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Effects of a Chronic Low Carbohydrate High Fat Diet on Markers of Cholesterol and Lipoprotein Metabolism in Elite Level Ultra-endurance Male Runners

Brent C. Creighton

Unniversity of Connecticut, BrentCreighton@gmail.com

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Brent C. Creighton, P.hD.

University of Connecticut, 2015

The long-term safety of low carbohydrate high fat diets (LCHFD) continues to remain in question. Previous research has shown marked variability in the cholesterol response among those who adopt a LCHFD, raising concern, as elevated cholesterol is associated with increased risk for cardiovascular disease. More in-depth analyses are necessary to determine if elevated cholesterol associated with chronic consumption of a LCHFD increases atherogenic risk. Assessment of lipoprotein subfractions and noncholesterol sterol biomarkers provide a greater ability to quantify atherogenic risk. Twenty highly trained male ultra marathoners habitually consumed either a traditional high-carbohydrate HC: $n=10$, %carbohydrate:protein:fat = 57:15:27) diet or a low-carbohydrate (LC; $n=10$, 10:19:70) diet for an average of 20 mo (range 9 to 36 mo). Serum total (TC), low-density lipoprotein (LDL-C), and high-density lipoprotein (HDL-C) cholesterol were all greater in the LCHF group (TC: 277.7 ± 50.6 vs 168.7 ± 24.4 mg/dL; $P=0.0000$, LDL-C: 161.3 ± 37.4 vs 88.1 ± 13.7 mg/dL; $P=0.0000$, HDL-C: 102.3 ± 26.2 vs 63.9 ± 18.0 mg/dL; $P=0.0007$). Noncholesterol sterol biomarkers were used to calculate fractional cholesterol balance and was not significantly different between groups (0.60 ± 0.16 vs 0.66 ± 0.22). Men in the LCHF group presented less atherogenic lipoprotein profiles. Mean VLDL particle size was significantly reduced in the LC group (38.2 ± 4.1 vs 43.65 ± 1.7 nm; $P=0.0022$), while mean LDL (21.5 ± 0.34 vs 20.1 ± 0.35 nm; $P=0.0102$) and HDL (10.2 ± 0.47 vs $9.5 \pm$

0.47 nm; $P=0.0053$) particle size were greater. Total LDL particle count was significantly greater in the LCHF group (1363 ± 343 vs 893 ± 196 nmol/L; $P=0.0021$). Although not statistically different ($P=0.0021$), the LCHF group had 126% less small LDL particles (160.6 ± 134.8 vs 363.0 ± 245.2 nmol/L; $P=0.1013$). Total HDL particles were not significantly different between groups ($P=0.1369$). HDL particle distribution was comprised of significantly more atheroprotective large particles (14.8 ± 4.4 vs 8.1 ± 3.4 nmol/L; $P=0.0013$). These results provide further evidence that compared to HCD, habitual LCHF diets, irrespective of high circulating cholesterol, do not significantly alter fractional cholesterol balance and shift the lipoprotein profile towards a more atheroprotective state.

Effects of a Chronic Low Carbohydrate High Fat Diet on Markers of Cholesterol and
Lipoprotein Metabolism in Elite Level Ultra-endurance Male Runners

Brent C. Creighton

B.A., University of Connecticut, 2009

M.A., University of Connecticut, 2011

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Brent C. Creighton

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APPROVAL PAGE

Doctor of Philosophy Dissertation

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Presented by

Brent C. Creighton, B.S., M.A.

Major Advisor _____
Carl M. Maresh, Ph.D.

Associate Advisor _____
Jeff S. Volek, Ph.D., R.D.

Associate Advisor _____
William J. Kraemer, Ph.D.

Associate Advisor _____
Jeffrey M. Anderson, MD

Associate Advisor _____
Elaine C. Lee, Ph.D.

University of Connecticut

2015

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Chapter 1

Review of Literature

Current Perspective

Low carbohydrate high fat diets (LCHFD) continue to be a therapeutic means for improving metabolic and atherogenic risk factors and although no official definition of LCHF diets exist, arbitrary levels have been defined as less than 50 grams of carbohydrates per day or less than 10% of total energy intake¹. Many professional organizations^{2,3} continue to discourage LCHFD as long-term health outcomes remain in question. Though few, recent long-term studies, greater than six months in duration, have shown remarkably promising results for up to twelve months following a LCHFD⁴⁻⁷. These longitudinal studies, however, measured only mass cholesterol levels and were conducted with overweight, diabetic subjects where weight loss was a contributing factor. What remains to be seen, are the implications a LCHFD may have on cholesterol levels and cardiovascular risk when maintained for chronic periods of time (6 < months) in healthy normolipidemic individuals.

Published studies investigating the effects of LCHF diets on metabolic health in healthy, normolipidemic individuals, have shown variable responses in circulating cholesterol levels [total (TC) and low-density lipoprotein cholesterol (LDL-C)] not attributable to weight loss⁸⁻¹³. Numerous cross sectional and prospective investigations have illustrated that LCHF diets improve cardiometabolic risk factors¹⁴. Many if not all of these studies report data on subjects who followed a LCHF diet for no greater than 4-12 weeks. To our knowledge, no study to date has assessed measures of cardiovascular disease (CVD) risk and cholesterol balance in healthy, fit, normolipidemic men, who have followed a LCHFD for longer than six months.

Cholesterol

The importance of cholesterol in the human body is unquestionable as disorders in synthesis result in a myriad of adverse syndromes¹⁵. Still, elevated levels of total serum cholesterol and LDL-C are associated with increased risk of cardiovascular diseases (CVD) such as atherosclerosis, myocardial infarction, and stroke^{16,17}. Current international guidelines^{16,17} continue to weigh heavily on cholesterol markers for the evaluation of CVD risk. It is reasonable to suggest that total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) metrics are well known by the lay public, but not well understood. Published guidelines and practitioners advise patients to keep TC below 200mg/dL, LDL-C under 130mg/dL, and HDL-C over 40mg/dL¹⁶ depending on one's potential risk for CVD. Arguably, the question needing to be asked is whether or not these values and subsequent guidelines pertaining to serum cholesterol really help predict CVD risk. Although still a minority, a growing body of researchers and practitioners are beginning to argue that measuring circulating cholesterol levels have no relevance for accurately predicting CVD.

Circulating cholesterol levels are themselves highly variable as are indirect surrogate markers, particularly in response to dietary changes in cholesterol¹⁸ such as can occur on a LCHFD. Cholesterol levels following increased dietary consumption may decrease, increase, or stay the same. Of particular concern are variations or increases in LDL-C following cholesterol feeding, which have similarly been shown under metabolic ward conditions by Sehayek et al 1998¹⁹.

It is well established that endogenous cholesterol metabolism is inversely regulated. Reduced intestinal absorption of cholesterol causes an up-regulation of cholesterol synthesis, whereas increased intestinal absorption causes the liver to suppress cholesterol synthesis²⁰. Various human studies have suggested that an individual's ability to down-regulate endogenous cholesterol synthesis in response to increased cholesterol intake helps limit their plasma lipoprotein responsiveness^{21,22}. Animal data helps provide further evidence. Species that tolerate and adapt more efficiently to an increased dietary cholesterol load include those, which have an innately greater rate of hepatic cholesterol synthesis (rats, mice, squirrel monkeys)²³. These species down regulate synthesis more readily and are less responsive to dietary changes of cholesterol. Importantly, these species, which are frequently used in experiments for studying metabolism, may not translate into suitable models for human comparison. Humans have lower rates of hepatic cholesterol synthesis and so are more responsive to dietary changes in cholesterol. Endogenous cholesterol consumed in the diet mixes with biliary cholesterol in the intestines. Overall, most cholesterol within the intestinal lumen is actually derived from endogenous sources (bile), with dietary cholesterol contributing very little. Although highly variable, the average American consumes approximately 0.4g of cholesterol per day²⁴. Ultimately, the amount of cholesterol absorbed equals the amount lost (i.e., the body synthesizes an amount approximately equal to the amount it absorbs²⁴).

Surrogate Markers of Cholesterol Synthesis and Absorption

Serum cholesterol concentrations are derived from two sources; endogenous cholesterol from hepatic and extra-hepatic synthesis, and exogenous cholesterol derived from intestinal absorption of dietary and biliary cholesterol²³. Absolute quantification of cholesterol synthesis is difficult and time consuming, requiring measures of cholesterol output (fecal measurements and bile acids) and dietary intake to quantify total sterol balance [Output – Intake = Synthesis]. In response to these methodological difficulties, more recent, less arduous approaches to quantify cholesterol balance have been developed. These measurements vary but can include assessment of serum non-cholesterol sterols, synthesis markers (lathosterol and desmosterol), and absorption markers (beta-sitosterol, campesterol, and cholestanol). These sterol-based biomarkers are currently used primarily to assess the cause of hypercholesterolemia and can help to determine therapy selection. In addition, noncholesterol sterols can be extremely useful in determining and better understanding disease states²⁵.

The cholesterol balance score is calculated by taking the ratio of cholesterol synthesis or production measurements and dividing it by cholesterol absorption measurements. Pre-cholesterol sterols leak into lipoproteins at a rate proportional to that of their formation in the cholesterol synthetic pathway, thus is the rational for using them as indicators of synthesis²⁶. In addition, all sterols including plant sterols are transported in lipoproteins, predominantly the LDL and HDL subfractions²⁷. Plasma non-cholesterol sterols are frequently reported as a ratio to cholesterol in

order to normalize the differences caused by variable levels of lipoproteins that transport cholesterol and the sterols²⁸.

Cholesterol Synthesis Pathway

The process of cholesterol synthesis is complex, highly regulated, and controlled by a myriad of factors. Cholesterol or cholesterol that is synthesized in mammalian cells originates from acetate. **Figure 1** illustrates an overly simplified version of the important steps in the cholesterol synthesis pathway²⁹.

