LIPOCALIN 2 DEFICIENCY DIMINISHES THE METABOLIC EFFECT OF THE KETOGENIC DIET IN FEMALE MICE

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ABSTRACT

Obesity is a worldwide public health concern, and prevalence in the United States is continuously increasing. Treatment of obesity ranges from dietary approaches to gastric surgery, which can be life-threatening. Research has identified gene expression as a component worthy of consideration when deciding the appropriateness of a dietary approach. Expression of the Lipocalin-2 (Lcn2) gene has been suggested as a potential regulatory mechanism whose expression appears to be correlated to obesity. Identifying a diet that could help regulate the expression of this gene could be beneficial for management of weight and obesity-related diseases. In recent years the ketogenic diet has become popular for weight loss. By eliminating carbohydrates from the diet, it requires the body to use fat as its primary form of energy which is thought to be the cause of the wight loss observed as a result of this diet. Determining the affect the ketogenic diet has in the absence of the LCN2 gene could help identify potential metabolic pathways the ketogenic diet may affect, if any, that leads to weight loss. Both Lcn2 knockout (Lcn2 -/-) and wild type mice were examined after being fed either a normal chow or a ketogenic diet. Tissue weight and metabolic markers were then examined in the attempt to determining the affect, if any, that the ketogenic diet has in the absence of the LCN2 gene. Discovering weather or not there is a difference between these two groups could help identify potential metabolic pathways the ketogenic utilizes that leads to weight loss. In this study, wild type and Lcn2-/- female mice at 10 months of age were fed either a ketogenic diet or normal chow diet for 10 weeks, followed by the metabolic assessments including food intake, body weight, fat mass, and histology of tissues.

Our results showed that there was no significant difference in daily food intake between wild type and Lcn2-/- mice under either the normal chow or ketogenic feeding condition. However, we found that after 10 weeks on the ketogenic diet mice has significantly decreased inguinal and gonadal fat mass when compared with control mice fed a normal chow diet. Interestingly, the effect of ketogenic diet on body weight and fat mass was not observed in Lcn2-/- mice; the body weight and tissue weight of inguinal and gonadal fat depots were not significantly different between normal chow- and ketogenic diet-fed Lcn2-/- mice. From the histology of liver and adipose tissue, the ketogenic diet led to significantly increased small adipocytes in inguinal adipose tissue, suggesting a healthy remodeling of inguinal adipose tissue. Intriguingly, this adipose tissue remodeling effect of the ketogenic diet was completely absent in Lcn2-/- mice. Moreover, we found caused increased lipid accumulation in liver of mice fed the ketogenic diet compared to those fed a normal chow diet, which reflects increased utilization of fatty acids and production of ketogenesis. More strikingly, the ketogenic diet resulted in liver lipid accumulation more profoundly in Lcn2-/- mice, which may be caused by mitochondrial dysfunction and defective fatty acid oxidation in the absence of Lcn2.

Together, we demonstrate that Lcn2 deficiency diminishes the metabolic effect of ketogenic diet. This suggests that activating the function of Lcn2 gene in regulating mitochondrial metabolism, thermogenesis, and other unknown physiological activities may be an important mechanism by which the ketogenic stimulates the utilization of fat leading to weight loss.

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CHAPTER 1

LITERATURE REVIEW

Introduction

This review aims to assess the effects of the ketogenic diet on obesity and metabolic health. Upon the identification of the Lipocalin 2 (Lcn2) gene as a potential regulatory mechanism whose function appears to be correlated with obesity, lipid metabolism, and mitochondrial metabolism, examining the potential involvement of Lcn2 in the metabolic effect of ketogenic diet, is a topic of interested. Research suggests strong relationships between obesity, metabolic dysfunction, and inflammation in the body. The ketogenic diet could be a useful tool when implementing treatment plans for obesity and other metabolic disorders.

Energy Metabolism – An Overview

In the body there is a metabolic hierarchy that determines how the energy consumed from in the form of food is used. In respect to macronutrient, after consuming a meal the body prioritizes dietary carbohydrates and proteins first to create energy. Once these energy substrates have been sufficiently utilized, dietary fats, stored in the form of triglycerides, are then broken down to meet any remaining energy needs[1].

Dietary proteins are broken down into amino acids which are used to synthesize endogenous proteins. These endogenous proteins can then be used to maintain cellular and musculoskeletal structure, catalyze enzyme reactions, and transport molecules, amongst many other things[2]. Any excess amino acids are

then used as energy or excreted in the form of urea because there is no mechanism in place to store them within the body.

In the US and most other developed countries, carbohydrates make up a majority of the energy consumed from food. Carbohydrates are broken down into glucose which is then used to maintain blood glucose levels and replenish glycogen stores. Once these roles have been completed the remaining glucose is used to promote glycolysis and to be completely oxidized via the TCA cycle for ATP production. Once the body's basic energy needs are met, any excess glucose is converted into fatty acids via lipogenesis and stored in the form of triglycerides as fat, primarily in adipose tissue. Unlike the other energy sources, fat is not an essential fuel for any particular tissue in the fed state and can be stored in seemingly endless amounts. If the body's energy needs have been met from breaking down dietary carbohydrates and proteins, all of the fat consumed will be stored. During the fasting, stored fat can be broken down to release fatty acids which can used as energy fuels for many tissues such as muscle, heart, and kidneys.

Utilization of Glucose and Fatty Acids via Glycolysis, β-Oxidation, and Ketogenesis

As a primary energy source for all tissues and cells, glucose is completely or partially oxidized in the process of ATP production. Glycolysis is the metabolic pathway by which glucose and other sugars are broken down into pyruvate which can then be fed into the TCA cycle to produce ATP. Fatty acids and ketone bodies

become important energy sources for the body when carbohydrates are unavailable, for example in the fasted state. β-oxidation, part of the ketogenic pathway, is the metabolic process by which fatty acids are broken down to produce ketone bodies (ketones). Under normal conditions, the body is constantly producing small amounts of ketones, mainly regulated by insulin[3]. For instance, a low insulin state such as fasting triggers the ketogenic process. When dietary carbohydrate levels decrease and glucose stores are depleted, ketogenic pathways are upregulated to compensate for the reduced availability of glucose. During β-oxidation, fatty acids derived from either dietary fats or stored triglycerides are broken down and turned into acetyl-CoA. As concentrations of Acetyl-CoA build up in the liver, they are used to produce ketones (acetoacetate and β-hydroxybutyrate). These ketones are then transported from the liver to other tissue where they are reconverted to Acetyl-CoA and fed into the TCA cycle in order to synthesize ATP[2]. Even under ketogenic conditions the body continues to produce low levels of glucose via gluconeogenesis in order to maintain blood glucose levels and provide energy to brain and other tissues that only use glucose as energy, such as red blood cells[3].

Ketogenesis mainly occurs during a prolonged fasted state when the body transits from using glucose via glycolysis to using fatty acids via β-oxidation. This transition of fuel utilization can also be achieved by adhering to a low carbohydrate- high fat “ketogenic” diet, which is referred to as “nutritional ketosis”. The theory is that when both dietary and endogenous carbohydrates

from stored glycogen and gluconeogenesis are no longer available, the body will switch to using stored fat and to making ketone bodies to generate energy. Dietary fats are used first and if energy needs are not met stored fat will be used as well. This is an idyllic situation for weight loss as the body is unable to store excess carbohydrates as fat and is also burning the stored fat that has accumulated over time to produce energy. While benefits of inducing ketogenesis have been found, it can also be detrimental. During starvation, or in some cases of uncontrolled diabetes, the body begins to break down fats and producing ketones at a rate faster than energy can be produced. This can lead to an excess buildup of ketones in the body which can shift blood pH to acidotic levels[4]. This is known as ketoacidosis and can be deadly if left untreated.

Ketogenic Diet and Its Health Effects

The Ketogenic Diet or ketogenic diet is a high-fat, low-carb diet that mimics a fasted state by prompting ketogenesis through decreasing the availability of carbohydrates in the body while still providing adequate calories. The decrease in dietary carbohydrate favors a metabolic transition from using glucose to using fatty acids (FA's) as a source of energy5. This leads to a decrease in glycolysis and an increase in β-oxidation. When β-oxidation is increased, the Kreb Cycle is decreased, leading to excess Acetyl-CoA accumulation. During Ketogenesis, this excess Acetyl-CoA is used to produce Ketone bodies that convert NAD to NADH, which is then used in the electron transport chain to produce ATP.

There are 4 different variations of the ketogenic diet that are accepted in both traditional and alternative medical practice. These variations include the Traditional Keto Diet, the Medium Chain Triglyceride Diet, the Modified Atkins Diet, and the Low-Glycemic Index Diet [9]. The Traditional Ketogenic Diet is defined by a fixed ratio by weight of fats (90%), proteins (6%), and carbs (4%). This version of the diet is very restrictive and hard to maintain outside of a controlled hospital setting where dietitians, nurses, and doctors closely monitor proportions as well as caloric intake[3]. The Medium Chain Triglyceride (MCT) Diet calls for a diet consisting of 10% dietary fat, 60% MTC fat, 20% protein, and 10% carb. MCT oil is used as a source of fat decreasing the need for increased dietary fat consumption. This shift away from dietary fats allows for more carbs and proteins to be introduced into the diet[3]. This provides more variety in overall food choices. The Modified Atkins Diet proposes a dietary ratio of 65% fat, 25% protein, 10% carb. 10- 20g of carbs can be consumed per day and fat intake is highly encouraged[3]. This option is free of calorie restriction and meticulous weighing of food. The Low glycemic Index Diet is a low carb diet that adheres to a ratio of 60% fat, 30% protein, and 10% carb. While this diet allows for 40-60g of carbs to be consumed per day the carbs have to have a low glycemic index (Glycemic Index value <50).

