Effects of Incremental Dietary Macronutrient Changes on Fat Oxidation and Body Composition

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Effects of Incremental Dietary Macronutrient Changes on Fat Oxidation and Body Composition

Laura Kunces

University of Connecticut, 2014

**ABSTRACT:** Despite evidence that low-carbohydrate diets (LCD) can improve metabolic syndrome (MetS) characteristics and risk for cardiovascular disease, concerns remain regarding the potential deleterious effects of higher fat intake. If higher fat intake (in the context of lower carbohydrate intake) is accompanied by higher fat oxidation, there would likely be more efficient fat loss and improved health parameters. Our aim was to examine how diets spanning a broad range of carbohydrate levels ranging from very low (<50 g/day) to current dietary guidelines (~350 g/day) affect substrate oxidation patterns and changes in body composition within the same person while keeping caloric and protein intake constant. After an initial 3-wk run-in LCD, 16 adults with MetS (age 44.9 ± 9.9 yr, BMI 37.9 ± 6.3 kg/m²) were fed six sequential moderately hypocaloric 3-wk diets that progressively increased carbohydrate (CHO) (from 47 to 344 g/day) with concomitant decreases in total fat. Body composition was determined by dual-energy X-ray absorptiometry (DXA) and respiratory quotient (RQ), fat oxidation, and resting energy expenditure (REE) were determined by indirect calorimetry after each diet phase. Subjects lost significantly more fat mass (-2.32±1.53 kg) on the free-living LCD phase, but overall fat mass loss was variable between subjects and on average less than expected from the calculated caloric deficit (-8.3±4.5 vs 12.7±15.8 kg, respectively). There was a significant decrease in REE, but no significant change in relative REE (kcals/kg/day). Fat oxidation rates significantly increased when consuming diets with 7% CHO, but decreased to below baseline by the highest CHO phase. RQ decreased on the LCD (0.75±0.04), and increased linearly as CHO increased, up to 0.84 ±0.05. Body mass was significantly correlated with CHO consumption (r=0.49), insulin (r=0.34), fat consumption (r=-0.49) and ketones (r=0.46). These findings suggest that it is difficult to estimate weight loss within an individual, even with a constant caloric deficit, since individuals vary in their substrate oxidation response to reintroduction of dietary carbohydrate. Those who can maintain a higher fat oxidation to a greater
extent as carbohydrates are increased (and fat decreased) may possess an enhanced ability for fat loss on higher carbohydrate diets.
Effects of Incremental Dietary Macronutrient Changes on Fat Oxidation and Body Composition

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2014
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REVIEW OF LITERATURE

“Eat less, exercise more” is the advice from many public health experts to obese people. Clearly the failure to curtail the burgeoning obesity epidemic suggests this paradigm may be flawed. An alternative perspective is that obesity is due to excess accumulation of energy in adipose tissue, and that factors controlling flux of energy in and out of adipocytes is fundamental in understanding obesity. This view emphasizes the importance of macronutrient composition on fat balance.

The simple law of thermodynamics in the form of the equation ‘calories in minus calories out’ equaling weight loss has been challenged with recent research regarding the efficacy of hypocaloric diets. As the obesity epidemic around the world grows, a dietary strategy to reduce the incidence of metabolic syndrome continues on the research forefront and macronutrient composition of diets has been a point of contention. Research continues to test low carbohydrate diets (LCDs) and their ability to trump the recommended low calorie or low-fat (LF) diet for weight loss and body composition improvements in those who may benefit the most.

Metabolic Syndrome

Obesity is one of the most common diseases among adults in the world. More than 35% of American adults (defined as BMI >30 kg/m²) and 17% of children are obese (defined as >95th percentile for BMI), with numbers on the rise each year (Ogden, Carroll, Kit, & Flegal, 2014). Obesity is known to upset lipid and glucose metabolism and disrupt cellular metabolism by releasing numerous cytokines contributing to comorbidities (Redinger, 2007). Metabolic syndrome (MetS) is a diagnosis representing a multitude of complex pathways emergent from
the growing obesity epidemic. It is estimated more than 34% of the United Stated has MetS (Mozumdar & Liguori, 2011), and although the risk factors are seriously significant to your health, dietary modifications (Andersen & Fernandez, 2013) and lifestyle changes can help reduce body weight and manage the dyslipidemias associated. MetS is a combination of metabolic abnormalities that increase the risk for type 2 diabetes mellitus (T2DM) and coronary heart disease (CHD) exponentially (Meigs et al., 2007).

