Conservation Concerns for the Candy Darter (Etheostoma osburni) with Implications Related to Hybridization

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Feeding Soy Protein Isolate and/or Omega-3 Polyunsaturated Fatty Acids on the Spleen-Liver Axis in a Female Rat Model of Autosomal Recessive Polycystic Kidney Disease with Liver Steatosis

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Thesis submitted
to the Davis College of Agriculture, Natural Resources and Design
at West Virginia University

In partial fulfillment of the requirements for the degree of

Master of Science in
Nutritional & Food Sciences

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Keywords: soy protein, omega-3 fatty acids, spleen, splenomegaly, non-alcoholic fatty liver disease, autosomal recessive polycystic kidney disease

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ABSTRACT

Feeding Soy Protein Isolate and/or Omega-3 Polyunsaturated Fatty Acids on the Spleen-Liver Axis in a Female Rat Model of Autosomal Recessive Polycystic Kidney Disease with Liver Steatosis

Lauren Brooke Gibson

Autosomal recessive polycystic kidney disease (ARPKD) is a congenital hepatorenal fibrocystic syndrome with a mortality rate of 30% during the first year of life. The most common extra-renal manifestation of ARPKD is liver disease with a greater rate in females due to their higher estrogen levels. Abnormal spleen enlargement (splenomegaly) has been found to occur in 60% of ARPKD patients. In the absence of effective medications to treat the hepatic and splenic complications of ARPKD, diet offers a potentially efficacious, safe, and cost-effective therapy option. Soy protein isolate (SPI) has been shown to reduce cyst proliferation associated with its anti-estrogenic and anti-inflammatory actions. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have anti-inflammatory effects and influence mediators of de novo lipogenesis (DNL) through up-regulation of β-oxidation. Young (age 28 days) female PCK rats, an orthologous animal model of ARPKD, were randomly assigned to one of four diets (n=12/group) and fed for 12 weeks. Diet groups consisted of 1) casein + corn oil (Casein + CO), 2) casein + soybean oil (Casein + SO), 3) soy protein isolate + soybean oil (SPI + SO), or 4) soy protein isolate + a 1:1 soybean: salmon oil blend (SPI + SB) rich in long chain n-3 PUFAs. Unexpectedly, SPI + SB fed rats had the highest histological evidence of hepatic steatosis (p=0.003) suggesting non-alcoholic fatty liver disease (NAFLD). NAFLD is the leading cause of chronic liver disease in adults and children consuming Westernized diets. The SPI + SB group also had increased (p=0.03) inflammation and up-regulated (p=0.03) expression of fibrosis related genes suggesting progression of NAFLD to non-alcoholic steatohepatitis (NASH). The spleen was also significantly (P=0.02) elongated in the SPI+SB fed group compared to the Casein + CO and Casein + SO groups. However, spleen weight was not significantly different among diet. We hypothesize the spleen-liver axis is responsible for the development of steatosis and fibrosis in our rats. Given the close anatomical proximity of the spleen to the liver bioactive compounds produced by the spleen such as splenic fatty acids, inflammatory and immune genes can directly access the liver via the splenic and portal veins. The objective of this study was to determine the effect of SPI and/or n-3 PUFAs on splenic DNL, lipolysis, inflammatory response, and immune gene expression. Results of DNL gene expression and splenic fatty acid content of DNL fatty acids were not significantly different among diet groups. Rats fed the SPI+SB diet containing the highest (P<0.001) dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exhibited the highest (P<0.001) splenic EPA and DHA, and the lowest (P=0.03) splenic arachidonic acid (AA) showing diet did effect the spleen with potentially anti-inflammatory effects. However, no significant differences in gene expression regulating inflammation or immunity were found among dietary treatment groups. Based on our results we concluded that diet had no effect on splenomegaly and splenomegaly did not significantly contribute to the development of liver steatosis in a female PCK rat model.
Acknowledgments

I would like to wholeheartedly thank my advisor Dr. Janet Tou for my graduate experience here at West Virginia University. Working with such a wonderful mentor and knowledgeable professional I know I have learned so very much.

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Introduction

Autosomal recessive polycystic kidney disease (ARPKD) is a congenital form of polycystic kidney disease (PKD) [Sweeney et al., 2014; Dell et al., 2011]. It is a rare genetic disorder that occurs in 1 in 20,000 live births [Telega et al., 2013; Zerres et al., 1998; Dell et al., 2011] with a mortality rate of approximately 30% during the neonatal period or within the first year of life [Sweeney et al., 2014]. ARPKD causes congenital hepatorenal fibrocystic syndromes resulting in significant renal and liver-related morbidity and mortality in children [Sweeney et al., 2014]. Liver cysts are the most common extra-renal manifestation of ARPKD [Dell et al., 2009; Sweeney et al., 2011; 2014] with increased cyst proliferation in females due to their higher estrogen levels [Arnold et al., 2005]. In the absence of effective medications for the treatment of liver disease in ARPKD, diet offers a potentially efficacious, safe, and cost-effective therapeutic option [Maditz et al., 2014 & 2015].

Soy protein isolate (SPI) contains isoflavones with anti-estrogenic properties [Aukema et al., 1999; Vitale et al., 2013] and has been shown to reduce renal cyst proliferation in PKD [Peng et al., 2009]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), have been extensively studied for their anti-inflammatory properties [Huang et al., 2011] and for their role in down-regulating de novo lipogenesis (DNL) through up-regulation of β-oxidation. EPA and DHA can be de novo synthesized from the essential α-linolenic acid (ALA) however, consuming rich dietary sources of EPA and DHA such as fish oil is recommended due to a poor conversion rate of about 2% [Simopoulos et al., 2000; St-Jules et al., 2013]. Maditz et al., [2015] examined the effects of feeding SPI and/or n-3 PUFAs on the severity of liver cysts in young female PCK rats, an orthologous animal model of human ARPKD [Nagao et al., 2012]. Unexpectedly, female
PCK rats fed SPI and n-3 PUFA enriched diets resulted in the highest liver cysts, steatosis, and inflammation [Maditz et al., 2015]

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic steatosis [Araya et al., 2004; Fon Tacer et al., 2011] which can progress to non-alcoholic steatohepatitis (NASH) characterized by inflammation and fibrosis and ultimately cirrhosis in the liver [Fon Tacer et al., 2011; Valenzuela et al., 2011]. Steatosis occurs when the rate of hepatic fatty acid (FA) uptake from plasma and DNL increases [Fabbrini et al., 2010], and is associated with increased transport of FAs for triglyceride (TG) synthesis to the liver [Tamura et al., 2005]. Hepatic uptake of FA is not regulated and therefore, the concentration of FA in the blood is directly related to an influx of FA to the liver through the portal vein [Tamura et al., 2005].

In addition to NAFLD, spleen enlargement was observed in our ARPKD rat model. Additionally, splenomegaly has been shown to increase with the rate of hepatic lipid accumulation in NAFLD [Wang et al., 2015]. Although the mechanism has not been defined [Alberti et al., 2012], the spleen has an important role possibly through regulating gene expression associated with lipid metabolism in the liver [Petroianu et al., 2006; Paulo et al., 2007; Paulo et al., 2009]. We have the novel aim to investigate the effect of diet on gene expression regulating lipogenesis and lipolysis in spleen tissue to compare with development of liver steatosis and fibrosis. This has important implications since NAFLD is the most common cause of liver disease associated with the Western diet [Bellentani et al., 2010].

Splenomegaly has also been reported to be present in 60% of ARPKD patients under the age of five [Gunay Aygun et al., 2013]. Sugawara et al., [2000] reported that splenectomy slows the progression of hepatic fibrosis to cirrhosis. We hypothesize that due to the close anatomical
proximity of the spleen to the liver, bioactive compounds produced by the spleen can directly access the liver through the splenic and portal veins [Akahoshi et al., 2002]. Therefore, we conducted an exploratory study to determine the potential role of the spleen in NAFLD.

Splenic response is controlled by splenic chemokines that organize the tissue of the white pulp and regulate spleen function [Sweeney et al., 2014]. The spleen responds to infection and disease by mobilizing splenic lymphocytes that control inflammatory gene expression [Wang et al., 2015], and FAs stored in splenic macrophages [Schmidt et al., 1997]. Vuppalanchi et al., [2009] reported that splenic cytokines regulate inflammatory responses in the liver [Pestka et al., 2014]. Therefore, in addition to investigating splenic lipid metabolism, the current study also has the novel aim to investigate the effect of diet on the expression of genes regulating inflammation and immunity in the spleen in order to better understand the relationship of splenomegaly to the development and progression of NAFLD.

1.0 Literature review

1.1 Prevalence and pathogenesis of background disease conditions

PKD is an incurable genetic disorder that is characterized by the development of multiple fluid-filled renal cysts, kidney enlargement, structural damage, and loss of renal function that can progress to end stage renal disease (ESRD) [Halvorson et al., 2010; Maditz et al., 2014]. PKD manifests in two main forms as autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) [Sweeney et al., 2014]. The majority of children diagnosed with ADPKD are asymptomatic until adulthood [Sweeney et al., 2014; Dell et al., 2011]; while, children with ARPKD present symptoms in infancy of enlarged kidneys and hepatobiliary abnormalities [Dell et al., 2011; Sweeney et al., 2014].
Greater than 50% of children with ARPKD progress to ESRD within the first decade of life [Telega et al., 2013; Sweeney et al., 2014]. Liver cysts are the most common extra-renal manifestation of PKD accounting for 80-90% of all polycystic liver disease (PCLD) cases with greater susceptibility and severity in females due to higher estrogen levels [Gabow et al., 1990]. Hepatomegaly due to cyst growth and portal hypertension due to hepatic fibrosis are the main clinical symptoms of PCLD [Quain et al., 2003; Maditz et al., 2015]. Progressive portal hypertension or high blood pressure in the portal vein is presents in 70% of ARPKD patients [Gunay-Aygun et al., 2013; Sweeney et al., 2014] and occurs early in disease progression [Roy et al., 1997]. Liver cysts originate from biliary ducts and peribiliary glands surrounding the intracellular bile ducts [Chauveau et al., 2000]. The invariant liver lesion of ARPKD also known as congenital hepatic fibrosis is a developmental abnormality of biliary ductal plate remodeling [Sweeney et al., 2014]. Individuals with congenital hepatic fibrosis develop progressive portal hypertension, hypersplenism, protein-losing enteropathy, and gastrointestinal bleeding [Gabow et al., 1990; Dell et al., 2009; Telega et al., 2013].