The ketogenic diet emerged in modern science in 1920 as a way to control epileptic seizures in children6. Epileptics often fasted as a way to prevent seizures and the ketogenic diet was introduced as a less restrictive alternative. Two doctors

at the Mayo Clinic established that a low-carb, high-fat diet would mimic a fasted state in the body while easing the concern of developing nutritional deficiencies in patients[3]. After the 1970's the use of the ketogenic diet to treat epilepsy drastically declined due to modern medicine and developments in the treatment of the disease[6]. Recently this diet has be adopted widely by the public as a break though weight loss tool with potential benefits of improved glycemic control and reduced insulin resistance[7]. Other studies have found potential application to improve cognitive development and even reverse neurodegeneration as a consequence of diseases[8].

Recent research has examined the ketogenic diet for its potential aid in the treatment of some diseases, as well as its potential physical and cognitive benefits. The ability for the ketogenic diet to aid in the reduction of epileptic symptoms drew researchers to look into the diets influence on other neurodegenerative diseases such as Alzheimer's and Parkinson's[8]. These two diseases are both neurodegenerative and have no known cure. It is known that oxidative stress and mitochondrial dysfunction are central features of brain degenerative disease. The ketone bodies produced during ketogenesis are known to increase mitochondrial respiration because they increase ATP production and decrease the production of free radicals[9]. It is hypothesized that implementation of the ketogenic diet could help restore some mitochondrial function in patience with these diseases. The ketogenic diet is also thought to increase antioxidant and glutathione peroxidase activity which in turn might help protect the hippocampus from neurodegenerative

changes, slowing the progression of some of these diseases. The ketogenic diet is restrictive in nature in that it limits what types of foods can be consumed, which usually leads to a decrease in caloric intake. Calorie restrictive diets are known to have anti-inflammatory effects on the body which can lead to an increase in neuroprotective factors that could be beneficial to people suffering from neurodegenerative diseases[9]. Although no conclusive evidence has been found, studies using human subjects have observed a reduction of symptoms in patients with both Alzheimer's and Parkinson's diseases while on the ketogenic diet.

The ketogenic diet has also been linked to its effect on patients struggling with Type II Diabetes. Type II Diabetes arises from a decrease in the body's sensitivity to insulin which regulates glucose uptake into the cells/tissues from the circulation[4]. When the body can't take up glucose that is absorbed from the diet into the cells and tissues, circulating glucose rises leading to high blood glucose levels and diabetes. The ketogenic diet aids in the control of blood glucose levels by regulating glucose intake, via decreasing carbohydrate consumption. As the body transitions from using glucose to using fatty acids as a source of energy, the glucose that the body does produce can be dedicated to maintaining blood glucose levels because it is not needed to produce energy. Studies have found that people with diabetes who abide by the ketogenic diet significantly reduce their use of insulin[10]. The restrictive nature of this diet is also beneficial to diabetic patients because it promotes weight loss. Losing weight is one of the main goals in the

treatment of Type II Diabetes because it has been known to cure the disease all together.

Apart from the beneficial effects on neurodegenerative diseases and type II diabetes, it has been shown that the ketogenic diet can improves survival, memory, and health span in aging mice[11]. as well as physical activity. In aged mice, only those consuming a ketogenic diet displayed preservation of physiological function and an elevation of motor functions. One study found that the ketogenic diet showed improved memory/cognitive function during maze testing[12] . Another study looking into the effects of the ketogenic diet on children with epilepsy has observed an increase in alertness and better cognitive function when on the ketogenic diet[13]. Physical activity benefits have also been associated with the ketogenic diet. One study looking at the effects of the ketogenic diet on endurance found a significant increase in the endurance of mice fed the ketogenic diet compared to the controls[12]. This study hypothesized that the improvement seen in physical performance could be due to the thermodynamic advantages of ketone oxidation. They also suggested that the ketogenic diet could be supporting increased muscle work during physiologic stress by sparing alternative energy sources, particularly glycogen, as muscle glycogen content correlates with endurance exercise in humans. Another study looking into the metabolic effect of ketogenic diet found that smaller lipid droplets contained in the brown adipose tissue (BAT) of mice on a ketogenic diet[14]. Observable decrease in the size of lipid droplets in BAT suggest an increase in BAT mitochondrial activity. Increase BAT metabolism has been

proposed as an approach to combat obesity via its ability to increase basal energy expenditure which can lead to weight loss.

Potential drawbacks associated with this diet are diabetic ketoacidosis and malnutrition. Ketoacidosis occurs when the body produces too many ketones as a result of trying to keep up with the body's energy need. When the body produces ketones faster than it can use them the blood can become acidic, which can be life-threatening. This rapid breakdown of fatty acids is most commonly seen in individuals who enter into ketogenesis unintentionally, usually induced when the body is in a state of starvation or cannon supply the body with adequate amounts of glucose. As mentioned before when the body has adequate amount of dietary fatty acids to use to produce energy it does not need to enter into a fasted state, using stored fat as energy. Therefore when people intentionally induce ketogenesis while on the ketogenic diet ketoacidosis rarely occurs. However, because it can happen ketoacidosis is something that researchers need to keep an eye out for when administering the ketogenic. Due to the restrictive nature of the Keto diet, malnutrition, especially in the elderly population is a concern. Not only can this diet lead to a decrease in caloric intake but it has also been linked with appetite suppression[9]. Elderly patients with neurodegenerative diseases are known to forget meals and also have a hard time finishing them. These two factors in conjunction with each other can make it hard to ensure that elderly patients, especially those with neurodegenerative disease, are getting all the nutrients they need from their diet. The ketogenic diet has been around for many years to aid in

the treatment of epilepsy. In recent years there has been a reemergence of interest in the ketogenic diet because of its potential physical and cognitive benefits, as well as its potential implementation to help treat a number of diseases. While a lot of research has been done in looking at the association between the ketogenic diet and its influence in these areas, more research needs to be done in order to identify the mechanism by which this diet is imposing these effects

While there is a clear association between the ketogenic diet and weight loss the mechanism by which this is achieved is highly contested[15]. While the mechanisms are unclear, there are a few theories. One hypothesis is that while on this diet more protein is being used to create energy then on a typical high carbohydrate diet. Producing energy from proteins is more energetically expensive than using glucose. This added expense requires more calories and thus leads to more weight loss when compared to other diets[11]. Another theory also attributing effects to proteins states that this diet may rely on the satiety effects of proteins or appetite suppressing hormones[16]. Other studies propose a decrease in lipogenesis and increase in lipolysis or an increase in metabolic efficiency via reduction in resting respiratory [17,18]. As research continues to investigate this diet, we should be able to create a clearer picture as how exactly weight loss is achieved.

Lipocalin2

Lipocalin 2 (LCN2) has been identified as an adipose-derived cytokine from previous studies on adipocyte secretion[19]. This protein functions as a transporter, sideophore, and acts as an activator/repressor of iron-responsive genes. High levels of LCN2 are found in tissues that are often exposed to microorganisms such as the uterus, prostate, salivary glands and stomach. LCN2 is an acute phase protein that is substantially activated by lipopolysaccharides through activating the inducible transcription factor NF-κB[19] LCN2 expression is increased during inflammatory activity and is thought to be a component of the innate immune response due to increased sensitivity to infection in LCN2- deficient mice models[19]. An increase in expression of LCN2 has been seen not only during inflammatory activity but has also been exhibited in rodents as well as human subjects with obesity. In these subjects, this increased expression seems to be correlated to insulin resistance and remodeling of adipose tissue. The presence of LCN2 seems to be an indication for a number of different obesity-related metabolic pathways and appears to be linked to inflammation.

LCN2 is a modulator of hepatic lipid homeostasis and has a significant effect on the formation of lipid droplets. Lipid droplets are the subcellular organelles that make up the fat cells in adipose tissue. Excess energy is stored as triglycerides in lipid droplets which are essential in maintaining the balance between lipogenesis and lipolysis, the metabolic pathways of storing fatty acids and breaking down triglycerides respectively[20]. Lipid droplet size is directly influenced by triglyceride levels and the amount of stored lipids[20]. Balancing lipid size and storage capacity

is important in maintaining normal biological functions and dysregulation of this balanced system can result in the development of metabolic disease like obesity and diabetes[20]. However, it is unknown how LCN2 regulates the formation and size of lipid droplets and levels of lipids in adipocytes.

Lipocalin 2 in Ketogenic Diet Effect

A study looking at the general physiology of BAT found that mice fed a Ketogenic diet had smaller lipid droplets contained in their BAT compared to mice fed a standard chow diet[14]. An observable decrease in the size of BAT lipid droplets suggest an increase in BAT mitochondrial activity. Increasing BAT metabolism has been proposed as an approach to combat obesity via its ability to increase basal energy expenditure which can lead to weight loss. Since both the ketogenic diet and LCN2 expression have been linked to lipid droplet metabolism and noted for its weight loss and anti-inflammatory effects, it seems as if LCN2 could be a contributing factor to the metabolic process that leads to weight loss via the ketogenic diet.