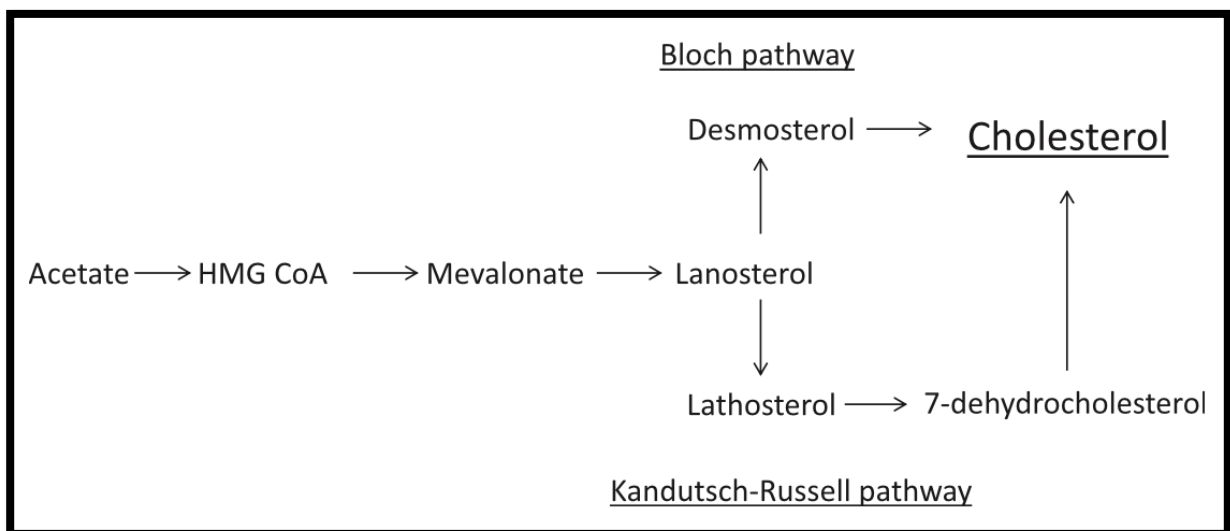


Figure 1: Cholesterol synthesis pathways

As shown above, acetate is converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) by HMG-CoA synthase and then to mevalonate by HMG-CoA reductase. This conversion step, HMG-CoA reductase, is where statin drugs are utilized to stop cholesterol synthesis. Mevalonate is converted to squalene and finally to lanosterol. The final stages of cholesterol synthesis shuttle lanosterol through two distinct

synthetic pathways, the Kandutsch-Rusell³⁰ pathway and the Bloch pathway. Accounting for nearly 80% of endogenous cholesterol synthesis, the Kandutsch-Rusell pathway converts lanosterol first to lathosterol, then to 7-dehydrocholesterol, and finally cholesterol. Conversely, the Bloch pathway accounts for the remaining 20%, converting desmosterol to cholesterol. Although yet to be fully mapped out, it is thought that both pathways are likely independently regulated and share many of the same enzyme pathways¹⁵. Intermediary sterols, squalene, lathosterol, desmosterol can and are used as biomarkers to evaluate cholesterol synthesis^{31,32}. Elevated concentrations of these markers indicate increased endogenous production. The value of measuring cholesterol precursors lies with the assumption that serum they leak into plasma lipoproteins at a rate relative to that of their formation in the synthetic pathway²⁶.

Lathosterol and desmosterol are two of the major cholesterol precursors which can be used as markers of hepatic synthesis^{33,34}. Both precholesterol sterols maintain very low circulating levels while transported and distributed in a very similar manner to that of cholesterol. Inherited metabolic diseases or abnormal clinical conditions (fatty liver disease or insulin resistance) may cause an elevation in either of these sterols³⁵. In particular, cholesterol precursors show a high degree of association with fecal cholesterol synthesis, making them a strong alternative option.

Mechanism of Cholesterol Absorption

The manner by which cholesterol is absorbed from the gut lumen is a tightly regulated process which has been studied extensively^{36,37}. Intestinal cholesterol originates from dietary absorption, the hepatobiliary system, and intestinal epithelial sloughing, contributing roughly 2000mg/day³⁷. The standard western diet on average contributes about 300-500 mg of this total through intestinal absorption. Absorbed cholesterol is transported in micelles, which form in conjunction with other newly absorbed lipids (phospholipids and monoglycerides) and the help of bile salts. Micelles then facilitate transport of cholesterol to the brush border of the small intestines where cholesterol is removed and unesterified cholesterol passes through to the enterocytes by way of the Niemann-Pick C1-like (NPC1) transporter. Once absorbed, cholesterol is then esterified by acyl-CoA cholesterol acyltransferase (ACAT). Efflux from the enterocytes is mediated by intestinal ATP-binding cassette transporters (ABC) G5 and G8 proteins, which are highly sensitive to plant sterols. Genetic variances in this protein can result in hyperabsorption of cholesterol³⁸ and considering an estimated 5% of plant sterols are absorbed from the gut³⁹, elevated levels can be considered toxic.

Once absorbed and esterified, cholesterol is then packaged and transported to the liver in chylomicrons, where newly absorbed cholesterol has a direct impact on lipoprotein production and removal pathways^{23,40}. Between individuals, differences in enzyme kinetics (K_m and/or V_{max}) related to cholesterol absorption is a likely factor influencing cholesterol flux as is Apo E genotype⁴¹; this has not been found true in all cases¹⁹. Overall, the precise mechanism regulating cholesterol homeostasis has yet

to be fully mapped out. Still, effects of circadian rhythm, body weight, and diet on cholesterol response clearly demonstrate the presence of genetically determined differences that control a person's cholesterol responsiveness^{19,42}.

The assessment of hypercholesterolemia can be conducted using plant sterol biomarkers β -sitosterol and campesterol, the major phytosterols present in human serum and tissues. Phytosterols serve a supporting role in plant membrane function, not unlike cholesterol in animals but serve no function in human physiology. Naturally more hydrophobic than animal sterols, plant sterols consumed in the diet endogenously inhibit cholesterol absorption by competing for incorporation into micelles and transport through the NPC1 transporter⁴³. Ratios of serum phytosterols to cholesterol largely depend on absorption efficiency, biliary secretion, and the amount consumed in the diet. Quantification of β -sitosterol and campesterol, when normalized to cholesterol, act as surrogates for evaluating cholesterol absorption because humans do not produce phytosterols⁴⁴.

Although less commonly used, cholestanol can also be used to assess absorption rates. Cholestanol is a synthesized metabolite of cholesterol which exists ubiquitously throughout the body at a concentration of roughly 1/500 to 1/800 of cholesterol⁴⁵. Similar to other noncholesterol sterols, accumulation of cholestanol results in adverse pathological conditions. Similar to plant sterol absorption, elevated levels of cholestanol in the circulation is indicative of increased absorption at the gut.

Considerations for Lipid Assessment

In low carbohydrate studies, weight loss is frequently both a desired outcome as well as a useful tool for subject recruitment. Still, caution is advised when interpreting changes in lipids (cholesterol and triglycerides). A meta-analysis conducted by Dattilo and Kris-Etherton⁴⁶ concluded that for every kilogram of body weight lost, there is an associated 0.015 mmol/L reduction in serum triacylglycerols. Furthermore, whether subjects are actively losing weight or weight stable has been shown to have an effect on both the magnitude and direction of cholesterol levels⁴⁷ making weight loss, independent of diet, a potential confounding factor when evaluating lipid levels in dietary studies. In addition, weight loss itself likely plays a large role in influencing the lipoprotein profile. Wood et al.¹⁴ illustrated that a LCHFD in conjunction with weight loss helped improve lipoprotein subclasses. Similarly, Katzel et al.⁴⁸ showed weight loss to aid in the favorable redistribution of lipoprotein subclasses. Thus, it is imperative to consider weight loss when assessing changes in lipids.

An additional consideration when evaluating changes in lipids is whether or not obese and normal weight individuals respond differently to dietary cholesterol. The mechanism of dietary cholesterol has been well established^{49,50}. Increased consumption of dietary cholesterol contributes to the already recycled biliary cholesterol pool and increases the volume of cholesterol entering the liver. Upon exposure to increased cholesterol levels, the liver down regulates expression of LDL receptors on hepatocytes. Subsequently, clearance of LDL from the plasma decreases and circulating cholesterol increases until a new steady has been

