Implications of a low-carbohydrate, high-fat diet on heart size in a young murine model.

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ABSTRACT

Implications of a low-carbohydrate, high-protein diet on heart size in a young murine model.

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Objective: The incidence of childhood obesity in North America and around the world has risen significantly over the past decade leaving clinicians constantly searching for effective weight loss strategies. Despite the growing popularity or carbohydrate restricted diets, consequences of long term use remain widely unknown. The objective of the current investigation is to evaluate the effects of LCHF diets on heart size and cardiac glycogen content in young mice. **Methods:** Young mice (age 21 d, n = 24) and mature mice (age 84 d, n = 18) were placed on either a LCHF diet, a WD diet or a control diet for 12 weeks. Activity levels, body weight, and glucose values were measured during the investigation. At the competition of the dietary intervention, wet heart weights were measured to compute the heart weight-to-tibia length ratio, cross sectional area was calculated and sections of cardiac tissue were stained with periodic acid and Schiff reagent to visualize glycogen. **Results:** No differences in activity levels or glucose measures were noted between groups. Mice following the LCHF diet displayed a smaller heart weight-to-tibia length ratio when compared to controls. The trend was observed in both young ($p = 0.012$) and mature ($p = 0.024$) mice. No differences in cross sectional area were detected. Cardiac tissue from mice consuming the LCHF diet had a reduced percentage of total area stained positive for glycogen when compared to mice following the WD diet ($p = 0.016$). **Conclusions:** A LCHF diet can lead to the development of a smaller heart in young and mature mice. Alterations in intercellular cardiac glycogen content may contribute to differences observed in heart weight. Cardiac restricted diets should be recommended with caution as long term cardiac developmental impairments are unknown.

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INTRODUCTION

The focus of this thesis is to investigate the potential effects that low-carbohydrate, high-fat (LCHF) diets may have on cardiac development in young subjects. All research contributing to this project was performed in the Cardiovascular Physiology Lab under the supervision of Dr. Andreas Bergdahl with the Department of Exercise Science at Concordia University. This thesis contains one manuscript entitled *"Implications of a low-carbohydrate, high-fat diet on heart size in a young murine model,"* currently in process of submission (Journal of Pediatric Gasteroenterology and Nutrition).

In 2010, approximately 6.7% of all children were considered to be overweight or obese with prevalence expected to rise to 9.1% in the year 2020 [de Onis, 2010]. With such a large percentage of the Western world struggling with childhood obesity, clinicians are constantly searching for healthy and effective weight management strategies. Many of these strategies focus on diets involving caloric restriction or macronutrient manipulation. By lowering the carbohydrate content in an individual's diet, glycogen stores become depleted; forcing the body to use fats as a primary source of ATP production. These diets have been associated with accelerated weight loss, decreases in systolic and diastolic blood pressure, improved LDL and HDL cholesterol profiles, and reduced insulin levels [Samaha *et al*, 2003 & Foster *et al*, 2003]. During short-term use, a LCHF diet does not appear to have any negative effects on overall cardiac function [Holloway *et al*, 2011], but over a longer time period, the effects of a prolonged state of ketosis on the heart and the overall growth are unknown.

It is well accepted that the heart will rely predominantly on fatty acids for energy production; however various stresses such as ischemia, hypertrophy or exercise can stimulate a greater reliance on glycogen [Wambolt *et al*, 1999, Schaefer *et al,* 1997 & Goodwin *et al*, 1996]. More recent studies have shown that glycogen in the developing embryonic heart is crucial for survival, with impairments in glycogen production being related to congenital heart disease [Pederson *et al*, 2004].

The content of this thesis will evaluate the effects of a LCHF diet, a high-fat western diet (WD) and a control diet on heart size and glycogen content in young hearts. As a more detailed introduction to the topic, a literature review on the current understandings of LCHF diets and cardiac metabolism has been included. Following chapters address all aspects of the research that was undertaken for this project.

CHAPTER 1: REVIEW OF THE LITERATURE

1. 1 The low carbohydrate, high fat diet

The first documented successful low carbohydrate diet was described by William Banting in the 1860s. Banting claimed that he was never hungry and that he was able to lose 46 pounds; decreasing his weight from 202 pounds to 156 pounds over the course of one year [Banting, 1863].

Rationale behind a low carbohydrate, high fat diet for weight loss

By lowering carbohydrate content in an individual's diet, glycogen stores become depleted. As a result of an increased concentration of ketones in the blood formed through beta-oxidation of fats, excessive urination and ultimately, increased water loss will amplify weight loss, particularly in the early months of dietary intervention [Kappagoda *et al*, 2004]. This time frame coincides with data showing that low-carbohydrate, high-fat (LCHF) diets are more effective than low-fat diets in reducing body weight during the first three to six months of dietary intervention [Samaha *et al*, 2003, Keogh *et al*, 2008].

When lowering the carbohydrate content of a diet, in order to maintain the same caloric value it is necessary to concomitantly increase the protein and fat content of the diet. If protein and fat consumption do not increase when restricting carbohydrate intake, daily metabolic energy requirements will not be met. It has been suggested that increasing protein content of a diet promotes weight loss through inducing satiety and increasing energy expenditure [Westerterp-Plantenga *et al*, 2009]. The mechanism behind this observation is

that induced satiety is mainly due to oxidation of excess amino acids amplified by the fact that some amino acids serve as precursors for neurotransmitters involved in appetite or body weight regulation (ie: tryptophan for serotonin, tyrosine for dopamine and norepinepherine, histidine for histamine) [Westerterp-Plantenga *et al*, 2009]. Protein-induced changes in energy demands are suggested to affect resting energy expenditure as well as diet-induced thermogenesis as a result of urea synthesis and gluconeogenesis [Westerterp-Plantenga *et al*, 2009].

Positive effects of a low carbohydrate, high fat diet on health status

A number of randomized trials suggest that LCHF diets may accelerate weight loss in the short term with little effect on cardiac risk factors.

Obesity

In diet interventions lasting less than six months it has been clearly shown that participants following a LCHF diet will 1) decrease their body weight more than participants following a high carbohydrate diet [Rankin & Turpyn, 2007], a low-fat diet [Samaha *et al*, 2003, Keogh *et al*, 2008] or 2) decrease their body weight to the same degree as participants following an isocaloric low-fat diet [Phillips *et al*, 2008, Keogh *et al*, 2007, Meckling *et al*, 2004], control diet [Brinkworth *et al*, 2004].

Unfortunately, it has also been shown that individuals who lose weight following an LCHF diet will tend to regain some of the weight lost during the initial three to six months [Brinkworth *et al*, 2004]. Evidence suggests that after one

year of dietary intervention, the amount of weight loss is similar between LCHF diets, high carbohydrate diets and low-fat diets as long as the total caloric consumptions are comparable [Dansinger *et al*, 2005, Brinkworth *et al*, 2004].

Dyslipidemia

In participants with normal cholesterol levels, LCHF diets appear to have no effect on total cholesterol levels, HDL-c or LDL-c levels [Phillips *et al*, 2008, Brinkworth *et al*, 2004, Samaha *et al*, 2003]. In obese individuals following a LCHF diet for six months, triglyceride levels have been shown to decrease more than individuals who followed a low-fat diet [Samaha *et al*, 2003]. Obese individuals also tend to show an improvement in HDL-c levels after 8 weeks and one year on a LCHF diet, where individuals following a low-fat diet do not [Keogh *et al*, 2008, Dansinger *et al*, 2005]. In these same obese individuals, LDL-c has been shown to decrease following a LCHF diet [Keogh *et al*, 2007, Keogh *et al*, 2008, Dansinger *et al*, 2005], however typically greater decreases in LDL-c are seen in low fat diets [Keogh *et al*, 2008, Dansinger *et al,* 2005]. In opposition to the above findings, in a murine model Non-Esterfied Fatty Acids (NEFAs) are shown to increase in response to a LCHF diet [Foo *et al*, 2009].

Hypertension

In interventions comparing LCHF diets to low-fat diets involving obese participants, decreases in both systolic and diastolic blood pressures after 6 to 12 weeks are similar between groups [Phillips *et al*, 2008, Keogh *et al*, 2008, Keogh *et al*, 2007]. In normotensive individuals as well as individuals taking

antihypertensive therapies, a LCHF does not appear to have an effect on systolic or diastolic blood pressures [Samaha *et al*, 2003, Brinkworth *et al,* 2004, Dansinger *et al,* 2005].

Insulin

In most cases, fasting insulin has been shown to be significantly reduced in individuals following a LCHF diet [Brinkworth *et al*, 2004, Keogh *et al*, 2007, Meckling *et al*, 2004]. Significant changes have been seen in as little as 4 weeks [Phillips *et al*, 2008]. Although one large randomized trial published in the Journal of the American Medical Association found that fasting insulin levels did not decrease in overweight and obese individuals after one year on a LCHF diet [Dansinger *et al*, 2005].

Biochemical Factors

The effects of a LCHF diet on CRP, an index of chronic low-level inflammation, are conflicting. One intervention comparing a LCHF diet and a high-carbohydrate diet found that after 4 weeks, serum CRP levels increased from baseline in individuals following a LCHF diet while simultaneously decreasing from baseline in individuals following a high-carbohydrate diet [Rankin & Turpyn, 2007]. In other trials comparing a LCHF diet to a low fat diet, there was no detectable change in CRP levels [Phillips *et al*, 2008, Keogh *et al*, 2007]. Conversely, after one year on a LCHF diet, CRP levels have been shown to decrease from baseline [Brinkworth *et al*, 2004, Dansinger *et al*, 2005]. In a smaller trial, significant decreases in CRP have been have been shown after

following a LCHF for six weeks [Sharman & Volek, 2004].

In a smaller randomized cross over study, it was shown that LCHF diets induce the same decreases in inflammatory cytokines as low-fat diets in overweight men [Sharman & Volek, 2004]. Significant decreases in high sensitivity CRP, high sensitivity Tumor Necrosis Factor Alpha (TNF-α), and high sensitivity Interleukin-6 (IL-6), inflammatory cytokines, as well as soluble Intercellular Adhesion Molecule (ICAM), an adhesion molecule involved in monocyte recruitment, were measured after 6 weeks [Sharman & Volek, 2004]. Decreases in soluble ICAM have also been detected at 8 weeks [Keogh *et al*, 2008], and 68 weeks [Brinkworth *et al*, 2004].

The trials described in support of the LCHF diet are quite heterogeneous; significantly varying in length, sample population and diet composition. To be able to comfortably state that there are distinct benefits to following a LCHF diet as opposed to a traditional low-fat diet, higher quality studies, such as the randomized trial by Dansinger et al (2005), need to be completed.

Potential negative effects of a low carbohydrate, high protein diet

Inadequacy of Current Literature

In a systematic review of 94 peer reviewed dietary intervention articles, it was found that there is insufficient evidence to make recommendations for or against the use of LCHF diets [Bravata *et al*, 2003]. This was the first publication to objectively summarize present data on the efficacy and safety of LCHF diets. Based on their results, reviewers concluded that there are four significant gaps in

the published research at the time of the review [Bravata *et al,* 2003]. The first was that there is a lack of long-term follow-up data, limiting the knowledge about safety and long-term efficacy of these diets [Bravata *et al,* 2003]. This remains true today; very few studies have followed participants beyond one year post-intervention. The second gap in literature at the time was the effect of LCHF diets on different racial and ethnic groups [Bravata *et al,* 2003]. There was an absence of data regarding the safety and efficacy of LCHF diets on different populations making it impossible to provide individualized recommendations [Bravata *et al,* 2003]. Although all trials in the analysis controlled for total caloric intake, few studies included a measure of energy expenditure, making it impossible to include as a covariate during the analysis [Bravata *et al,* 2003]. Reviewers recommended that future studies investigating the efficacy of LCHF diets on weight loss control for energy expenditure of the participants [Bravata *et al*, 2003]. Lastly and most importantly, studies investigating LCHF diets are very heterogeneous [Bravata *et al,* 2003]. Macronutrient composition was quite varied between studies as well as the amount of support received by participants enrolled in the interventions. These four factors outlined by the reviewers highlight why it was difficult at the time to make any recommendations for or against the use of LCHF diets.

At present time, little has changed. Dietary intervention trials still do not have large groups of participants. Diet safety is measured in terms of positive changes in risk factors, rather than actual measures of disease. Long-term

efficacy of LCHF diets remains unknown as follow-up measurements still rarely exceed one year post-intervention.