LCN2 has been considered a potential biomarker for pathological conditions including rheumatic diseases, cancer in human organs, hepatic steatosis, hepatic damage, and inflammation.[15] Overnutrition activates metabolic inflammation mediator IKKβ/NF-κB.[15] Obesity is known to lead to a state of chronic low-grade inflammation which is thought to contribute to metabolic disorders. KKβ/NF-kB activity in the hypothalamus appears to be associated with the energy imbalance

and underly obesity. Protein kinases JNK1 and IKKβ have been found to serve as critical molecular links between obesity, metabolic inflammation, and disorders of glucose homeostasis[18]. The presence of LCN2 in inflammatory cells involved in the NF-kB pathway strongly suggests involvement in the stimulation of this pathway. This further alludes to a relationship between LCN2 and obesity.

The presence of LCN2 during metabolic inflammation is likely an anti-inflammatory response as opposed to being a part of the inflammation cascade itself. However, when it comes to lipid metabolism, there may be a more causal relationship. A study performed on mice found that when Lcn2 knockout mice that did not express the LCN2 gene were fed a high fat diet; exacerbated dyslipidemia, diet-induced obesity, fatty liver disease, and insulin resistance were observed[21] One study suggests that the Ketogenic diet disrupts NF-kB signaling leading to the suppression of inflammatory gene expression pathways[21]. This paper admits that there is some contradictory evidence in the literature due to the scarcity of research on the topic. While the observation suggests that the ketogenic diet may affect inflammation through LCN2 expression, more research should be done in this area to insure more concrete conclusions. In a corresponding study, when abnormally elevated LCN2 expression was decreased to within normal range in obese rodent models, improved insulin sensitivity was observed[10]. Expression of LCN2 has also been correlated with issues concerning the liver such as hepatic inflammation, mitochondrial malfunction, and oxidative stress[22]. It is hypothesized that

implementation of the Ketogenic diet could help restore some mitochondrial function in patients with excess inflammation because the ketone bodies produced during ketogenesis are known to increase mitochondrial respiration production of free radicals[23].

LCN2 has also been indicated as a modulator of hepatic lipid homeostasis which controls the formation of intercellular lipid droplets via regulation of PLIN5 expression[24]. This study showed that LCN2 deficiency results in abnormal lipid droplet accumulation which indicates dysfunction in the pathways that regulate lipid metabolism. As previously mentioned, proper regulation of lipolytic pathways is essential to proper energy production while in a state of metabolic ketosis. Thus, playing a critical role in reducing lipid droplet size, in the event on weight loss, and preventing abnormal lipid accumulation in places like the liver while on a ketogenic diet. Most importantly, our previous studies have shown that LCN2 plays an important role in the regulation of mitochondrial function and BAT thermogenesis. LCN2 deficiency causes mitochondrial dysfunction and impaired thermogenesis. All the information mentioned above suggests a potential role of LCN2 in regulating the metabolic effect of ketogenic diet in lipid and mitochondrial metabolism in adipose tissue.

Conclusion

LCN2 is a protective anti-inflammatory protein that is activated in response to inflammation and is an important part of the innate immune response. It has

also, more recently, been noted for its role in lipid droplet homeostasis and mitochondrial metabolism. When LCN2 is not expressed, metabolic inflammation control is lost leading to intensified disease outcomes. When insulin sensitivity is improved, LCN2 levels in obese, insulin resistant mice are decreased. Suggests a direct link between LCN2 and glucose homeostasis. LCN2 appears to be a good marker for the indication of metabolic function and inflammatory state. The Ketogenic diet-induced disruption of NF-κB signaling insinuates its ability to influence LCN2 expression as NF-κB regulates LCN2 transcription. Therefore, it is reasonable to hypothesize that Lcn2 may be involved in the ketogenic diet effect on insulin sensitivity, obesity, and weight loss. Currently there is no published data that examines a relationship between these two mechanisms but exploring this potential association further could help aid in the development of promising interventions for some metabolic disease.

CHAPTER 2

LIPCALIN 2 DEFICIENCY DEMINISHES THE METOBOLC EFFECT OF THE KETOGENIC DIET IN FEMALE MICE

Introduction

Obesity is a worldwide public health concern, and prevalence in the United States in continuously increasing. Currently, more than 2/3 of adults in the US and 1/3 of children are adolescence are overweight or obese[25]. In an attempt to address this problem, physicians, nutritionists, public health officials, and many others have tried, over the years, to come up with ways promote weight loss. While some methods are better than others, dietary intervention is the approach that people tend to latch onto the most. Most “diets” are meant to be short term solutions intended to kickstart a person weight loss journey and facilitate in easing them into sustainable lifestyle changes. One type of diet that has gotten the attention of researcher, clinicians, and the general public is a low-carbohydrate high-fat ketogenic diet. While dietary fat has been stigmatized as part of the problem for many years there have been lot of studies published that challenge this assumption.

The ketogenic diet is low-carbohydrate high-fat diet that dates as far back as 500 bc as a ancient method to treat epilepsy. Fast forward to the 1920 and the diet reemerged as an epilepsy treatment[3]. In the last decade interest in this diet has increased yet again but this time as a weight loss treatment. Recent research has appreciated a relationship between the ketogenic diet and weight loss in both human and mouse models. Although the mechanism by which this diet leads to weight loss to relatively unknown there are several theories that explaining the metabolic efficiency of the ketogenic diet. First, the ketogenic diet is known for to

suppress appetite via effecting the release of appetite control hormones and possibly direct via the ketones production. Second, is the theory that this diet promotes a reduction in lipogenesis and increase in lipolysis. Third, it leads to greater metabolic efficiency in utilizing fatty acids for energy. The forth theory suggests that weight loss is secondary to increased energetic toll of gluconeogenesis and thermogenesis[26]. It is known is that the ketogenic diet promotes the mobilization and utilization of fatty acids from adipose tissue to provide metabolic energy to the body as well as precursors such as acetyl-CoA for ketogenesis via β-oxidation. Fatty acids are metabolized to acetoacetate which is then converted to beta-hydroxybutyrate and acetone, which are ketone bodies, in the liver[7]. These ketone bodies are transported from the liver into circulation where they can be taken up into by peripheral tissues, converted into acetyl CoA and fed into the TCA cycle to produce ATP[27]. The metabolic state that results from the production of these ketone bodies as a ketogenic diet is sustained is called "nutritional ketosis". However, ketoacidosis occurs the production of ketones bodies at a rate that is greater than the body can use for energy production, for example under poorly controlled diabetic conditions. This leads to excessive concentrations of ketone bodies which alter the blood pH to an acidotic level. In contrast nutritional ketosis produces small concentrations of ketone bodies as a steady rate, without altering blood pH.

Previous studies have shown that high fat diets, such as the Ketogenic Diet can induce weight loss and increase mitochondrial capacity by using lipids as

metabolic fuel [26,28]. Other have indicated that that ketogenic diets can lead to greater weight loss when compared to high fat diets[29] . The main mechanism for the weight loss caused by this diet is thought to promote the body to use fat as its primary form of energy by eliminating carbohydrates from the diet. Researchers attempts to understand the molecular mechanism for the metabolic effect of the ketogenic diet is crucial for determining the appropriateness of this dietary approach. At the cellular and molecular level, the ketogenic diet has been known to activate mitochondrial function and thermogenesis in thermogenic adipose tissue to stimulate fat utilization[30]. However, specific molecules and genes that are involved in the metabolic regulation of the ketogenic diets effect remain largely unknown.

Lipocalins are a part of a broad family of proteins that are involved in different metabolic processes. The lipocalin subfamily are transporter proteins that transport small lipophilic molecules in circulation[31]. The lipocalin 2 or Lcn2 protein that belongs to the lipocalin subfamily It is best known for its association with inflammation and immune defense. In recent years, Lnc2 has been has been found to contribute to the regulation of lipid metabolism[32]. In mice Lcn2 has been found to suppress food intake and act as a satiety signal[33]. Previous research has also demonstrated that Lcn2 directly influences the expression of lipid droplet proteins which is important in fatty acid oxidation. Lipid droplets provide fatty acids to mitochondria for β-oxidation[34]. Disruption of Lcn2 expression in mice has been seen to significantly potentiated diet induced obesity as well as fatty liver disease[32].

Moreover, Lcn2 has been found to have a thermogenic effect in brown adipose tissue and browning of white adipose tissue (WAT) though regulating mitochondrial function in mice; Lcn2 deficiency leads to decreases in thermogenesis and an increase in weight gain and fat mass in Lcn2 knock out mice[35]. Since the ketogenic diet has been shown to promote weight loss by stimulating thermogenesis and mitochondrial oxidation of fatty acids, it would be of interest to determine if Lcn2 plays a role in the mechanisms for ketogenic diet-induced fatty acid metabolism and ketogenesis by establishing if Lcn2 deficiency has an impact on the metabolic effects of the ketogenic diet.

Results

Lcn2 deficiency does not impact ketogenic diet-induced weight loss

We determined if Lcn2 deficiency (Lcn2 -/-) could impact the beneficial effect of a ketogenic diet on weight loss. To that end, the body weight of all mice involved in the study was recorded on a weekly basis for 10 weeks. We then calculated the overall change in weight from week 1 to week 10 and used the average of this change in each group to determine statistical significance. While the mice fed the ketogenic diet lost weight on average and the mice fed the normal chow diet gained weight, the difference in body weight between these two groups was not statistically significant (Fig 1). When comparing wild-type to Lcn2 knockout (Lcn2-/-) mice fed the normal chow diet, the Lcn2 knockout mice gained more weight on average but there was no significant difference between the two groups

(Fig 1). In addition, when comparing wild type to Lcn2-/- mice fed the ketogenic diet, the Lcn2 knockout mice lost more weight on average. But again, there was no significant difference found between these two groups (Fig 1).