Maditz et al. [2015], reported hepatic steatosis, characteristic of NAFLD presented in PCK rats, an orthologous model of ARPKD. NAFLD is the leading cause and most common form of chronic liver disease in adults and children [Tiniakos et al., 2010]. NAFLD is defined as greater than 5% fat accumulation in the liver [Rahimi et al., 2013], with the absence of hepatitis B or C viruses, and without excessive alcohol consumption (conventionally defined as an intake of ethanol > 20 g/day) [Bellentani et al., 2010].

NAFLD affects one-third of the global population [Scorletti et al., 2013]. NAFLD has surpassed alcoholic liver disease and hepatitis C as the most common form of liver disease in the United States [Hasse et al., 2013]. The prevalence of NAFLD in adults in Western countries is
20-30%, with 2-3% progressing to NASH [Bellentani et al., 2010]. In the U.S., 3-10% of all children have NAFLD making this the most common liver disease in children [Nobili et al., 2013]. Additionally, the prevalence of NAFLD is expected to increase with consumption of the Western diet [Tiniakos et al., 2010].

Steatosis in PKD was suggested to be caused by cyst obstruction; however, there was little evidence of cholangitis. Maditz et al., [2015] suggested this was because rats lack a gallbladder and their biliary ducts are more superficial than in humans [Martins et al., 2007]. Gunay-Aygun et al., [2013] determined that kidney and liver complications from ARPKD are independent of one another. Based on reports that hepatosplenomegaly has been observed in many individuals with ARPKD, the spleen may be involved in liver complications [Roy et al., 1997]. Gunay Aygun et al., [2013] reported splenomegaly in 65% of ARPKD patients between the ages of one and 56. A study by Tsushima et al., [2000] showed increased (p<0.001) spleen volume in NAFLD patients compared to normal liver subjects. Therefore, we investigated the role of the spleen in an ARPKD model with consequential liver steatosis.

1.2 Spleen anatomy and physiology

In humans, the spleen is a dark red to blue-black organ located in the left cranial abdomen to the lower right of the stomach [Cesta et al., 2006]. The hepatic artery and the splenic artery branch off from the descending aorta. The splenic artery is noted for its large size and tortuosity [Tarantino et al., 2011] that brings and diffuses oxygenated blood from the heart to the spleen [Mebius et al., 2005]. Blood leaves the spleen through the splenic vein, which drains into the hepatic portal system [Cesta et al., 2015]. The hepatic portal system supplies up to 75% of total hepatic blood flow and 60-70% of hepatic oxygen by directing partially deoxygenated
blood through the portal vein into the liver. With the close anatomical proximity of the spleen to the liver, bioactive compounds produced by the spleen can directly access the liver via the portal vein [Akahoshi et al., 2012].

The spleen is comprised of two functionally and morphologically distinct compartments comprised of the red pulp and the white pulp. The afferent splenic artery branches into two central arterioles sheathed by white pulp areas that end in cords in the red pulp (Fig.1). Arterial blood arrives into the chords of the red pulp, which consists of fibroblasts and reticular fibers and forms an open blood system free of restriction from endothelium [Groom et al., 1991]. The red pulp filters blood by removing oxygen, foreign material, and damaged erythrocytes [Tarantino et al., 2011; Mebius et al., 2005]. The blood passes into the venous sinuses from the cords in the red pulp [Mebius et al., 2005]. In the sinuses of the red pulp the unusual arrangement of stress fibers together with the parallel arrangement of the endothelial cells, forces the blood from the cords into the sinuses through the slits that are formed by the stress fibers [MacDonald et al., 1987]. The passage becomes difficult for aging or damaged erythrocytes as their stiffened membranes adhere to the cords and are subsequently phagocytized by red pulp macrophages [Mebius et al., 2005]. The red pulp has also been recognized as a reservoir for undifferentiated monocytes used for regulating inflammation at injured sites throughout the body [Swirski et al., 2009]. Pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are produced by monocytes as well as by other cell types in response to injury and infection. Pro-inflammatory cytokines can also be produced by immune-mediated activation to induce immune cell trafficking to the site of injury [Harbige et al., 2003].
Unlike the red pulp, the white pulp closely resembles the structure of a lymph node [Nolte et al., 2000]. The key difference between the spleen and the lymph nodes is that molecules that enter the tubular network of the white pulp are present in the blood; whereas, molecules that enter the lymph nodes are transported by the lymph [Nolte et al., 2003; Palframan et al., 2001; Mebius et al., 2005]. The white pulp is responsible for the production of white blood cells [Tarantino et al., 2013], which can include neutrophils, eosinophils, basophils, lymphocytes, and monocytes. The spleen contains about one-fourth of the body’s lymphocytes [Cesta et al., 2006]. Lymphocytes are grouped into T- and B-cells [Harbige et al., 2003].

The omega-6 polyunsaturated fatty acids (n-6 PUFAs), arachidonic acid (AA), and to a lesser extent the essential FA, linoleic acid (LA), are major fatty acid components of lymphocyte
membranes and phospholipids [Cinader et al., 1983]. Growth and development of lymphoid tissues as well as the structural and functional integrity of T- and B-cells are affected by essential fatty acid deficiency [Boissonneault et al., 1984; Dewille et al., 1979; Dvorak et al., 1992; Harbige et al., 2003]. Histology of a healthy spleen shows clearly defined T-cell zones and B-cell follicles while these areas become less defined in a dysfunctional or hyperactive spleen [Cesta et al., 2006]. Immune signaling chemokines attract T and B-cells to their respective zones which function in maintaining the organization of the white pulp (Fig.2). Regulation of the immune signaling white pulp chemokines is partly controlled by TNFα [Ngo et al., 1999]. B cells are attracted to the B cell zone follicles in response to CXC-chemokine ligand 13 (CXCL13) [Ansel et al., 2000]; whereas, T cells are directed to the T-cell zone in response to CC-chemokine ligand 19 (CCL19) and CC-chemokine ligand 21 (CCL21) [Gunn et al., 1999; Förster et al., 1999]. Macrophages also depend on CCL19 and CCL21 for their localization [Mebius et al., 2005]. When the spleen is injured, gene expression of chemokines CXCL13, CCL19, and CCL21 is reduced and disorganization of the white pulp occurs [Ngo et al., 1999].
The spleen is organized in such a way to efficiently facilitate both innate and adaptive immunity responses [Tarantino et al., 2013]. The white pulp is strictly involved in adaptive immunity, while the marginal zone is involved in both innate and adaptive immunity through its specific macrophage populations and marginal-zone B cells making it an important organ for immune homeostasis [Mebius et al., 2005]. Activation of lymphocyte proliferation is central to the adaptive immune responses that specifically recognize and bind antigens through receptors and thus, providing the basis for the molecular identification of specific antigens [Harbige et al., 2003]. The red pulp is the site where plasmablasts and plasma cells lodge after antigen-specific differentiation in the white pulp [Mebius et al., 2005]. B cells and plasma cells mature in the spleen, and have key functions in humoral immunity [Tanabe et al., 2015]. Moreover, T cells and
cytokines produced by T-cells play important roles in modifying the maturation process of lymphocytes in adaptive immunity [Tanabe et al., 2015].

The overall structure of the immune system in rodents and humans is similar [Mestas et al., 2004]. The main difference between the rat spleen and the spleen of humans is the structure of the marginal zone surrounding the white pulp [Mebius et al., 2005]. In contrast to rodents, humans have an inner and outer marginal zone encompassed by a large perifollicular zone (Fig 3). CCL9 is a rat specific chemokine examined in the current study to compare with CXCL13, CCL19, and CCL21 homologous in humans and rats.

Figure 3. Structure of White Pulp in Rodent Spleen vs. Human Spleen. Adapted from Mebius et al. [2005]

The perifollicular zone is a dynamic region that can be either a part of the red pulp or of the follicles in humans [Mebius et al., 2005]. Unlike in rats, the human splenic marginal zone does not contain a marginal sinus. It is assumed that in humans the perifollicular region is the compartment where antigen and recirculating lymphocytes enter the organ [Mebius et al., 2005]. The effect of these differences on efficiency of spleen function between rodents and humans is
not clear. Despite these differences rodent models remain essential for providing a better understanding of biological function of the spleen in human in healthy and disease states [Mestas et al., 2004].

1.3 Overview of spleen function

An overview of the spleen’s functions is provided in Table 1. In general the spleen functions as a blood filter removing unwanted material and eliminating abnormal, damaged, or old blood cells. A major function of the spleen is its role in immunity through the production and storage of antibodies and lymphocytes [Tarantino et al., 2011]. The spleen serves as a reservoir for different white blood cells (leukocytes) synthesized by the white pulp and platelets that can be mobilized when a metabolic challenge occurs [Tarantino et al., 2011]. Although important to health maintenance, the spleen is not critical to survival. Splenectomy is a treatment for certain medical situations such as liver fibrosis and cirrhosis. In the absence of the spleen the liver can compensate for the loss of splenic immune function [Baykal et al., 1999].
1.4 Spleen disorders

Splenomegaly refers to abnormal spleen enlargement. The gross appearance and size of the spleen is variable depending on species [Cesta *et al.*, 2006]. In a human study, Tsushima *et al.*, [2000] reported significantly larger (p<0.0001) spleen volumes in patients diagnosed with NAFLD compared to healthy patients. Splenomegaly has been found to increase with degree of hepatic fatty infiltration and serum IL-6 [Wang *et al.*, 2015]. Tsushima *et al.*, [2000] using multivariate linear regression analysis observed liver-to-spleen ration (p<0.0001) and age

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<td><strong>Red Pulp</strong></td>
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<td>• Facilitates shedding of solid waste materials from erythrocytes</td>
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<td>• Blood filter: foreign material, damaged and/or aging blood cells</td>
</tr>
<tr>
<td>• Storage site: iron, erythrocytes, platelets, plasmablasts, and plasma cells</td>
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<tr>
<td>• Produce antigen-specific antibodies for released into circulation</td>
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<tr>
<td>• Defense against sepsis: red pulp macrophages metabolize iron to defend against harmful bacteria</td>
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<tr>
<td><strong>White Pulp</strong></td>
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<tr>
<td>• Storage and synthesis of B and T lymphocytes upon antigenic challenge</td>
</tr>
<tr>
<td>• Release of immunoglobulins (antibodies) from B lymphocytes in response to infection</td>
</tr>
<tr>
<td>• Production of immune mediators involved in clearance of bacteria</td>
</tr>
<tr>
<td><strong>In the white pulp marginal zone</strong></td>
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<tr>
<td>• Macrophages phagocytize circulating microorganisms and immune complexes</td>
</tr>
<tr>
<td>• Produce B lymphocytes in response to TI and T-2 antigenic challenge</td>
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<td>• Release of immunoglobulins from B lymphocytes upon antigenic infection</td>
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(p<0.01) correlated with spleen volume. Using forward selection stepwise regression, the liver-to-spleen ratio entered first (β=-0.634) and age second (β=-0.293) [Tsushima et al., 2000].