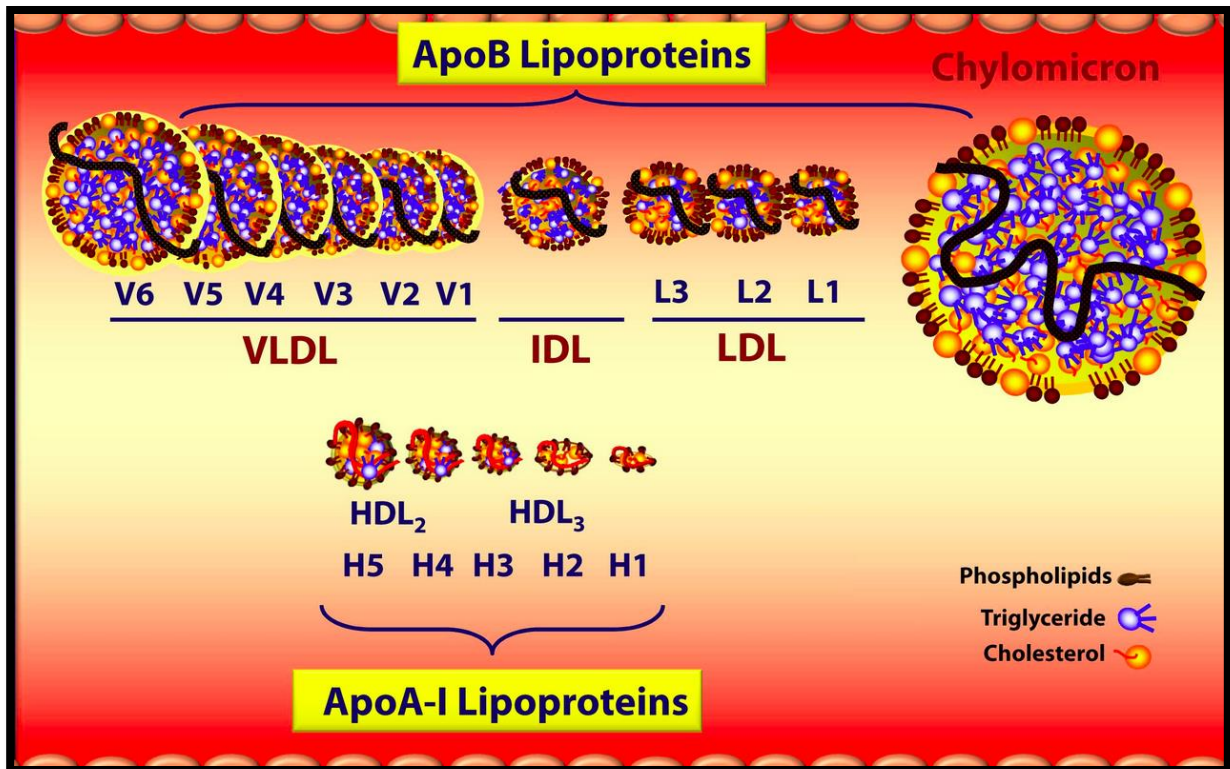
established. This mechanism offers an explanation as to why obese individuals or individuals with naturally elevated cholesterol, may be less responsive to changes in dietary cholesterol. That is, people with elevated cholesterol, such as obese persons, tend to have higher rates of total body cholesterol synthesis and a larger pool of cholesterol circulating from the gut back to the liver⁵¹. Thus, a person with elevated cholesterol would have a lower response to dietary cholesterol because the amount taken in with the diet would be small compared with the large amount already present in the enterohepatic cholesterol pool and it would, therefore, not contribute to further suppression of the LDL receptor²⁰. In addition, there may be a link between obesity, insulin resistance, and diminished cholesterol absorption, however this has yet to be seen. Apart from genetic influence, the opposite can likely be said about non-obese, normolipidemic individuals, that dietary cholesterol is likely to more greatly effect cholesterol levels due to a lower circulating cholesterol pool.

Lipoproteins

Lipoproteins are particles that function to carry and transport TGs and cholesterol throughout the body. Essential to cell structure and metabolism, both TGs and cholesterol are hydrophobic molecules, which cannot move freely within the fluidic cardiovascular system. Thus, the body has engineered an eloquent system to transport these molecules. Lipoproteins are comprised of apolipoproteins, which function as ligands and in structural integrity of the molecule, a phospholipid bilayer (hydrophilic on the outside and hydrophobic on the inside), TGs, and cholesterol. The

major apolipoproteins present and the relative contents of all the lipid components vary among different classes of lipoproteins.

Following the ingestion of a meal, the intestines packages consumed lipids into chylomicrons for transport around the body. Chylomicrons are primarily TG filled lipoproteins, which make their way towards the liver depositing fatty acids along the way in adipose, cardiac, and skeletal muscle tissues^{52,53}. The liver produces VLDLs which function as the primary transport vehicle of TGs and free fatty acid delivery⁵². Removal of TGs from the VLDL core produces metabolic by-products known as LDLs. In most normal physiological conditions, these are the primary carries of circulating plasma cholesterol. All three of these lipoproteins, chylomicrons, VLDLs, and LDLs, carry apoB, in addition to other apolipoproteins. HDLs carry other apolipoproteins known as apoAI and apoAII. Nascent HDL particles are produced by the both liver and intestines. These newly formed HDL particles mature as they become enriched with additional other apolipoproteins and lipids through an exchange with chylomicrons and VLDLs^{52,53}. Size and density of the various lipoproteins vary with chylomicrons being the largest and least dense, to the smallest and most dense HDL. Each lipoprotein class can further be broken down into a spectrum of distinct particles that vary in size, density, relative proportions of lipid and protein, as well as atherogenicity⁵². **Figure 3** below illustrates the varying degree of subclasses, which have been discovered for each distinct lipoprotein class.



Low-density lipoproteins (LDL) are the chief cholesterol-carrying lipoproteins in the human plasma and thus play a central role in atherogenesis. LDL-C has long been touted as “bad cholesterol”, since elevated levels have been associated with increased risk of coronary heart disease (CHD)^{16,17}. Recently, the significance of LDL-C on its own as a marker of CVD risk has come into speculation within certain fields. Instead, LDL pattern type (A and B) has been receiving growing attention as a more accurate marker of CVD risk.

Krause and Blanche¹²² were the first to divide the LDL gel electrophoresis profile into two distinct phenotypes, which they referred to as patterns A and B. Pattern A is distinguished by predominantly large-sized LDL, whereas pattern B is composed of a greater proportion of small, dense LDL particles. Results from their studies showed that ~25% of the sample population reported the pattern B

phenotype but that this was less frequent in women and younger subjects (<40 yrs). Not surprisingly, pattern B was associated with moderate elevation in plasma TGs and low levels of HDL-C⁵⁴, a combination commonly termed the atherogenic lipoprotein phenotype. Patients with combined hyperlipidemia exhibit this atherogenic profile and are at an increased risk of CHD events regardless of their total LDL circulating mass⁵⁵.

In a similar fashion as mentioned previously, plasma TGs have been shown to be the major determinant of the appearance of small, dense LDL particle's, regardless of the method of analysis^{54,56,57}. Packard et al.⁵⁵ observed that the pattern B phenotype was infrequently seen in individuals with a plasma TG concentration less than 115 mg/dL, while above this level, small, dense LDL increased in proportion to the plasma TG concentration.

The concern over small, dense LDL is that it has been shown to be more readily oxidized, at least in vitro, than its larger pattern A counterparts. Additionally, because of its reduced size it is likely to penetrate the arterial wall more easily, and we have been able to demonstrate that LDL from pattern B subjects have an enhanced affinity for arterial wall proteoglycan, thus prolonging its residence time in the subendothelial space¹²⁴. All of these features contribute to the enhanced atherogenicity of this lipoprotein species.

Effects of Low Carbohydrate High Fat Diets on Blood Lipids

One of the most consistent and predictable changes seen in those following a low carbohydrate diet is a reduction in circulating triacylglycerols (TGs)^{9,123}. The primary mode of transport for TGs within the circulation is within very low-density lipoprotein particles (VLDLs). It is theorized that increased production or synthesis of TGs within the liver can increase the size and number of VLDLs synthesized and subsequently released into the circulation by the liver¹⁰. Thus, by restricting carbohydrates in the diet, lipogenesis within the liver, which occurs primarily from glucose derived sources (denovolipogenesis or DNL) from the standard American diet, is greatly reduced or entirely turned off. With DNL occurring at a much lower degree if it all, the liver has less TGs to package and traffic and needs to synthesize less VLDL particles.

Serum cholesterol has long been of interest to both doctors and researchers. Consistent among dietary intervention studies that measured TC, LDL-C and HDL-C as outcome measures, is the large degree of individual variability¹⁰. This variability has been shown in a range of studies looking at high and low fat intake⁵⁸ in the presence of carbohydrates, high and low cholesterol in the presence of fat and carbohydrates, and notably among low carbohydrate high fat intervention studies. It is thought that some individuals are sensitive to changes in dietary cholesterol and fat within the diet while others are insensitive. What's more is that some believe varying cholesterol responses may result largely from test error and that replication of the same studies generates varying results⁵⁹. The notion that cholesterol levels fluctuate based on diet and activity is largely overlooked on a day-to-day basis. Large

variability among cholesterol responses has been shown in those consuming various diets.

Specifically, LCHFDS have been shown to reduce both fasting and postabsorptive TGs in both over weight/obese individuals as well as healthy, normolipidemic individuals¹⁰. This can largely be attributable to a reduction in carbohydrates as was previously described. The cholesterol response to LCHFDS as noted previously has been shown to be highly variable and is largely dependent on cholesterol levels prior to the adherence of a LCHF. On average, it appears both TC and LDL-C increase following the consumption of a LCHF in healthy normolipidemic individuals but among those who have higher levels of cholesterol the response is different. HDL-C cholesterol has a more predictable response as it increases in nearly all individuals¹⁰.

Effects of Low Carbohydrate High Fat Diets on Lipoproteins

Proposed Physiological Mechanism

A proposed theoretical mechanism for the effects of LCHF diets on circulating lipoproteins has been previously described by Volek et al.¹⁰ (**Figure 2**).

This metabolic hormonal shift promotes TAG hydrolysis and increases fatty acid (FA) rate and appearance throughout the body.

Increased skeletal muscle LPL is likely to result in LPL-mediated lipolysis of newly formed chylomicrons, resulting in a release of FA that is either taken up by the underlying tissue or escapes into the circulation. These circulating FAs are taken up by the liver and preferentially diverted away from esterification to TAG and toward mitochondrial oxidation to acetyl CoA. Accumulation for acetyl CoA exceeding the capacity for mitochondrial oxidation results in the formation of ketones. Decreased glucose availability for use by skeletal muscle shifts dependence towards fatty acids. Thus, any increase in FA delivery to skeletal muscle is balanced (utilized) by an increase in fat oxidation, as has been seen by postabsorptive respiratory exchange ratios close to or below 0.7 (unpublished data).

Reduced hepatic production of TAG, which normally occurs from excess fatty acids and glucose (Standard American diet), results in less VLDL synthesis and secretion into the circulation. Furthermore, LPL-mediated lipolysis of VLDL results in the transfer unesterified cholesterol, phospholipids (PL), and apolipoproteins to form mature HDL-C. Remaining remnant particles are either taken up by the liver or converted to LDL. Decreased circulating VLDL, particularly in the postprandial state, results in less cholesterol ester transfer protein (CETP)-mediated neutral lipid exchange with LDL-C. A reduction in hepatic lipase (HL) prevents larger LDL-C from being delipidated to smaller, dense (atherogenic) LDL, producing a predominance of larger LDL particles.