Lcn2 deficiency does not impact adipose tissue or organ weight of mice fed either a normal chow diet or a ketogenic diet

To test the impact of Lcn2 deficiency on ketogenic diet-induced changes in tissue and organ weight, we measured the tissue and organ weight of the mice fed either the normal chow or ketogenic diet. Mice were sacrificed after 10 weeks of the normal chow or ketogenic diet and their brown adipose tissue (BAT), inguinal white adipose tissue (ING-WAT), gonadal white adipose tissue ((GON-WAT), and perirenal white adipose tissue (PWAT) was weighted. Regardless of the diet, the difference in BAT, ING-WAT, and GON-WAT weight between wild type and Lcn2-/- mice was not statistically significant. We also recorded kidney, liver, spleen, heart, and muscle weight. While there appeared to be a slight increase in the liver weight of Lcn2-/- mice, there was no significant difference found in the weight of any other types of tissues and organs between wild type and Lcn2-/- mice fed either the normal chow or ketogenic diet (Fig 3).

Lcn2 deficiency attenuates ketogenic diet-promoted reduction in inguinal adipose tissue

As shown above, Lcn2 deficiency did not seem to impact the weight of adipose tissues and organs in mice fed the normal chow diet. The average weight of ING-WAT, GON-WAT, and PWAT was 3.3g, 3.9g, and 2.3g respectively for wild type mice and 1.9g, 2.3g, and 1.9g respectively for Lcn2-/- mice. While the wild type mice had increased weight of ING-WAT, GON-WAT and PWAT on average compared to the Lcn2-/- mice, no significant genotypic difference was found (Fig 2). As for BAT weight, the difference between wild type and Lcn2 -/- mice was less than 0.03g. Moreover, the difference in organ weight between wild type and Lcn2-/- mice was also not statistically significant (Fig 3).

Interestingly, we found there was a statistically significant effect of the ketogenic diet on inguinal white adipose tissue. When mice were grouped together by diet and the difference in inguinal adipose tissue weight was analyzed, the ING-WAT weight of mice fed the ketogenic diet was significantly reduced by 0.9g compared to that of mice fed the normal chow diet. While it appears that the average weight of other tissues in the ketogenic diet-fed mice were also reduced compared to the normal chow diet-fed mice, the difference was not statistically significant (Fig4). Moreover, the weight of ING-WAT and GON-WAT had a trend to decrease after ketogenic diet feeding, which was only observed in wild type mice but not Lcn2-/- mice (Fig 2 and Fig 3).

Lcn2 deficiency does not impact blood glucose lowering effect of the ketogenic diet

To determine if the ketogenic diet affects blood glucose, we measured blood glucose levels of the mice after 10 weeks of their respective diets. Under the normal chow diet-fed condition, Lcn2-/- mice had a slightly higher blood glucose level compared to the wild type mice. After 10 weeks of ketogenic diet feeding, both wild type and Lcn2-/- mice fed the ketogenic diet had reduced blood glucose levels compared to their control mice fed the normal chow diet. There was no significant difference between two genotypes or diets (Fig 5). However, when all of the ketogenic diet-fed mice, regardless of the phenotype, were grouped and compared to all the normal-chow-fed mice, the ketogenic diet-fed mice had significantly lower blood glucose levels compared to normal chow diet mice (Fig 6). These results indicate that there is a dietary effect but no genotypic effect on blood glucose levels, and a ketogenic diet is able to effectively promote the reduction in blood glucose levels in this study.

Lcn2 deficiency reduces ketone body levels but not ketogenic diet-promoted ketogenesis

To determine if Lcn2 deficiency affects the ketogenic capabilities, we measured blood ketone body levels in wild type and Lcn2-/- mice fed either the normal chow diet or the ketogenic diet. We showed that serum levels of β-hydroxybutyrate were significantly increased in both wild type and Lcn2-/- mice after 10 weeks of ketogenic diet feeding (Fig 7). However, the levels of serum β-hydroxybutyrate were similar between wild type and Lcn2-/- mice fed the ketogenic diet (Fig 8). This result indicates that both wild type and Lcn2-/- mice have similar

capability to achieve ketogenesis. Interestingly, Lcn2-/- mice fed the normal chow diet had significantly decreased β-hydroxybutyrate levels compared to wild type mice (Fig 8). The reason as to why Lcn2-/- mice have lower ketone body levels warrants further investigation.

Lcn2 deficiency diminishes ketogenic diet-promoted reduction in adipose cell size in inguinal and gonadal white adipose tissue

The above results have demonstrated that ketogenic diet feeding caused a reduction in inguinal adipose tissue weight in wild type mice but not Lcn2-/- mice. To determine the morphological changes, occur in adipose tissue, we performed the histology, i.e. hematoxylin & eosin (H&E) staining of adipose tissue. As shown in Fig 8, inguinal adipose tissue from wild type mice fed the ketogenic diet had an increased population of smaller fat cells compared to that from wild type mice fed a normal chow diet. The similar effect of ketogenic diet feeding was also observed in gonadal adipose tissue in wild type mice (Fig 9). Interestingly, the ketogenic diet did not seem to have an effect on fat cell size in both inguinal and gonadal adipose tissue in Lcn2-/- mice. This indicates that Lcn2 deficiency diminishes the reducing effect of the ketogenic diet on fat cell size.

Lcn2 deficiency diminishes ketogenic diet-promoted shift of fat cell size distribution in inguinal and gonadal adipose tissue

To quantitatively assess the fat cell size distribution, a particle size analyzer was used to determine the size distribution of adipose cells in inguinal and gonadal adipose tissue of wild type and Lcn2-/- mice. Adipose tissue was collected and

fixed in osmium tetroxide, and adipose cell size was determined using the Bluewave particle analyzing system.

The size distribution data showed that inguinal adipose tissue from ketogenic diet-fed wild type mice had increased small fat cells with the size between 25-75 µl and decreased large fat cells with the size between 100-125 µl compared to normal chow diet-fed wild type mice (Fig 11a). However, ketogenic diet feeding did not cause a shift of fat cell size distribution in Lcn2-/- mice (Fig 11b). Instead, ketogenic diet feeding resulted in a decrease in small fat cells with the size between 50-75 µl (Fig 11b).

In gonadal adipose tissue, *the analysis of fat* cell size distribution similarly showed that ketogenic diet feeding increased the population of small fat cells with the size between 50-75 µl but decreased the population of large fat cells with the size between 100-150 µl in wild type mice (Fig 12a). Similar to what we have observed in inguinal adipose tissue, ketogenic diet feeding did not shift the fat cell size distribution but caused a significant decrease in small fat cells with the size between 50-100 µl in Lcn2-/- mice (Fig 12b).

Lcn2 deficiency exacerbates ketogenic diet-induced lipid accumulation in the liver

While short-term ketogenic diet feeding has a beneficial effect on liver steatosis and reduces lipid content in the liver[36]. Long-term ketogenic diet feeding has been known to increase lipid accumulation in the liver[37]. To determine how Lcn2 deficiency affects the effect of the ketogenic diet on lipid metabolism in the liver,

we assessed the histology of the liver and compared the lipid accumulation in the liver of wild type and Lcn2-/- mice. In line with the literature report, we found that the long-term (10-week) ketogenic diet feeding caused an evident increase in lipid accumulation in the liver in wild type mice compared to normal chow-fed control mice (Fig 13). Interestingly, this ketogenic diet-induced liver lipid accumulation was significantly exacerbated in Lcn2-/- mice (Fig 13). These results suggest that Lcn2 deficiency potentiates the detrimental effect of long-term ketogenic diet feeding on lipid metabolism in the liver.

Discussion

Low-fat diets have been used for years to treat obesity, perpetuating the notion that consuming a high fat diet leads to weight gain and obesity. However, in recent years the use of low- carbohydrate, high-fat diets have exploded and become seen as somewhen of a “weight loss miracle”. While there are studies that support these kinds of diets as being good for weight loss, there are also studies that suggest that in the long term these types of diets my not be sustainable. Studies in both human and rodent subjects have shown low-carbohydrate, high-fat ketogenic diets to be effective in short term weight loss or prevention of weight gain[38,39,29,37]. However, a study looking at the long term effects of ketogenic diet found that while mice lost weight during the first few weeks of the study, this weight loss effect is no longer seen over an extended period of time[7].

Apart From previous studies that only look at the effects of low–carbohydrate high–fat ketogenic diet on weight loss, this study evaluated the potential role of Lcn2 as a contributing factor to the metabolic and weight loss effect of ketogenic diet. Although WT mice lost more weight when fed a ketogenic diet compared to a normal diet, the ketogenic diet effect on body weight was not significant difference between wild type and

Lcn2-/- mice. These two groups of mice exhibited similar trends in weight loss, and there was no significant difference in the average weight between wild type and Lcn2-/- mice fed a ketogenic diet. This suggests that Lcn2 does not play a significant mechanistic role in the weight loss effect of ketogenic diet. A study looking into the role of osteoblast-derive Lcn2 in satiety and appetite found that Lcn2 inhibits food intake and activates an MC4R-dependent anorexigenic pathway in mice[40]. In our study, an increase in food intake can be observed when comparing ketogenic to normal chow diet in both wild type and Lcn2-/- mice, but there was no significant difference between wild type and Lcn2-/- mice.