Prolonged splenomegaly can result in hypersplenism a condition characterized by accelerated filtering resulting in the spleen removing cells that are still useful and functioning normally [Gunay-Aygun et al., 2013]. Hypersplenism decreases plasma platelet needed for blood clotting, increases mobilization of inflammatory monocytes [Gunay-Aygun et al., 2013; Swirski et al., 2009], and mobilization of free FAs from the spleen to the liver [Tarantino et al., 2013]. Splenectomy reduces the number of spleen specific B cells important in immunity [Tarantino et al., 2013]. In patients with a malfunctioning spleen, splenectomy has also been shown to slow the progression of liver fibrosis to cirrhosis [Sugawara et al., 2000]. Yamada et al., [2015] reported splenic macrophages and cytokines, such as IL-6, promote the progression of fibrosis via the liver-spleen axis and showed splenectomy improves liver function by removing these bioactive compounds.

1.5 Role of spleen in lipid metabolism

Splenectomy has been shown to cause dyslipidemia [Paulo et al., 2007; Petroianu et al., 2006; Paulo et al., 2009]. Baykal et al., [1999] reported higher (p=0.01) serum CHL levels in splenectomized compared to a sham-operated rats. Low serum CHL levels have also been observed in patients with hypersplenism suggesting the spleen plays a role in lipid metabolism related to NAFLD [Cesta et al., 2006; Mejias et al., 2010; Wang et al., 2015]. Maditz et al., [2015] reported rat fed SPI + SB diet had the lowest (p<0.001) circulating CHL and highest (p<0.05) hepatic steatosis.
NAFLD occurs when the rate of hepatic FA uptake from plasma and DNL is greater than the rate of FA oxidation and export as TGs in VLDLs [Fabbrini et al., 2010; Chan et al., 2003]. VLDL production and secretion of TGs from DNL in the liver is increased in NAFLD; however, the rate of secretion does not compensate for the rate TG production from free FA uptake [Fabbrini et al., 2010]. NAFLD encompasses the spectrum of liver injury from steatosis to fibrosis and cirrhosis. NAFLD patients have increased serum TGs and VLDL levels while NASH patients have decreased serum TG and VLDL [Fon Tacer et al. 2011]. Female PCK rats fed SPI + SB diet had the lowest total TG (p=0.003) and lowest (p=0.04) serum VLDL among the diet groups consistent with the lipid profile of NASH patients.

Chronic up-regulation of DNL in the liver is major mechanism for NAFLD and progression to cirrhosis and liver failure [Lamas et al., 2004]. The rate of DNL is regulated by nuclear transcription factors associated with lipid uptake and synthesis; sterol regulatory element-binding protein 1-c (SREBP1-c) and carbohydrate response element-binding protein (ChREBP), that regulate lipogenic enzymes including fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD-1). FAS is a multi-enzyme protein complex needed for elongation of FA carbon chains. SCD-1 is the rate-limiting enzyme required for the production of unsaturated FAs.

Peroxisome proliferator-activated receptor alpha (PPARα) is a transcription factor for the activation of β-oxidation [Pestka et al., 2014]. Up-regulation of PPARα drives FA oxidation for energy and has been suggested to be beneficial in NAFLD through breakdown of hepatic TGs [Scorletti et al., 2013]. The expression of these transcription factors and lipogenic enzymes may be the link between splenic lipid metabolism and TG accumulation in the liver. Schmidt et al., [1997] theorized the spleen as a lipid reservoir that can be exacerbated to promote NAFLD. We hypothesize that FA produced by DNL in the spleen may directly access the liver through the
portal vein to provide a source of FA for enhanced hepatic DNL resulting in NAFLD. Currently, there is no evidence of lipid metabolism in spleen cells despite evidence of spleen storage of phospholipids and effect of splenectomy on dyslipidemia [Alberti et al., 2012]. To our knowledge the effect of diet on the spleen and development of splenomegaly has not been investigated.

1.51 Diet and lipid metabolism

The Western diet is rich in n-6 PUFAs and low in n-3 PUFAs [Popescu et al., 2013]. This is due to higher dietary consumption of n-6 PUFAs rich foods such as corn oil, compared to n-3 PUFA rich foods such as flaxseed and fatty fish such as salmon [Vitale et al., 2013]. In our study lipid sources included corn oil a rich source of LA, soybean oil, a rich source of both LA and ALA, and a soybean and salmon oil blend providing LA and ALA, but also rich in EPA and DHA. The ratio of n-6 to n-3 PUFAs in the Western diet is 15-20:1 [Arnold et al., 2005]. The optimal n-6 to n-3 PUFA ratio is 1:1 to 4:1 [Gotoh et al., 2012]. The n-6 to n-3 PUFA ratio in the salmon oil blend diet was approximately 1:1.

The Western diet is high in n-6 PUFAs and has been associated with increased onset of hepatic injury [Popescu et al., 2013]. Feeding n-3 PUFA may offer a possible safe and effective way to ameliorate NAFLD [Spoto et al., 2013]. NAFLD has been associated with depleted hepatic n-3 PUFA tissue concentrations [Altunkaynak et al., 2007; Scorletti et al., 2013]. Also, n-3 PUFAs have been shown to regulate hepatic lipid metabolism by down-regulating the expression of SREBP-1c and ChREBP and up-regulating lipid oxidation by activation of PPARα [Gotoh et al., 2012]. In the liver both n-3 and n-6 PUFAs down-regulate SREBP-1c expression. Of the PUFAs, long-chain n-3 PUFA, DHA has been reported to cause the greatest reduction of
SREBP-1c and ChREBP activity and to decrease lipogenesis in NAFLD [Gotoh et al., 2012]. Diet can effect spleen FA content shown by increased (p<0.01) percentage of fat in spleen tissue when fed saturated FA-rich and PUFA-rich diet [Guimaraes et al., 1990]. Splenic n-3 PUFA content has been shown to increase (p<0.05) in spleen tissue to reflect that fed in the diet [Peterson et al., 1998; Kelley et al., 2006]. We hypothesize FAs produced and then mobilized by the spleen can access the liver directly through the splenic and portal veins promoting the development of hepatic steatosis. In the presence of increased inflammation, hepatic steatosis can progress to fibrosis and NASH.

1.6 Diet and Inflammation and Immunology

1.6.1 Dietary proteins

High protein consumption has been suggested to negatively affect PKD leading to higher renal perfusion and kidney hyperfiltration in the compromised organ [Tomobe et al., 1994]. However, Infant and children with ARPKD have a higher protein requirement since they are in a rapid growth and developmental stage. Therefore, protein restriction can lead to malnutrition and increased susceptibility to infections in children [Rice et al., 2000]. The lack of a recommendation for the amount of dietary protein to safely limit in order to delay disease progression has led to investigating protein source rather than reducing the amount of protein on ARPKD.

SPI was investigated in our female PCK rat study based on previous studies reporting attenuate of early stages of cyst pathogenesis indicated by reduced epithelial cell proliferation [Aukema et al., 1999; Peng et al., 2009]. Also, SPI contains isoflavones with anti-estrogenic properties [Vitale et al., 2013]. More extensive PCLD in women than men has been attributed to
higher estrogen levels in females [Gabow et al., 1990; Arnold et al., 2005]; therefore, the anti-
estrogenic SPI diet may lessen PCLD in the PCK female rat model. Soy protein is thought to
prevent adipocyte hypertrophy and hyperplasia which may prevent the release of excessive
amounts of fatty acids into the circulation associated with NAFLD [Torres et al., 2007].
Additionally, epidemiological, clinical, and experimental evidence shows that soy protein may
prevent lipotoxicity in non-adipose tissues during obesity [Torres et al., 2007]. SPI when fed to a
rat model of PKD showed anti-inflammatory effects as indicated by reduced renal expression of
inflammatory genes and enzyme activity [Peng et al., 2009]. Based on these studies, dietary soy
protein reduced NEFA and inflammation. To our knowledge no study has investigated the effect
of feeding SPI on splenic inflammatory gene expression.

1.62 Dietary fatty acids

Omega-6 PUFA, LA and omega-3 PUFA, ALA are the two essential amino acids. EPA
and DHA conversion rate from ALA is poor and therefore consuming rich dietary sources of
EPA and DHA is recommended [Simopoulos et al., 2000; St-Jules et al., 2013]. EPA and DHA
are synthesized from ALA through processes on elongation and desaturation. The n-6 PUFA AA
is synthesized from LA and competes with long-chain n-3 PUFAs for enzyme receptors during
synthesis [Scorletti et al., 2013]. The result of this competition favors n-3 PUFAs and down-
regulate AA synthesis resulting in less inflammatory effects. The Western diet which is high in
n-6/n-3 PUFA ratio has been related to an increase in PKD [Kant et al., 2005], and incidence of
NAFLD [Di Minno et al., 2012]. It has been reported that n-6 PUFA rich diets cause
immunodeficiency possibly through reduced immune cell proliferation in the spleen [Jeffrey et
al., 1997].
N-3 PUFAs have been extensively studied for their anti-inflammatory properties. N-3 PUFAs regulate the production of mediators of inflammation, activation of transcription factors, and expression of inflammatory genes in liver [Calder et al., 2015; Tou et al., 2015], adipose tissue [Scorletti et al., 2013], and potentially spleen. Dietary n-3 PUFAs have been reported to inhibit nuclear transcription factor NFκβ resulting in down-regulation of pro-inflammatory cytokines including TNF-α, IL-6, and MCP-1 [Huang et al., 2011]. In studies of NAFLD, n-3 PUFA supplementation had anti-inflammatory effects through stimulation of fatty acid oxidation in the liver via activation of PPARα [Scorletti et al., 2013; Popesco et al., 2013].