Ketone Production

When following a LCHF diet, individuals rely on lipid and protein metabolism for adenosine triphosphate (ATP) production. Excessive Acetyl CoA unable to enter the citric acid cycle due to a shortage of glucose is converted to acetoacetate which in turn yields β hydroxybuterate and acetone. Together acetoacetate, β hydroxybuterate and acetone are known as ketone bodies. Increased concentrations of ketones in the blood create a significant solute load on the kidneys resulting is osmotic diuresis. During urinary excretion, ketone bodies remain bound to cations to maintain electrical neutrality, increasing elimination of calcium, magnesium and potassium ions [Reddy *et al,* 2002]. Long term loss of these cations without proper replenishment could significantly increase the individual's risk of developing osteoporosis [Reddy *et al,* 2002]. More immediate side effects seen in individuals undergoing prolonged osmotic diuresis or water depletion are postural hypotension, fatigue, constipation and kidney stones [Reddy *et al,* 2002]. These immediate side effects have been documented in individuals following a LCHF diet [Westman *et al*, 2002, Reddy *et al*, 2002].

Endothelial Dysfunction

A compromised endothelial layer in the artery is an important trigger in the atherosclerotic process. Abnormal endothelial function is marked by a reduced

dilation in response to an increased blood flow. In a recent study measuring flow mediated dilation (FMD), obese participants followed either a low-fat or low-carbohydrate diet, it was shown that after six weeks on a low-carbohydrate diet, FMD was decreased [Phillips *et al*, 2008]. This decrease was not seen in participants following the low fat diet [Phillips *et al*, 2008]. This is an interesting finding as it is one of the few studies on humans that look beyond traditional serum risk factors for cardiovascular disease.

Atheromatous Plaque Development

Due to the fact that it is difficult to directly measure the development of atherosclerotic lesions in humans, accepted animal models are often used. Recent data obtained from a murine model demonstrated that traditional serum markers of cardiac risk may not be sufficient in evaluating the safety of LCHF diets [Foo *et al*, 2009]. Apolipoprotein E (ApoE) knockout mice, a well-accepted animal model of atherosclerosis, fed a LCHF diet for 12 weeks were shown to have more aortic atherosclerotic lesions compared to a control group [Foo *et al*, 2009]. More interestingly, when compared to mice following a high-fat western diet, the LCHF group still had significantly more atherosclerotic lesions [Foo *et al*, 2009].

Prevention and treatment of cardiovascular diseases requires permanent lifestyle modifications, meaning that any dietary intervention recommended to at-risk patients should be applicable for life. Recommendation of diet strategies such as

the LCHF diet should be cautioned until more is known on the safety of these diets over the long term.

Impaired Cognitive Function

The importance of glucose in brain metabolism has been well documented [Benton *et al*, 1994, Hoyland *et al*, 2008]. As a result, carbohydrate restriction can have a negative impact on brain function leading to a host of cognitive impairments. In an animal model, rats following a low carbohydrate, ketogenic diet displayed decreased brain growth and severe visual-spatial memory impairment when compared to controls following a standard chow diet [Zhoa *et al*, 2005]. Cognitive impairments have also been documented in human models. After following a LCHF diet for only 5 days, participants displayed impaired attention, speed, and decreased mood when compared to scores achieved on an isocaloric standard diet [Holloway *et al*, 2011]. A longer-term study following participants for one year displayed similar outcomes; with those following the LCHF diet exhibiting higher anger, depression and confusion compared to controls following a standard low fat diet at the end of the intervention [Brinkworth *et al*, 2009].

The use of the low carbohydrate, high fat diet in children and adolescents

The rise in popularity and information available on the LCHF diet, carbohydrate restriction is now being recommended to children and adolescents of treat a variety of adverse health conditions.

Epilepsy

The LCHF diet is currently being used as a non-pharmacologic mechanism to control intractable childhood epilepsy. In 2009, a panel of 26 pediatric epilepsy specialists and dieticians published a consensus report agreeing that LCHF diets should be strongly considered as a treatment option in children that have not responded to classical anticonvulsant therapies [Kossoff *et al*, 2009]. It should be noted that the majority of the literature in support of LCHF diets for the treatment of epilepsy are observational. In a single randomized controlled trial evaluating the effectiveness of a LCHF diet to reduce seizure frequency it was confirmed that severe carbohydrate restriction can significantly reduce the number of seizures experienced by children with drug-resistant epilepsy [Neal *et al*, 2008]. On a less positive note, children following the LCHF displayed some rather severe side effects such as extreme drowsiness, vomiting, diarrhea, and in some cases developed hematuria [Neal *et al*, 2008].

Unfortunately to achieve the continued benefit of reduced seizure frequency, children need to maintain the LCHF diet. The secondary effects of prolonged reduction of carbohydrate intake are unknown and could have a significant impact on the child's growth and development throughout the years.

Childhood Obesity

In 2010, approximately 6.7% of all children were considered to be overweight or obese with prevalence expected to rise to 9.1% in the year 2020 [de Onis *et al*, 2010]. Carbohydrate restriction is suggested to be an effective

strategy for weight loss in obese children. The LCHF diet has been applied to this population in medically supervised treatment centers as well as school-based and home-based intervention programs. In each case, the LCHF diet was shown to be equally effective or more effective at reducing the BMI-Z score in obese children than traditional low-fat diets [Krebs *et al*, 2010, Seigel *et al*, 2009 & Seigel *et al*, 2011]. Similarly to the trends seen in adults, LCHF diets have been shown to improve both LDL-c and HDL-c cholesterol levels, in addition to insulin sensitivity in adolescents [Krebs *et al*, 2010]. Although these results may appear to be promising, at present, the longest follow-up to evaluate the long-term benefits and risks of this diet in adolescents has been 36 weeks leaving true long-term effects in this population unknown.

Children with Diabetes

Due to the positive changes in weight and insulin sensitivity seen in obese children, LCHF diets are now being considered in the treatment of Type I and Type II diabetes. A large randomized controlled trial based in New Zealand is investing the capacity of a LCHF diet to improve glucose tolerance in prediabetic children at risk of developing type II diabetes [Garnett *et al*, 2010]. This will be the first investigation with a LCHF diet intervention for children that will follow participants regularlyfor one year; the longest to date. Less frequently LCHF Diets are being prescribed in children with Type I diabetes to manage glycemic control in addition to other concomitant conditions. A case study following a young child with type I diabetes for 15 months found that a LCHF diet could be safely used in this population and was not associated to hypoglycemia [Dressler

et al, 2010]. The child displayed normal glycosylated hemoglobin levels throughout the intervention, indicating that the diabetes was well managed [Dressler *et al,* 2010].

1.2: Cardiomyocyte Metabolism

A healthy human heart is an efficient machine that pumps approximately 5 liters of blood per minute, equating to over 7000 liters per day [Taegtmeyer, 2004]. The energy required to perform this work is generated from the transfer of chemical energy from nutrients in the blood stream to mechanical energy within the cardiomyocytes. From a metabolic point of view, cardiac tissue is considered to be 'omnivorous,' meaning that it will use both fats and carbohydrates in different ratios to generate ATP depending on substrate availability and disease state [Randall, 1998].

Fatty Acids and Energy Production

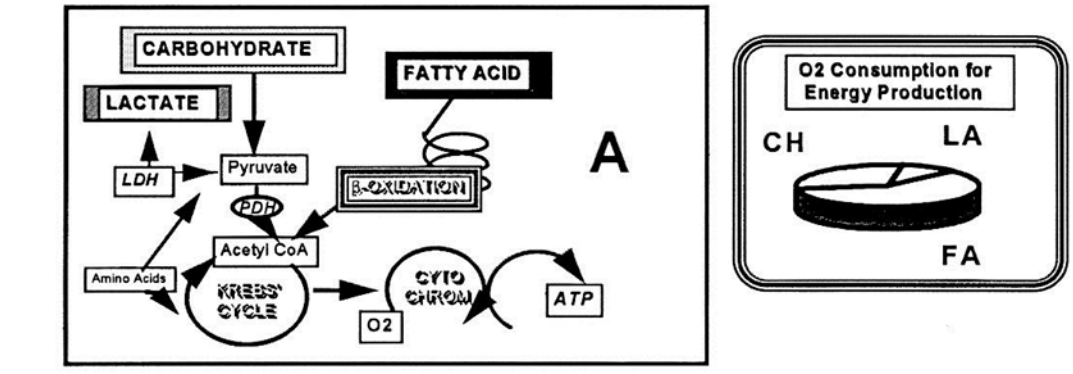
Considered to be the primary substrate used in energy production in human cardiac tissue, fatty acids account for approximately 60-70% of oxygen consumption within the tissue. The fatty acids are first acylated into acyl CoA by acyl CoA synthase. The acyl CoA will then enter the mitochondria using the carnitine shuttle where it will undergo β-oxidation to produce hydrogen atoms and acetyl CoA. The Acetyl CoA then enters the Citric Acid cycle and is further converted to hydrogen atoms. The hydrogen atoms produced by these reactions allow the production of NADH2 and FADH2, which allow for the production of

ATP through the cytochrome chain and the mitochondrial ATPase system [Grynberg & Demaison, 1996]. Refer to Figure 1.

Carbohydrate and Energy Production

Maintaining the homeostasis of glucose in the bloodstream is essential for survival of all human beings. Because glucose concentration is maintained within a narrow range in the blood, glucose becomes a very reliable substrate for energy production in the heart. Glucose or glycogen will be converted to glucose-6-phosphate and then proceed through the multistep glycolysis reactions, resulting in 2 pyruvate molecules. The pyruvate will then be further metabolized into acetyl CoA by pyruvate dehydrogenase to enter the Citric Acid cycle at the same point as the acetyl CoA produced from the β-oxidation of fatty acids leading to the generation of ATP [Grynberg & Demaison, 1996]. Refer to Figure 1.

Figure 1: Schematic of cardiomyocyte substrate metabolism



[Grynberg & Demaison, 1996]

The equilibrium of substrate use for energy production can be shifted following a high-carbohydrate meal or during a fasted state. Immediately following a meal rich in carbohydrates, serum levels of glucose and insulin will be elevated. Insulin will promote the uptake of glucose into the cardiomyocyte and inhibit the uptake of fatty acids. By decreasing the availability of acyl CoA and there for acetyl CoA from β-oxidation, glucose from carbohydrates will now become the main substrate used in energy production. Conversely, in a fasted state, fatty acids are oxidized preferentially to glucose. The enhanced contribution of β-oxidation is a result of the increased availability of intracellular fatty acids [Neely & Morgan 1974].

The process of generating ATP through the Citric Acid cycle and mitochondrial ATPase system is an aerobic process and requires oxygen. Without the presence of oxygen, the heart is still able to generate ATP via anaerobic respiration. It is during anaerobic respiration that the heart will rely more heavily on glucose and glycogen to meet energy demands. In humans, glycogen occupies about 2% of the cell volume in an adult cardiomyocyte and up to 30% of cell volume in fetal and newborn cardiomyocytes [Shelley, 1961].

Metabolism of the developing heart

The heart is one of the first organs to develop in utero and assume its definitive function [Ascuitto & Ross-Ascuitto, 1996]. Throughout fetal development, the heart is able to meet the energy requirements needed to beat in an environment of oxygen insufficiency. During gestation, the fetal heart will derive its energy primarily from the breakdown of carbohydrates [Fisher, 1984].

In the developing years after birth, the heart will go through a series of changes involving the differentiation of myocardial enzymes and changes to mitochondrial morphology and function related to the alterations in oxygen and substrate availability [Rolph & Jones, 1985]. Glycogen has also been shown to play an integral role in cardiac development. Stores are initially rather high throughout early and mid-gestational periods and then decrease slowly until birth [Gutierrez-Correa *et al,* 1991]. Studies involving the disruption of the GSY1 gene, which encodes for glycogen synthase, have shown that mice that were unable to produce glycogen had a very poor survival rate of less than 10% [Pederson *et al*, 2004].

Metabolism of the stressed heart

Ischemia

During ischemia, substantial changes occur in the energy metabolism of the heart as a consequence of the reduced oxygen availability. After a considerable decrease in accessible oxygen, endogenous glycogen becomes the main energy source for ATP production through glycolysis [Lloyd *et al,* 2004 & Wang *et al*, 2005]. The impairment of this process has been shown to lead to greater ischemic injury in the heart [Askenasy, 2001].

Hypertrophy

In cases of pathological cardiac hypertrophy, it has been well demonstrated that the heart will regain some characteristics of the fetal heart; one of which being a shift towards increased glucose metabolism [Barger & Kelly,

1999]. The increased rate of glycolysis seen in pathological cardiac hypertrophy is associated with an increased rate of insulin-independent glucose uptake into the cell with no significant alterations in transporter proteins or glycolytic enzymes, suggesting that substrate availabilityis essential in meeting energy demands [Razeghi *et al*, 2001]. In addition, hypertrophied hearts have also been shown to preferentially oxidize glucose from internal glycogen stores as opposed to exogenous glucose, further emphasizing the importance of glycogen in the stressed heart [Allard *et al,* 1997].