We also observed a decrease in inguinal, gonadal, and parametrial white adipose tissue (PWAT) in wild type mice fed a ketogenic diet compared to the control mice fed a normal chow diet. Notably, differences in inguinal adipose tissue weight were the only significant comparisons found. This is in opposition of previous finding which showed an increase in fat mass without change in body weight[37]. However, when looking more closely into the composition of the ketogenic diet, the diet they used has a much higher fat content than those used

in our study, as well as in other literature. In terms of overall weight loss, our findings seem to be in line with recent literature when looked at fat mass changes in obesity on the basis of sexual dimorphism. Most of the available literature assessing the ketogenic diet and its effects on weight loss are studying male mice. One study looking into lipid metabolism and metabolic dysfunction found that when obesity was induced, males mice gained less total fat but higher visceral on subcutaneous fat ratio compared to female mice[41]. This suggests that the lipid metabolism in male mice may be different in some way than in female mice. Similar finding have also been appreciated in human studies evaluating the differences in weight loss between males and females[42,43]. The small effect of ketogenic diet on weight loss and fat mass in our study could be attributive to the sexual dimorphism.

β-hydroxybutyrate concentrations are commonly used to estimate ketone body levels and to verify successful transition into ketosis[44]. As expected, we found that ketogenic diet-fed mice had significantly higher β-hydroxybutyrate concentrations compared to the control mice fed a normal chow diet. Thus, confirming that the mice were in a state of ketosis and assuring that the diet could be assessed as such. One thing that it is important to understand is that although β-oxidation is not the primary energy producing pathway under normal conditions, the body still produces small amounts of ketones[1]. Interestingly in our study β-hydroxybutyrate concentrations were almost identical between wild type and Lcn2-/- mice fed the ketogenic diet but significantly lower in Lcn2-/- mice fed a normal chow diet compared to wild type mice. These findings were previously described

in our lab with comparisons between standard chow and high-fat dies in Lcn2-/- mice[45]. Similarly significant differences in ketone regulation have been described in the literature. As previously mentioned, Lcn2 is known to regulate fatty acid oxidation and is a critical regulator of thermogenesis [19,45]. This infers that fatty acids may not be accessible for β-oxidation in the absence of Lcn2. Given that ketones are not needed for energy under normal conditions, we speculate that our findings could be a result of the bodies reliance on β-oxidation to produce energy under ketogenic conditions. It would thus make sense that under normal conditions the synthesis of ketones would decrease if a facilitator of the β-oxidation pathway was eliminated making the process energetically unfavorable. Further investigation into the mechanisms of this process may provide more insight into these findings.

The ketogenic diet has been shown to regulate glucose levels and help improve glycemic control[7,29,37]. Poor glycemic control is associated with insulin resistance which is the most prevalent metabolic abnormality associated with diabetes. Insulin resistance is predominantly associated with body fat and the progression of this condition can lead to metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes[46]. The primary characteristics of insulin resistance are decreased glucose uptake and decreased ability to lower glucose output[26]. This typically results in widely fluctuating blood glucose levels which is indicative of poor glycemic control. Decreasing insulin resistance is an important aspect of regaining glycemic control[10]. In consistent with the literature, we found

that ketogenic diet-fed mice had significantly lower serum glucose levels comparted to normal chow diet-fed mice. In support of the literature our findings suggest that the ketogenic diet can be a useful aid to lower blood glucose levels in subjects that may not be deemed “obese” but are still on the verge of developing insulin resistance. However, we did not see the effect of Lcn2 deficiency on the blood glucose-lowering effect of ketogenic diet in our study.

Triglyceride levels are directly related to lipid droplet size and number[20]. Adipocyte size is strongly correlated with energy intake and expenditure[47]. During weight loss adipocytes decrease in size as the triglycerides that make up the cell are broken down to release fatty acids and mobilized to the liver to be utilized in β-oxidation. Analysis of inguinal adipocyte size and quantity revealed that compared to the control diet, the ketogenic diet decreases adipocyte size in wild type mice from a distribution of 50-125μm to 25-100μm and decreased the quantity of adipocytes in Lcn2-/- mice from 50% to ~33% of adipocytes distributed between 25-100μm. This is consistent with our inguinal tissue weight findings previously discussed. As we know, while under ketogenic conditions the body needs to tightly regulate fatty acid metabolism in order to decrease the risk of metabolic dysfunction, which is known to accompany increased circulations of free fatty acids as a result of an increased dependence on β-oxidation. Based on our findings, lipolysis appears to be well medicated under normal conditions. This is indicated by a normal distribution of adipocyte size in both ketogenic and normal chow diet fed mice (25-125 μm, 50-150 μm) and decreased adipocyte size under ketotic

conditions. However, it appears that when there is a deficiency of Lcn2, regulation of lipids size is disrupted leading to an increasingly broadened distribution of adipocyte sizes ranging from 50-175μm. This is similar to what was observed in the liver. Where we saw increased accumulation of lipids which is indicative of metabolic dysfunction, specifically in lipolytic pathways.

There is strong evidence to suggest that the ketogenic diet could have beneficial applications for inducing weight loss. That being said, there has also been evidence to suggest that a high-fat low carbohydrate diet could lead to liver dysfunction and increased risk of developing metabolic conditions such as NAFLD[10,29]. Various examples show elevated hepatic lipid accumulation in the liver as well as distinct evidence of NAFLD. Hepatic steatosis is a precursor to NAFLD. While our study did not analyze intrahepatic triglyceride concentrations our findings are consistent with previous literature that has observed hepatic steatosis via histological staining in mice fed a ketogenic diet. A one such study examining the effects of low-carbohydrate ketogenic diets on glucose homeostasis and NAFLD demonstrated that hepatic lipid accumulation could be seen as early as 3-weeks after starting the diet and after 12 weeks mice has fully developed NAFLD[48]. A study comparing gene expression in the liver of mice fed a ketogenic diet illustrated that fatty acid synthesis, cholesterol synthesis, and glucose-handling pathways were decreased comparted to high-fat diet fed mice[29]. In fact, the expression of all the genes that were analyzed appears starkly inverse to one another when compared. This prevalent distinction in metabolic pathways further validates the

difference between the effects that these two diets have on metabolism. Thus, suggesting that hepatic lipid accumulation may be controlled by some other mechanisms outside of normal lipogenic pathways. Lcn2 is a modulator of lipid homeostasis and recent investigations have shown that it may play a protective role in acute liver injury[24,50]. This interplay can be seen in results from our study. In conjunction with previous references our results demonstrate that a high-fat ketogenic diet increases lipid accumulation in the liver. Furthermore, the absence of Lcn2 appeared to increase the amount of lipids appreciated in the liver when compared to the control. We previously discussed that hepatic steatosis is a consequence of liver dysfunction which draws us to the conclusion that in the absence of Lcn2, the increased progression of hepatic steatosis compared to the control is likely secondary to dysregulation of lipid homeostasis exacerbated by the ketogenic diets reliance on lipids to create energy.

Conclusion

The ketogenic diet has immense potential in the context of weight loss and obesity. Its effects have been examined under numerous conditions but there is still room for skepticism. These are strong implications that in the short term this diet can help with weight loss, but prolonged adherence could induce or perpetuate metabolic dysfunction, which is the very thing this diet is supposed to control. Deficiency of lipocalin-2 seems to have some physiological effects under ketogenic conditions but due to the nature of our analysis these connections remain

speculative. More research into these kinds of associations continues to be a need as the mechanisms underlying this diet are still highly debated. Nutrition and weight loss are multifaceted concepts that need to be addressed with the expectation that in order to provide people with the best information for their individual needs one has to be willing to adjust their perspective and accept new ideas.

Materials and methods

Animals

Lcn2-deficeint mice were provided by Dr. Alan Aderem, Institute for systems Biology, Seattle, Washington, USA. Heterozygous mating scheme was used to generate WT and Lcn2-/- mice as previously described[1]. Animals were housed in a pathogen-free facility at the University of Minnesota. The room was maintained at 22 ˚C on a 12:12 light-dark cycle with free access to water. All mice were group housed based on their genotype, which was determined via PCR analysis, up to 4-5 mice per cage. Food was distributed in either a ceramic ramekin or in a recess with a metal wire lid at the top of the cage depending on the experimental diet. Food was changed weekly, and mice were allowed to eat freely. Food in the ramekins were changed bi-weekly and the food in the wire lid was changed weekly along with the lid.

Mice were fed a ketogenic diet F3666 (75% calories from fat, 8.6% from protein, 3.2% from carbohydrates, Bio-serv F3666, Flemington, NJ, USA) at 14

weeks of age for 10 weeks. 6 Lcn2 -/- mice and 7 WT mice were fed the ketogenic diet. Control mice were fed a regular chow diet (22% calories from fat, 19% from protein, 44.9% from carbohydrates Envigo 2019, Madison, WI, USA). 4 Lcn2-/- and 8 WT mice were fed the regular chow diet. Body weight was monitored weekly. After 10 week, mice were sacrificed and tissues were collected and immediately frozen in liquid nitrogen and stored at - 80 ˚C for future analysis. This protocol was repeated and a total of two independent cohort animal studies were conducted with the approval of the University of Minnesota Animal Care and Use Committee and conformed to National Institute of Health guidelines for laboratory animal care.