Chapter 2

Introduction

Low carbohydrate high fat diets (LCHFD) continue to show promise as a way to improve the cardiometabolic profile, not only in at-risk populations, but also within healthy individuals. Even so, long term safety and efficacy remain in question, as longitudinal LCHF studies are limited⁵⁻⁷ and contradict current health recommendations^{2,16}. Variation in the cholesterol responses following adoption of a LCHFD^{8-13,60,61} warrant concern as elevated cholesterol levels have been associated with increased risk for cardiovascular diseases (CVD) and atherogenesis⁶². Advances in our understanding that atherogenesis is lipoprotein mediated, not cholesterol driven necessitate the need for more in depth comprehensive investigations be conducted.

Cholesterol homeostasis (serum cholesterol levels) within the body reflects a balance between endogenous cholesterol synthesis (hepatic and extra-hepatic) and intestinal absorption (dietary and biliary cholesterol). Tight regulation of cholesterol balance helps prevent a net accumulation of cholesterol both within the tissues and circulation^{24,63} and disturbances in either input or output can increase atherogenic progression^{39,42,64}. Considering some individuals consuming a LCHF diet present with increased cholesterol, whether or not this increase results from changes in absorption or synthesis has yet to be confirmed. To date, no previous studies have examined the response of a chronic low carbohydrate high fat diet on surrogate markers of cholesterol balance in healthy, fit men.

Quantification of lipoprotein subfractions has become a well-recognized means of assessing atherogenic risk^{65,66} and is arguably better⁶⁷. Distribution of lipoprotein subfractions towards a larger, more buoyant phenotype may help reduce

atherogenic risk, where as increased remnant lipoproteins (VLDL) and small, dense LDL particles may increase risk⁵⁵. Smaller LDL particles are considered more atherogenic because of their ability to more easily penetrate the arterial wall, and their reduced binding affinity to the LDL receptor. This results in a prolonged circulating half-life and thus increased susceptibility to oxidation⁶⁸⁻⁷¹. In addition, growing evidence suggests greater levels and size of triglyceride-rich lipoprotein (TRL) particles, primarily VLDL remnants, are likely to contribute to atherogenic risk⁷²⁻⁷⁴ through eventual conversion to small LDL particles^{68,75}. Finally, it appears the protective effects of HDL may be more largely attributable to large HDL particle subfractions⁷⁶.

Lipoprotein subfractions and surrogate markers of cholesterol balance provide viable measures for evaluating the impact LCHF have on overall long-term cardiometabolic health. Such measures provide a far more detailed analysis of metabolic risk than current clinical biomarkers (fasting total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerides). Thus, the primary purpose of this study was to compare the effects of a chronic (>6 months) LCHF diet to that of a high carbohydrate (HC) diet on markers of lipoprotein metabolism and cholesterol absorption and synthesis. We first hypothesized that increased consumption of cholesterol among men following a chronic LCHF diet would not demonstrate a significant shift in fractional cholesterol balance compared with those consuming a chronic HC diet. Secondly, we hypothesized that a chronic LCHF diet would produce a less atherogenic lipoprotein profile when compared to a HC diet. Despite increased

circulating cholesterol levels among individuals consuming a chronic LCHF diet, we propose that this cholesterol is carried in larger, less atherogenic lipoproteins.

Chapter 3

Methods

Experimental Approach

The following data is an extension of a larger cross-sectional investigation {Volek et al 2015 *pending*} that reported on metabolic responses in two groups of elite ultra-marathoners habitually consuming either a LC or HC diet. The purpose of the current analysis was to more closely examine the effect of diet on markers of lipoprotein metabolism and surrogate markers of cholesterol balance.

Participants

Twenty highly trained male ultra-endurance runners consuming a LC ($n=10$) or a HC ($n=10$) diet 21-45 years of age were selected for participation. Athletes competed in sanctioned running events ≥ 50 km and/or triathlons of at least half iron man distance (113 km) and where in the top 10% of finalists. Athletes were carefully matched for age, physical characteristics, primary competition distance, and competition times. With one exception, all athletes lived in the United States and traveled via plane to our laboratory for two days of testing. Interested athletes completed questionnaires to assess their medical, diet, training, and running competition history. At least one phone call was scheduled to review this information and determine eligibility and availability. Diet information was entered into commercial nutrient analysis software (Nutritionist Pro™, Axxya Systems, Stafford, TX). Subjects consuming a LC diet, defined as $<20\%$ energy from carbohydrate and $>60\%$ energy from fat, consistently for at least 6 months were eligible for the LC group. Subjects consistently consuming $>55\%$ energy from carbohydrate were considered for the HC group. Athletes were excluded if they did not consume the appropriate diet for the allotted amount of time or had any health

issues, including, but not limited to diabetes, heart disease, kidney, liver, or other metabolic or endocrine dysfunction, current injury, anti-inflammatory medication use, anabolic drug use, or prone to excessive bleeding. Subjects were informed of the purpose and possible risks of the investigation prior to signing an informed consent document approved by the Institutional Review Board.

Measurements

Full methods have been described previously {*Volek et al 2015 pending*}. Briefly, athletes arrived at the laboratory at 0600 after a 10 hr overnight fast and were asked to restrict caffeine, over the counter medications, and alcohol. The night prior and morning before testing, subjects were encouraged to liberally consume water to ensure hydration. A small urine sample was provided to assess specific gravity (Model A300CL, Spartan, Japan) as a measure of hydration (all subjects had a USG ≥ 1.025). Body composition was determined by dual-energy X-ray absorptiometry (DXA) (Prodigy, Lunar Corporation, Madison, WI). Height was measured to the nearest 0.1 cm and total body weight was recorded to the nearest 0.1 kg on a digital scale (OHAUS Corp., Fordham Park, NJ).

Blood Collection and Analysis

All blood samples were obtained with a 21G butterfly needle from an antecubital vein of the subject. After resting quietly for 15 min in a supine position, blood was collected into ethylenediaminetetraacetic acid (EDTA) and serum separator vacutainer tubes (Vacutette, Greiner Bio-one North America, Inc., Monroe NC). EDTA

tubes were immediately spun while serum tubes remained at room temperature for 15 min prior to centrifugation to allow clotting to occur. Whole blood was centrifuged (1500 x g for 15 min at 4°C), promptly aliquoted into cryostorage tubes, snap frozen with liquid nitrogen, and stored at -80°C for later analysis. One 10mL serum tube was immediately sent on ice to a certified medical laboratory (Quest Diagnostics, Wallingford, CT) for determination of glucose and other blood analytes using automated enzymatic procedures (Olympus America Inc., Melville, NY). Frozen samples were thawed only once before analysis of all variables. Baseline EDTA plasma was analyzed for total cholesterol, HDL cholesterol, and triglycerides using enzymatic methods on an automated analyzer (Cobas C 111, Roche Diagnostics, Indianapolis, IN). Concentrations of total and LDL cholesterol were also measured using standard enzymatic methods^{77,78}.

Analysis of lipoprotein (high, low, and very low density lipoproteins [HDL, LDL, and VLDL] particle size and number was conducted by Liposciences Inc. (Raleigh, NC) using hydrogen nuclear magnetic resonance (H-NMR) on a 400 MHz NMR analyzer (Bruker BioSpin Corp, Billerica, MA) as previously described by Jeyarajah and colleagues^{79,80}. Lipoprotein subclasses were grouped based on particle diameters: large VLDL (>60 nm), medium VLDL (35–60 nm), small VLDL (27–35 nm), intermediate- density lipoproteins [IDL] (23–27 nm), large LDL (21.2–23 nm), medium LDL (19.8–21.2 nm), small LDL (18–19.8 nm), large HDL (8.8–13 nm), medium HDL (8.2–8.8 nm), and small HDL (7.3–8.2 nm). Lipoprotein insulin resistance index (LP-IR) was calculated by Liposciences Inc. (Raleigh, NC) combining

six lipoprotein measures (VLDL, LDL, and HDL size, large VLDL-P, small LDL-P, and large HDL-P) into a single algorithm⁸¹.

Concentrations of noncholesterol sterol precursors (lathosterol, desmosterol, cholestanol, sitosterol, and campesterol) were also quantified by Boston Heart Diagnostics using a gas chromatography mass-spectrometry (GCMS) method similar to that previously described⁸². To eliminate the effect of different cholesterol levels, absolute values were standardized and expressed in terms of $10^2 \times \text{mol/mmol}$ of cholesterol (ratios to cholesterol values).

Statistical Analysis

Means and standard deviation were calculated for all variables using conventional methods. Differences between groups for demographic characteristics, diet, lipoprotein subfractions, and surrogate markers of cholesterol balance were assessed using independent samples t-tests. Normality testing was performed using the Shapiro-Wilks test and non-normal data were analyzed with the Mann-Whitney U test. Normal data with significant variance was analyzed using the Welch's unequal variance t-test. Relationships between variables were examined using Pearson's product-moment correlation coefficients. The level of significance was set at $p \leq 0.05$. All statistical analyses were performed using Prism Graphpad (Software Version 6.0, La Jolla, CA).

Chapter 4

Results

Subject Characteristics and Diet

There were no significant differences between groups in physical characteristics or aerobic capacity (**Table 1**). Two athletes in each group were triathletes and all others competed in events largely ranging from 80 to 161 km (50 to 100 miles).