Exercise

It has been suggested that the contractile performance of the heart at a given VO2 is greater when the heart is oxidizing more glucose and lactate, and less fatty acids [Korvald *et al*, 2000 & Burkhoff *et al*, 1991]. One study in isolated rat hearts demonstrated that the mechanical power of the left ventricle is less at a given rate of oxygen consumption when fatty acids rather than glucose are the sole exogenous substrate [Burkhoff *et al*, 1991]. A similar decrease in cardiac mechanical efficiency with elevated plasma concentration of free fatty acids was seen in health humans [Simonsen & Kjekshus, 1978]. However the mechanisms behind the impaired efficiency due to increased fatty acid concentration are unclear.

With all things considered, it can easily be concluded that glucose and glycogen play an essential role in developing and stressed hearts. Although fatty acids are the principal energy substrate for ATP production in mature, healthy hearts,

altering the availability of glucose for energy metabolism, as during the consumption of a low-carbohydrtate, high-fat diet, could have severely detrimental effects on the more fragile younger and pathological populations.

CHAPTER 2: OBJECTIVES AND METHODS

2.1 Objectives

The long term effects of LCHF diets in children and adolescents merit further investigation. It remains unclear whether the consumption of a LCHF diet will have a negative impact on physical and mental development during the childhood years. As glucose and glycogen are significant contributors to the metabolism of developing and stressed hearts; altering glucose availability through decreased carbohydrate intake could be potentially harmful to young children. To date, there are few quality studies investing the consequences of LCHF diets on cardiac tissue directly. The intent of this project is to investigate the effects of a LCHF diet on the heart in young subjects during the developmental years of life prior to maturity. More specifically, we will evaluate the effects of a LCHF diet on heart size in young mice.

It is hypothesized that heart size in young mice will be greatly affected by a LCHF diet and that differences observed will be a result of the low carbohydrate content in the diet and not the increased fat content. To further this hypothesis, we suspect that any variations in heart size from a LCHF diet will be a result of alterations in the glycogen content of the cardiomyocytes, and that all observed changes in young mouse hearts will parallel differences seen in mature adult mouse hearts.

To better understand the role of carbohydrate restriction on cardiac development in young mice, heart size was measured in response to three different diets; a LCHF diet, a typical high-fat North American diet and a control

diet. A murine model was used due to the invasiveness of the experimental measures. As this was our initial investigation, no preliminary data was available to estimate the effect size or variance. To determine the number of animals required to prove the hypothesis we were required to rely on comparable studies performed on rats [Wang *et al*, 2008]. With the significance value set to 0.05 and the power set at 0.8, it was calculated that 4 animals per group would be adequate to determine any differences in our dependent variables. Based on this information it was decided that 5 or 6 animals would be used to measure each parameter to account for any animals unable to complete the 12-week dietary intervention due to deformity of disease.

2.2 Interventions

Young animals will follow their assigned diet for 12 week, at which point they will be euthanized and the hearts will be resected. The procedure will be repeated in mature mice to determine whether age plays a role in any observed changes in response to the diet.

Animals

C57BL/6 mice were chosen as a suitable murine model for this investigation. In 2007, Kennedy *et al* demonstrated that C57BL/6 mice fed a carbohydrate restricted diet display similar characteristics to humans following a LCHF diet; including weight loss, improved glucose tolerance, and decreased serum insulin levels [Kennedy *et al*, 2007]. To represent the young murine model, male C57BL/6 pups were weaned at 21 days after birth and placed immediately

on their assigned diet. For representation of the mature murine model, at age 12 weeks, male C57BL/6 mice had their standard chow replaced with the assigned diet. The diet for each experimental animal was randomly assigned. Only male mice were used due to the known cardio protective effects of estrogens [Nathan & Chaudhuri, 1997]. Mice were housed in a temperature and humidity controlled room with alternating 12-hour periods of light and dark in accordance with Concordia University's animal research ethics committee. In addition, mice will be provided with *ad-libitum* access to water and assigned diet with food and water intakes monitored.

Diets

Three different diets were used throughout the investigation. The control was Agribrands 5075 Charles River Rodent Diet. This formula is considered to be a healthy maintenance diet for rodents and is the standard chow used in Concordia University's Animal Care Facility. The macronutrient composition is similar to what is recommended to humans during a maintenance phase of weight loss. The diet used to simulate a typical high-fat North American diet, and to control for fat content, is the Harlan Teklad TD.110229. This diet is a modification of the manufacturer's more commonly used TD.88137 to include additional L-methionine to represent the same total methionine content in the LCHF diet. Elevated levels of methonine have been suggested as a possible contributor to cardiovascular disease [Alvarez-Maqueda *et al*, 2004]. Lastly, the diet used to simulate the LCHF diet was the Harlan Teklad TD.04524. This diet is also a modification of the commonly used TD.88137 to decrease carbohydrate

content while maintaining fat content. The high-fat North American diet and the LCHF diet have similar fat content and cholesterol content to ensure that any differences observed in outcome measures will be due to variations in macronutrient composition. Refer to Table 1 for specific diet composition details.

Table 1. Detailed Diet Compositions

Product Name	Agribrands 5075 Charles River Rodent Diet	Harlan Teklad TD.110229	Harlan Teklad TD.04524
	CON	WD	LCHF
Calories from Carbohydrate (%)	63	42	11
Calories from Protein (%)	24	16	46
Calories from Fat (%)	14	42	43
Caloric Density (kcal/g)	4.1	4.5	4.4
Methionine Content (%)	0.39	1.3	1.3
Cholesterol (g/kg)	0.01	1.5	1.5

CON = Control diet, WD = high-fat, Western diet, LCHF = Low-carbohydrate, high-fat diet

2.3 Outcome Measures

Activity Level

To ensure that any differences in heart size are due to the altered macronutrient composition of the diets and not changes in liveliness, activity levels of the mice were measured. A smaller sample of 3 mice per group was used to estimate activity levels of the groups. At week-6 and week-12 mice were placed in cages equipped with running wheels capable of counting wheel turns per set unit of time. Mice were allowed free access to the wheels while in the cages. At each time point, data was collected for 24-hours for 3 consecutive days. To adjust for learning and adaptation, a mean running distance per day will be used in data analysis.

Body Weights

Mice were weighed throughout the duration of the investigation to monitor for any deformities or defects. Initial body weights were measured at baseline. Afterwards, weights were taken once per week. At the end of the investigation, body weights were measured prior to and following a 12-hour fast. Pre-fast weights were used in all calculations and analyses. All weights were measured to 0.1 g with the Dial-o-gram balance (OHAUS, Florham Park, NJ).

Fasting and Non-Fasting Glucose

To monitor the effects of the intervention diets on glucose tolerance, fasting and non-fasting glucose measures were taken. Both fasting and non-fasting glucose measures were taken at the conclusion of the dietary intervention, prior to euthanasia. Fasting glucose measures were obtained following a 12-hour fast. All values were collected with the Precision Xtra glucometer (Abbott Laboratories, Alamaeda, CA). Blood samples for both measures were acquired from a tail bleed. Measures were recorded to an accuracy of 0.1 mmol/L.

Heart Size

A commonly used measure to determine the presence cardiac hypertrophy or heart size is the heart weigh-to-tibia length ratio [Yin *et al*, 1982]. Wet heart weights were taken in accordance with the standardized procedures set forth by Animal Models of Diabetic Complications Consortium [AMDCC, 2004]. Immediately following euthanasia by carbon dioxide gas, mice were dissected and blood was extracted from the heart by means of a cardiac

puncture. Following removal of blood from the ventricles, hearts were resected and blotted prior to being weighed. Heart weights were measured to an accuracy of 0.1 mg with the Pioneer balance (OHAUS, Pine Brook, NJ).

At the time of dissection, the left hind leg of the mouse was removed at the level of the femur and stored at -20 degrees Celsius to await further processing. At a later date, mouse legs were heated to 125 degrees Celsius to allow for easy removal of the soft tissues surrounding the tibia. Once tibiae had been isolated and given time to dry, length measurements were taken from the mid-point between the medial and lateral condyles to the mid-point between the medial and lateral maleoli. Tibia lengths were measured to an accuracy of 0.01 mm with a Neiko digital calliper (Montevideo, Uraguay). The heart weight-to-tibia length ratio was computed by dividing the heart weight in milligrams, by the tibia length in millimeters.

Cross Sectional Area

Following euthanasia by means of carbon dioxide gas, young mice were immediately dissected and blood was removed from the heart via cardiac puncture. A tie was place around the superior vena cava, aortic arch, and pulmonary truck to close off the major vessels exiting the heart. At this time, the heart was injected with a 5% gelatin solution to expand the ventricles, and then covered with ice to allow the gelatin to set. Once gelatin had solidified, hearts were resected and instantly imbedded in Optimal Cutting Temperature medium. Hearts were slowly cooled in isopentane immersed in liquid nitrogren to limit any cracking, and stored at -80 degrees Celsius for further processing. Thirty

micrometer sections of the heart were taken at the level of the papillary muscles with the MICROM HM560 cryostar (Thermo Scientific, Layfayette, CO) and placed on Superfrost slides (Fisher Scientific, Pittsburgh, PA). Sections were laid flat to adhere for 2 hours. As soon as sections had completely adhered, slides were stained with Harris' Hematoxylin and then counterstained with 5% eosin solution to better visualize boundaries. Cross Sectional Area was computed using ImageJ software (NIH, Besthesda, MD) and reported in millimetres squared. Sections were imaged on the Amscope MD900E microscope (Amscope, Fairbanks, CA) at 40x magnification.

Glycogen Visualization

Following the measurement of the wet heart weight, young mouse hearts were imbedded in Optimal Cutting Temperature medium, and then slowly cooled with isopentane immersed in liquid nitrogen. Eight micrometer sections were cut with MICROM HM560 cryostar (Thermo Scientific, Layfayette, CO). All sections were taken at the level of the papillary muscles and then placed on Superfrost slides (Fisher Scientific, Pittsburgh, PA). Slides were laid flat for 2 hours to adhere. To visualize intracellular glycogen, sections were stained with a periodic acid solution and Schiff reagent staining kit 395B (Sigma Aldrich, St Louis, MO). The stain was applied according to the recommended guidelines provided by the manufacturer. Sections were then counter stained with Harris' Hematoxylin to visualize nuclei. Sections were imaged at 200x magnification in established 16-bit colour settings. Colour intensity was determined by setting software to identify all pixels with a given red wavelength associated to the positive staining and

presence of glycogen in a set area. All analyses were performed using ImageJ software (NIH, Besthesda, MD) and are based on a mean of 3 images per sample.

2.4 Statistical Analysis

All data is presented as a mean (±SD), and the SPSS 13.0 statistical package (SPSS Inc, Chicago, IL) was used for analysis. One-way analysis of variance was used to analyze the differences among the groups, and the differences between 2 groups were evaluated by repeated t-tests with a bonferoni correction. For analysis of cross sectional area where only 2 groups are compared, a student's t-test was used to evaluate any differences. $P<0.05$ was considered to be statistically significant.

CHAPTER 3: RESULTS AND DISCUSSION

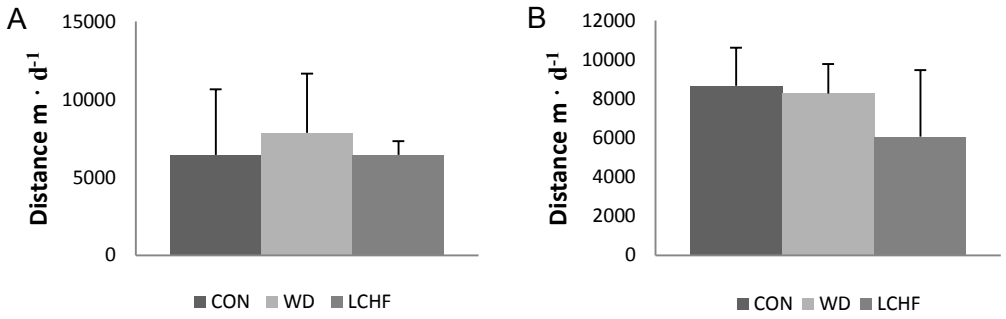
3.1 Results

A total of 42 mice were used in this investigation; 24 young mice and 18 mature mice. Of the 24 young mice, 9 were assigned to the Control diet, 6 were assigned to the Western diet, and 9 were assigned to the LCHF diet. Of the 18 mature mice, 6 were assigned to the Control diet, 6 were assigned to the Western diet, and 6 were assigned to the LCHF diet. Two of the 42 mice were removed from their intervention diet due to dental deformities (1 from mature Western diet group and 1 from mature LCHF diet group). Two of the 42 mice died during the 12-week intervention before completion (1 from mature Western diet group and 1 from mature LCHF diet group); cause of death was unknown.