Histology and immunochemistry

Tissues were fixed in a 10% neutral buffered formalin (Thermo Scientific, Rockford, IL), washed in 1X PBS and block in paraffin. After deparaffinization and rehydration, tissues were sectioned and stained with H&E using a standard protocol.

Serum Assays

Blood glucose

Mice were fasted for 4 hours prior to checking blood glucose levels. Blood glucose levels were measured using an Ascensia Contour glucometer (Ascensia Diabetes Care, Parsippany, NJ).

Ketone body

Serum ketone body levels were measured using β-Hydroxybutyrate Assay Kit (SIGMA-Aldrich MAK014, St Louis, MO, USA). The assay was performed following the manufacturer's instruction. Briefly, a dilution series of 1mM β-hydroxybutyrate standard (0,2,4,6,8 and 10 µl of standards) was prepared following the standard procedure and added to a 96 well plate. Assay Buffer was added to each well, followed by adding serum samples to the wells to bringing the final volume to 50 µl. Reaction mixes were prepared and added to the well following standard procedure and incubated for 30 minutes at room temperature away from the light. Absorbance was measured using a spectrophotometer and sample concentrations were calculated.

Fat cell size distribution analysis

Osmium fixation

2% Osmium tetroxide solution was prepared following a protocol as previously described[51]. After tissue collection, 20-30mg of inguinal and gonadal adipose tissue samples were fixed in 2% osmium solution and incubated in a water both at 37 ˚C for 48 hours. Fixed samples were washed through 25 µm nylon mesh to remove osmium tetroxide solution and then 250µm nylon mesh using 0.9% saline. All fixed cells that passed though the 250µm mesh were collected into a 50 ml conical tube for particle size analysis.

Cell size analysis

Bluewave laser diffraction system was used to analyze the size and total number of fat cells in each sample. Both the PartAn SI and Micortrace computer

systems were set up following standard operating procedures for adipose cells. Samples were shaken vigorously and then allowed to settle at the bottom of the tube before pipetting. 200µm was initially loaded into the sample collection apparatus. If needed, more samples were added to the apparatus in 50µm increments until the analyzer indicated that there was an optimal concentration of the sample for a reliable measurement. Raw data, particle images, and size distribution graphs were saved for further analysis.

Statistical analysis

Values were reported as Mean +/- standard error of the mean. Statistical significance between WT and Lcn2-/- mice based on diet was determined by running an ANOVA and Tukey's range test in R studios. *P* value less than 0.05 was considered significant.

FIGURE

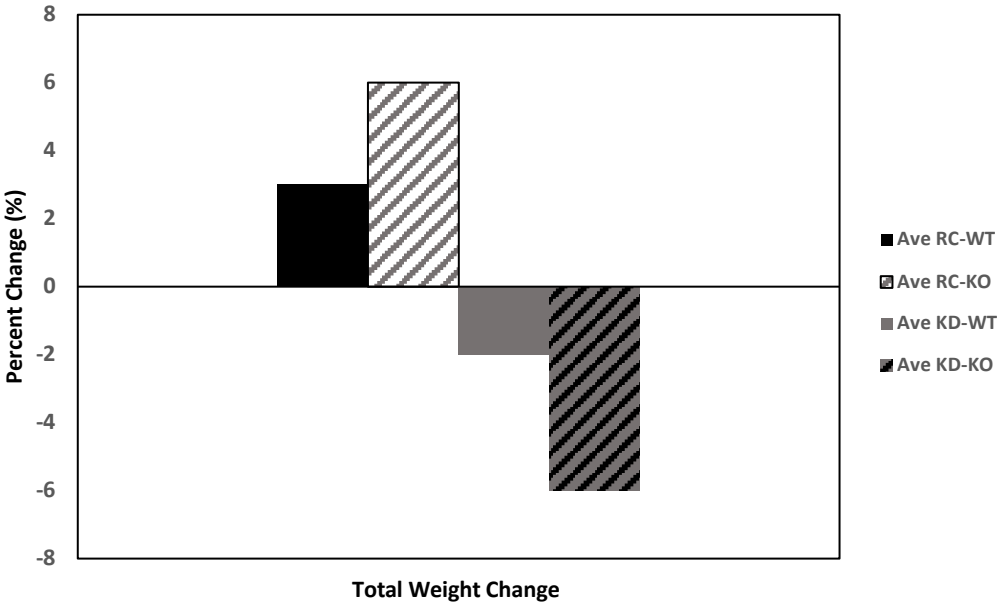

Figure 1. Difference in average weight loss between wild type and Lcn2-/- mice comparing normal chow diet vs ketogenic diet.

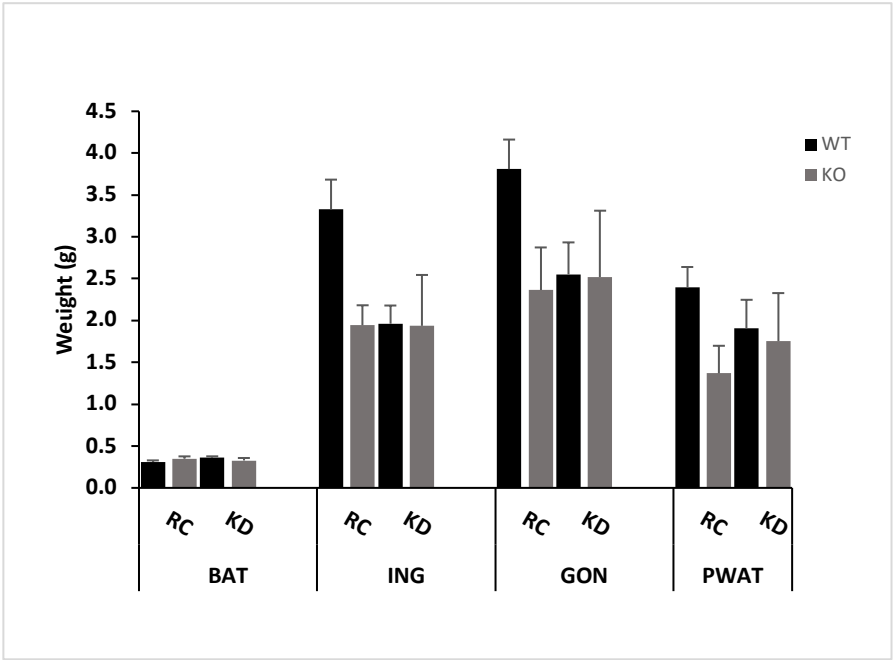

Figure 2. Effect of the normal chow diet (RC) and ketogenic diet (KD) on Brown Adipose Tissue (BAT), Inguinal Adipose Tissue (ING), Gonadal Tissue (GON), and Parametrial White Adipose Tissue (PWAT) weight between wild type (WT) and Lcn2 knockout (KO) mice.

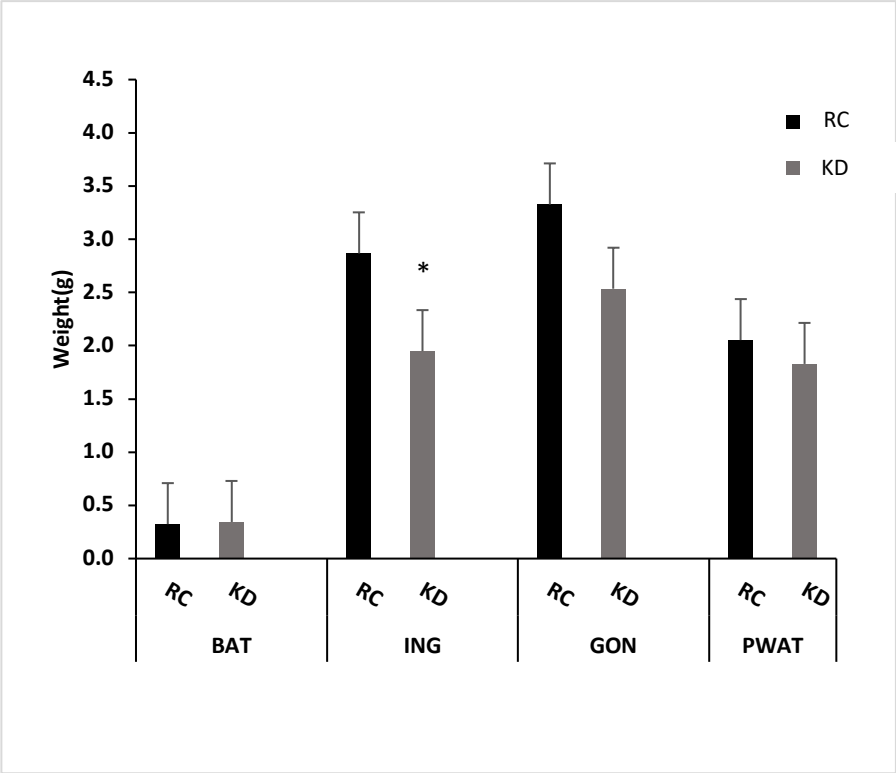

Figure 3. Effect of the normal chow diet (RC) and ketogenic diet (KD) on Brown Adipose Tissue (BAT), Inguinal Adipose Tissue (ING), Gonadal Tissue (GON), and Parametrial White Adipose Tissue (PWAT) weight. A significant difference (*P>.05) in inguinal tissue