Primary differences between groups were among diet (**Table 2**). Total dietary caloric intake and total macronutrient grams consumed (protein + fat + carbohydrates) did not differ between groups (**Table 2**). Total grams of carbohydrate ($p < 0.0001$) and fat ($p < 0.0001$) consumed were significantly different between groups for both the habitual and pre-testing diets (**Table 2, 3 and Figure 1**). Individuals in the LC group derived a majority of their energy from fat (70%), predominantly in the forms of saturated and monounsaturated fatty acids. Only ~10% of energy intake was from carbohydrate sources. Conversely, individuals in the HC group consumed over half their energy in the form of carbohydrates (57%). Protein was not significantly different between groups. Notably, dietary cholesterol intake was significantly greater ($p < 0.003$) in the LC group (844 mg/day) compared with the HC group (291 mg/day) (**Table 2**). The average duration on a LC diet ranged from 9 to 36 months.

Plasma Lipids

Men consuming a LCHF diet had significantly greater ($p < 0.0001$) serum total cholesterol (64.6%), LDL-C ($p < 0.0001$) (83.1%), and HDL-C ($p < 0.0007$) (52.9%) compared to the HC group (**Table 4, Figure 2**). This did not however adversely affect the HDL-C to triglyceride ratio. Serum triglycerides were not different between groups, although LCHF men had a significantly ($p < 0.0113$) lower TG/HDL ratio.

Fractional Cholesterol Balance and Noncholesterol Sterols

No significant differences existed between groups following the calculation of fractional cholesterol balance [Fractional Cholesterol Balance = (lathosterol \times 0.8 + desmosterol \times 0.2)/(beta-sitosterol \times 0.5 + campesterol \times 0.5)]. Absolute and normalized sterol levels are presented in (**Table 8**). Non-cholesterol sterols are transported in plasma by lipoproteins and to correct for variations in lipoprotein levels, sterol concentrations are frequently expressed relative to the concentration of total cholesterol ($\mu\text{mol}/\text{mmol}$ of cholesterol). Absolute levels of each measured sterol were greater in the LCHFD group with desmosterol ($p < 0.0003$) and cholestanol ($p < 0.0087$) being significant. When normalized to cholesterol, all sterol values apart from desmosterol were greater in the HCD group. Normalized values of lathosterol ($p < 0.0158$), desmosterol ($p < 0.0455$), cholestanol ($p < 0.0026$), and campesterol ($p < 0.0185$) were significantly different between groups.

Noncholesterol Sterols Related to Serum Cholesterol

The serum concentrations of all noncholesterol sterols were significantly correlated with HDL cholesterol (**Table 9**). In addition, all sterols but campesterol were significantly correlated with total and LDL cholesterol. When values were normalized to cholesterol, noncholesterol sterol levels were no longer correlated with total cholesterol and only desmosterol and campesterol remained significantly correlated with LDL cholesterol. Cholestanol was the only sterol to remain correlated with HDL cholesterol.

VLDL, LDL, and HDL Particle Size and Quantity and LP-IR

VLDL particle size, but not total particle number, was significantly lower ($p < 0.0022$) (-14.3%) in men who consumed a LCHF diet (**Table 5**). Of the varying particle sizes, only large VLDL particles were significantly ($p < 0.0062$) lower (-126.8%) in the LCHF group compared to the HC. Both groups presented with large pattern A particles (**Table 6**). Those following a LCHF diet had significantly larger ($p < 0.0102$) LDL particles. Total LDL particle number was significantly higher ($p < 0.0021$) by 52.7% in the LCHF group; however, this significant difference was caused by a 124.8% increase ($p < 0.0001$) in the large and a 140.2% increase ($p < 0.0021$) in the intermediate particles. Although not statistically significant ($p < 0.1013$) due to large variation within the HC group and outliers in the LCHF group, median small particle size (most atherogenic), was 126% greater in the HC group. Upon removal of two LCHF outliers, however, the difference between groups becomes significant ($p < 0.083$) and the percent change more than doubles (264.8%). Men consuming a LCHF diet had HDL particles significantly ($p < 0.0053$) larger (7.3%) than those consuming a HC diet (**Table 7**). Total HDL particles were not different between groups; however, men in the LCHF group had significantly greater ($p < 0.0013$) larger particles (82.6%), and significantly ($p < 0.0038$) less (-56%) medium particles compared to the HC group. Small HDL particles were not different between groups. The lipoprotein insulin resistance index (LP-IR) was significantly lower in the LCHF group by 360% ($p < 0.0001$) revealing a significantly reduced predictive risk for insulin resistance (**Table 4, Figure 3**).

Chapter 5

Discussion

In the present study, we sought to compare the lipoprotein and cholesterol responses in healthy, active, normal-weight men who were chronically adapted to either a low carbohydrate high fat diet (LCHFD) or a high carbohydrate diet (HCD). LCHFDs continue to remain controversial in regards to their impact on health and long-term safety⁸³, and while few long-term studies (> 6 months) have sought to validate LCHFD in this realm, only cholesterol mass responses in obese participants have been measured⁴⁻⁷. Thus to date, this is the first study to measure surrogate markers of cholesterol synthesis and absorption as well as the lipoprotein profile in healthy, active, normal-weight men adapted to a LCHFD (> 6 months).

LCHFDs have received criticism for their potential to increase circulating serum cholesterol mass. Previously conducted short-term LCHFD studies in healthy, normolipidemic subjects corroborate these findings⁸⁻¹³, however, frequently disregarded is the notion that individual cholesterol responses following a LCHFD can be highly variable¹³ and are elevated as a result of significant increases in HDL-C. Despite significant increases in dietary and circulating cholesterol, we proposed men following a long-term (> 6 month) LCHFD would not demonstrate altered fractional cholesterol balance and would exhibit favorable changes in circulating lipoproteins subfractions.

Plasma Lipids

Mean dietary cholesterol intake among men following a LCHFD was 190% greater (553 mg/d), or more than triple those consuming a HCD. Based on previously conducted LCHF studies in healthy normolipidemic men⁸⁻¹³, elevated cholesterol in our LCHF group was not unexpected; TC, LDL-C, and HDL-C were significantly higher by 64.6%, 83.1%, and 52.9% respectively. Correspondingly, we found dietary cholesterol intake demonstrated significant correlations with cholesterol measures (TC: $r = 0.75$, $p < 0.0001$; LDL-C: $r = 0.71$, $p < 0.0005$; HDL-C: $r = 0.54$, $p < 0.0135$). Elevated cholesterol in the LCHF group suggests a potential hyper responsiveness to dietary cholesterol, however this was not possible to determine given the cross-sectional nature of the study. Regardless, hyper-absorption in response to increased dietary cholesterol consumption is unlikely to explain such a dramatic elevation in cholesterol levels in men as fit as ours⁸⁴.

Consistent with variable cholesterol responses, men in the LCHFD group displayed a greater range of TC values (LCHF = 162, HC = 65) than men in the HC group. Cholesterol feeding has previously been shown to produce large interindividual variability in circulating cholesterol¹⁹. Furthermore, leanness of the subjects may have contributed to a more dramatic response^{18,85}. Such disparity in the responsiveness of blood cholesterol following the adoption of a LCHFD is thought to occur largely from individual variations in hormonal factors, obesity, and genetic predisposition¹⁹, although this has yet to be fully determined. Both groups had extremely favorable HDL-C levels ($> 40\text{mg/dL}$), a trait likened to a the elite fitness level of our subjects⁸⁶.

High HDL-C levels among athletes is not uncommon⁸⁷. It has been well established that endurance training increases HDL-C cholesterol^{88,89} and promotes enhanced cholesterol efflux⁹⁰. Various physiological mechanisms have been attributed to this adaptation including increased LPL activity⁹¹, decreased cholesterol ester transfer protein (CETP)⁹², and increased expression of ABCA1 transporters necessary for HDL genesis⁹³. Even more notable were the significantly greater HDL-C levels among men in the LCHF group. To our knowledge, further increases in HDL-C beyond those seen with exercise can only be attributed to differences in diet as TG levels were not significantly different between groups. Short term LCHFDs consistently show positive changes in circulating HDL-C levels among normal weight^{11,60} and overweight individuals⁹⁴⁻⁹⁶. Studies evaluating the long-term (> 6 mo) HDL-C response in healthy men has yet to be conducted, however those carried out in obese patients have show increases in HDL-C⁵⁻⁷. Considering this fact, it is not unreasonable to expect significantly elevated HDL-C following chronic LCHFD in healthy fit men.

Surrogate Markers of Cholesterol Synthesis and Absorption

Plasma non-cholesterol sterols can be measured to assess cholesterol homeostasis and correlate strongly with absolute cholesterol synthesis and absorption^{44,97,98}. With varied cholesterol responses previously reported in men consuming LCHFDs⁸⁻¹³, we sought to assess whether a chronic (> 6 months) free-living LCHFD shifts cholesterol homeostasis in favor of synthesis or absorption. The ratio of cholesterol synthesis markers (lathosterol and desmosterol) to absorption markers (beta-sitosterol and campesterol) can be used to calculate fractional

cholesterol balance. A reduced ratio (< 0.5) is indicative of greater serum phytosterol concentrations and increased cholesterol absorption at the gut lumen where as raised fractional values (> 1.1) are representative of elevated cholesterol precursors and imply increased synthesis of cholesterol. To date, no previous long-term LCHF studies have utilized these surrogate markers to assess cholesterol balance in healthy, fit, individuals.

Quite remarkably, calculated mean fractional cholesterol balance was similar between groups (LCHF = 0.66, HC = 0.60) and was not indicative of increased absorption or synthesis. Given that men in the LCHF group consumed significantly greater levels of dietary cholesterol and maintained significantly greater levels of circulating cholesterol, this finding was noteworthy. Extrapolation of these findings may suggest men who follow a chronic LCHF expand their endogenous cholesterol pool before establishing a new circulating homeostatic level^{18,99}. Similar to cholesterol levels, we found surrogate markers in both groups to be greatly variable, in line with other findings³², and further substantiating an individualized, likely genetic, response to diet.

