrobial) sepsis, obesity was associated with hyperleptinemia and improved innate immune response as well as survival rates (Seigl et al. 2014). In addition, leptin administration to normal weight septic mice stabilized body temperature, reduced pro-inflammatory cytokine levels and improved survival rates (Seigl et al. 2014).

Our aim was to explore the potential role of exogenous leptin on organ function during sepsis. Mice were fed normal chow diet and housed in static cages for 10-12 weeks, for consistency with our original model. We then investigated if administration of exogenous leptin to wild type mice one hour before sepsis surgery, will have any effects
on lung MPO, and organ pathology. We found that leptin treatment one hour before sepsis surgery was associated with reduced lung MPO levels (figure 8). Upon assessment of lung histology scores, septic mice with leptin treatment had significantly less injury compared to saline treated septic mice. Leptin treated septic mice had less congestion and alveolar damage as indicated by H&E staining (figure 10). Our findings are supported by a recent study by Negrin et al., where subcutaneous leptin administration (2.5ug/g and 5ug/g) was associated with dose dependent anti-inflammatory effects and improved survival in a murine model of trauma and sepsis (Negrin et al. 2017). Leptin administration was associated with an increase in CD4 and CD8 levels in the spleen as well as a decrease in TNF-α and IL-6 (Negrin et al. 2017). In addition, these effects associated with leptin did not occur in IL-6 knock out mice during sepsis, highlighting the role of IL-6 (Negrin et al. 2017). The authors suggested leptin administration as a therapeutic and preventative intervention for sepsis (Negrin et al. 2017). A possible mechanism for how leptin may reduce lung inflammation is through neutrophils. Leptin has been shown to have contrasting affects on neutrophil function. In chronic renal failure, leptin inhibited chemotaxis of neutrophils (Ottonello et al. 2004), while increasing neutrophil infiltration to the gut, in response to Entamoeba histolytica (Naylor et al. 2014). These findings suggested that leptin treatment may prevent the development and progression of lung injury during early sepsis.
8.6 Conclusion

To summarize, we found that there is more than one mechanism involved in protection from lung injury during early sepsis. PCSK9 expression was associated with less inflammation in normal weight mice. In addition, regardless of PCSK9 expression levels, obesity was associated with less lung injury in our sepsis and obesity model. Leptin treatment prior to surgery was also associated with reduced lung injury. Therefore, protection from lung injury during sepsis is a multifactorial process that involves both leptin and PCSK9.
**Figure 1: Body weight in PCSK9 knockout, over expressing and wildtype mice 15 weeks post diet introduction:**

Mice with differential PCSK9 expression were fed a WD or NCD and weight gain was assessed. Data is presented as the mean (SE) (n=10/group). *p<0.0001 WD groups compared to NCD groups.
Figure 2: Glucose tolerance test at 15 weeks of WD and NCD fed mice with differential PCSK9 expression: Results are expressed as changes in glucose levels after an I.P challenge of 2g/kg glucose:

Mice with differential PCSK9 expression were fed a WD or NCD and response to an intraperitoneal glucose challenge was examined after 15 weeks on respective diets. Data is presented as the mean (SE) (n=5 for all groups). * p<0.05 for PCSK9 Tg+ WD vs. PCSK9 -/- WD and wt WD. ^p<0.0001 for NCD groups compared to WD groups.
Figure 3: Lung myeloperoxidase levels after 15 weeks on WD or NCD diet, 6 hours post sepsis surgery:
Mice with differential PCSK9 expression were subjected to either sepsis or sham surgeries. Lung inflammation was quantified using MPO levels as a surrogate marker of inflammation. Data is presented as the mean (SE) (n=5-6/group). * p<0.001 NCD Tg+ compared to NCD KO. #p<0.001 for WD compared to NCD of the same PCSK9 expression level (i.e WD KO vs NCD KO). ^p<0.0001 CLP versus sham groups of the same diet and PCSK9 expression level.
Figure 4: Histopathology scores of lung samples from WD and NCD fed, CLP and sham operated animals with differential PCSK9 expression:
CLP compared to sham groups of the same PCSK9 expression level. Organs harvested from septic and sham WD and NCD groups with differential PCSK9 expression were scored for injury by two blinded experts in pathology. Tissues were scored for inflammatory cell infiltrate, interstitial edema, cell necrosis, vascular congestion and assigned a score from 0-3. Healthy sham animals were assigned a score of 0. Data is presented as the mean (SE) (n=5/ group). * p<0.0001 NCD KO compared to NCD wt. ^ p<0.01 NCD KO compared to NCD Tg+. # p<0.05 for CLP compared to sham groups.
5A. WD KO CLP  
5D. NCD KO CLP  
5B. WD wt CLP  
5E. NCD wt CLP  
5C. WD Tg+ CLP  
5F. NCD Tg+ CLP
Figure 5: H&E staining of lung samples from CLP and sham operated WD and NCD mice with differential PCSK9 expression:

WD and NCD groups with differential PCSK9 expression were subjected to CLP or sham surgeries. Tissues were harvested six hours post surgery and stored in buffered formalin. Lung tissues were processed, embedded in paraffin wax and 5 μm thick sections were stained with hematoxylin and eosin to visualize overall morphology. Photomicrographs of stained tissues were examined under 200x magnification (25μm). Figures A-C represent WD CLP, D-F represent NCD CLP, G-I represent WD sham and J-L represent NCD sham groups. Data is presented as the mean (SE) (n=5/group).
Figure 6: Histopathology scores of liver samples from WD and NCD fed, CLP and sham operated animals with differential PCSK9 expression:
Organs harvested from septic and sham WD and NCD groups with differential PCSK9 expression were scored for injury by two blinded experts. Tissues were scored for inflammatory cell infiltrate, interstitial edema, cell necrosis, vascular congestion and assigned a score from 0-3. Healthy sham animals were assigned a score of 0. In WD groups, significant fat infiltration and damage to hepatic microvasculature contributed to the high histopathology scores in this group. Data is presented as the mean (SE) (n=5/group). *p<0.05 CLP compared to sham groups on diets and PCSK9 expression level. # p<0.01 WD Tg+ CLP compared to NCD KO CLP. ^ p<0.01 NCD Tg+ CLP compared to NCD KO CLP.
Figure 7: H&E staining of liver samples from CLP and sham operated WD and NCD fed mice with differential PCSK9 expression:

WD and NCD groups with differential PCSK9 expression were subjected to CLP or sham surgeries. Tissues were harvested six hours post surgery and stored in buffered formalin. Liver tissues were processed, embedded in paraffin wax and 5 μm thick sections were stained with hematoxylin and eosin to visualize overall morphology. Photomicrographs of stained tissues were examined under 200x magnification (25 μm). Figures A-C represent WD CLP, D-F represent NCD CLP, G-I represent WD sham and J-L represent NCD sham groups.
Figure 8: Lung myeloperoxidase levels post leptin and saline treatment in CLP and sham operated mice:
Normal weight mice were treated with leptin or saline one hour prior to surgery and MPO levels were quantified. Data is presented as the mean (SE) (n=5/ group). *p<0.01 for Lep CLP compared to Sal CLP. ^p<0.001 CLP compared to sham groups.
Figure 9: Histopathology scores of lung samples from saline and leptin treated CLP and sham operated mice:

Organs harvested from saline and leptin treated septic and sham animals were scored for injury by two blinded experts. Tissues were scored for inflammatory cell infiltrate, interstitial edema, cell necrosis, vascular congestion and assigned a score from 0-3. Healthy sham animals were assigned a score of 0. Data is presented as the mean (SE) (n=5/group). *p<0.05 Sal CLP vs. Lep CLP. ^p<0.05 CLP vs. Sham.
Figure 10: H&E staining of lung samples from saline and leptin treated CLP and sham operated animals:
Normal weight mice were treated with leptin or saline and subjected to CLP and sham surgeries. Tissues were harvested six hours post surgery and stored in buffered formalin. Tissues were processed, embedded in paraffin wax and 5 μm thick sections were stained with hematoxylin and eosin for visualizing overall morphology. Photomicrographs of stained tissues were examined under 200x magnification (25μm). Figure 10A represents leptin and CLP treatment, 10B saline and CLP treatment, 10C leptin and sham treatment and 10D saline and sham treatment.
Figure 11: Histopathology scores of the liver samples from saline and leptin treated CLP and sham animals:
Organs harvested from saline and leptin treated septic and sham mice were scored for injury by two blinded experts. Tissues were scored for inflammatory cell infiltrate, interstitial edema, cell necrosis, vascular congestion and assigned a score from 0-3. Healthy sham mice were assigned a score of 0. Data is presented as the mean (SE) (n=5/group). ^p<0.05 for CLP compared to sham groups.
Normal weight mice were treated with leptin or saline and subjected to CLP and sham surgeries. Tissues were harvested six hours post surgery and stored in buffered formalin. Tissues were processed, embedded in paraffin wax and 5 µm thick sections were stained with hematoxylin and eosin for visualizing overall morphology. Photomicrographs of stained tissues were examined under 200x magnification (25µm). Figure 12A represents leptin and CLP treatment, 12B saline and CLP treatment, 12C leptin and sham treatment and 12D saline and sham treatment.
8.11 References