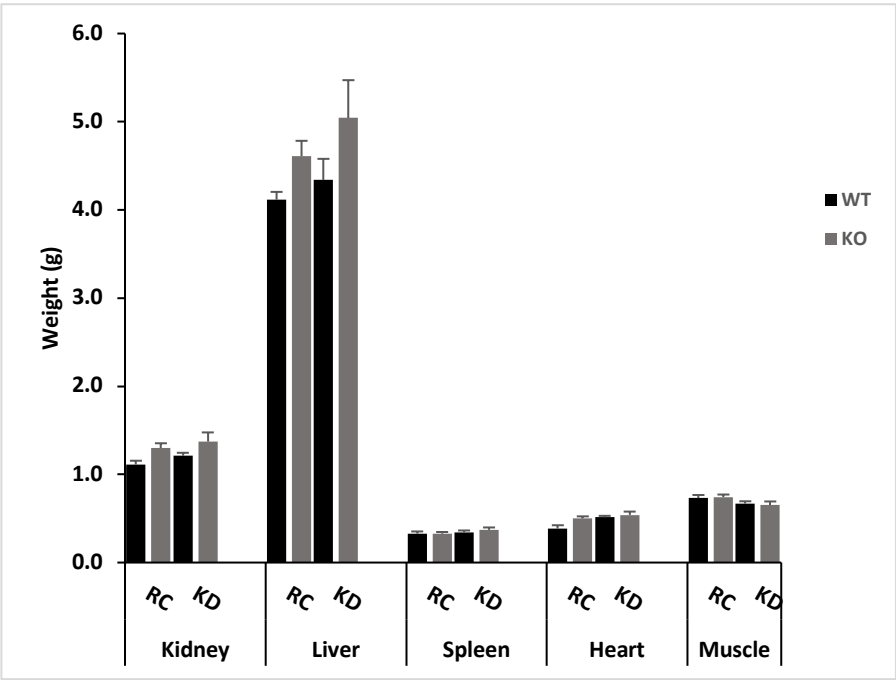

Figure 4. Effect of the normal chow diet and ketogenic diet on organ weight in Lcn2 knockout and wild type mice.

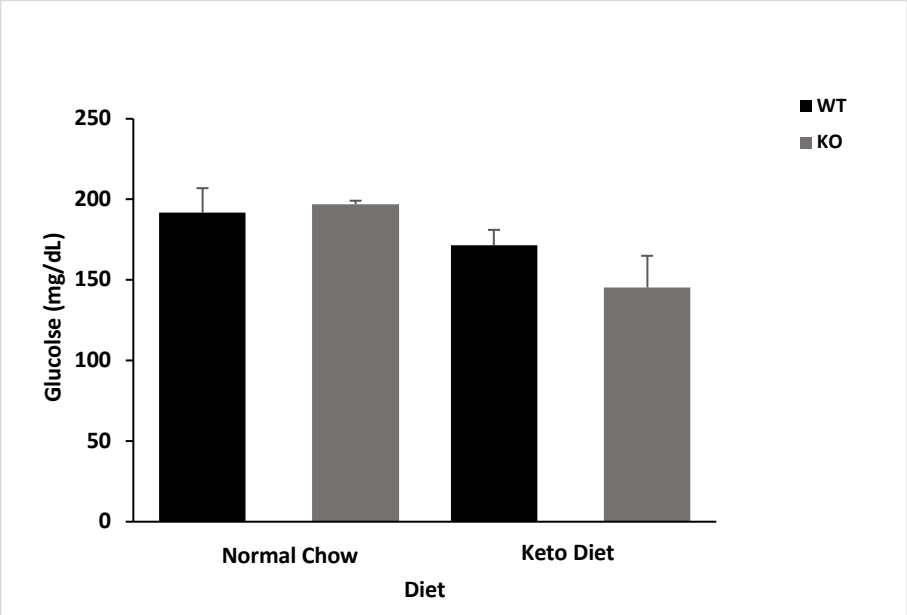

Figure 5. Changes in blood glucose level in wild type (WT) and Lcn2 knock out (KO) mice after 10 weeks of a normal chow or ketogenic diet feeding.

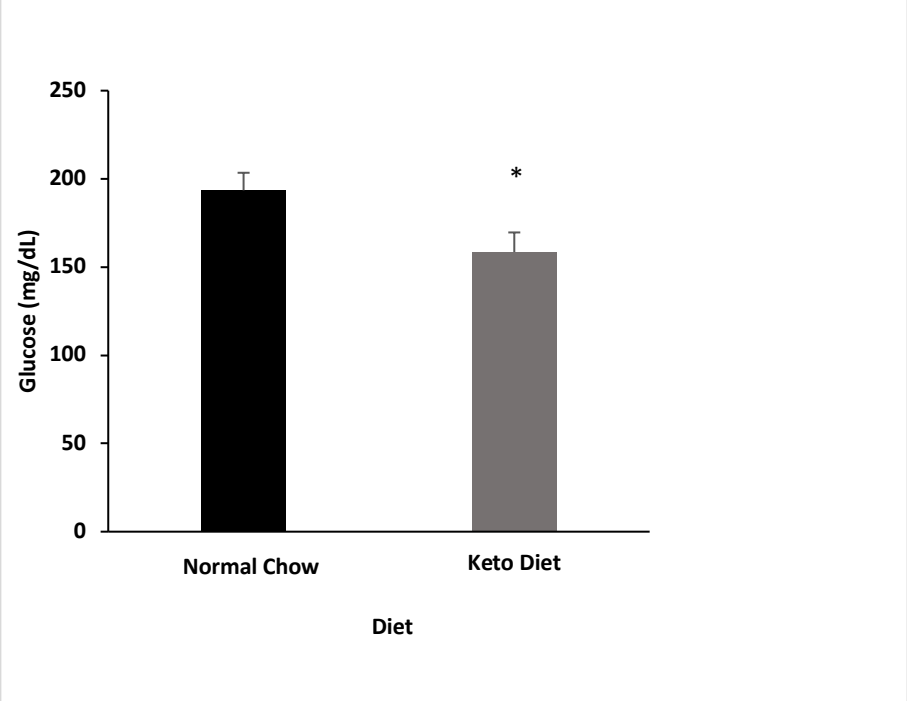

Figure 6. Change in blood glucose levels after 10 weeks of normal chow diet and ketogenic diet feeding. Participants in the ketogenic diet group showed a significant decrease in glucose level compared to in the normal chow diet group.

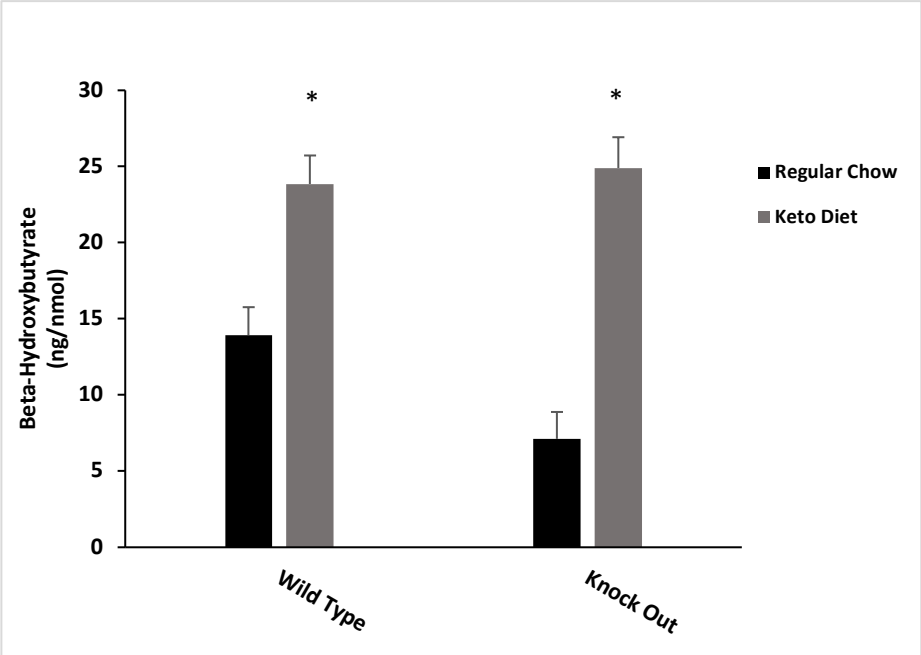

Figure 7. Effect of ketogenic diet and normal chow diet on β-hydroxybutyrate levels between lcn2 knockout and wild type mice. A significant difference (*P>0.05) was noticed in β-hydroxybutyrate levels in the ketogenic diet groups compared to the normal chow diet groups.