Activity level

A smaller sample of 3 animals per group were used to estimate activity levels. There were no associations between intervention diets and average distance ran per day at both Week 6 and Week 12. This would suggest that any differences in heart size or glycogen stores are not due to disparities in activity over the 12 weeks of the intervention. Data from Week 12 is presented in Figure 2.

Figure 2. Mean distance ran per day at Week 12 in young and mature mice (n = 18)



A) Mean distance per day in young mice (CON = 6438.4 ± 4213.8, WD = 7856.0 ± 3793.1, LCHF = 6450.5 ± 869.6) **B)** Mean distance per day in mature mice (CON = 8663.9 ± 1945.0, WD = 8267.3 ± 1514.7, LCHF = 6063.7 ± 3405.1).
Data presented as means ± SD.

Body weights

As shown in Table 2, there were no differences in baseline weights between groups in both young and mature animals. At the conclusion of the 12-week dietary intervention, young mice following the LCHF diet gained less weight than mice following both the Control diet and Western diet ($P < 0.05$). There were no notable differences in weight gain between young mice following the Control diet and Western diet after the 12-weeks. Following the 12-hour fast, young mice placed on the LCHF diet still weighed less than young mice placed on the Western diet ($P < 0.05$), however in the fasted state there were no differences in weight between young mice fed the Control diet and young mice fed the LCHF diet or young mice fed the Control diet and young mice fed the Western diet. In mature mice at the conclusion of the 12-week intervention, no significant

differences in weight gain were noted between groups. There was a trend towards mature mice fed the LCHF diet gaining less weight than the mature mice fed the Control diet or Western diet however the values did not reach significance (P = 0.059). Following the 12-hour fast, there remained no notable differences in weight between the groups.

Fasting and Non-Fasting Glucose

At completion of the 12-week dietary intervention, non-fasting glucose values did not differ between groups in both young and mature mice. In addition, both young and mature mice displayed no notable differences between groups in fasting glucose values after consumption of their assigned diets for 12-weeks. Data is displayed in Table 2.

Table 2. Body weights and glucose measures for young and mature mice after 12-weeks on diet

	Young (n = 24)				Mature (n = 14)			
	CON	WD	LCHF	p-value	CON	WD	LCHF	p-value
Baseline Weight (g)	11.1 ± 1.3	12.0 ± 1.0	11.6 ± 1.3	0.346	28.0 ± 1.0	27.7 ± 1.3	27.2 ± 1.5	0.612
Pre-Fast Weight (g)	33.7 ± 3.8	37.3 ± 4.3	28.9 ± 2.4	<0.001*	40.0 ± 6.7	42.0 ± 4.2	32.7 ± 2.3	0.059
Post-Fast Weight (g)	30.9 ± 3.5	34.8 ± 3.7	27.5 ± 3.1	<0.010**	36.2 ± 6.2	37.6 ± 3.2	30.2 ± 2.0	0.089
Non-Fasting Glucose (mmol/L)	11.6 ± 2.1	10.4 ± 1.6	10.0 ± 1.9	0.216	11.9 ± 3.5	10.1 ± 1.1	8.2 ± 1.4	0.130
Fasting Glucose (mmol/L)	7.8 ± 1.7	7.7 ± 1.6	7.8 ± 1.4	0.984	7.4 ± 2.2	7.6 ± 2.7	6.0 ± 1.6	0.563

*P<0.05 for LCHF compared to CON and WD. **P<0.05 for LCHF compared to WD only.

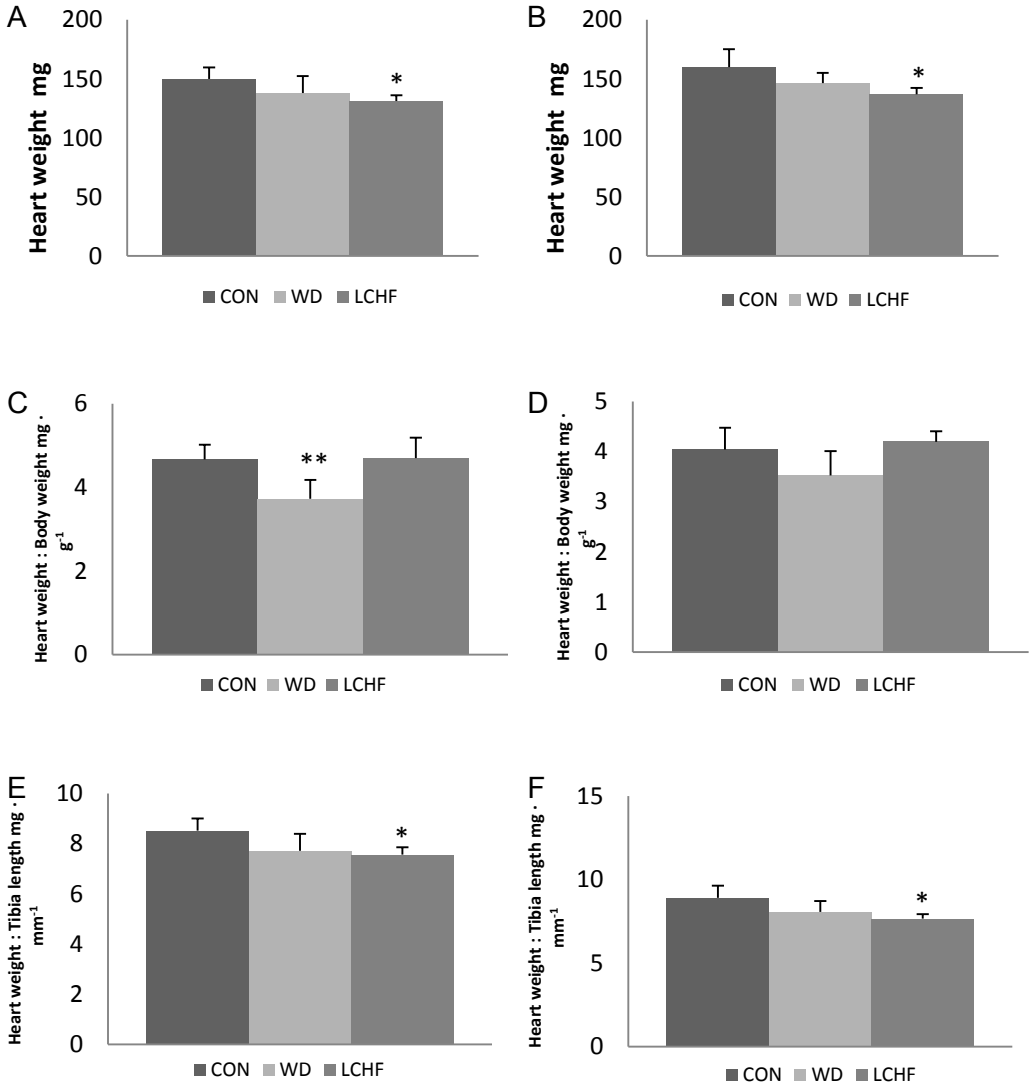
Heart Size

After 12-weeks of following the assigned diets, there were notable differences between groups present in both young and mature mice. Young mice

following the LCHF diet had significantly smaller hearts (131.2 ± 4.9 mg) than mice following the Control diet (150.1 ± 9.6 mg). There were no notable differences in heart size of young mice following the Western diet (138.0 ± 14.4 mg) when compared to both Control and LCHF mice. Mature mice expressed similar differences in heart size after 12-weeks on the assigned diets. Mature mice following the LCHF diet had significantly smaller hearts (137.0 ± 5.5 mg) than mice following the Control diet (160.0 ± 15.1 mg). Parallel to trends seen in young mice, there were no notable differences in heart size of mature mice following the Western diet (146.5 ± 8.6 mg) when compared to both Control and LCHF mice. When the heart weights of the young mice were normalized to their body weight, differences were noted between mice following the Western diet (3.73 ± 0.46 mg/g) and both the mice following the Control diet (4.67 ± 0.35 mg/g) and mice following the LCHF diet (4.70 ± 0.49 mg/g). When the heart weights were normalized to body weights in mature mice, there were no significant differences between mice following the Control diet (4.04 ± 0.43 mg/g), mice following the Western diet (3.52 ± 0.49 mg/g), and mice following the LCHF diet (4.19 ± 0.21 mg/g). Lastly, when heart weights were normalized to tibia length in young mice, trends were similar to the initial comparisons of the absolute heart weights. Young mice following the LCHF diet exhibited a smaller heart weight-to-tibia length ratio (7.56 ± 0.30 mg/mm) than mice following the Control diet (8.53 ± 0.49 mg/mm). The heart weight-to-tibia length ratio of young mice following the Western diet (7.72 ± 0.69 mg/mm) did not differ significantly from values seen in Control mice or LCHF mice. In the mature mice, parallel

trends in heart weight-to-tibia length ratio were observed. Mature mice assigned to the LCHF diet displayed a smaller heart weight-to-tibia length ratio (7.66 ± 0.27 mg/mm) when compared to mice assigned the Control diet (8.90 ± 0.74 mg/mm). Similarly, the heart weight-to-tibia length in mature mice assigned the Western diet (8.07 ± 0.65 mg/mm) did not differ significantly from Control mice or LCHF mice. Refer to Figure 3 for a summary of the data.

Figure 3. Heart weight in young and mature mice normalized to body weight and tibia length

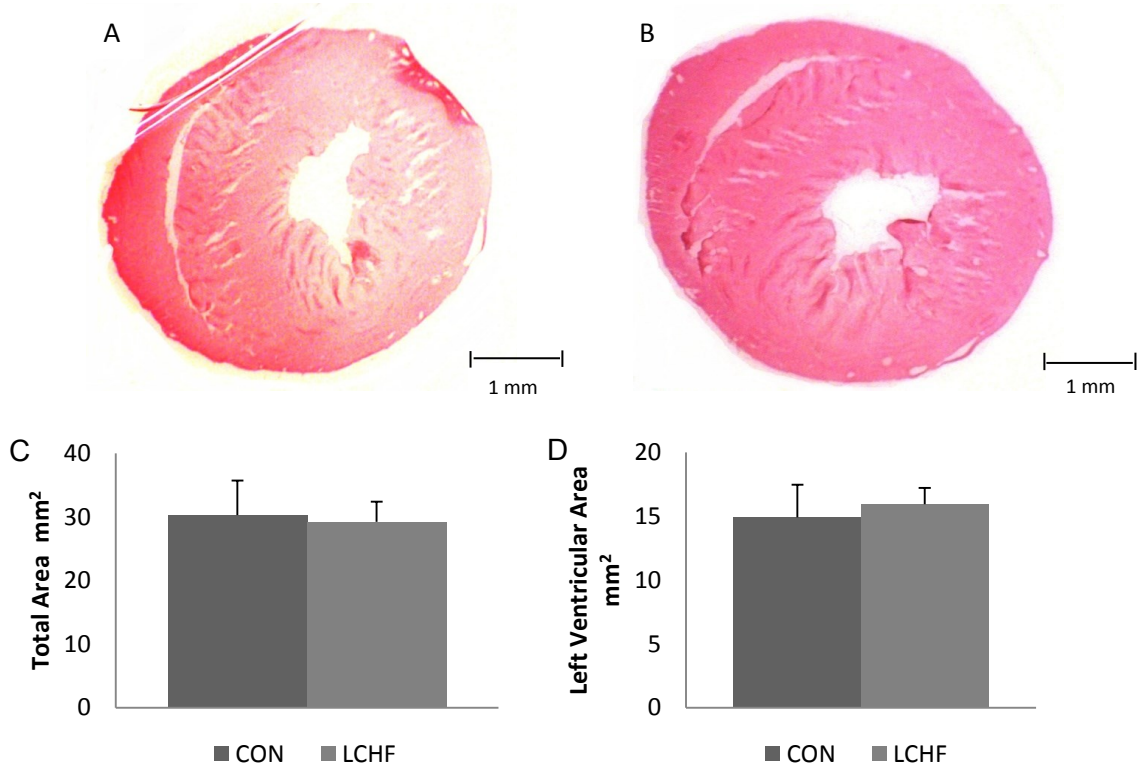


A) Heart weight in young mice (n = 18). **B)** Heart weight in mature mice (n = 14). **C)** Heart weight normalized to body weight in young mice (n = 18). **D)** Heart weight normalized to body weight in mature mice (n = 14). **E)** Heart weight normalized to tibia length in young mice (n = 18). **F)** Heart weight normalized to tibia length in mature mice (n = 14). * P<0.05 for LCHF compared to CON. **P<0.05 for WD compared to CON and LCHF.