Unexpectedly, Maditz et al., [2015] found female PCK rats fed SPI +SB had the greatest (p<0.03) histological evidence of hepatic cyst obstruction, portal inflammation and up-regulation (p=0.03) of fibrosis related genes. Additionally, many rats had a noticeably enlarged spleen (Fig. 5) [Maditz et al., 2015]. Splenectomy slows the progression of hepatic fibrosis to cirrhosis in the liver [Yamada et al., 2015]. The most effective treatment for ARPKD and subsequent PCLD remains kidney and liver transplantation. Splenectomy is performed when severe antibody-mediated rejection occurs post-organ transplant [Locke et al., 2006]. However, splenectomy has been reported to cause a lifelong increased risk of infection with a mortality rate of 50% [Jones et al., 2010; Chu et al., 2014]. This is because the spleen is the major site for storage and rapid deployment of undifferentiated monocytes used to regulate inflammation [Swirski et al., 2009]. IL-10, an anti-inflammatory cytokine produced by the B cells in the marginal-zone of the spleen, play a role in suppressing harmful immune responses [Gotoh et al., 2012]. Splenectomy significantly reduces serum IL-10 involved in inducing steatosis and inflammatory responses in the liver [Vuppalanchi et al., 2009; Wang et al., 2015].
Obesity is associated with low chronic inflammation [Donath et al., 2011] and hepatic steatosis [Fabbrini et al., 2010]. Adults who are obese increase their risk of NAFLD by 80-90% [Bellentani et al., 2010] while risk in obese children is 40-70% [Bellentani et al., 2010]. Obese rats have increased inflammation in the liver and anti-inflammatory IL-10 expression is reduced in the spleen [Gotoh et al., 2012]. Obesity decreases splenic expression of pro-inflammatory cytokines, such as TNF-α, monocyte chemoattractant protein (MCP)-1, and IL-6 leading to chronic systemic low-grade inflammation [Gotoh et al., 2012]. In diet-induced obese rats, mRNA expression levels of TNF-α and IL-6 were down-regulated in the spleen [Lamas et al., 2004]. Bellentani et al., [2010] showed a correlation (p<0.001) between high fat diet induced obesity and splenomegaly [Altunkaynak et al., 2007; Pestka et al., 2014] suggesting that dietary fat affects the spleen. Pestka et al., [2014] showed n-3 PUFA supplementation resulted in anti-inflammatory properties associated with decreased splenic expression of TNF-α, and ablated increased expression of IL-10.

Adipose lipolysis plays an important role in the development of liver steatosis. To our knowledge no studies have investigated the role of adipose tissue in ARPKD. Gonadal fat is the largest adipose depots in rodents [Bjørndal et al., 2011]. Adipocytes are dynamic endocrine cells that secrete bioactive adipokines including TNF-α and IL-6 [Kanda et al., 2006]. Adipose tissue inflammation is due to recruitment of T-cells and macrophages and leads to a disturbed adipokines-balance and an uncontrolled release of FFAs, TNF-α, (MCP)-1 and IL-6 which can affect lipoprotein metabolism in other organs such as the liver [Bjørndal et al., 2011]. The inflammatory process can lead to an excess of TGs in the adipocytes stimulating lipolysis and uncontrolled release of NEFAs in to the circulation [Spoto et al., 2013] that are transported to the liver and promote the development of NAFLD. NEFAs are also activators of key molecules in
the innate immune response [Spoto et al., 2013]. N-3 PUFAs can affect adipose tissue by the up-regulation of PPARα, leading to decreased lipolysis, improved lipid storage capacity in subcutaneous adipose tissue, as well as anti-inflammatory effects possibly due to inhibitory action on NFκβ [Singer et al., 2008]. Based on this dietary n-3 PUFA can alter adipose lipid metabolism and subsequently reduce free FAs and inflammatory factors that circulate to the liver and contribute to NAFLD. Given this effect on adipose tissue, we hypothesize that feeding n-3 PUFAs can also alter spleen lipid metabolism and inflammation to ameliorate NAFLD.
2.0 Study hypothesis and objectives

We hypothesize the direct connection of the spleen and liver by the splenic and portal veins allows the transport of bioactive compounds such as fatty acids and inflammatory and immune factors produced by the spleen to the liver. In turn, the spleen-liver axis may promote the development of liver steatosis and progression to fibrosis.

The objective of the present study was to investigate the effect of diet on the role of splenic lipid metabolism in the development of liver steatosis. Additionally, we explored the effect of diet on gene expression of inflammation and immunity in the spleen on the progression
of steatosis to fibrosis in the liver. Finally, we extended our research to the FA content of gonadal adipose tissue from our female PCK rats to include the known effects of adipose lipolysis in our discussion for the development of steatosis in a model of ARPKD.
3.0 Chapter 1

Feeding Soy Protein Isolate and/or Omega-3 Polyunsaturated Fatty Acids on the Spleen-Liver Axis in a Female Rat Model of Autosomal Recessive Polycystic Kidney Disease with Liver Steatosis

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3.1 Abstract

Autosomal recessive polycystic kidney disease (ARPKD) is a congenital hepatorenal fibrocystic syndrome. Splenomegaly occurs in 60% of ARPKD patients. Splenomegaly correlates with hepatic lipid accumulation in non-alcoholic fatty liver disease (NAFLD). We hypothesized the close anatomical proximity of the spleen to the liver allows bioactive compounds produced by the spleen including fatty acids, inflammatory and immune factors to access the liver through the splenic and portal veins. With the absence of effective medications diet offers a potentially efficacious, safe, and cost-effective therapy option. Both soy protein isolate (SPI) and omega-3 polyunsaturated fatty acids (n-3 PUFAs) exhibit anti-inflammatory properties. The objective of this study was to investigate the effect of SPI and/or n-3 PUFA on gene expression regulating lipogenesis, lipolysis, inflammation, and immunity in splenic tissue using RT-qPCR. Young (age 28 d) female PCK rats randomly assigned (n=12/group) to diets consisting of casein + corn oil (Casein + CO), casein + soybean oil (Casein + SO), soy protein isolate + soybean oil (SPI + SO), or soy protein isolate + a 1:1 soybean: salmon oil blend (SPI + SB) were fed for 12 weeks. Unexpectedly, spleen was significantly (P=0.02) elongated and liver steatosis was previously reported highest (p=0.003) in SPI+SB fed rats compared to Casein + CO and Casein + SO diets. No significant differences in expression of genes regulating lipid metabolism, inflammation or immunity were found among dietary treatments. Based on our results we concluded that diet had no effect on splenomegaly, and splenomegaly did not significantly contribute to the development of liver steatosis. Interestingly, relative gonadal adipose fad pad weight was significantly lower (p=0.03) in SPI + SB fed rats compared with Casein + SO fed rats possibly indicating increased mobilization of FAs from adipose tissue to the liver in the SPI + SB fed group was the major contributor to NAFLD.
**Keywords:** rat, autosomal recessive polycystic kidney disease, splenomegaly, omega-3 fatty acids, soy protein isolate, non-alcoholic fatty liver disease.
3.2 Introduction

Autosomal recessive polycystic kidney disease (ARPKD) is a congenital form of polycystic kidney disease (PKD) [Sweeney et al., 2014; Dell et al., 2011]. It is a rare genetic disorder that occurs in 1 in 20,000 live births [Telega et al., 2013; Zerres et al., 1998; Dell et al., 2011] with a mortality rate of approximately 30% during the neonatal period or within the first year of life [Sweeney et al., 2014]. ARPKD causes congenital hepatorenal fibrocystic syndromes resulting in significant renal and liver-related morbidity and mortality in children [Sweeney et al., 2014]. Liver cysts are the most common extra-renal manifestation of ARPKD [Dell et al., 2009; Sweeney et al., 2011; 2014] with increased cyst proliferation in females due to their higher estrogen levels [Arnold et al., 2005]. In the absence of effective medications for the treatment of liver disease in ARPKD, diet offers a potentially efficacious, safe, and cost-effective therapeutic option [Maditz et al., 2014 & 2015].

Soy protein isolate (SPI) contains isoflavones with anti-estrogenic properties [Aukema et al., 1999; Vitale et al., 2013] and has been shown to reduce renal cyst proliferation in PKD [Peng et al., 2009]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), have been extensively studied for their anti-inflammatory properties [Huang et al., 2011] and for their role in down-regulating de novo lipogenesis (DNL) through up-regulation of β-oxidation. EPA and DHA can be de novo synthesized from the essential α-linolenic acid (ALA) however, consuming rich dietary sources of EPA and DHA such as fish oil is recommended due to a poor conversion rate of about 2% [Simopoulos et al., 2000; St-Jules et al., 2013]. Maditz et al., [2015] examined the effects of feeding SPI and/or n-3 PUFAs on the severity of liver cysts in young female PCK rats, an orthologous animal model of human ARPKD [Nagao et al., 2012]. Unexpectedly, female
PCK rats fed SPI and n-3 PUFA enriched diets resulted in the highest liver cysts, steatosis, and inflammation [Maditz et al., 2015]

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic steatosis [Araya et al., 2004; Fon Tacer et al., 2011] which can progress to non-alcoholic steatohepatitis (NASH) characterized by inflammation and fibrosis and ultimately cirrhosis in the liver [Fon Tacer et al., 2011; Valenzuela et al., 2011]. Steatosis occurs when the rate of hepatic fatty acid (FA) uptake from plasma and DNL increases [Fabbrini et al., 2010], and is associated with increased transport of FAs for triglyceride (TG) synthesis to the liver [Tamura et al., 2005]. Hepatic uptake of FA is not regulated and therefore, the concentration of FA in the blood is directly related to an influx of FA to the liver through the portal vein [Tamura et al., 2005].

In addition to NAFLD, spleen enlargement was observed in our ARPKD rat model. Additionally, splenomegaly has been shown to increase with the rate of hepatic lipid accumulation in NAFLD [Wang et al., 2015]. Although the mechanism has not been defined [Alberti et al., 2012], the spleen has an important role possibly through regulating gene expression associated with lipid metabolism in the liver [Petroianu et al., 2006; Paulo et al., 2007; Paulo et al., 2009]. We have the novel aim to investigate the effect of diet on gene expression regulating lipogenesis and lipolysis in spleen tissue to compare with development of liver steatosis and fibrosis. This has important implications since NAFLD is the most common cause of liver disease associated with the Western diet [Bellentani et al., 2010].

Splenomegaly has also been reported to be present in 60% of ARPKD patients under the age of five [Gunay Aygun et al., 2013]. Sugawara et al., [2000] reported that splenectomy slows the progression of hepatic fibrosis to cirrhosis. We hypothesize that due to the close anatomical
proximity of the spleen to the liver, bioactive compounds produced by the spleen can directly access the liver through the splenic and portal veins [Akahoshi et al., 2002]. Therefore, we conducted an exploratory study to determine the potential role of the spleen in NAFLD.

Splenic response is controlled by splenic chemokines that organize the tissue of the white pulp and regulate spleen function [Sweeney et al., 2014]. The spleen responds to infection and disease by mobilizing splenic lymphocytes that control inflammatory gene expression [Wang et al., 2015], and FAs stored in splenic macrophages [Schmidt et al., 1997]. Vuppalanchi et al., [2009] reported that splenic cytokines regulate inflammatory responses in the liver [Pestka et al., 2014]. Therefore, in addition to investigating splenic lipid metabolism, the current study also has the novel aim to investigate the effect of diet on the expression of genes regulating inflammation and immunity in the spleen in order to better understand the relationship of splenomegaly to the development and progression of NAFLD.