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9.0 Summary of major findings and future directions

Obesity rates in Canada are escalating every year and in 2014, 20.2% of Canadian adults, approximately 5.3 million, were categorized as obese (Statistics Canada 2016). With the rise in obesity, there will be an increasing need to treat obese patients with critical illness such as sepsis. Despite the utilization of both animal models and clinical studies, how obesity shapes immune responses during critical illness is not entirely clear. Animal models have been useful tools in establishing and understanding the effects of obesity on sepsis associated immune responses. However, one of the drawbacks of current models is the lack of clinical relevance, which prevents findings from being translated to the clinical settings.

Firstly, majority of these models utilize young naïve and transgenic animals, in the absence of comorbid conditions, with considerable variation in methods of sepsis induction. Animal studies are also limited by the assumption of simplicity in a model (Suratt 2016). For instance, studies examining specific features of a metabolic syndrome such as hyperglycemia, may not consider the potential implications of obesity or the microbiome on the outcomes (Suratt 2016). And it is not entirely clear what features are directly or indirectly responsible for the outcome. Secondly, different strains of mice have variations in susceptibility to weight gain and glucose intolerance (Alexander et al. 2005). BABL/c mice for instance, do not develop metabolic syndrome compared to C57BL/6 which are more susceptible but have a delayed onset (Alexander et al. 2005).
Lastly, there are discrepancies in the definition of obesity among animal models. In humans, obesity is usually defined using BMI, a measure that is not available for mice. In mice, obesity may be defined by features of metabolic syndrome including adiposity, increased glucose levels, increase in triglyceride and reduced HDL levels (Suratt 2016). Therefore, the disease process of obesity varies among animal models based on the type and duration of diet, strain and age, all making it challenging to compare findings between studies.

Keeping these limitations in mind, we developed a clinically relevant murine model of diet induced obesity, and examined inflammatory response during early sepsis. In this model, we utilized 4 weeks old C57BL/6 mice and fed them a WD for 6, 15 or 27 weeks. At 15 and 27 weeks, mice had significant weight gain and developed hyperglycemia. At these time points, sepsis was induced using the CLP technique that involves ligating and puncturing half a centimeter of the cecum from the distal end. CLP is considered the gold standard for sepsis, because it closely mimics the characteristics and progression of clinical sepsis (Rittirsch et al. 2008). Sham surgery was used as control and only the laparotomy was performed. Six hours post sepsis or sham surgeries, mice were euthanized and tissues were harvested and stored at -80°C. We examined organ specific inflammatory responses primarily focusing on the lungs and liver. Proinflammatory cytokines, glutathione, MPO and histopathology were used as markers of injury. We determined that obesity independent of sepsis was associated with severe
hepatic steatosis and non-alcoholic fatty liver disease. We did not find any differences in other markers of hepatic injury (proinflammatory cytokine and glutathione). The highlight of our study was that at both 15 and 27 weeks, obese animals had significantly less lung inflammation as indicated by low lung MPO levels. Based on these findings, we concluded that obesity conferred a protection from lung injury in our murine model of obesity and early sepsis.

The obesity paradox refers the phenomena that counterintuitively obesity may be protective and associated with better outcomes, and is supported by reports from both animal models and clinical studies. Contrary to previous reports that obesity increases risk for critical illnesses, Gaulton et al. reported from a retrospective cohort study that there was no association between obesity and mortality risk in severe sepsis (Gaulton, MacNabb and Mikkesen 2015). They proposed that the limitations of previous studies were comparisons between non ICU and ICU, and children and adult subjects (Huttunen et al. 2007). Controlling for these confounding factors, Pepper et al., designed a meta analysis study in ICU adult patients being treated for sepsis, severe sepsis or shock, and observed impact of BMI on acute outcome (Pepper at al. 2016). Using four retrospective (n=6609 patients) and two prospective (n=555) studies, being overweight or obese was associated with reduced odds ratio of mortality (Pepper et al. 2016).