Noncholesterol sterols are novel biomarkers not yet endorsed by international guidelines, but can be useful in determining clinical approaches for elevated cholesterol (hypercholesterolemia)²⁹. Although men following a LCHF were hypercholesterolemic (TC > 200 mg/dL), all normalized sterol levels except desmosterol were greater in the HC group. Considering current recommended ranges for normalized sterol levels have been established using data from patients consuming a traditional American diet, normalizing noncholesterol sterols many not

be appropriate for assessment of over absorption and synthesis in LCHF adapted individuals. Greater absolute sterol values in the LCHF group were not surprising given the elevated cholesterol levels in those men. Significantly greater absolute desmosterol levels in the LCHF men would seem to imply greater production of cholesterol, however, the pathway through which desmosterol forms cholesterol is thought to contribute far less to overall synthesis than the lathosterol, which was not significantly different between groups. Apart from campesterol, absolute values of noncholesterol sterols showed significant positive correlations with total, LDL, and HDL cholesterol supporting the relationship between elevated sterol biomarkers and circulating cholesterol levels. We are the first to measure noncholesterol sterols in healthy, fit men, making it difficult to fully elucidate our findings. The effect exercise training has on surrogate cholesterol markers remains to be seen and further research is necessary to understand how noncholesterol sterols levels respond to long term LCHFDs.

Lipoprotein Particle Distribution, Size, and LP-IR

NMR analysis revealed favorable, pattern A, lipoprotein profiles in both groups. Considering the elite fitness level among men recruited for this study, we acknowledge that adaptation to endurance exercise influences lipoprotein particle distribution independent of diet^{86,88}. Nonetheless, we found a LCHFD resulted in an even more favorable lipoprotein profile (less atherogenic) compared to consuming a HCD. Though pattern type (A or B) is influenced by genetic inheritance⁵⁴, dietary change can also alter particle distribution, particularly atherogenic LDL subclasses⁵⁶.

In support of previous literature^{11,14,100}, reduced carbohydrate consumption among men following a LCHFD (< 100 g/day), in combination with less circulating TGs (-10.4%) and VLDLs (-27.9%), is likely the primary contributing factor towards an atheroprotective shift in lipoprotein metabolism (less atherogenic LDL particles [LDL-P] and greater atheroprotective HDL particles [HDL-P]). Still, cholesterol feeding with eggs has been shown to favorably alter LDL subclasses⁸⁴. Dietary carbohydrate reduction has consistently been shown to result in a significant decrease in both fasting and postprandial TGs^{1,11,14,100}. Due to the natural metabolism of TG rich VLDLs to LDLs, a reduction in TGs results in less VLDL synthesis by the liver and therefore a direct reduction in circulating LDL particles¹⁰. In addition, reduced circulating TGs decrease cholesterol-TG transfer between TG rich-VLDLs and LDL via CETP, allowing for HDL and LDL to carry a greater capacity of cholesterol, resulting in larger, more buoyant lipoproteins¹⁰¹.

Differences in TGs and VLDL subfractions among groups is likely attributable to a combination of reduced carbohydrate consumption, reduced VLDL production rate, shown to increase on a high carbohydrate diet¹⁰², and an increase in TG removal given high-fat diets (> 45% total energy) promote increased postheparin plasma and skeletal muscle LPL activity in humans^{56,103,104}. Reduced TGs are likely responsible for a significant 14.3% reduction in mean VLDL particle (VLDL-P) size, reduced total number, and decreased size of VLDL-P subfractions. Significant positive correlations between circulating TGs and VLDL particle count and subfractional size support this assessment (Total VLDL-P: $r = 0.94$, $p < 0.0001$; Large VLDL-P: $r = 0.66$, $p < 0.0016$; Medium VLDL-P: $r = 0.88$, $p < 0.001$; Small VLDL-P: $r = 0.64$, $p < 0.0026$). Although

LCHF adapted men had less total, large, medium, and small VLDL-P compared to men in the HCD group, only large VLDL-P were found to be significantly lower as a result of wide particle variation among participants.

Reduced VLDL-P size among men adapted to LCHFDS affected LDL particle size. Smaller denser LDL particles evolve from elevated levels of TGs and thus larger VLDL particles^{22,68,105,106}, primarily as a result of hepatic lipase¹⁰¹. This was evident in the men adapted to a LCHF as supported by the strong negative relationship between the two measures ($r = -0.70$, $p < 0.006$). Convincingly, mean LDL particle size was significantly greater in the LCHF group compared to the HCD group. Total LDL particle count was 52.7% greater among men consuming a LCHF, however, the distribution of LDL particles was dominated by large and intermediate particles (88.2% of total particles) while in the HC group, large and intermediate particles made up 59.3% of total LDL particles.

Of considerable importance are the findings related to small LDL-P, those attributed with an increased risk of CVD^{54,57,107}. Two men within the LCHF group prevented small LDL particles from being statistically significant between groups. Regardless, small LDL particles comprised 40.7% of total particles in men who consumed a HCD compared to only 11.8% in men on a LCHF. As a whole, HC men had 126% more small LDL particles than those in the LCHF group. Considering LDL-C levels in the LCHF group were nearly double those in the HC groups (161.3 vs 88.1 mg/dL), these findings suggest elevated LDL-C in men consuming a chronic LCHF does not contribute to the development of atherogenic small LDL-P and that elevated LDL-C may not be a good predictor of atherogenesis in those who follow a

LCHF. These findings support previous LCHF studies which demonstrate restricting dietary carbohydrate increases mean LDL particle size, reduces small LDL-P, and subsequently shifts particle pattern^{11,14,60,94,108-112}.

Carbohydrate restriction, reduced TGs, and increased dietary cholesterol can similarly be attributed to the positive shift in HDL lipoproteins. Mean HDL size was significantly larger in the LCHF group, suggesting a reduced uptake of HDL particles by the liver and thus increased circulating HDL-C concentrations^{111,112}. Although not statistically different, total HDL-P were greater in the LCHF group. Large atheroprotective HDL particles^{76,113} were significantly greater in the LCHF group accounting for 39.6% of total particles, in contrast to 23.9% in men consuming a HCD. Although not measured in the current study, cholesterol ester transferase likely plays a role increasing large HDL particles in the LCHF group¹¹⁴. Interestingly, men on a HCD had a 56% greater number of medium HDL particles compared to the LCHF group. Because men following a LCHF had reduced circulating TGs, it is probable that neutral lipid exchange between VLDL and HDL lipoproteins was reduced, resulting in the formation of fewer TG-rich HDL particles. If HDL particles contained less TGs, it would reduce their susceptibility to remodeling by hepatic lipase, yielding fewer small HDL particles¹⁴.

The lipoprotein insulin resistance index (LP-IR) is an effective tool to summarize the NMR results. The LP-IR index is an algorithmic system which gives numeric weight to six parameters of the NMR lipoprotein profile test associated with insulin resistance¹¹⁵⁻¹¹⁷ (VLDL, LDL, and HDL size, large VLDL-P, small LDL-P, and large HDL-P). The final LP-IR score is calculated by summing the six NMR parameter

scores⁸¹. Shalaurova et al.⁸¹ recently demonstrated the value of the index in predicting insulin resistance. Although both groups in the present study were comprised of healthy fit males who's mean LP-IR scores fell just at or below the 25th percentile (< 25), those following a LCHFD showed an astoundingly lower (-360%) LP-IR score. Knowing that insulin resistance precedes the development of obesity, metabolic syndrome, and type 2 diabetes mellitus, such a significantly lower LP-IR score in men consuming a LCHFD, even among healthy fit individuals, provides further evidence for the therapeutic potential of LCHFDs in treating these metabolic abnormalities.

Despite significantly greater consumption of dietary cholesterol by men consuming a chronic LCHFD, surrogate markers of cholesterol synthesis and absorption did not reveal a direct cause (increased synthesis or absorption) for elevated serum cholesterol (TC, LDL-C, and HDL-C) within the LCHF group. Although speculative, individuals who adopt a LCHFD may experience an expansion of their endogenous cholesterol pool during the adaptation phase of the diet, after which they maintain greater circulating cholesterol levels but no increased risk for CVD. Increased cholesterol consumption, elevated cholesterol levels, and low rates of heart disease in indigenous Inuit populations suggest this may be true¹¹⁸⁻¹²¹. In further support of this theory, we found that regardless of an increase in total and LDL cholesterol within the LCHFD group, these men had more favorable lipoprotein profiles in regards to CVD risk than those consuming a HCD, notably less small LDL particles. Current evidence suggests CVD is lipoprotein-mediated, leaving measurements of circulating cholesterol nearly obsolete in their efficacy for predicting

heart disease. Additional research is necessary to fully determine if elevated LDL cholesterol in LCHF individuals presents an increased risk for CVD.

Limitations of this study include small sample size, cross-sectional design, surrogate measures of cholesterol balance rather than absolute cholesterol turnover, and lack of measured variables such as enzymes important in lipoprotein metabolism and markers of LDL oxidation. These data should be regarded as pilot data requiring future, more in-depth studies be conducted to fully determine the meaning of these data. Increased cholesterol did not occur as a result of over absorption or synthesis as measured by surrogate markers and suggests the body may maintain a new homeostatic set point upon adaptation to a LCHFD. Our findings support the notion that long-term LCHFD can increase circulating cholesterol mass but in so doing, shift lipoprotein size and particle distribution towards a more favorable less atherogenic profile.

Tables & Figures

Table 1. Baseline Subject Demographics¹

	High Carb	Low Carb
Age (yr)	32.9 ± 6.0	34.1 ± 7.1
Height (cm)	173.9 ± 5.3	175.7 ± 6.5
Body Mass (kg)	66.5 ± 6.8	68.8 ± 8.2
Body Fat (%) ²	9.6 ± 4.3	7.8 ± 2.4
Lean Mass (kg) ²	57.3 ± 5.0	60.9 ± 7.1
Fat Mass (g)	6.5 ± 3.6	5.46 ± 1.8
VO ₂ max (mL/kg•min)	64.6 ± 6.1	64.6 ± 4.3

¹Values are mean ± SD (n=10).