3.3 Materials and Methods

3.31 Animals and diets

All tissues examined in this study were obtained from a previous feed study conducted by Maditz et al., [2014]. All animal procedures were conducted in accordance with the National Research Council for the Care and Use of Laboratory Animals Guidelines [1996] and approved by the Animal Care and Use Committee at West Virginia University. Young (age 28 days) female PCK rats were purchased from Charles River Laboratories (Wilmington, MA, USA). All rats were individually caged in a room kept at standardized environmental conditions of a consistent 21 °C, with a 12-hour light/dark cycle throughout the 12-week study. After a 7-day
acclimation period, rats were randomly assigned to different experimental diets (n = 12 rats/group).

Diet differed by protein and lipid sources. Protein sources consisting of 200-g/kg diet were fed as either casein or SPI. Both protein sources consisted of 87% crude protein by proximate analysis (Table 3). Soy protein isolate was donated by DuPont Nutrition and Health (St Louis, MO, USA). Lipid sources in a 70-g/kg diet were fed as corn oil (CO; low in n-3 PUFAs), soybean oil (SO; containing essential fatty acids, n-6 PUFA, linoleic acid (LA; 18:2n-6), and n-3 PUFA, ALA or salmon oil, a rich source of long-chain n-3 PUFAs, EPA and DHA as a 1:1 soybean oil + salmon oil blend (SB) (Table 4). Salmon oil was purchased from Jedwards International Inc. (Quincy, MA, USA). Rats were randomly assigned to (1) Casein + CO, (2) Casein + SO, (3) SPI + SO, or (4) SPI + SB. Caloric value was 38 Kcal/g and metabolized energy was 16.1± 0.3kJ/g for all diets.

To prevent variability in food intake, female rats were restricted to 15 ± 2 g of powdered diet daily based on previous studies showing this amount was sufficient to support growth in young female Sprague-Dawley rats [Tou et al., 2011]. All diets were stored at −20 °C until fed to the rats. Food intake was measured and replaced with fresh diet daily. Rats were provided deionized distilled water ad libitum. Body weight and food consumption were recorded weekly. At the end of the 12-week feeding study, rats were euthanized by CO₂ inhalation. The spleen and liver were perfused and excised. Gonadal fat, the largest adipose deposit in rodents [Bjørndal et al., 2011], was excised. Spleen, liver, and adipose tissues were weighed, and immediately frozen in liquid nitrogen and stored at -80 C until analyzed.

3.32 Serum lipids and lipoproteins
Rats were fasted overnight then euthanized by CO₂ inhalation. Blood was collected by aortic puncture. Blood was centrifuged at 1,500 g for 10 min at 4°C. Collected serum samples were stored at -80°C until analyzed. Fasting serum total cholesterol, triglyceride (TG), very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) were determined by lipid test rotor enzymatic colorimetric assays and measured using a Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics Inc., Columbia, MD).

3.33 Splenic gene expression analysis

Primers were designed using the Primer 3 program (Howard Hughes Medical Institute). Shown in Table 2 are mRNA sequences for all primers obtained from NCBI showing forward and reverse primer sequences for genes. Primers for lipid metabolism consisted of sterol regulatory element-binding protein 1-c (SREBP1-c), carbohydrate response element-binding protein (ChREBP), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD-1), and peroxisome proliferator-activated receptor alpha (PPARα). Primers for inflammation genes included nuclear factor-κβ (NF-κβ), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein 1 (MCP-1), and mammalian target of rapamycin (mTOR). Primers for immunity genes included C-X-C motif chemokine ligand 13 (CXCL13), C-C motif chemokine ligand 19 (CCL19), C-C motif chemokine ligand 21 (CCL21), and C-C motif chemokine ligand 9 (CCL9). All primers were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Table 2).
Table 2: Primer designs and identifications

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<tr>
<th>Gene</th>
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<th>Reverse primer (5' to 3')</th>
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Table abbreviations: sterol regulatory element-binding protein 1-c (SREBP1-c), carbohydrate response element-binding protein (ChREBP), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD-1), peroxisome proliferator-activated receptor alpha (PPARα), nuclear factor-κβ (NF-κβ), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein 1 (MCP-1), mammalian target of rapamycin (mTOR), C-X-C motif chemokine ligand 13 (CXCL13), C-C motif chemokine ligand 19 (CCL19), C-C motif chemokine ligand 21 (CCL21), C-C motif chemokine ligand 9 (CCL9), glyceraldehyde 3-phosphate dehydrogenase (GADPH)
Spleens were frozen in liquid nitrogen and stored at −80 °C until analysis. Total RNA was extracted following the Total RNA Isolation procedure of the mirVana miRNA Isolation Kit (Ambion, TX, USA). Isolated mRNA integrity was visualized on a 1.5% agarose gel, and isolated mRNA concentration quantified by spectrophotometry (Thermo Scientific, DE, USA). After DNase treatment of the isolated mRNA (Applied Biosystems, CA, USA), total messenger RNA (mRNA) was amplified using the Superscript III First Strand Synthesis assay with oligo dT primers [Invitrogen, CA, USA]. The procedure for total RNA extraction, quantification, and amplification is detailed in Maditz et al., [2014].

Polymerase chain reaction consists of 2.5 μL of SYBR Green Master Mix (Applied Biosystems, CA, USA), 1 μL of complementary DNA, 1 μL of respective forward and reverse primers, and 0.5 μL of ddH₂O, for a total reaction volume of 5 μL. The thermal profile consisted of 50 °C for 2 minutes, 95 °C for 10 minutes, and then 40 cycles of 95 °C for 15 seconds and at 60 °C for 1 minute. Primer efficiencies (>1.8) were determined using LinReg PCR [Ramakers et al., 2013] on pooled mRNA samples serially diluted in ddH₂O up to 1:10,000 according to the procedure outlined by Bari et al., [2006]. Data abundance was normalized using log10 transformation, and all samples were performed in triplicate.

3.34 Fatty acid analysis

Total lipids were extracted from the spleen and adipose samples using a modified method of Bligh and Dyer [1959]. Nonadecenoate (19:1) was added to all adipose (0.025-0.03g) or spleen (0.2-0.5g) samples (n=6/ diet group) as an internal standard. Extracted lipid samples were transmethylated according to Fritsche and Johnston [1990]. Extracted lipids were dried under nitrogen gas and diluted in isooctane to a concentration of 5 mg fatty acid methyl esters/mL isooctane. Fatty acid methyl ester samples were analyzed by gas chromatography (CP-3800;
Varian, CA, USA) as described in Gigliotti et al., [2013]. Initial temperature was 140°C held for 5 minutes and then increased 1°C per minute to a final temperature of 220°C with nitrogen used as the carrier gas. Quantitative 37 component fatty acid methyl ester Sigma Mix (Supelco, Bellefonte, PA, USA) was used as a standard to identify FAs. FAs were identified by retention time and quantified using peak area counts. All samples were reported as % of total FAs.

3.35 Statistical analyses

Results are expressed as means ± standard error of the mean (SEM). For gene expression, mRNA abundance was calculated as 
\[ A = \frac{1}{(\text{efficiency of GADPH} \times \text{DCT of GADPH}) - (\text{efficiency of the gene of interest (GOI)} \times \text{DCT of GOI})} \]
where DCT of GOI= the average CT of Casein+CO group minus the geometric mean of the triplicate for each rat, and the DCT of GADPH= the average CT for the GADPH of the Casein+CO group minus the geometric mean of the triplicate for GADPH of each sample. This equation was derived from those Jacomo et al., [2014].

One-way analysis of variance (ANOVA) was used to determine differences among diet treatment groups. Post hoc multiple comparison tests were performed using Tukey’s test. Differences are considered significant at \( P < 0.05 \). All statistical analyses were performed using SigmaPlot 13.0 statistical software program (Systat Software Inc., San Jose, CA, USA).

3.4 Results

3.41 Spleen lengths and organ/tissue weights

Figure 4 shows evidence of splenomegaly in female PCK rats. Spleen length was longer (p=0.02) in the SPI+SB compared to the Casein+CO and Casein+SO fed groups (Table 5). However, there were no significant differences in spleen weight among diet groups. Maditz et
al., determined no significant differences in liver weight among diet groups (Table 5). Relative gonadal fat pad weight was lowest (p=0.03) in rats fed SPI+SB compared to Casein+SO diet (Table 5).

3.42 Serum lipid and lipoproteins

Shown in Table 6 rats fed SPI+SB diet had the lowest (p≤0.003) total serum cholesterol (CHL), total serum triglycerides, serum VLDL among the diet treatment groups (Table 6).

3.43 Spleen lipid metabolism gene expression

Shown in Table 7, there were no significant differences in gene expression of DNL transcription factors ChREBP and SREBP-1c, or lipogenic enzymes FAS and SCD-1. Gene expression of transcription factor PPARα, a marker of splenic lipolysis, showed no significant differences among diet groups (Table 7).

3.44 Splenic fatty acid composition

Shown in Table 8, splenic total lipid content was not significantly different between diet groups. Splenic content of saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0) and monounsaturated fatty acids, palmitoleic acid (C16:1n-7) and oleic acid (C18:1n-9), were not significantly different between diet groups. Splenic long-chain n-3 PUFAs, EPA and DHA content was highest (p<0.001) in rats fed SPI+SB diet. Splenic long-chain n-6 PUFA AA content was lowest (p=0.037) in rats fed SPI+SB diet.

3.45 Spleen inflammation gene expression
There were no significant differences in the expression of Pro-inflammatory genes including NFκβ, TNFα, IL-6, MCP-1, and mTOR or anti-inflammatory gene IL-10 among diet groups (Table 9).

3.46 Spleen immune gene expression

There were no significant differences in the expression of immunity genes including CCL9, CXCL13, CCL19, and CCL21 among diet groups (Table 10).

3.47 Gonadal adipose fatty acid composition

Gonadal adipose tissue of rats fed SPI + SB diet had the highest (p<0.004) palmitic acid (16:0), stearic acid (18:0), and palmitoleic acid (16:1n-7) content. Rats fed SPI + SB diet had the lowest (p<0.001) n-6 PUFA LA content and lower (p=0.009) AA content compared to rats fed Casein + CO and SPI +SO diets. Rats fed SPI + SB diet had higher (p<0.001) ALA content compared to rats fed Casein + CO diet and the highest (p<0.001) DHA content among groups (Table 11).