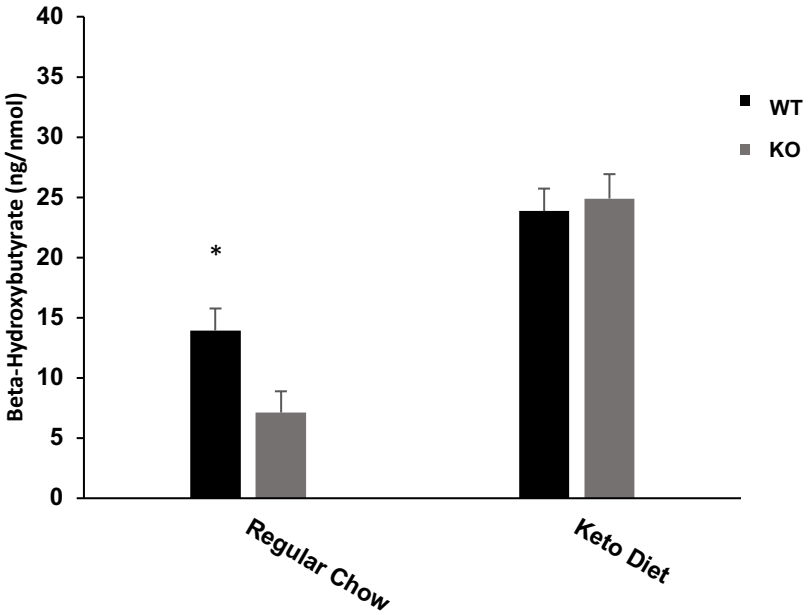

Figure 8. Serum β-hydroxybutyrate levels in wild type and Lcn2 knockout mice after 10 weeks of a normal chow diet or ketogenic diet feeding. A significant difference (*P>0.05) was noticed in the Lcn2 knockout mice compared to the wild type mice that were fed a normal chow diet.

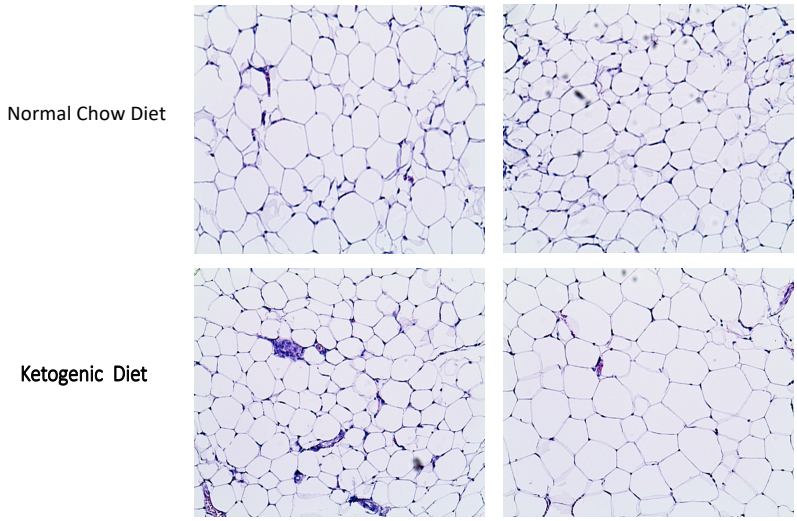

Figure 9. Histology of inguinal adipose tissue: Effect of the ketogenic diet and normal chow diet on morphology of inguinal adipose tissue in Lcn2 knockout and wild type mice (hematoxylin and eosin staining, 4X magnification).

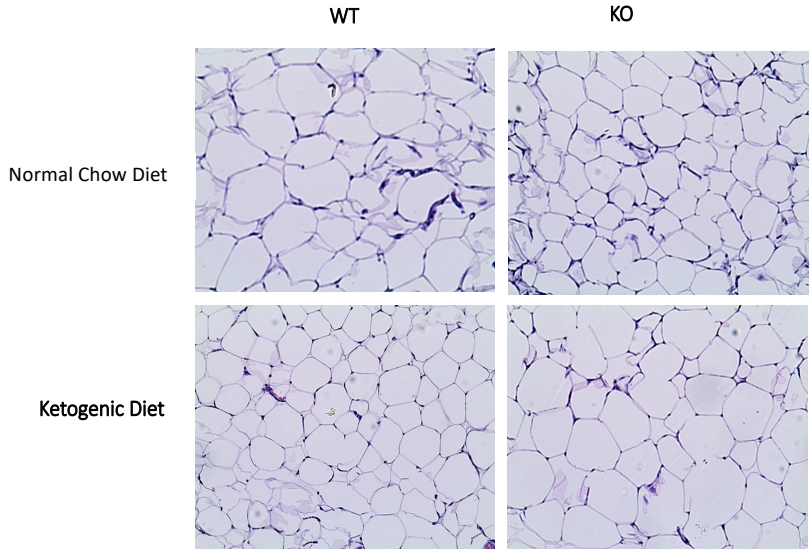

Figure 10. Histology of gonadal adipose tissue: Effect of the ketogenic diet and normal chow diet on morphology of gonadal adipose tissue in Lcn2 knockout and wild type mice (hematoxylin and eosin staining, 4X magnification).

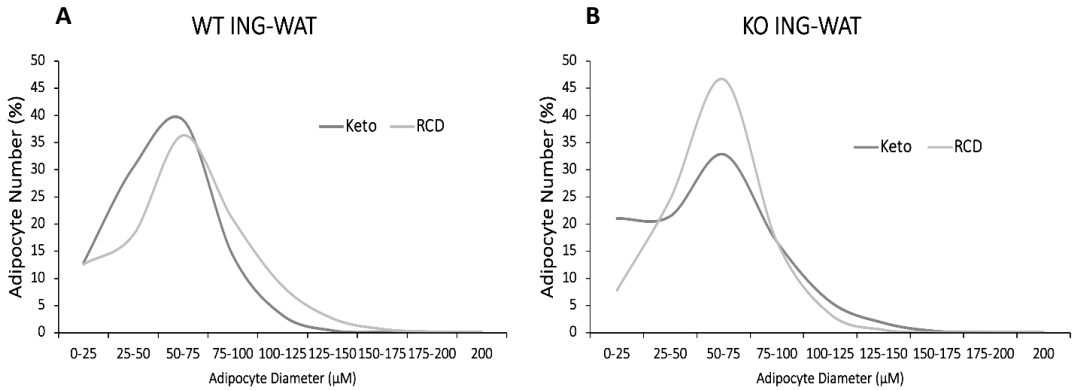

Figure 11. (A) Comparative inguinal adipocyte size distribution of wild type mice fed a ketogenic diet vs. a normal chow diet. (B)Comparative adipocyte size distribution of knockout mice fed a ketogenic diet vs. a normal chow diet.

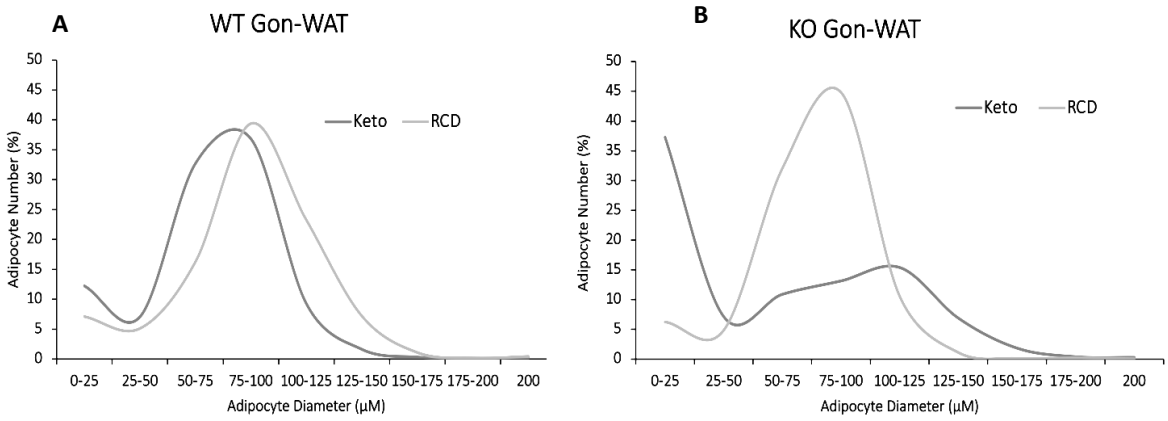

Figure 12. (A)Comparative gonadal adipocyte size distribution of wild type mice fed a ketogenic diet vs. a normal chow diet. (B) Comparative adipocyte size distribution of knockout mice fed a ketogenic diet vs. a normal chow diet.

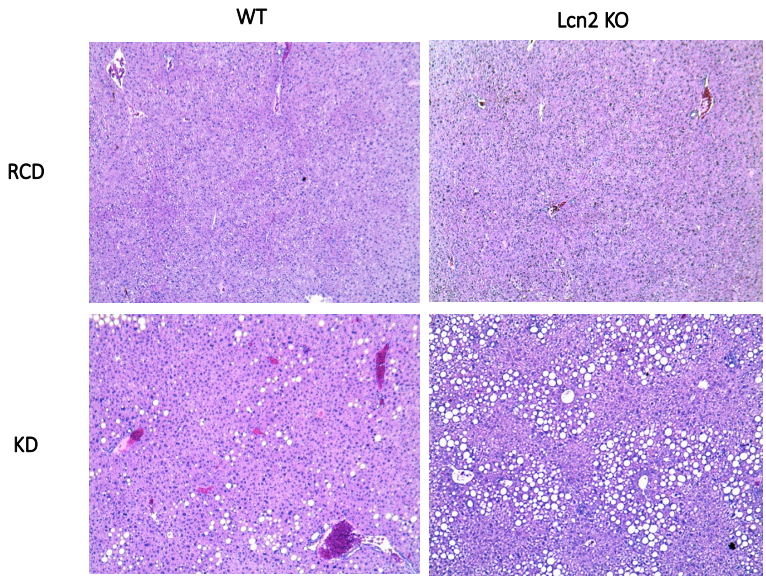

Figure 13. Histology of liver: Effect of ketogenic diet on hepatic lipid accumulation in Lcn2 knockout and wild type mice (hematoxylin and eosin staining, 4X magnification).

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