²Determined by dual-energy X-ray absorptiometry.

Table 2. Daily macronutrient breakdown for habitual diet¹

Habitual Diet ²	High Carb	Low Carb
Energy (kcal/day)	3044 ± 604	2884 ± 814
Protein (g/day)	117 ± 39	139 ± 32
Protein (%en)	14.9 ± 3.9	19.4 ± 2.4
Protein (g/kg)	1.7 ± 0.4	2.1 ± 0.6
Carbohydrates (g/day)	442 ± 116	82 ± 62****
Carbohydrates (%en)	56.5 ± 10.2	10.4 ± 4.9****
Fat (g/day)	94 ± 33	226 ± 66****
Fat (%en)	26.7 ± 7.5	69.5 ± 6.0****
Saturated fat (g/day)	22 ± 10	86 ± 22****
Monounsaturated fat (g/day)	32 ± 16	82 ± 42***
Polyunsaturated fat (g/day)	17 ± 9	28 ± 17
Alcohol (%en)	1.8 ± 2.7	0.7 ± 1.4
Cholesterol (mg/day)	291 ± 256	844 ± 351**
Fiber (g/day)	48 ± 15	23 ± 17**

¹Values are mean ± SD (n=10).

²3-Day 24-Hr food record including one weekend day.

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

Table 3. Daily macronutrient breakdown for pre-testing diet¹

Pre-testing Diet²	High Carb	Low Carb
Energy (kcal/day)	2856 ± 846	2572 ± 597
Protein (g/day)	118 ± 41	148 ± 34
Protein (%en)	16.4 ± 3.5	23.2 ± 5.7
Protein (g/kg)	1.7 ± 0.4	2.2 ± 0.7
Carbohydrates (g/day)	371 ± 107	64 ± 27****
Carbohydrates (%en)	50.9 ± 5.0	10.4 ± 5.4****
Fat (g/day)	95 ± 26	195 ± 65**
Fat (%en)	29.5 ± 2.8	66.0 ± 8.8****
Saturated fat (g/day)	25 ± 9	79 ± 37**
Monounsaturated fat (g/day)	29 ± 17	69 ± 29*
Polyunsaturated fat (g/day)	16 ± 3	19 ± 7
Alcohol (%en)	3.2 ± 4.2	0.3 ± 0.7*
Cholesterol (mg/day)	302 ± 364	883 ± 320**
Fiber (g/day)	37 ± 13	20 ± 12*

¹Values are mean ± SD (n=10).

²2-Day 24-Hr food record prior to test day.

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

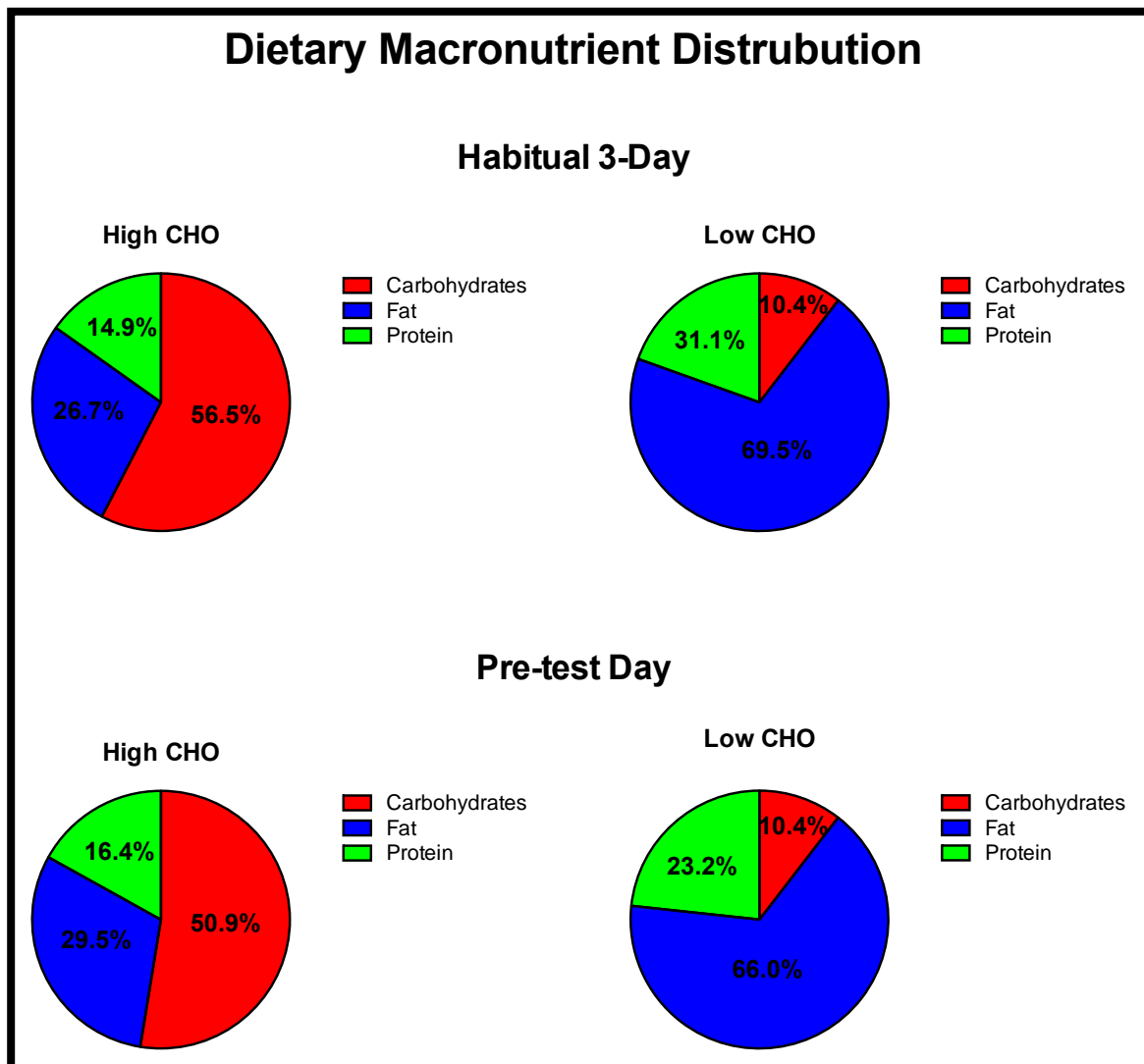


Figure 1: No statistical differences between Habitual Diet and Pre-test Day

Table 4. Serum Cholesterol and Lipids ¹

Variable	High Carb	Low Carb
Total Cholesterol (mg/dL)	168.7 ± 24.4	277.7 ± 50.58****
LDL Cholesterol (mg/dL)	88.1 ± 13.68	161.3 ± 37.38****
HDL Cholesterol (mg/dL)	63.9 ± 17.99	102.3 ± 26.21**
Total Cholesterol/HDL Cholesterol	2.74 ± 0.47	2.81 ± 0.53
Triglycerides (mg/dL)	70.0 ± 25.09	63.4 ± 17.26
Triglycerides/HDL Cholesterol	1.15 ± 0.49	0.67 ± 0.23*
Lipoprotein Insulin Resistance Index ²	26.7 ± 10.47	5.8 ± 5.75***

¹Values are mean ± SD (n=10).

²Combined algorithm of six lipoprotein measures.

To convert to SI units, multiply total cholesterol, LDL-C, HDL-C (mg/dL) × 0.0256 = mmol/L;

multiply triglycerides (mg/dL) × 0.0113 = mmol/L.