3.5 Discussion

In the current study, SPI+SB fed rats had elongated (p=0.02) spleens compared to rats fed Casein + CO and Casein + SO diets. Suzuki et al., [2010] found spleen length was longest (p=0.0001) in individuals with NASH compared to individuals with NAFLD or a normal liver. Spleen volume was increased (p<0.0001) with hepatic TG accumulation in NAFLD patients and the liver-to-spleen ratio was also positively correlated (p<0.0001) with spleen volume [Tsushima et al., 2000]. Hepatic steatosis occurs when the rate of hepatic FA uptake and DNL is greater than the rate of FA oxidation and export in VLDLs [Fabbrini et al., 2010]. Rats fed SPI+ SB diet
had the highest (p<0.05) hepatic TG accumulation and the lowest (p=0.003) serum TGs, VLDL (p=0.04), and cholesterol (p<0.01). Schmidt et al., [1997] reported hypolipidemia resulting from splenic macrophages accumulating large quantities of fat due to an increase in phagocytic ability. A spleen-liver axis was suggested where the enlarged spleen acting as a lipid reservoir results in an influx of splenic FAs to the liver [Schmidt et al., 1997].

Rats fed SPI+SB diet had the highest (p<0.001) splenic EPA and DHA content. Dietary n-3 PUFAs may affect lipid metabolism by inhibiting nuclear transcription factors and enzymes that stimulate DNL while up-regulating transcription factors that stimulate β-oxidation [Scorletti et al., 2013]. In our study, gene expression of transcription factors regulating DNL, ChREBP and SREBP-1c [Filhoulaud et al., 2013; Shao et al., 2012] were not significantly different in the rats fed SPI and/or n-3 PUFA diets. The spleen of rats fed different diet treatments also showed no significant differences in expression of lipogenic genes FAS, an enzyme complex that produces palmitic acid (16:0) which can then be elongated to stearic acid (18:0). Gene expression of or SCD-1, a rate-limiting enzyme which desaturates palmitic acid to palmitoleic acid (16:1n-7) and stearic acid to oleic acid (18:1n-9) was not significantly different among the diet treatment groups. A study by Jeffrey et al. [1997], showed Lewis rats had increased cytotoxic T cell activity with increased dietary palmitic acid and decreased cytotoxic T cell activity as dietary oleic acid increased. Oleic acid decreases splenic lymphocyte proliferation [Jeffrey et al., 1997]. In our study, splenic content of oleic, stearic, palmitic, and palmitoleic acid were not significantly different among diet treatment groups.

PPARα stimulates the β-oxidative degradation of FAs [Chinetti et al., 2000] which prevents TG accumulation in NAFLD [Scorletti et al., 2013]. In our study, there higher hepatic lipid accumulation, but no significant differences in gene expression of PPARα in the spleen of
rats fed SPI and/or n-3 PUFAs. Based on our results study, splenic DNL was not significantly increased and did not provide a significant source of FAs contributing to NAFLD in female PCK rats.

NAFLD can progress to NASH with increased inflammation [Fon Tacer et al., 2011]. Splenectomy has been shown to slow the progression of fibrosis to cirrhosis [Akahoshi et al., 2002; Yamada et al., 2015] possibly due to splenic lymphocytes migrating into the liver to promote liver fibrosis by increasing inflammatory cytokine expression [Tanabe et al., 2015]. Female PCK rats fed SPI + SB diet showed up-regulated (p=0.003) expression of fibrosis related genes [Maditz et al., 2015]. Dietary n-3 PUFAs have been reported to inhibit NFκβ, a nuclear transcription factor regulating pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 [Huang et al., 2011]. In our study, gene expression of NFκβ was not significantly different among diet groups. Pestka et al., [2014], showed n-3 PUFA supplementation exhibited anti-inflammatory properties associated with decreased expression of pro-inflammatory TNF-α and IL-6, and ablated expression of anti-inflammatory IL-10 in the spleen. Splenectomy significantly reduces serum IL-10 which induces steatosis and inflammatory responses in the liver [Vuppalanchi et al., 2009; Wang et al., 2015]. In our study, expression of genes regulating inflammation, TNF-α, IL-6, and MCP-1 and anti-inflammatory IL-10 in the spleen were not significantly different among rats fed SPI and/or n-3 PUFAs despite elongated spleen length and increased NAFLD.

Sustained liver injury results in liver fibrosis regardless of the cause [Tanabe et al., 2015]. The spleen produces and stores antibodies and immune cells that mobilize upon infection or injury [Tarantino et al., 2013]. Chemokines function as immune signaling proteins that attract splenic T and B lymphocytes to different areas of the spleen to control immune cell proliferation and maintain organization of the white pulp [Mebius et al., 2005]. Splenic CXCL13 is
responsible for attracting B lymphocytes to the follicles [Ansel et al., 2000] while CCL19 and CCL21 attract T lymphocytes to T-cell zones of the white pulp [Gunn et al., 1999]. Diets rich in n-6 PUFAs have been reported to cause immunodeficiency through reduced immune cell proliferation in the spleen [Jeffrey et al., 1997]. Despite higher n-3 PUFAs, EPA and DHA and reduced (p=0.04) n-6 PUFA, AA in the spleen of rats fed SPI+SB diet, there was no significant differences in gene expression of CXCL13, CCL19, CCL21, and CCL9 among diet groups.

Recently, Oishi et al., [2011], and Inoue et al., [2012], found that splenectomy exacerbated TG deposition in the liver, suggesting a role for the spleen in preventing instead of promoting progression of hepatic steatosis to steatohepatitis. Rather than splenomegaly being a contributor to NAFLD, it may provide protective effects. Brunt et al., [2009], reported a correlation between portal inflammation and progressive hepatic steatosis. Maditz et al., [2015], observed highest (p=0.03) portal inflammation in the SPI + SB group. Portal hypertension has been suggested to contribute to splenomegaly through congestion of the portal vein into the spleen [Sweeney et al., 2014].

Inhibition of mTOR is identified as a potential therapeutic intervention for splenomegaly secondary to portal hypertension [Biecker et al., 2005]. Mejias et al., [2010] showed mTOR blockade by rampamycin ameliorated splenomegaly indicated by a 44% decrease in spleen size. In our study there were no significant differences in the splenic gene expression of mTOR between among diet groups. This may be because the spleens were elongated in rats fed SPI+SB diet, but spleen weight was not significantly increased compared to the other dietary groups.

NAFLD results when the rate hepatic FA uptake and DNL is greater than the rate of FA oxidation and FA export in VLDLs [Fabbrini et al., 2010]. Maditz et al., [2015], showed feeding
SPI+SB diet had no significant effect on transcription factors and enzymes regulating DNL and β-oxidation in the liver, but serum TG and VLDL was significantly reduced. Rats fed SPI+SB diet had lower (p=0.03) relative gonadal adipose tissue weight compared to rats fed Casein+SO suggesting increase adipose lipolysis. Lipolysis of adipose is the largest contributor of serum FA [Tamura et al., 2005]. Release of these FAs into serum is uncontrolled and increased when inflammation presents [Bjorndal et al., 2011]. Adipocytes are also known to secrete inflammatory mediators [Liang et al., 2013] and bioactive adipokines including TNF-α, IL-6, and MCP-1 [Bjorndal et al., 2011; Kanda et al., 2006]. Inflammation increases TG content of adipocytes leading to an increase in lipolysis and the subsequent release of NEFAs to be transported to the liver and promote NAFLD [Spoto et al., 2013]. SPI + SB fed rats had gonadal adipose fat pad FA content indicating increased DNL when compared to feed FA content. SPI + SB fed rats had the highest DNL FAs including palmitic, stearic, and palmitoleic acid content in the gonadal adipose fat pads. Rats fed SPI + SB diet had the lowest (p<0.001) n-6 PUFA, LA content of any group and lowest (p=0.009) n-6 PUFA, AA content compared to rats fed Casein + CO and SPI +SO diets. SPI + SB fed group had the highest n-3 PUFA (p<0.001) DHA content, and higher (p<0.001) ALA content known to drive β-oxidation compared to rats fed Casein + CO diet rich in LA. Exploratory results suggest increased adipose lipolysis and reduced TG export from the liver potentially contributed to the NAFLD development in our rats over splenic FA transport through the spleen-liver axis.
3.6 Summary and conclusions

We anticipated that feeding SPI + SB diet to the ARPKD rat model would have the best outcome among diet treatment groups based on reported benefits of soy protein and n-3 PUFA rich diets. Unexpectedly, rats fed SPI + SB diet showed spleen elongation, a marker of spleen enlargement, and exacerbated NAFLD symptoms compared to rats fed Casein + CO diet, typical of a Western diet. Based on our results, dietary n-3 PUFAs and SPI did not significantly affect gene expression of splenic lipid metabolism, inflammatory or immunity in female PCK rats with evidence of NAFLD. Therefore, we disproved our hypothesis that the direct connection of the spleen and liver by the splenic and portal veins contributed to the transport of bioactive compounds produced by the spleen to promote the development of hepatic steatosis and progression to fibrosis in ARPKD. Metabolic dysfunction associated with the disease state in ARPKD may be responsible for the unexpected study results. Cyst obstruction and steatosis known to cause blockage of the biliary ducts and congestion of the portal vein may have led to the development of splenomegaly. Hyperactivity of the spleen due to disease and possibly liver dysfunction may have also contributed to splenomegaly in the female PCK rat model. More studies on the role of diet on ARPKD are needed to determine the mechanism by which the spleen-liver axis influences hepatic lipid metabolism and development of liver steatosis and splenomegaly.
3.7 References


National Research Council (US) Institute for Laboratory Animal Research. . 1996.


### 3.8 Tables

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Casein ± CO</th>
<th>Casein ± SO</th>
<th>SPI ± SO</th>
<th>SPI ± SB</th>
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</thead>
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</tr>
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<td>50</td>
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<tr>
<td>Calories</td>
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<td>377</td>
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All ingredients are from Harlan Teklad except for SPI from DuPont Nutrition and Health and salmon oil from Jedwards International.