Shah et al., have suggested from invitro studies that obesity may enhance susceptibility to lung injury by disrupting expression of endothelial junctional proteins,
and increase in adhesion molecule that enhance vascular permeability, and leukocyte movement from intravascular space (Shah et al. 2015). However, Shah et al., were unable to observe these findings in their studies with obese mice and suggested that in the absence of a secondary insult, the barrier properties of the lung endothelium are functional in obese mice (Shah et al. 2015). They further reported increase in permeability of lung endothelium and influx of neutrophils in response to LPS. The limitation of these experiments is use of a mouse model of obesity with normal insulin sensitivity, and therefore negating possible effects of hyperglycemia (Shah et al. 2015). Clinical studies have shown that type II diabetes protects against development of ARDS, possibly due to the immunosuppressive properties of diabetes (Gu et al. 2014). In addition, db/db and DIO mice in an inhaled LPS model of ALI, had reduced neutrophil chemotaxis and low levels of IL-6 and MCP-1 in lungs (Kordonowy et al. 2012). Therefore, based on these findings obesity may confer protection from lung injury.

We attempted to establish our obesity and sepsis model in a new facility with certain deviations from our original model, the most important one being use of ventilated cages as opposed to standard static ones. In ventilated cages, mice on WD did not gain significant weight nor develop hyperglycemia. In addition, the pulmonary inflammatory response to sepsis was also altered. To understand the potential cause for these changes, we set up a new cohort of mice in the same facility, however used static cages for housing. With the switch to static cages, our original model was replicated,
both in terms of developing metabolic syndrome and the specific inflammatory responses.

We proposed that one of the reason for this variation in immune response and susceptibility to developing metabolic syndrome may be changes in gut microbiome. It has previously been established that gut microbiota plays an important role in developing obesity (Armougom et al. 2009; Walters, Xu, and Knight 2014). Beaumont et al., have also shown that the types of bacteria found in human fecal matter may influence the amount of visceral fat, and partially explain genetic predisposition of some individuals to developing obesity (Beaumont et al. 2016). In their study, individuals were split in different groups based on BMI and body fat, and fecal bacteria was compared (Beaumont et al. 2016). Individuals with greater diversity in fecal bacteria had low levels of visceral fat (Beaumont et al. 2016). The specific mechanisms for how bacteria can influence deposition of fat are not yet clear. However, it is hypothesized that less diversity might allow for domination of microbes that are involved in fat synthesis from carbohydrates (Beaumont et al. 2016).

Using bioilluminescence assays to identify the composition of fecal microbiota from static and ventilated cages, significant differences were observed. Firmicutes and Bacteroidetes are dominant phyla of bacteria in the gut and play important roles in fermentation of carbohydrates (den Besten et al. 2013). A greater ratio of Bacteroidetes compared to Firmicutes were found in the static group, while the opposite was the case
for the ventilated group. It is possible that mice in ventilated cages did not develop overt obesity and hyperglycemia due to a greater ratio of Firmicutes. In static cages, the higher ratio of *Bacteroidetes: Firmicutes* may have contributed to the development of metabolic syndrome. *Bacteroidetes* mainly produces acetate and propionate, while *Firmicutes* produces butyrate (Abdallah Ismail et al. 2011). Butyrate increase insulin sensitivity in mice (Hartstra et al. 2014), may protect against diet induced obesity (Lin et al. 2012) and increases leptin gene expression (Harris et al. 2012). Our findings were supported by a large study conducted by Schwiertz, where obesity was associated with greater ratio of *Bacteroidetes* to *Firmicutes* (Schwiertz et al. 2010). This study highlights the importance of environment and gut microbiome for susceptibility to metabolic syndrome.