Cross Sectional Area

Cross sectional areas were only measured in young mice following control diets and LCHF diets as cardiac tissue from mice following the Western diet was unavailable at time of analysis. When comparing the total heart cross sectional area in young mice, there appear to be no significant differences between mice following the Control diet (30.27 ± 5.45 mm^2) and mice following the LCHF diet (29.21 ± 3.21 mm^2). Similarly, when isolating measurements to include only the cross sectional area of the left ventricle, no differences between Control mice (14.91 ± 2.55 mm^2) and LCHF mice (15.94 ± 1.29 mm^2) emerged. Data is represented in Figure 4.

Figure 4. Total cross sectional area and left ventricular cross sectional area in young mice



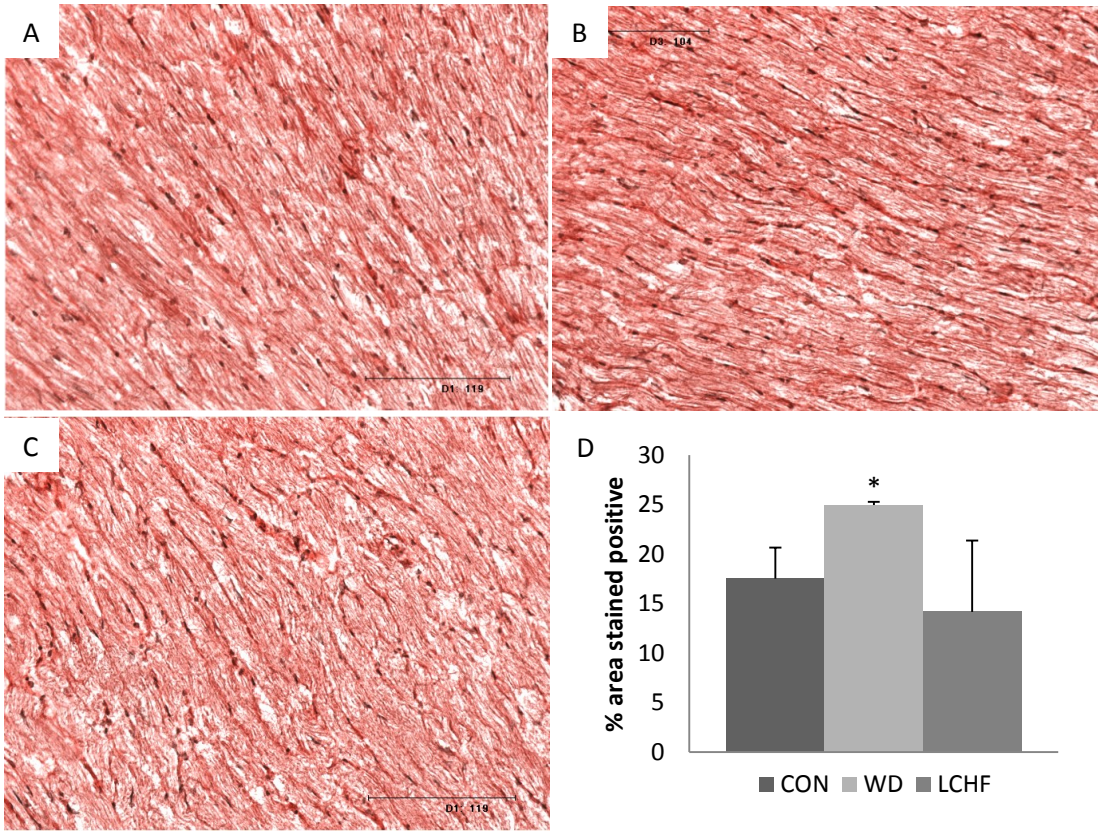
A) Young mouse cross section of heart. Control diet. 30 µm section stained with H&E. 40x magnification. **B)** Young mouse cross section of heart. LCHF diet. 30 µm section stained with H&E. 40x magnification. **C)** Total cross sectional area of young mouse hearts (n = 6). **D)** Left ventricular area of young mouse hearts (n = 6).

Glycogen Visualization

The percentage of cardiomyocyte area in young mice stained positive for glycogen with the Periodic Acid-Schiff Reagent protocol varied between groups. Significant differences were observed between cardiac sections taken from young mice following the Western diet (24.96 ± 0.31 %) and both young mice following the Control diet (17.54 ± 3.13 %) and mice following the LCHF diet

(14.18 ± 7.19 %) . It should also be noted that cardiac sections taken from young mice following the Control diet trend towards having an increased percentage of area stained positive for glycogen when compared to LCHF mice, but values did not reach significance. Data is illustrated in Figure 5.

Figure 5. Percentage of cellular area stained positive for glycogen



A)Section of young mouse cardiac tissue. Control diet. 8 µm section stained with PAS and Harris' Hematoxylin. 200x magnification. **B)** Section of young mouse cardiac tissue. Western diet. 8 µm section stained with PAS and Harris' Hematoxylin. 200x magnification. **C)** Section of young mouse cardiac tissue. LCHF diet. 8 µm section stained with PAS and Harris' Hematoxylin. 200x magnification. **D)** Percentage of cellular area stained positive for glycogen (n = 15). *P<0.05 for Western diet when compared to control and LCHF diets

3.2 Discussion

The objective of the investigation was to determine whether or not the consumption of a LCHF diet in young subjects effects heart size. In the case that differences in heart size were noted after 12 weeks of dietary intervention, our second objective was to determine whether differences in heart size were due to alterations in cross sectional area or disparities in glycogen content. The results of this study suggest that the heart size in both young and mature mice are greatly affected by a LCHF diet. Both young and mature mice following the LCHF diet displayed smaller heart weights than mice following the standard chow Control diet. Although values did not reach significance, there were evident trends that mice assigned to the LCHF diet had smaller heart weights than mice assigned to the high-fat Western diet. This would indicate that differences in heart weight are not solely related to the increased fat content in the diets, but rather a combination of the decreased carbohydrate content and the increased fat content together. With respect to heart weight, responses to the dietary manipulations in mature mice paralleled the responses observed in young mice. This would suggest that age does not have a significant impact on the factors contributing to the decreased heart weights. Our data further suggested that the differences in heart weight observed in young mice were due to alterations in glycogen content rather than cross sectional area. The intracellular glycogen content of the young mice assigned to the LCHF diet was decreased in comparison to mice assigned the high-fat Western diet. Again, although data did not reach significance, there is a trend towards the cardiomyocytes of mice

following the LCHF diet to retain less glycogen than the cardiomyocytes of mice following the Control diet. This finding suggests that differences in heart weight could be related to discrepancies in glycogen content. We would expect to see similar trends in glycogen content in the mature mice.

In a recent study published in 2013 [Liu & Lloyd, 2013], similar discrepancies in glycogen content of cardiomyocytes were noted after obese Sprague-Dawley rats were placed on a control diet or LCHF diet for 2 weeks. Previous work in rodents has also suggested that short term use of a LCHF diet can lead to derangements in glycogen stores within the heart [Wang *et al*, 2008]. Although the exact mechanisms behind the effects of the LCHF diet on heart size and glycogen stores are unknown, the conserved phosphoinositide 3-kinase (PI3K) pathway has been implicated in the determination of heart size [Shioi *et al*, 2000]. Because insulin signaling is an essential regulator for this pathway, alterations of serum insulin levels in response to a LCHF diet could be responsible for the alterations in heart size observed in our study. The PI3K pathway also well known to play a key role in glycogen synthase activity [Moule *et al*, 1995]. However further investigation into the exact mechanisms behind a decreased heart size in response to a LCHF diet will confirm present hypotheses. With complete picture of the phenomenon, more concrete recommendations can be made to clinicians.

The present study provides important information for clinicians to consider before prescribing a LCHF diet to adolescents or adults. As previously discussed, glycogen availability is extremely important for both developing and stressed

hearts. By decreasing cardiac glycogen stores with a LCHF diet, individuals are at risk for not being able to meet metabolic demands during events of cardiac stress, increasing the likelihood of myocardial ischemia. Clinicians prescribing this diet to children should also be cautioned, as prolonged use of a LCHF diet could lead to the development of a smaller heart over the years. One needs to consider all of the risks associated to this diet prior to initiating the intervention.

Strengths of the current study include the use of a second control group, the high-fat Western diet group, to control for the high-fat content of the LCHF diet. We were able to distinguish whether the effects of the dietary intervention were due to an increase in the consumption of fats, or the carbohydrate restriction. In addition, our intervention period of 12-weeks allowed for sufficient time for alterations in cardiac remodeling to occur. We noted differences in heart weight that were not apparent after only 2 weeks of dietary intervention [Liu & Lloyd, 2013]. Weaknesses of our investigation include the use of a non-obese murine model. The majority of individuals who are prescribed a LCHF diet are overweight or obese with the goal of weight loss in mind. This may limit the applicability of our findings. Secondly, cross sectional area and glycogen content measures were only performed in young mice. Although it is expected that mature mice would demonstrate similar trends as observed in young mice, the added data would strengthen the conclusions.

CHAPTER 4: MANUSCRIPT

Implications of a low-carbohydrate, high-fat diet on heart size in a young murine model.

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Abstract

Objectives: Despite the growing popularity or carbohydrate restricted diets, consequences of long term use remain widely unknown. The objective of the current study is to evaluate the effects of LCHF diets on heart size and cardiac glycogen content in young mice.

Methods: Young mice (age 21 d, n = 24) and mature mice (age 84 d, n = 18) were placed on either a LCHF diet, a WD diet or a control diet for 12 weeks. At the competition of the dietary intervention, wet heart weights were measured to compute the heart weight-to-tibia length ratio and sections of cardiac tissue were stained with periodic acid and Schiff reagent to visualize glycogen.

Results: Mice following the LCHF diet displayed a smaller heart weight-to-tibia length ratio when compared to controls. The trend was observed in both young ($p = 0.012$) and mature ($p = 0.024$) mice. Cardiac tissue from mice consuming the LCHF diet had a reduced percentage of total area stained positive for glycogen when compared to mice following the WD diet ($p = 0.011$).

Conclusions: A LCHF diet can lead to the development of a smaller heart in young and mature mice. Alterations in intercellular cardiac glycogen content may contribute to differences observed in heart weight. Cardiac restricted diets should be recommended with caution as long term cardiac developmental impairments are unknown.

Key Words

Carbohydrate restriction, Diet, Glycogen, Heart

Introduction

The incidence of childhood obesity in North America and around the world has risen significantly over the past decade. Childhood obesity is associated with altered bone development, hyperlipidemia, glucose intolerance, and hepatic steatosis (Dietz, 1998). In more severe, cases children are acquiring hypertension and sleep apnea (Dietz, 1998). In 2010, approximately 6.7% of all children were considered to be overweight or obese with prevalence expected to rise to 9.1% in the year 2020 (de Onis *et al*, 2010). With such a large percentage of the Western world struggling with childhood obesity, clinicians are constantly searching for healthy and effective weight management strategies. Many of these strategies focus on diets involving caloric restriction or macronutrient manipulation. A low-carbohydrate, high-fat diet (LCHF) popularized by Dr. Robert Atkins in the 1972 was the first of many fad diets where dieters will significantly limit the calories consumed from carbohydrates while simultaneously increasing caloric intake from fat and protein sources (Atkins, 1998). By lowering the carbohydrate content in an individual's diet, glycogen stores will become depleted leaving the body to use fats as a primary source of ATP production. In overweight and obese adults, this type of LCHF diet has been associated with accelerated weight loss, decreases in systolic and diastolic blood pressure, improved LDL and HDL cholesterol profiles, and reduced insulin levels (Samaha *et al*, 2003 & Foster *et al*, 2003). More recently, clinicians have been recommending LCHF diets to children and adolescents to help with weight loss. Improvements in body mass index Z-scores and lipid profiles in adolescents

following a LCHP diet parallel the changes seen in the adult population (Krebs *et al*, 2010). However there are still safety concerns that remain when this diet is being used in in the adult population that could be even more detrimental to children and adolescents. It has been suggested that LCHP diets lead to an accelerated atherosclerotic process due to increased cholesterol retention in the vascular walls (Foo *et al*, 2009). It has also been shown that LCHP diets impair cardiac phosphate metabolism and cognitive performance (Holloway *et al*, 2011).