*Based on the AIN-93G vitamin and mineral mixes.
<table>
<thead>
<tr>
<th>Fatty acids (relative %)</th>
<th>Casein ± CO</th>
<th>Casein ± SO</th>
<th>SPI ± SO</th>
<th>SPI ± SB</th>
</tr>
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<tbody>
<tr>
<td><strong>Saturated FAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic C16:0</td>
<td>11.08 ± 0.63ab</td>
<td>8.65 ± 0.37b</td>
<td>10.98 ± 0.57ab</td>
<td>13.02 ± 0.95a</td>
</tr>
<tr>
<td>Stearic C18:0</td>
<td>1.85 ± 0.06b</td>
<td>4.39 ± 0.18a</td>
<td>3.63 ± 0.13a</td>
<td>3.96 ± 0.32a</td>
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<tr>
<td><strong>MUFA s</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic C16:1n-7</td>
<td>0.19 ± 0.00b</td>
<td>0.00 ± 0.00b</td>
<td>0.12 ± 0.02b</td>
<td>3.46 ± 0.35a</td>
</tr>
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<td>Oleic C18:1n-9</td>
<td>27.45 ± 0.22a</td>
<td>21.16 ± 0.35b</td>
<td>17.91 ± 0.31c</td>
<td>14.93 ± 0.23d</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>ALA (18:3n-3)</td>
<td>0.59 ± 0.18c</td>
<td>7.69 ± 0.02a</td>
<td>7.68 ± 0.04a</td>
<td>4.85 ± 0.01b</td>
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<tr>
<td>EPA (20:5n-3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>7.40 ± 0.03</td>
</tr>
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<td>DHA (22:6n-3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.90 ± 0.10</td>
</tr>
<tr>
<td><strong>n-6 PUFAs</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>LA (18:2n-6)</td>
<td>52.93 ± 2.68a</td>
<td>55.78 ± 0.07a</td>
<td>56.09 ± 0.09a</td>
<td>35.19 ± 0.06b</td>
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<tr>
<td>AA (20:4n-6)</td>
<td>1.10 ± 0.04a</td>
<td>0.16 ± 0.01c</td>
<td>0.21 ± 0.01c</td>
<td>0.41 ± 0.01b</td>
</tr>
</tbody>
</table>

All ingredients are from Harlan Teklad except for SPI from DuPont Nutrition and Health and salmon oil from Jedwards International.

Different superscript letters a, b, and c within the same rows indicate significant differences at p < .05 by 1-way ANOVA followed by Tukey test. Abbreviations: alpha linolenic acid (ALA), eicosapentaenoic acid (EPA), decosahexaenoic acid (DHA), linoleic acid (LA), arachidonic acid (AA).
<table>
<thead>
<tr>
<th>Measurements</th>
<th>Casein + CO</th>
<th>Casein + SO</th>
<th>SPI + SO</th>
<th>SPI + SB</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen lengths (cm)</td>
<td>3.04 ± 0.08b</td>
<td>3.09 ± 0.06b</td>
<td>3.26 ± 0.13ab</td>
<td>3.60 ± 0.21a</td>
<td>0.02</td>
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<tr>
<td>Absolute spleen weight (g)</td>
<td>0.64 ± 0.01</td>
<td>0.63 ± 0.01</td>
<td>0.77 ± 0.14</td>
<td>0.92 ± 0.14</td>
<td>0.17</td>
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<tr>
<td>Relative spleen weight (mg/g bwt)</td>
<td>2.35 ± 0.05</td>
<td>2.34 ± 0.05</td>
<td>2.85 ± 0.48</td>
<td>3.26 ± 0.53</td>
<td>0.21</td>
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<tr>
<td>Absolute liver weight (g)</td>
<td>9.90 ± 0.20</td>
<td>10.50 ± 0.30</td>
<td>12.60 ± 2.00</td>
<td>13.90 ± 1.60</td>
<td>0.14</td>
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<tr>
<td>Relative liver weight (mg/g bwt)</td>
<td>36.70 ± 0.70</td>
<td>39.00 ± 0.90</td>
<td>46.70 ± 7.40</td>
<td>49.30 ± 5.20</td>
<td>0.17</td>
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<tr>
<td>Absolute Gondal adipose weight (g)</td>
<td>7.84 ± 0.50</td>
<td>8.01 ± 0.49</td>
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<td>6.10 ± 0.57</td>
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<td>Relative Gondal adipose weight (mg/g bwt)</td>
<td>28.8 ± 1.66ab</td>
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<td>29.34 ± 2.40ab</td>
<td>21.87 ± 2.23b</td>
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Values are the mean ± SEM of n=11-12 rats/group (liver) n=8-9 rats/group (spleen & adipose). Different superscript letters a, b within the same rows indicate significant differences at P<0.05 by one-way ANOVA followed by Tukey’s test.

Abbreviations: corn oil (CO), soybean oil (SO), soy protein isolate (SPI), 1:1 soybean oil:salmon oil blend (SB)
Table 6: Serum lipids and lipoprotein profiles in female PCK rats fed various protein and lipid sources

<table>
<thead>
<tr>
<th></th>
<th>Casein+CO</th>
<th>Casein+SO</th>
<th>SPI+SO</th>
<th>SPI+SB</th>
<th>p-value</th>
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<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>144.55 ± 8.74a</td>
<td>130.40 ± 5.96ab</td>
<td>124.09 ± 6.33b</td>
<td>77.73 ± 5.80c</td>
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<tr>
<td>Total TG (mg/dL)</td>
<td>21.81 ± 2.2a</td>
<td>21.25 ± 1.24a</td>
<td>18.16 ± 1.51a</td>
<td>13.37 ± 1.94b</td>
<td>0.003</td>
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<tr>
<td>VLDL (mg/dL)</td>
<td>29.80 ± 2.6a</td>
<td>27.89 ± 1.75a</td>
<td>29.06 ± 1.50a</td>
<td>18.60 ± 0.80b</td>
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<tr>
<td>LDL (mg/dL)</td>
<td>28.85 ± 2.31</td>
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<td>31.31 ± 2.87</td>
<td>31.26 ± 2.94</td>
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<tr>
<td>HDL (mg/dL)</td>
<td>35.09 ± 3.23</td>
<td>34.62 ± 2.96</td>
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<td>30.84 ± 2.20</td>
<td>0.2</td>
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</table>

Values are mean serum lipid ± SEM. Different letters a, b, c within the same rows indicate significant differences at p<0.05 by One Way Analysis of Variance followed by Tukey Test. Abbreviations: triglyceride (TG), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL).
### Table 7: Spleen lipid metabolism gene expression levels in relation to dietary treatment in female PCK rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Casein+CO</th>
<th>Casein+SO</th>
<th>SPI+SO</th>
<th>SPI+SB</th>
<th>p- value</th>
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<td>SREBP-1c</td>
<td>0.06 ± 0.006</td>
<td>0.06 ± 0.006</td>
<td>0.07 ± 0.003</td>
<td>0.06 ± 0.007</td>
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<tr>
<td>ChREBP</td>
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<td>0.04 ± 0.003</td>
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<td>FAS</td>
<td>0.12 ± 0.04</td>
<td>0.10 ± 0.02</td>
<td>0.09 ± 0.02</td>
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<tr>
<td>SCD-1</td>
<td>0.13 ± 0.03</td>
<td>0.16 ± 0.04</td>
<td>0.14 ± 0.04</td>
<td>0.12 ± 0.03</td>
<td>0.7</td>
</tr>
<tr>
<td>PPARα</td>
<td>0.05 ± 0.003</td>
<td>0.04 ± 0.004</td>
<td>0.05 ± 0.003</td>
<td>0.05 ± 0.003</td>
<td>0.7</td>
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</tbody>
</table>

Values are the means ± SEM of n=6 rats/group. Significant differences at P<0.05 by one way ANOVA. Abbreviations: sterol regulatory element-binding protein 1-c (SREBP1-c), carbohydrate response element-binding protein (ChREBP), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD-1), and peroxisome proliferator-activated receptor alpha (PPARα)
Table 8: Spleen fatty acid composition in female PCK rats fed various protein and lipid sources

<table>
<thead>
<tr>
<th></th>
<th>Casein+CO</th>
<th>Casein+SO</th>
<th>SPI+SO</th>
<th>SPI+SB</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>%Total lipid (g lipid/g spleen*100)</strong></td>
<td>2.42 ± 0.32</td>
<td>2.26 ± 0.21</td>
<td>2.21 ± 0.17</td>
<td>2.01 ± 0.041</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Saturated FAs (relative %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid 16:0</td>
<td>24.9 ± 2.03</td>
<td>26.5 ± 1.75</td>
<td>24.3 ± 1.39</td>
<td>26.6 ± 1.78</td>
<td>0.7</td>
</tr>
<tr>
<td>Stearic acid 18:0</td>
<td>15.7 ± 1.16</td>
<td>17.1 ± 1.78</td>
<td>15.6 ± 1.36</td>
<td>16.8 ± 0.97</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>MUFA</strong>s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid 16:1</td>
<td>1.22 ± 0.3</td>
<td>1.15 ± 0.42</td>
<td>1.37 ± 0.19</td>
<td>1.78 ± 0.32</td>
<td>0.5</td>
</tr>
<tr>
<td>Oleic acid 18:1 n-9</td>
<td>11.4 ± 1.5</td>
<td>8.93 ± 1.59</td>
<td>11.3 ± 0.68</td>
<td>11.9 ± 1.04</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>n-3 PUFAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALA 18:3n3</td>
<td>0.11 ± 0.05</td>
<td>0.33 ± 0.1</td>
<td>0.48 ± 0.19</td>
<td>0.17 ± 0.09</td>
<td>0.4</td>
</tr>
<tr>
<td>EPA 20:5 n-3</td>
<td>0.0 ± 0.00b</td>
<td>0.0 ± 0.00b</td>
<td>0.0 ± 0.00b</td>
<td>3.20 ± 0.027a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHA 22:6 n-3</td>
<td>0.55 ± 0.17c</td>
<td>1.20 ± 0.19bc</td>
<td>1.29 ± 0.15b</td>
<td>4.60 ± 0.15a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>n-6 PUFAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA 18:2</td>
<td>13.6 ± 2.4</td>
<td>10.8 ± 1.98</td>
<td>15.9 ± 1.54</td>
<td>14.3 ± 1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>AA 20:4</td>
<td>22.2 ± 2.3a</td>
<td>22.4 ± 3.5a</td>
<td>23.2 ± 0.88a</td>
<td>14.2 ± 1.6b</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values are % total fatty acid means ± SEM of n=8-9 rats/group. Different letters a, b, and c within the same rows indicate significant differences at P<0.05 by one way ANOVA followed by Tukey test. Abbreviations: alpha linolenic acid (ALA), eicosapentaenoic acid (EPA), decosahexaenoic acid (DHA), linoleic acid (LA), arachidonic acid (AA)
<table>
<thead>
<tr>
<th>Gene</th>
<th>Casein+CO</th>
<th>Casein+SO</th>
<th>SPI+SO</th>
<th>SPI+SB</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κβ</td>
<td>0.18 ± 0.04</td>
<td>0.20 ± 0.03</td>
<td>0.12 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.38</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.07 ± 0.006</td>
<td>0.07 ± 0.005</td>
<td>0.07 ± 0.006</td>
<td>0.07 ± 0.002</td>
<td>0.90</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.08 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.07 ± 0.006</td>
<td>0.08 ± 0.003</td>
<td>0.33</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.05 ± 0.003</td>
<td>0.07 ± 0.007</td>
<td>0.06 ± 0.003</td>
<td>0.05 ± 0.002</td>
<td>0.19</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.08 ± 0.006</td>
<td>0.09 ± 0.16</td>
<td>0.07 ± 0.005</td>
<td>0.09 ± 0.007</td>
<td>0.50</td>
</tr>
<tr>
<td>mTOR</td>
<td>0.14 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.03</td>
<td>0.12 ± 0.03</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Values are the means ± SEM of n=6 rats/group. Significant differences at P<0.05 by one way ANOVA. Abbreviations: nuclear factor-κβ (NF-κβ), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein 1 (MCP-1), and mammalian target of rapamycin (mTOR)
Table 10: Spleen immune gene expression levels in female PCK rats fed various protein and lipid sources