Insulin resistance is strongly influenced by obesity through elevated plasma fatty acid levels (Coppack, Jensen, & Miles, 1994). The excessive storage of fat that defines obesity, eventually leads to excessive stored and circulating fatty acids, creating oxidant stress to each cell’s mitochondria and endoplasmic reticulum. The stored and circulating fatty acids inhibit lipogenesis, preventing adequate clearance of serum triacylglycerols, contributing to hypertriglyceridemia. The release of fatty acids by endothelial lipoprotein lipase from serum triglycerides with elevated β-lipoproteins causes lipotoxicity and insulin receptor dysfunction. This condition is often followed by hyperglycemia, and compensated with gluconeogenesis. Increased circulating fatty acids are able to stimulate insulin secretion, however, fatty acid-induced insulin secretion is strongly dependent on carbohydrate levels of the diet (Stein et al., 1996). Hyperglycemia is further attenuated when fatty acids decrease the utilization of insulin-stimulated muscle glucose (Boden, Chen, Ruiz, White, & Rossetti, 1994). Lipotoxicity from the elevated circulating free fatty acids also decreases pancreatic β-cell ability to produce insulin (Unger, 1995), resulting in insulin resistance.
MetS is classified as having at least three of the parameters in Table 1 (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report, 2002). In addition, because of the associated increased risk for CHD and T2DM, MetS is often combined with imbalances of inflammation markers and hormones. Although there are various similar definitions of MetS defined by different health organizations, most can agree that diet and lifestyle should be addressed as the first option for prevention or treatment.

Table 1. NCEP defined metabolic syndrome diagnosis characteristics.

<table>
<thead>
<tr>
<th>Metabolic Characteristic</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>&gt;102 cm</td>
<td>&gt;88 cm</td>
</tr>
<tr>
<td>High Density Lipoprotein (HDL) Cholesterol</td>
<td>&lt;40 mg/dL</td>
<td>&lt;50 mg/dL</td>
</tr>
<tr>
<td>Fasting serum blood glucose</td>
<td>&gt;100 mg/dL</td>
<td></td>
</tr>
<tr>
<td>Blood pressure</td>
<td>&gt;130/85 mmHg*</td>
<td></td>
</tr>
<tr>
<td>Plasma triglycerides</td>
<td>&gt;150 mg/dL</td>
<td></td>
</tr>
<tr>
<td>* or on blood pressure medications</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Current Dietary Recommendations

Dietary fat and saturated fat have been the focus of nutritional recommendations for over three decades. The latest guidelines issued by the Dietary Guidelines Advisory Committee (DGAC) encourages Americans to consume less than 35% of their calories from fat, and to keep saturated fat consumption to less than 7% (Lichtenstein et al., 2006). Protein recommendations are set at a modest 0.8 g/kg body weight (15-20% of calories) and carbohydrates are to comprise of the remaining calories (55-65%). According to dietary trends, there has been a decrease in fat
and saturated fat consumption, but Americans have seemed to replace the fat they previously consumed with high carbohydrate options (Trends in intake of energy and macronutrients--United States, 1971-2000, 2004) and the incidence of obesity and MetS continues to rise.

**Low-Carbohydrate Diets**

The mainstream use of LCDs as an obesity treatment began around 1960s, often eliminating or reducing the numerous pharmaceutical prescriptions related to physical and clinical unhealthy diagnoses. It is challenging to associate metabolic responses and clinical outcomes to one nutrient, but carbohydrates have a complex link to plasma glucose and insulin, and cellular function. With lower glucose availability, changes in insulin and glucagon concentrations will shift the body away from storing fat, and start efficiently oxidizing fat. LCDs need to be operationally defined, but there is a threshold effect seen around 20-50g/day in clinical research where the body starts to produce ketones. Nutritional ketosis is reached when systemic ketone blood levels are maintained between 0.5 mM and ~3.0 mM. Metabolic changes and benefits are seen beyond this low threshold (Westman et al., 2007). Similar to many metabolic changes, this threshold is variable for individuals and is dependent on their level of carbohydrate tolerance.