During short-term use, a LCHF diet does not appear to have a negative effect on overall cardiac function (Holloway *et al*, 2011), but over a longer period the effects of a prolonged state of ketosis on the heart and the overall growth and development are unknown. It is well accepted that the heart will rely predominantly on fatty acids for energy production; however various stresses can stimulate a greater reliance on glycogen. The importance of glycogen in the ischemic heart and hypertrophied heart are well documented (Wambolt *et al*, 1999 & Schaefer *et al,* 1997), It has also been shown that when the workload of the heart is increased, such as after stimulation by epinephrine (Collins-Nakai*et al*, 1994) or with increased exercise (Goodwin *et al*, 1996 & Hemming *et al*, 1996), the heart will depend more heavily on glycogen as an energy source. More recent studies have shown that glycogen in the developing embryonic heart is crucial for survival, with impairments to glycogen synthase activity being related to congenital heart disease (Pederson *et al*, 2004).

The present investigation will evaluate the effects of a LCHF diet, a high-fat western diet (WD) and a control diet on heart size and glycogen content

in young hearts. We hypothesize that prolonged use of a LCHF diet will negatively impact the growth and development of the young heart through alterations in cellular arrangement and glycogen content. We expect to see similar changes in both young and mature hearts. Furthermore, we hypothesize that these changes will be more prevalent in the LCHF group than the WD and control groups.

Methods

Animals

C57BL/6 mice were chosen as a suitable murine model for this investigation as they are known to demonstrate similar characteristics to humans following a LCHF diet; including weight loss, improved glucose tolerance, and decreased serum insulin levels (Kennedy *et al*, 2007). For our young model, 24 male C57BL/6 pups were weaned at 21 days after birth and placed immediately on their assigned diet (refer to Table 1 for diet compositions). In our mature model, at age 12 weeks of age, 18 male C57BL/6 mice had their standard chow replaced with the assigned diet. All diets were randomly assigned. Only male mice were used due to the known cardio protective effects of estrogens (Nathan & Chaudhuri, 1997). Mice were housed in a temperature and humidity controlled room with alternating 12-hour periods of light and dark and allowed *ad-libitum* access to water and assigned diet in accordance with Concordia University's animal research ethics committee. Food and water intakes were recorded regularly. In addition, body weight, activity levels were measured throughout the

investigation. Fasting and non-fasting glucose measures were taken at the conclusion of the dietary intervention, prior to euthanasia. Fasting glucose measures were obtained following a 12-hour fast. All values were collected with the Precision Xtra glucometer (Abbott Laboratories, Alamaeda, CA). Blood samples for both measures were acquired from a tail bleed and recorded to an accuracy of 0.1 mmol/L.

Heart Size

Wet heart weights were taken in accordance with the standardized procedures set forth by Animal Models of Diabetic Complications Consortium (AMDCC, 2004). Immediately following euthanasia by carbon dioxide gas, mice were dissected and blood was extracted from the heart by means of a cardiac puncture. Following removal of blood from the ventricles, hearts were resected and blotted prior to being weighed. Heart weights were measured to an accuracy of 0.1 mg with the Pioneer balance (OHAUS, Pine Brook, NJ). The left hind leg of the mouse was removed at the level of the femur and stored at -20 degrees Celsius. Mouse legs were then heated to 125 degrees Celsius to allow for easy removal of the soft tissues surrounding the tibia. Once tibiae had been isolated and given time to dry, length measurements were taken from the mid-point between the medial and lateral condyles to the mid-point between the medial and lateral maleoli. Tibia lengths were measured to an accuracy of 0.01 mm with a Neiko digital calliper (Montevideo, Uraguay). The heart weight-to-tibia length ratio was computed by dividing the heart weight in milligrams, by the tibia length in millimetres.

Glycogen Visualization

Following the measurement of the wet heart weight, young mouse hearts were imbedded in Optimal Cutting Temperature medium (Fisher Scientific, Pittsburgh, PA), and then slowly cooled with isopentane immersed in liquid nitrogen. Eight micrometer sections were cut with MICROM HM560 Cryostar (Thermo Scientific, Layfayette, CO). All sections were taken at the level of the papillary muscles and then placed on Superfrost slides (Fisher Scientific, Pittsburgh, PA) and given time to adhere. To visualize intracellular glycogen, sections were stained with a periodic acid solution and Schiff reagent staining kit 395B (Sigma Aldrich, St Louis, MO). The stain was applied according to the recommended guidelines provided by the manufacturer. Sections were then counter stained with Harris' Hematoxylin to visualize nuclei. Sections were imaged at 200x magnification with the Olympus BX-60 microscope (Ann Arbour, MI) in established 16-bit colour settings. Colour intensity was determined by setting software to identify all pixels with a given red wavelength associated to the positive staining and presence of glycogen in a set area. All analyses were performed using ImageJ software (NIH, Besthesda, MD) and are based on a mean of 3 images per sample.

Statistical Analysis

All data is presented as a mean (±SD), and the SPSS 13.0 statistical package (SPSS Inc, Chicago, IL) was used for analysis. One-way analysis of variance was used to analyze the differences among the groups, and the

differences between 2 groups were evaluated by repeated t-tests with a bonferoni correction. $P<0.05$ was considered to be statistically significant.

Results

A total of 42 mice were used in this investigation; 24 young mice and 18 mature mice. Of the 24 young mice, 9 were assigned to the Control diet, 6 were assigned to the Western diet, and 9 were assigned to the LCHF diet. Of the 18 mature mice, 6 were assigned to the Control diet, 6 were assigned to the Western diet, and 6 were assigned to the LCHF diet. Two of the 42 mice were removed from their intervention diet due to dental deformities (1 from mature Western diet group and 1 from mature LCHF diet group) and 2 of the 42 mice died during the 12-week intervention before completion (1 from mature Western diet group and 1 from mature LCHF diet group); cause of death was unknown. There were no differences in weight at baseline between groups in both the young and mature mice. At the conclusion of the dietary intervention, young mice following the LCHF diet weighed less than young mice following the WD and Control diets (Table 2). Mature mice did not follow this trend with no significant differences in weight between groups noted at the conclusion of the dietary intervention. In both young and mature mice, there were no discrepancies in activity level, fasting glucose, or non-fasting glucose values observed between groups. Data is presented in Table 2.

Heart Size

After 12-weeks of following the assigned diets, there were notable differences between groups present in both young and mature mice. Young mice following the LCHF diet had significantly smaller hearts (131.2 ± 4.9 mg) than mice following the Control diet (150.1 ± 9.6 mg). There were no notable differences in heart size of young mice following the WD diet (138.0 ± 14.4 mg) when compared to both Control and LCHF mice. In addition, mature mice following the LCHF diet had significantly smaller hearts (137.0 ± 5.5 mg) than mice following the Control diet (160.0 ± 15.1 mg); with mice following the WD diet (146.5 ± 8.6 mg) not expressing any differences. When normalized to their body weight, differences in heart weight were noted between mice following the WD diet (3.73 ± 0.46 mg/g) and both the mice following the Control diet (4.67 ± 0.35 mg/g) and LCHF diet (4.70 ± 0.49 mg/g). In mature mice, there were no significant differences noted between mice following the Control diet (4.04 ± 0.43 mg/g), WD diet (3.52 ± 0.49 mg/g), and LCHF diet (4.19 ± 0.21 mg/g). Lastly, when normalized to tibia length young mice following the LCHF diet exhibited a smaller heart weight-to-tibia length ratio (7.56 ± 0.30 mg/mm) than mice following the Control diet (8.53 ± 0.49 mg/mm); with WD diet mice (7.72 ± 0.69 mg/mm) not differing significantly from values seen in Control mice or LCHF mice. Parallel trends were observed in mature mice. Mature mice assigned to the LCHF diet displayed a smaller heart weight-to-tibia length ratio (7.66 ± 0.27 mg/mm) when compared to mice assigned the Control diet (8.90 ± 0.74 mg/mm); with WD diet

mice (8.07 ± 0.65 mg/mm) not differing significantly. Refer to Figure 1 for a summary of the above data.

Glycogen Visualization

The percentage of cardiomyocyte area in young mice stained positive for glycogen with the Periodic Acid-Schiff Reagent protocol varied between groups. Significant differences were observed between cardiac sections taken from young mice following the Western diet (24.96 ± 0.31 %) and both young mice following the Control diet (17.54 ± 3.13 %) and mice following the LCHF diet (14.18 ± 7.19 %) . It should also be noted that cardiac sections taken from young mice following the Control diet trend towards having an increased percentage of area stained positive for glycogen when compared to LCHF mice, but values did not reach significance. Data is illustrated in Figure 2.

Discussion

The objective of the investigation was to determine whether or not the consumption of a LCHF diet in young subjects effects heart size. In the case that differences in heart size were noted after 12 weeks of dietary intervention, our second objective was to determine whether differences in heart size were due to differences in cross sectional area or disparities in glycogen content. The results of this study suggest that the heart size in both young and mature mice are greatly affected by a LCHF diet. Both young and mature mice following the LCHF diet displayed smaller heart weights than mice following the standard chow Control diet. Although values did not reach significance, there were evident

trends that mice assigned to the LCHF diet had smaller heart weights than mice assigned to the high-fat Western diet. This would indicate that differences in heart weight are not solely related to the increased fat content in the diets, but rather a combination of the decreased carbohydrate content and the increased fat content together. With respect to heart weight, responses to the dietary manipulations in mature mice paralleled the responses observed in young mice. This would suggest that age does not have a significant impact on the factors contributing to the decreased heart weights. Our data further suggested that the differences in heart weight observed in young mice were due to alterations in glycogen content. The intracellular glycogen content of the young mice assigned to the LCHF diet was decreased in comparison to mice assigned the high-fat Western diet. Again, although data did not reach significance, there is a trend towards the cardiomyocytes of mice following the LCHF diet to retain less glycogen than the cardiomyocytes of mice following the Control diet. This finding suggests that differences in heart weight could be related to discrepancies in glycogen content. We would expect to see similar trends in glycogen content in the mature mice.

In a recent study published in 2013 [Liu & Lloyd, 2013], similar discrepancies in glycogen content of cardiomyocytes were noted after obese Sprague-Dawley rats were placed on a control diet or LCHF diet for 2 weeks. Previous work in rodents has also suggested that short term use of a LCHF diet can lead to derangements in glycogen stores within the heart [Wang *et al*, 2008]. Although the exact mechanisms behind the effects of the LCHF diet on heart size

and glycogen stores are unknown, the conserved phosphoinositide 3-kinase (PI3K) pathway has been implicated in the determination of heart size [Shioi *et al*, 2000]. Because insulin signaling is an essential regulator for this pathway, alterations of serum insulin levels in response to a LCHF diet could be responsible for the alterations in heart size observed in our study. The PI3K pathway also well known to play a key role in glycogen synthase activity [Moule *et al*, 1995]. However further investigation into the exact mechanisms behind a decreased heart size in response to a LCHF diet will confirm present hypotheses. With complete picture of the phenomenon, more concrete recommendations can be made to clinicians.

The present study provides important information for clinicians to consider before prescribing a LCHF diet to adolescents or adults. As previously discussed, glycogen availability is extremely important for both developing and stressed hearts. By decreasing cardiac glycogen stores with a LCHF diet, individuals are at risk for not being able to meet metabolic demands during events of cardiac stress, increasing the likelihood of myocardial ischemia. Clinicians prescribing this diet to children should also be cautioned, as prolonged use of a LCHF diet could lead to the development of a smaller heart over the years. One needs to consider all of the risks associated to this diet prior to initiating the intervention.

Strengths of the current study include the use of a second control group, the high-fat Western diet group, to control for the high-fat content of the LCHF diet. We were able to distinguish whether the effects of the dietary intervention were due to an increase in the consumption of fats, or the carbohydrate

restriction. In addition, our intervention period of 12-weeks allowed for sufficient time for alterations in cardiac remodeling to occur. We noted differences in heart weight that were not apparent after only 2 weeks of dietary intervention [Liu & Lloyd, 2013]. Weaknesses of our investigation include the use of a non-obese murine model. The majority of individuals who are prescribed a LCHF diet are overweight or obese with the goal of weight loss in mind. This may limit the applicability of our findings. Secondly, glycogen content measures were only performed in young mice. Although it is expected that mature mice would demonstrate similar trends as observed in young mice, the added data would strengthen the conclusions.