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

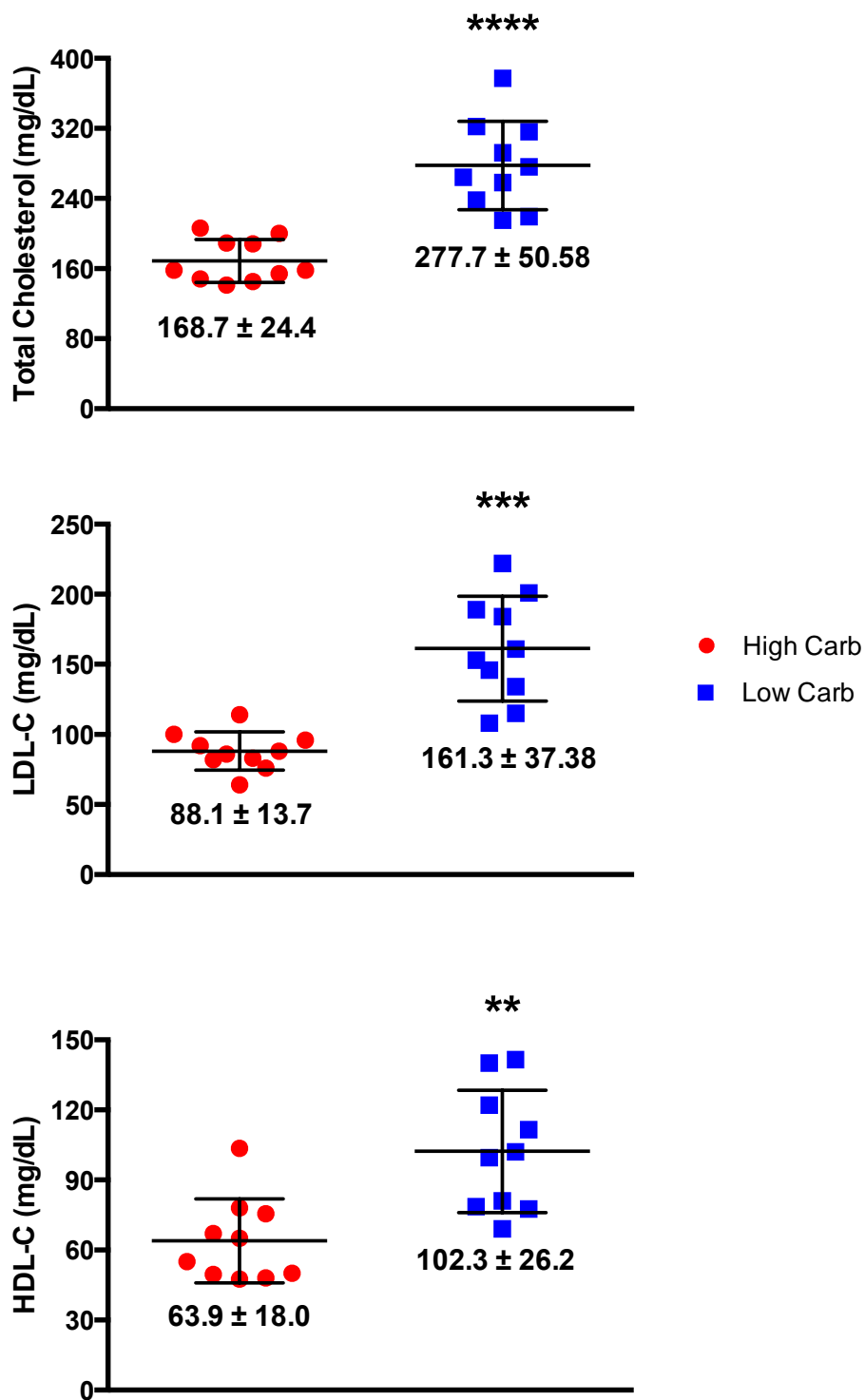


Figure 2: Total, LDL, and HDL cholesterol group spread values. Values are mean \pm SD ($n=10$). Asterisks indicate significant difference between groups: $**P < 0.01$; $***P < 0.001$; $****P < 0.000$

Table 5. VLDL size and concentration of total VLDL particles and VLDL subfractions¹

Variable	High Carb	Low Carb
VLDL Particle Size (nm)	43.65 ± 1.72	38.2 ± 4.09**
Total VLDL Particles (nmol/L)	48.10 ± 25.47	37.61 ± 5.90
Large VLDL Particles (nmol/L)	1.27 ± 0.70	0.56 ± 0.09*
Medium VLDL Particles (nmol/L)	13.55 ± 15.36	4.12 ± 3.70
Small VLDL Particles (nmol/L)	33.26 ± 14.29	32.94 ± 18.41

¹Values are mean ± SD (n=10).

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

Table 6. LDL size and concentration of total LDL particles and LDL subfractions¹

Variable	High Carb	Low Carb
LDL Particle Size (nm)	20.99 ± 0.35	21.47 ± 0.34**
Total LDL Particles (nmol/L)	892.8 ± 195.8	1363 ± 343.1**
Large LDL Particles (nmol/L)	456.4 ± 101.0	1026 ± 258.9****
Intermediate LDL Particles (nmol/L)	73.4 ± 76.92	176.3 ± 63.87**
Small LDL Particles (nmol/L)	363.0 ± 245.2	160.6 ± 134.8

¹Values are mean ± SD (n=10).

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

Table 7. HDL size and concentration of total HDL particles and HDL subfractions¹

Variable	High Carb	Low Carb
HDL Size (nm)	9.51 ± 0.47	10.2 ± 0.47**
Total HDL Particles (nmol/L)	33.96 ± 5.35	37.38 ± 4.43
Large HDL Particles (nmol/L)	8.1 ± 3.41	14.79 ± 4.41**
Medium HDL Particles (nmol/L)	12.9 ± 3.06	8.27 ± 3.18**
Small HDL Particles (nmol/L)	12.96 ± 5.54	14.31 ± 5.19

¹Values are mean ± SD (n=10).

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

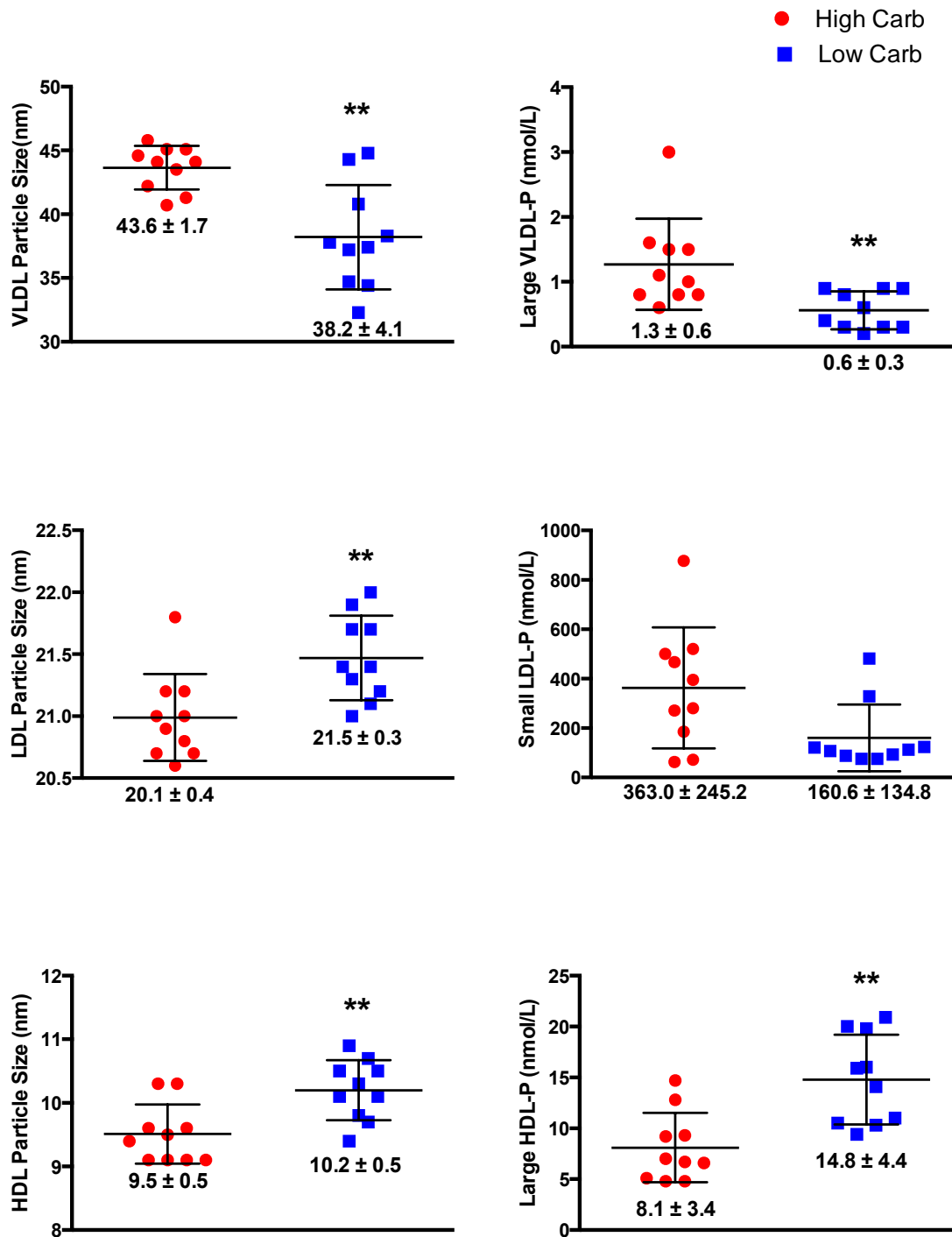


Figure 3: Lipoprotein Insulin Resistance Index Measures. Values are mean ± SD (n=10). Asterisks indicate significant difference between groups: **P < 0.01

Table 8. Fractional cholesterol balance and non-cholesterol sterols ¹

	High Carb	Low Carb
Fractional Cholesterol Balance ²	0.60 ± 0.16	0.66 ± 0.22
Absolute (mg/L)		
Desmosterol	1.42 ± 0.24	2.82 ± 0.80***
Lathosterol	1.84 ± 0.55	2.23 ± 0.61
Campesterol	3.5 ± 1.46	3.86 ± 1.37
Sitosterol	3.07 ± 1.03	4.34 ± 1.75
Cholestanol	2.23 ± 0.44	2.8 ± 0.43**
Normalized (10 ² x µmol/mmol of TC)		
Desmosterol	84.8 ± 11.55	101.9 ± 22.35*
Lathosterol	109.4 ± 30.67	79.6 ± 14.41*
Campesterol	195.0 ± 59.04	134.7 ± 43.98*
Sitosterol	167.7 ± 44.65	145.8 ± 55.03
Cholestanol	132.3 ± 24.44	101.5 ± 13.4**

¹Values are mean ± SD (n=10).

²Calculated with normalized values

Asterisks indicate significant difference between groups:

*P < 0.05; **P < 0.01; ***P < 0.001

Table 9. Correlations of serum noncholesterol sterols with serum total and lipoprotein cholesterol levels among healthy, fit men ($n=20$)

Sterols	Serum Cholesterol (mg/dL)		
	Total Cholesterol	LDL Cholesterol	HDL Cholesterol
Absolute (mg/L)			
Desmosterol	0.8851****	0.9169****	0.5972**
Lathosterol	0.6231**	0.5900**	0.5413*
Campesterol	0.4327	0.2528	0.5321*
Sitosterol	0.6179**	0.5042*	0.6268**
Cholestanol	0.7141***	0.6106**	0.6162**
Normalized ($10^2 \times \mu\text{mol}/\text{mmol}$ of TC)			
Desmosterol	0.3963	0.5262*	0.07971
Lathosterol	-0.4269	-0.3877	-0.3359
Campesterol	-0.3318	-0.4561*	-0.1279
Sitosterol	-0.1371	-0.2114	0.005034
Cholestanol	-0.6654	-0.0348	-0.5312*

Both groups ($n=20$), Low Carb ($n=10$), High Carb ($n=10$)

Asterisks indicate significant difference between groups:

** $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.*

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