PCSK9 expression and leptin were investigated as potential factors for reduced lung injury during early sepsis in our mouse model. PCSK9 mediates cholesterol homeostasis by regulating LDLR expression on hepatic cell surface by binding and degrading LDLR (Naureckiene et al. 2003; Seidah et al. 2003). For this study, KO, transgenic and wildtype PCSK9 animals were housed in static cages, and fed either a WD or NCD for 15 weeks. Sepsis was induced by CLP and tissues were harvested at 6 hours. The highlight of the study was that lack of PCSK9 was associated with reduced lung injury during early sepsis in normal weight mice. This finding is supported by a recent study by Dwivedi et al., where over expression of PCSK9 increased bacterial
dissemination, inflammation and organ pathology in a murine model of sepsis (Dwivedi et al. 2016). The mechanisms associated with differential effects of PCSK9 on organ function are not entirely clear. PCSK9 inversely regulates hepatic LDLR and adipose VLDLR, both clear circulating LPS, reducing systemic load (Topchiy et al. 2015). Interestingly, WD was associated with reduced lung injury irrespective of PCSK9 expression levels during early sepsis. These finding have important implications for anti-PCSK9 treatments administration to obese sepsis patients in the clinical setting.

Leptin is an adipose tissue derived hormone and is elevated in obese individuals. It also plays an important role in mediating immune responses. In wildtype and db/db (lack of functional leptin receptors) mice exposed to ozone (2ppm) for three hours, the pulmonary resistance and airway responsiveness were elevated (Lu et al. 2006). In addition, in db/db mice, there was also an increase in IL-6, KC, MIP-2 and neutrophils. This provides evidence that leptin mediates pulmonary immune responses to external stimuli. We administered leptin to young naïve mice 1 hour before sepsis surgery and induced sepsis using CLP. Mice treated with leptin had significantly less lung injury compared to saline treated controls, highlighting the effects of leptin for lung injury. Therefore, both PCSK9 and leptin play important roles in conferring protection from lung injury during sepsis.

In terms of future experiments, examining neutrophil function in obese animals would be useful to further elucidate the mechanisms associated with the protective
effects observed using our model. Neutrophils from whole blood of obese animals with sepsis can be isolated using standard gradient separation method as previously performed by Oh et al (Oh, Siano, and Diamond 2008). Neutrophils will then be incubated with different concentrations of chemotaxins such as Interleukin-8 (IL-8) and C5a, and chemotaxis of the cells will be examined and compared to neutrophils from non-obese mice. These experiments will elucidate if obese animals have impaired neutrophil chemotaxis in our mouse model. Furthermore, other functions of neutrophils such as phagocytosis and oxidative burst can also be explored.

Neutrophils can release both MPO and Neutrophil Extracellular Traps (NETs) during infection. NETs comprise of decomposed chromatin fiber and anti microbial peptides that trap and facilitate degradation of pathogens (Brinkmann et al. 2004). Moorthy et al. recently reported that when Balb/c mice fed high fat diet for 18 weeks, were administered with lethal dose of influenza virus, the HFD group had lower MPO activity and lung pathology scores, however had a higher amount of NET production which were thought to exacerbate injury. Therefore, it will be interesting to measure NETs in our mouse model and determine if obese mice have low MPO but greater NET production.

Our mouse model was utilized to study immune responses during early sepsis, and thus severe sepsis and septic shock were not examined. The immune functions drastically vary during different phases of sepsis. Early sepsis is characterized by hyper-inflammation and a cytokine storm created to eradicate pathogens (Casey 2017). The
late phase is a hypo inflammation and immunosuppressive phase, where the body ceases synthesis of proinflammatory cytokine to reduce damage to the tissues (Casey 2017). In our model, we only examined immune responses during early sepsis. It will be useful to determine if obese animals are also protected from lung injury during late sepsis (24-48 hours post surgery). In addition, for ethical reasons we were not able to determine the effects of obesity on mortality. It will add tremendously to our model, if we could perform these studies and determine if obesity confers protection against mortality during early or late sepsis.

The pneumonia model of sepsis is a clinically relevant model that involves intratracheal inoculation with *Klebsiella pneumoniae*. This model is characterized by lung injury, increase in inflammatory (mainly polymorphonuclear) leukocytes in bronchoalveolar lavage, leukopenia, hypotension and increase in pro-inflammatory cytokines at 6, 24 and 48 hours post inoculation (Sordi et al. 2013). The model we utilized for our studies was a polymicrobial peritonitis model and therefore it will be useful to examine the effects of obesity on lung injury in a pneumonia model of sepsis. WD and NCD fed animals can be inoculated intratracheally, and 6, 24 or 48 hours later, markers of organ inflammation can be quantified. This is especially interesting because according to a recent meta analysis study, overweight and obese subjects had reduced risk of pneumonia related mortality (Nie et al. 2014).
10. References for Discussion


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