Table 1. Detailed Diet Compositions

Product Name	Agribrands 5075 Charles River Rodent Diet	Harlan Teklad TD.110229	Harlan Teklad TD.04524
	CON	**WD**	**LCHF**
Calories from Carbohydrate (%)	63	42	11
Calories from Protein (%)	24	16	46
Calories from Fat (%)	14	42	43
Caloric Density (kcal/g)	4.1	4.5	4.4
Methionine Content (%)	0.39	1.3	1.3
Cholesterol (g/kg)	0.01	1.5	1.5

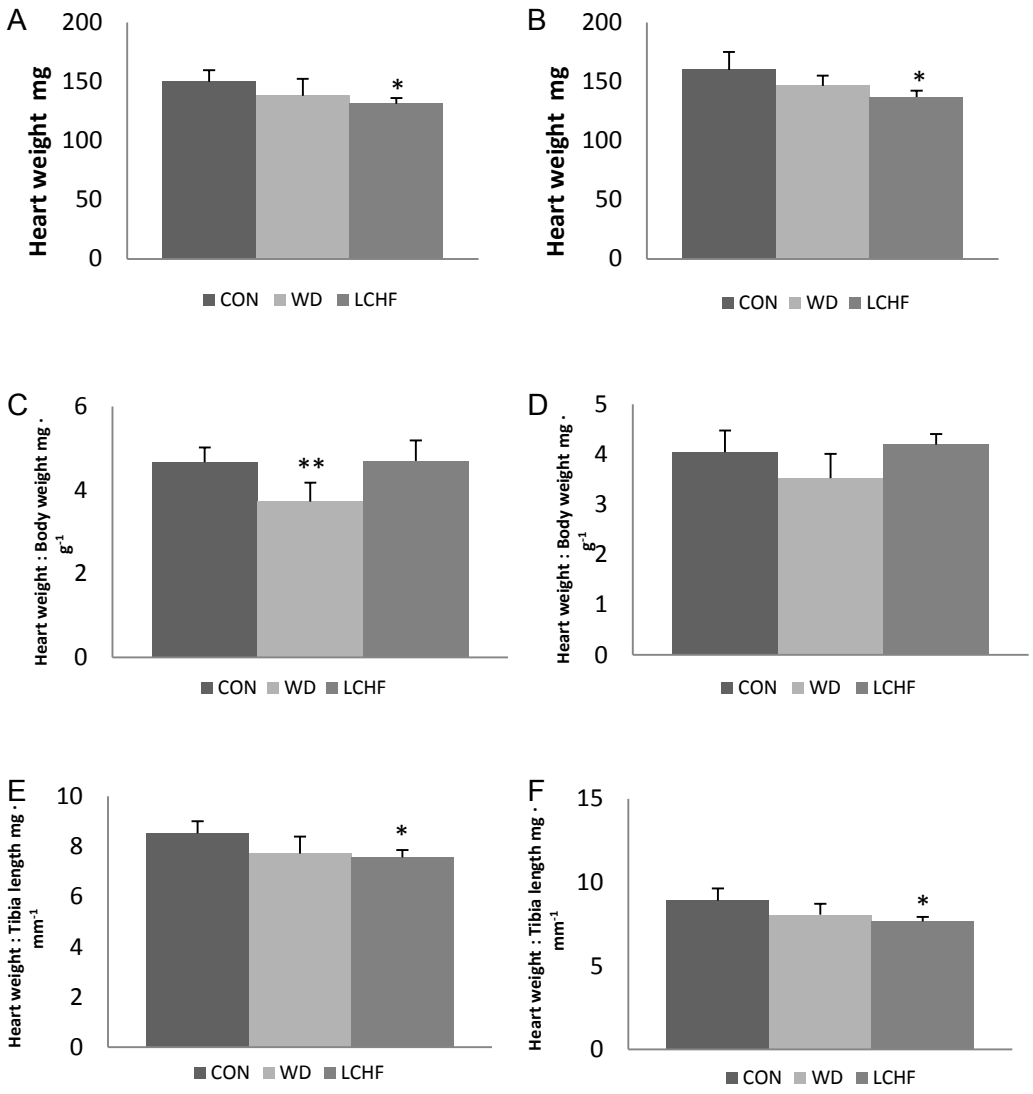
CON = Control diet, WD = high-fat, Western diet, LCHF = Low-carbohydrate, high-fat diet

Table 2. Body weights, activity level and glucose measures for young and mature mice after 12-weeks on diet

	Young (n = 24)				Mature (n = 14)			
	CON	WD	LCHF	p-value	CON	WD	LCHF	p-value
Baseline Weight (g)	11.1 ± 1.3	12.0 ± 1.0	11.6 ± 1.3	0.346	28.0 ± 1.0	27.7 ± 1.3	27.2 ± 1.5	0.612
Activity level (km/d)	6.43 ± 4.21	7.85±3.79	6.45± 8.69	0.838	8.66 ± 1.95	8.26 ± 1.51	6.06 ± 3.41	0.504
Pre-Fast Weight (g)	33.7 ± 3.8	37.3 ± 4.3	28.9 ± 2.4	<0.001*	40.0 ± 6.7	42.0 ± 4.2	32.7 ± 2.3	0.059
Post-Fast Weight (g)	30.9 ± 3.5	34.8 ± 3.7	27.5 ± 3.1	<0.010**	36.2 ± 6.2	37.6 ± 3.2	30.2 ± 2.0	0.089
Non-Fasting Glucose (mmol/L)	11.6 ± 2.1	10.4 ± 1.6	10.0 ± 1.9	0.216	11.9 ± 3.5	10.1 ± 1.1	8.2 ± 1.4	0.130
Fasting Glucose (mmol/L)	7.8 ± 1.7	7.7 ± 1.6	7.8 ± 1.4	0.984	7.4 ± 2.2	7.6 ± 2.7	6.0 ± 1.6	0.563

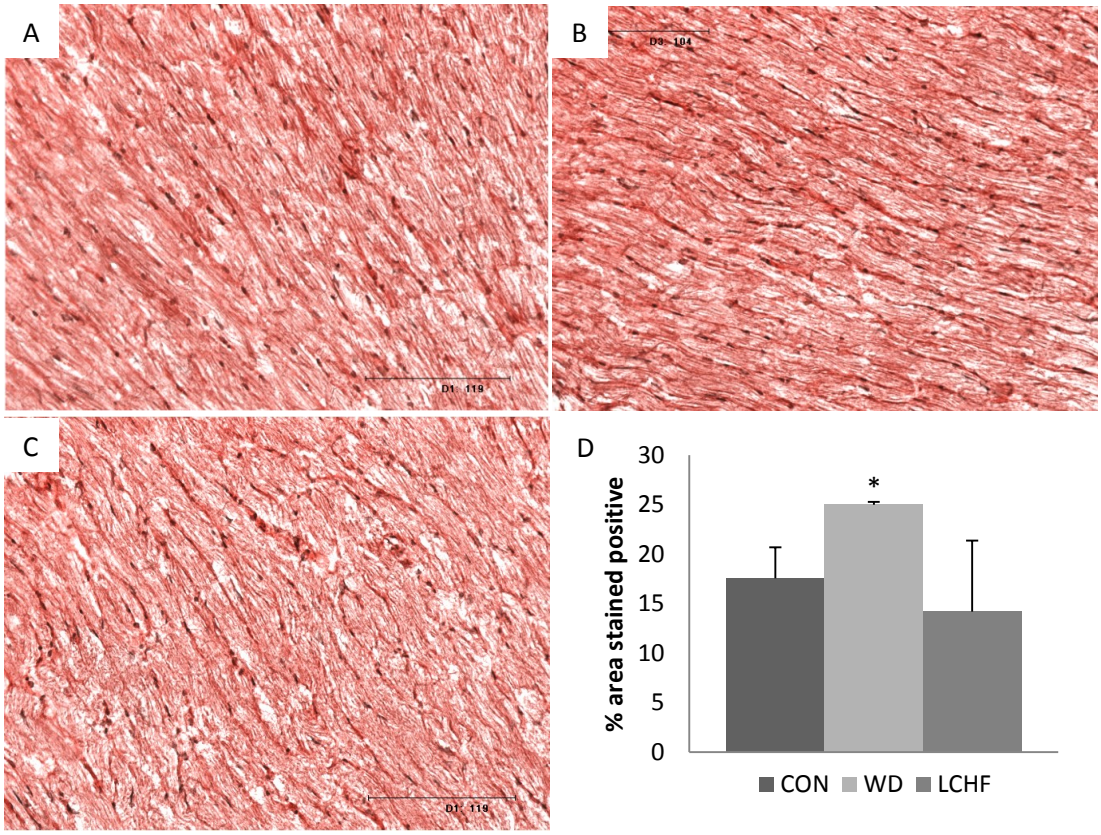
*P<0.05 for LCHF compared to CON and WD. **P<0.05 for LCHF compared to WD only.

Figure 1. Heart weight in young and mature mice normalized to body weight and tibia length



A) Heart weight in young mice (n = 18). **B)** Heart weight in mature mice (n = 14). **C)** Heart weight normalized to body weight in young mice (n = 18). **D)** Heart weight normalized to body weight in mature mice (n = 14). **E)** Heart weight normalized to tibia length in young mice (n = 18). **F)** Heart weight normalized to tibia length in mature mice (n = 14). * P<0.05 for LCHF compared to CON. **P<0.05 for WD compared to CON and LCHF.

Figure 2. Percentage of cellular area stained positive for glycogen



A)Section of young mouse cardiac tissue. Control diet. 8 µm section stained with PAS and Harris' Hematoxylin. 200x magnification. **B)** Section of young mouse cardiac tissue. Western diet. 8 µm section stained with PAS and Harris' Hematoxylin. 200x magnification. **C)** Section of young mouse cardiac tissue. LCHF diet. 8 µm section stained with PAS and Harris' Hematoxylin. 200x magnification. **D)** Percentage of cellular area stained positive for glycogen (n = 15). *P<0.05 for Western diet when compared to control and LCHF diets

CONCLUSIONS

The current findings suggest that the use of a LCHF diet can have negative implications for the heart. In this preliminary investigation it was shown that heart size and glycogen content are greatly affected by a LCHF diet in young subjects, with mature subjects demonstrating similar trends. Further investigations will need to examine the potential mechanisms behind this observed response. By understanding the processes involved in the alterations of cardiac glycogen content in response to LCHF diets, more concrete conclusions can be drawn about the overall safety of their use in both children and adults. Clinicians must still remain cautious when prescribing these diets to their patients as long term effects are still unclear. Should LCHF diets impair the heart's ability to perform under stressed conditions, their use in at-risk populations should be questioned.

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Appendix A – Statistical Tables

One way ANOVA- Young Activity Level

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	3	19315.32	6438.44	17756340
WD	3	23568.04	7856.013	14387263
LCHF	3	19351.36	6450.453	756246.4

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3985257	2	1992629	0.181699	0.838275	5.143253
Within Groups	65799698	6	10966616			
Total	69784955	8				

One way ANOVA –Mature Activity Level

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	2	17327.82	8663.91	3783175
WD	3	24801.88	8267.293	2294258
LCHF	2	12127.46	6063.73	11594905

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8160246	2	4080123	0.81739	0.503925	6.944272
Within Groups	19966596	4	4991649			
Total	28126842	6				

One Way ANOVA – Young baseline weights

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	9	99.6	11.06667	1.63
WD	6	72.2	12.03333	0.926667
LCHF	9	104	11.55556	1.810278

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.422778	2	1.711389	1.117666	0.345732	3.4668
Within Groups	32.15556	21	1.531217			
Total	35.57833	23				

One Way ANOVA - Mature baseline weights

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	167.8	27.96667	0.926667
WD	4	110.6	27.65	1.776667
LCHF	4	108.6	27.15	2.39

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.600952	2	0.800476	0.513924	0.611823	3.982298
Within Groups	17.13333	11	1.557576			
Total	18.73429	13				

One way ANOVA – Young pre-fast weights

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	9	303.2	33.68889	14.33861
WD	6	223.7	37.28333	18.59367
LCHP	9	260.6	28.95556	5.875278

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	260.7268	2	130.3634	10.74932	0.00061	3.4668
Within Groups	254.6794	21	12.12759			
Total	515.4063	23				

	CON	*WD*
Mean	33.68889	37.28333
Variance	14.33861	18.59367
Observations	9	6
Pooled Variance	15.97517	
Hypothesized Mean Difference	0	
df	13	
t Stat	-1.70632	
P(T<=t) one-tail	0.055854	
t Critical one-tail	1.770933	
P(T<=t) two-tail	0.111708	
t Critical two-tail	2.160369	

	WD	LCHP
Mean	37.28333	28.95556
Variance	18.59367	5.875278
Observations	6	9
Pooled Variance	10.76697	
Hypothesized Mean Difference	0	
df	13	
t Stat	4.815415	
P(T<=t) one-tail	0.000169	
t Critical one-tail	1.770933	
P(T<=t) two-tail	0.000337	
t Critical two-tail	2.160369	