<table>
<thead>
<tr>
<th>Gene</th>
<th>Casein+CO</th>
<th>Casein+SO</th>
<th>SPI+SO</th>
<th>SPI+SB</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL13</td>
<td>0.31 ± 0.08</td>
<td>0.16 ± 0.04</td>
<td>0.16 ± 0.04</td>
<td>0.21 ± 0.03</td>
<td>0.15</td>
</tr>
<tr>
<td>CCL19</td>
<td>0.69 ± 0.09</td>
<td>0.75 ± 0.11</td>
<td>0.76 ± 0.07</td>
<td>0.85 ± 0.02</td>
<td>0.6</td>
</tr>
<tr>
<td>CCL21</td>
<td>0.08 ± 0.012</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.005</td>
<td>0.07 ± 0.005</td>
<td>0.64</td>
</tr>
<tr>
<td>CCL9</td>
<td>0.04 ± 0.003</td>
<td>0.05 ± 0.007</td>
<td>0.05 ± 0.006</td>
<td>0.05 ± 0.004</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Values are the means ± SEM of n=6 rats/group. Significant differences at P<0.05 by one way ANOVA. Abbreviations: C-X-C motif chemokine ligand 13 (CXCL13), C-C motif chemokine ligand 19 (CCL19), C-C motif chemokine ligand 21 (CCL21), and C-C motif chemokine ligand 9 (CCL9)
### Table 11-Gonadal adipose fat pad fatty acid composition in female PCK rats fed various protein and lipid sources

<table>
<thead>
<tr>
<th>Fatty Acids (relative %)</th>
<th>Casein+CO</th>
<th>Casein+SO</th>
<th>SPI+SO</th>
<th>SPI+SB</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated FAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic 16:0</td>
<td>24.16 ± 0.35b</td>
<td>24.36 ± 0.67b</td>
<td>24.32 ± 1.03b</td>
<td>28.27 ± 1.11a</td>
<td>0.004</td>
</tr>
<tr>
<td>Stearic 18:0</td>
<td>3.28 ± 0.17c</td>
<td>3.64 ± 0.14bc</td>
<td>3.87 ± 0.11b</td>
<td>4.69 ± 0.17a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>MUFA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic 16:1</td>
<td>6.20 ± 0.24b</td>
<td>6.29 ± 0.32b</td>
<td>5.17 ± 0.31b</td>
<td>7.27 ± 0.45a</td>
<td>0.001</td>
</tr>
<tr>
<td>Oleic 18:1n-9</td>
<td>35.50 ± 0.38a</td>
<td>33.78 ± 0.52a</td>
<td>30.43 ± 0.63b</td>
<td>32.49 ± 1.40ab</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>n-3 PUFAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALA 18:3</td>
<td>0.48 ± 0.04c</td>
<td>2.39 ± 0.08ab</td>
<td>2.73 ± 0.22a</td>
<td>2.00 ± 0.13b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHA 22:6</td>
<td>0.00 ± 0.00b</td>
<td>0.02 ± 0.01b</td>
<td>0.07 ± 0.02a</td>
<td>0.95 ± 0.20a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>n-6 PUFAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA 18:2</td>
<td>26.90 ± 0.52a</td>
<td>25.73 ± 0.83a</td>
<td>28.47 ± 1.25a</td>
<td>18.25 ± 0.95b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ARA 20:4</td>
<td>0.35 ± 0.03a</td>
<td>0.24 ± 0.02ab</td>
<td>0.37 ± 0.05a</td>
<td>0.21 ± 0.04b</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Values are % total fatty acid means ± SEM of n=8-9 rats/group. Different letters a, b, and c within the same rows indicate significant differences at P<0.05 by one way ANOVA followed by Tukey test. Abbreviations: alpha linolenic acid (ALA), decosahexaenoic acid (DHA), linoleic acid (LA), arachidonic acid (AA)
3.9 Figures

*Figure 5: Splenomegaly in female PCK rats*
4.0 Future studies

Future studies should investigate the role of adipose tissue in ARPKD based on our findings of significant differences in DNL FA content. Serum NEFA released from the adipose tissue lipolysis should be measured using an isotope tagging method to determine potential relationship to hepatic steatosis and progression to fibrosis. Adipose tissues n-6: n-3 PUFA was highest in Casein + CO fed rats. In addition to NEFA, adipose tissue also releases adipokines that can contribute to inflammation and progression of NAFLD to NASH. Insulin sensitivity should also be explored given the established connection between insulin resistance and hepatic steatosis. The effects of mTOR inhibition should be investigated in ARPKD and NAFLD models to gain better insight to the role of spleen in disease progression. Also, spleen studies on ARPKD models using varying dietary doses of different FAs and/or medical treatments may also assist in the develop therapies that preserve the spleen and avoid splenectomy since the spleen may play a protective role.
5.0 Bibliography


Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol. 1993 Jan 15;150(2):353-60.


National Research Council (US) Institute for Laboratory Animal Research. . 1996.


6.0 Resume

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1168 Agricultural Sciences Building

West Virginia
Morgantown WV, 26508

304.319.1599
PO Box 6108, Morgantown, WV

26506  lauren.gibson21@gmail.com

Summary of Qualifications

Self-motivated highly productive team member with a passion for collaboration and efficiency. Extensive science background in nutrition and nutrition in disease, testing dietary intervention in the rat model for human application. Lab techniques include gene expression through RT-qPCR and tissue fatty acid analysis. Applied project management skills in the lab in order to keep projects on time, within budget, and meeting objectives while managing a small lab group of diverse personalities and skill sets. Excellent communication and time management skills, and ability to work effectively with others or independently. Confident in effectively communicating concepts to team members, professors, and colleagues with varying backgrounds.

Education

West Virginia University, Morgantown, WV, M.S., Nutritional and Food Sciences
Expected May, 2016

West Virginia University, Morgantown, WV, B.S. Human Nutrition and Foods/Minor in Food Science and Technology. August, 2014

Relevant Technical & Business Skills

- RT-qPCR
- Developing and delivering proposals
- Strategic planning

- mRNA extraction
- Detailing materials and methods
- computer knowledge (Microsoft office)

- cDNA synthesis
- Emotional intelligence
- Communication

- Gas chromatography
- Mentoring students
- Building and sustaining relationships

- Fatty acid extraction
- Project and lab management
- Innovative thinking

- Fatty acid methylation
- Oral & written Communications
- Problem solving
<table>
<thead>
<tr>
<th>Educational &amp; Professional Development</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Master's thesis project, Mentor/ Advisor:</strong> Dr. Janet C. Tou, Janet. <a href="mailto:Tou@mail.wvu.edu">Tou@mail.wvu.edu</a> Davis College, West Virginia University, Morgantown, WV Feeding Soy Protein Isolate and/or Omega-3 Polyunsaturated Fatty Acids on the Spleen-Liver Axis in a Female Rat Model of Autosomal Recessive Polycystic Kidney Disease with Liver Steatosis. -Manuscript in progress; 2014-present.</td>
</tr>
<tr>
<td><strong>Poster Presentation @ Experimental Biology Conference: Networking and promotion; April 2016.</strong></td>
</tr>
<tr>
<td><strong>Graduate Research Assistant, Mentor:</strong> Dr. Janet C. Tou (see above). Various laboratory skills, proficient in statistical software and data analysis. Organization using Excel. Teaching assistant for fundamental nutrition (HNF 271) and advanced nutrition: metabolism (HNF 460). Good lab management; 2014-present.</td>
</tr>
<tr>
<td><strong>Dietetic Assistant, Mon General Hospital, Morgantown, WV-June 2011-July 2013.</strong> Worked with patients to order hospital meals that adhered to specific dietary restrictions. Delivered food to patients in a timely and organized manner paying special attention to food safety and sanitation.</td>
</tr>
<tr>
<td><strong>Volunteer Work:</strong></td>
</tr>
<tr>
<td>• 2013-present: Sunday school teacher for kindergarten through first grade. Learned patience.</td>
</tr>
<tr>
<td>• 2009-2014: member of the student dietetic association at West Virginia University, acting as the AND liaison for the final year. Developed public speaking and networking skills.</td>
</tr>
<tr>
<td>• 2009-2011: volunteer work with the Special Olympics in track and field and bowling.</td>
</tr>
<tr>
<td>• 2009-2011: volunteer for the stepping stones golf program at mountaineer golf course.</td>
</tr>
</tbody>
</table>
7.0 List of symbols, abbreviations, and nomenclature

AA; arachidonic acid

ADPKD; autosomal dominant polycystic kidney disease

ALA; alpha-linolenic acid

ARPKD; autosomal recessive polycystic kidney disease

CCL9; Chemokine (C-C motif) ligand 9

CCL19; Chemokine (C-C motif) ligand 19

CCL21; Chemokine (C-C motif) ligand 21

CHL; cholesterol

ChREBP; carbohydrate-responsive element-binding protein

CO; corn oil

CXCL13; CXC- chemokine ligand 13

DHA; docosahexaenoic acid

DNL; de novo lipogenesis

EPA; eicosapentaenoic acid

FA; fatty acid

FAS; fatty acid synthase
HDL; high-density lipoprotein

IL-6; interleukin 6

IL-10; interleukin 10

LA; linoleic acid

LDL; low-density lipoprotein

MCP-1; monocyte chemoattractant protein-1

mTOR; mammalian target of rapamycin

n-3 PUFA; omega-3 polyunsaturated fatty acid

NAFLD; non-alcoholic fatty liver disease

NASH; non-alcoholic steatohepatitis

NFκβ; necrosis factor kappa-beta

PLCD; polycystic liver disease

PKD; polycystic kidney disease

PPARα; peroxisome proliferator–activated receptor alpha

RT-qPCR; quantitative polymerase chain reaction

SB; soybean oil/salmon oil blend

SCD-1; stearoyl-CoA desaturase-1
SO; soybean oil

SPI; soy protein isolate

SREBP-1c; sterol regulatory element binding protein

TG; triglyceride

TNFα; tumor necrosis factor alpha

VLDL; very low-density lipoprote