	CON	*LCHP*
Mean	33.68889	28.95556
Variance	14.33861	5.875278
Observations	9	9
Pooled Variance	10.10694	
Hypothesized Mean Difference	0	
df	16	
t Stat	3.158373	
P(T<=t) one-tail	0.003044	
t Critical one-tail	1.745884	
P(T<=t) two-tail	0.006087	
t Critical two-tail	2.119905	

One way ANOVA – Mature pre-fast weights

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	240.5	40.08333	45.20967
WD	4	167.9	41.975	17.56917
LCHP	4	130.9	32.725	5.149167

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	196.7402	2	98.37012	3.677971	0.059826	3.982298
Within Groups	294.2033	11	26.74576			
Total	490.9436	13				

One way ANOVA – Young post fast wt

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	9	278	30.88889	12.15861
WD	6	208.5	34.75	13.707
LCHF	9	247.3	27.47778	9.901944

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	191.8056	2	95.90278	8.219586	0.002309	3.4668
Within Groups	245.0194	21	11.66759			
Total	436.825	23				

	WD	*CON*
Mean	34.75	30.88889
Variance	13.707	12.15861
Observations	6	9
Pooled Variance	12.75415	
Hypothesized Mean Difference	0	
df	13	
t Stat	2.051341	
P(T<=t) one-tail	0.030475	
t Critical one-tail	1.770933	
P(T<=t) two-tail	0.060949	
t Critical two-tail	2.160369	

	CON	*LCHF*
Mean	30.88889	27.47778
Variance	12.15861	9.901944
Observations	9	9
Pooled Variance	11.03028	
Hypothesized Mean Difference	0	
df	16	
t Stat	2.178758	
P(T<=t) one-tail	0.022323	
t Critical one-tail	1.745884	
P(T<=t) two-tail	0.044645	
t Critical two-tail	2.119905	

	WD	*LCHF*
Mean	34.75	27.47778
Variance	13.707	9.901944
Observations	6	9
Pooled Variance	11.36543	
Hypothesized Mean Difference	0	
df	13	
t Stat	4.092847	
P(T<=t) one-tail	0.000635	
t Critical one-tail	1.770933	
P(T<=t) two-tail	0.00127	
t Critical two-tail	2.160369	

One way ANOVA – Mature post-fast weights

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	217.4	36.23333	38.51067
WD	4	150.4	37.6	10.16
LCHF	4	120.6	30.15	4.07

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	130.0738	2	65.0369	3.041132	0.088851	3.982298
Within Groups	235.2433	11	21.38576			
Total	365.3171	13				

One way ANOVA – Young non-fasting glucose

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	9	104.2	11.57778	4.466944
WD	6	62.4	10.4	2.544
LCHF	9	89.8	9.977778	3.626944

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12.16222	2	6.081111	1.648399	0.216291	3.4668
Within Groups	77.47111	21	3.689101			
Total	89.63333	23				

One way ANOVA – Mature non-fasting glucose

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	71.2	11.86667	12.45867
WD	4	40.4	10.1	1.186667
LCHF	4	32.9	8.225	1.915833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	32.10274	2	16.05137	2.465964	0.130372	3.982298
Within Groups	71.60083	11	6.509167			
Total	103.7036	13				

One way ANOVA – Young fasting glucose

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	9	69.9	7.766667	2.9775
WD	6	46.1	7.683333	2.625667
LCHF	9	70.5	7.833333	2.0975

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.08125	2	0.040625	0.015878	0.984259	3.4668
Within Groups	53.72833	21	2.558492			
Total	53.80958	23				

One way ANOVA – Mature fasting glucose

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	44.7	7.45	4.951
WD	4	30.3	7.575	7.475833
LCHF	4	24.2	6.05	2.55

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.044643	2	3.022321	0.606311	0.562614	3.982298
Within Groups	54.8325	11	4.984773			
Total	60.87714	13				

One way ANOVA – Young heart weight

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	900.3	150.05	92.731
WD	6	827.9	137.9833	207.2497
LCHP	6	787	131.1667	23.71867

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1097.303	2	548.6517	5.084827	0.020611	3.68232
Within Groups	1618.497	15	107.8998			
Total	2715.8	17				

	CON	*WD*
Mean	150.05	137.9833
Variance	92.731	207.2497
Observations	6	6
Pooled Variance	149.9903	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.706539	
P(T<=t) one-tail	0.059358	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.118716	
t Critical two-tail	2.228139	

	CON	*LCHP*
Mean	150.05	131.1667
Variance	92.731	23.71867
Observations	6	6
Pooled Variance	58.22483	
Hypothesized Mean Difference	0	
df	10	
t Stat	4.286326	
P(T<=t) one-tail	0.000798	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.001595	
t Critical two-tail	2.228139	

	WD	*LCHP*
Mean	137.9833	131.1667
Variance	207.2497	23.71867
Observations	6	6
Pooled Variance	115.4842	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.098681	
P(T<=t) one-tail	0.148828	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.297655	
t Critical two-tail	2.228139	

One way ANOVA – Mature heart weight

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	959.9	159.9833	227.5657
WD	4	585.9	146.475	76.61583
LCHP	4	548	137	30.34

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1320.958	2	660.4792	4.980662	0.028831	3.982298
Within Groups	1458.696	11	132.6087			
Total	2779.654	13				

	CON	*WD*
Mean	159.9833	146.475
Variance	227.5657	76.61583
Observations	6	4
Pooled Variance	170.9595	
Hypothesized Mean Difference	0	
df	8	
t Stat	1.600519	
P(T<=t) one-tail	0.074076	
t Critical one-tail	1.859548	
P(T<=t) two-tail	0.148151	
t Critical two-tail	2.306004	

	CON	*LCHP*
Mean	159.9833	137
Variance	227.5657	30.34
Observations	6	4
Pooled Variance	153.606	
Hypothesized Mean Difference	0	
df	8	
t Stat	2.87286	
P(T<=t) one-tail	0.010369	
t Critical one-tail	1.859548	
P(T<=t) two-tail	0.020738	
t Critical two-tail	2.306004	

	WD	*LCHP*
Mean	146.475	137
Variance	76.61583	30.34
Observations	4	4
Pooled Variance	53.47792	
Hypothesized Mean Difference	0	
df	6	
t Stat	1.832344	
P(T<=t) one-tail	0.058304	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.116609	
t Critical two-tail	2.446912	

One way ANOVA – Young HW:BW

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	27.9989	4.666483	0.127587
WD	6	22.35476	3.725793	0.209327
LCHP	6	28.20338	4.700563	0.240961

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.67247	2	1.836235	9.532684	0.00213	3.68232
Within Groups	2.889377	15	0.192625			
Total	6.561847	17				

	CON	*WD*
Mean	4.666483	3.725793
Variance	0.127587	0.209327
Observations	6	6
Pooled Variance	0.168457	
Hypothesized Mean Difference	0	
df	10	
t Stat	3.969741	
P(T<=t) one-tail	0.001322	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.002644	
t Critical two-tail	2.228139	

	CON	*LCHP*
Mean	4.666483	4.700563
Variance	0.127587	0.240961
Observations	6	6
Pooled Variance	0.184274	
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.13751	
P(T<=t) one-tail	0.446678	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.893357	
t Critical two-tail	2.228139	

	WD	*LCHP*
Mean	3.725793	4.700563
Variance	0.209327	0.240961
Observations	6	6
Pooled Variance	0.225144	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.55822	
P(T<=t) one-tail	0.002598	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.005197	
t Critical two-tail	2.228139	

One way ANOVA – Mature HW:BW

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	24.25875	4.043125	0.182341
WD	4	14.08747	3.521867	0.240702
LCHP	4	16.78165	4.195412	0.045712

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.024016	2	0.512008	3.180265	0.081295	3.982298
Within Groups	1.77095	11	0.160995			
Total	2.794966	13				

One way ANOVA – Young HW:TL

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	51.18642	8.53107	0.236623
WD	6	46.33349	7.722248	0.471321
LCHP	6	45.39221	7.565369	0.092243

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.222766	2	1.611383	6.041271	0.011899	3.68232
Within Groups	4.000937	15	0.266729			
Total	7.223704	17				

	CON	*WD*
Mean	8.53107	7.722248
Variance	0.236623	0.471321
Observations	6	6
Pooled Variance	0.353972	
Hypothesized Mean Difference	0	
df	10	
t Stat	2.354664	
P(T<=t) one-tail	0.020159	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.040319	
t Critical two-tail	2.228139	

	CON	*LCHP*
Mean	8.53107	7.565369
Variance	0.236623	0.092243
Observations	6	6
Pooled Variance	0.164433	
Hypothesized Mean Difference	0	
df	10	
t Stat	4.124853	
P(T<=t) one-tail	0.001031	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.002061	
t Critical two-tail	2.228139	

	WD	*LCHP*
Mean	7.722248	7.565369
Variance	0.471321	0.092243
Observations	6	6
Pooled Variance	0.281782	
Hypothesized Mean Difference	0	
df	10	
t Stat	0.511883	
P(T<=t) one-tail	0.309923	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.619846	
t Critical two-tail	2.228139	

One way ANOVA – Mature HW:TL

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CON	6	53.42818	8.904697	0.54306
WD	4	32.28028	8.07007	0.42318
LCHP	4	30.6572	7.664301	0.070502

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.019919	2	2.00996	5.268765	0.024836	3.982298
Within Groups	4.196345	11	0.381486			
Total	8.216264	13				

	WD	*CON*
Mean	8.07007	8.904697
Variance	0.42318	0.54306
Observations	4	6
Pooled Variance	0.498105	
Hypothesized Mean Difference	0	
df	8	
t Stat	-1.83205	
P(T<=t) one-tail	0.052154	
t Critical one-tail	1.859548	
P(T<=t) two-tail	0.104308	
t Critical two-tail	2.306004	

	LCHP	*CON*
Mean	7.664301	8.904697
Variance	0.070502	0.54306
Observations	4	6
Pooled Variance	0.365851	
Hypothesized Mean Difference	0	
df	8	
t Stat	-3.17698	
P(T<=t) one-tail	0.006528	
t Critical one-tail	1.859548	
P(T<=t) two-tail	0.013057	
t Critical two-tail	2.306004	

	LCHP	*WD*
Mean	7.664301	8.07007
Variance	0.070502	0.42318
Observations	4	4
Pooled Variance	0.246841	
Hypothesized Mean Difference	0	
df	6	
t Stat	-1.15501	
P(T<=t) one-tail	0.146002	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.292004	
t Critical two-tail	2.446912	

Two-tailed student's t-test – Young total CSA

	CON	*LCHF*
Mean	30.272	29.214
Variance	29.75647	10.33412
Observations	3	3
Pooled Variance	20.0453	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.289418	
P(T<=t) one-tail	0.393322	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.786643	
t Critical two-tail	2.776445	

Two-tailed student's t-test – Young left ventricular CSA

	CON	*LCHF*
Mean	14.91533	15.94433
Variance	6.502204	1.674352
Observations	3	3
Pooled Variance	4.088278	
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.62329	
P(T<=t) one-tail	0.283436	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.566872	
t Critical two-tail	2.776445	

One way ANOVA – Young %Area positive for PAS

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	70.148	17.537	9.826365
WD	5	124.81	24.9636	0.095068
LCHF	4	56.715	14.17875	51.7614

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	277.676 4	2	138.8382	7.498948	0.01024 4	4.10282 1
Within Groups	185.143 6	10	18.51436			
Total	462.82	12				

	Control	*WD*
Mean	17.537	24.9636
Variance	9.826365	0.095068
Observations	4	5
Pooled Variance	4.265624	
Hypothesized Mean Difference	0	
df	7	
t Stat	-5.36034	
P(T<=t) one-tail	0.000526	
t Critical one-tail	1.894579	
P(T<=t) two-tail	0.001052	
t Critical two-tail	2.364624	

	Control	*LCHF*
Mean	17.537	14.17875
Variance	9.826365	51.7614
Observations	4	4
Pooled Variance	30.79388	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.855846	
P(T<=t) one-tail	0.212468	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.424936	
t Critical two-tail	2.446912	

	WD	*LCHF*
Mean	24.9636	14.17875
Variance	0.095068	51.7614
Observations	5	4
Pooled Variance	22.23778	
Hypothesized Mean Difference	0	
df	7	
t Stat	3.409276	
P(T<=t) one-tail	0.005649	
t Critical one-tail	1.894579	
P(T<=t) two-tail	0.011299	
t Critical two-tail	2.364624	