A LCD would include the following foods: meat, eggs, fish, butter, and heavy cream, moderate amounts of cheeses, low-carbohydrate vegetables, and salad dressings, and small amounts of nuts and seeds. The diet restricts fruit and juices, breads, grains, pasta, cereal, starchy vegetables, high-carbohydrate desserts, and most dairy products. It is a common
misconception that a LCD increases protein consumption; contrarily, protein intake is set at normal recommendations (15-25% of total calories). Recommended carbohydrate amount is variable depending on the individuals’ tolerance and insulin sensitivity, but this diet is often referred to as less than 50-150 grams of carbohydrates per day (Westman, et al., 2007) or about 10-25% of dietary caloric intake. To be in nutritional ketosis and producing ketones, carbohydrate intake would likely need to be narrower (<10%), often referred to as a very-low-carbohydrate ketogenic diet (VLCKD). Dietary fat would comprise the rest of dietary calories (60-80% of calories). Micronutrients of sodium, potassium, and calcium should be monitored and supplemented, as necessary. There are very few studies to date that report appropriate length to be on a VLCKD to see optimal benefits from producing ketones, but the research concludes it is clearly longer than one week and more likely around 3-4 weeks. The adaption process is not dependent on weight, athleticism, or gender per research done to date (Phinney, 2004).

Ketone Physiology and Regulation

The rationale for reducing dietary carbohydrates derives from basic mechanisms: dietary carbohydrates are not essential and therefore are not needed to provide energy. Fatty acids provided from dietary fat or adipose stores, and ketone bodies created from dietary fat and adipose stores are all converted to be the main fuel sources in a LCD. Skeletal muscle can efficiently survive on burning fats as their main fuel source, however, fatty acids are unable to cross the blood brain barrier to be utilized by the central nervous system. Even though the brain would preferentially burn glucose, it easily adapts to using ketones just as efficiently. Ketones are produced in three forms: acetoacetate, B-hydroxybuterate, and acetone. The brain can use acetoacetate or B-hydroxybuterate, while acetone is excreted via the lungs as waste. The rate of
ketogenesis is regulated by hormone sensitive lipase (HSL). While very little to no dietary carbohydrates are consumed, glucose levels can be sustained by increases in pancreatic glucagon secretion, which phosphorylates adipose HSL, resulting in hepatic ketogenesis liberating fatty acids from adipose tissue. Additionally, gluconeogenesis can occur using dietary protein and fat. Gluconeogenesis depletes the Kreb’s cycle intermediates and increases acetyl-CoA, the substrate needed to initiate aerobic oxidation. Elevated insulin levels inhibit ketogenesis through dephosphorylation and inactivation of adipose HSL. The elevated levels of Malonyl-CoA produced from fatty acid synthesis are a negative feedback inhibitor, preventing fatty acids entrance to the mitochondria. This ultimately inhibits ketone production. The differences between a LCD and a starvation or low-calorie diet is the ability of the body to maintain serum glucose levels and spare the breakdown of endogenous protein (J. S. Volek & Westman, 2002) while consuming a LCD.

Body Composition Changes

Health benefits resulting from weight loss are linked to body fat loss and attenuation of muscle mass loss, however, diet regimens that focus on decreasing weight tend to decrease both lean and fat mass. This undesirable result is seen in about 25% of studies that focus on weight loss outcomes through LF diets (Kraemer et al., 1999; Layman et al., 2005) despite similar hypocaloric intakes across studies. Hypocaloric LF diets use protein inefficiently, so more dietary protein is needed to preserve lean body mass. Garrow and Summerbell (Garrow & Summerbell, 1995) created a regression analysis from a meta-analysis predicting that 71% of a 10 kg weight loss obtained by dieting alone comes from fat-mass. This suggests dieting can cause a 29% weight loss from tissue other than adipose. The more desired outcome is a result of
a diet that can decrease fat mass but preserve lean body mass, which is seen in certain circumstances when sufficient dietary protein is provided in a hypocaloric LCD (Krieger, Sitren, Daniels, & Langkamp-Henken, 2006; J. S. Volek et al., 2002).

A comprehensive meta-analysis looked at 87 diet trials regarding dietary macronutrient composition on body composition and concluded diets that restricted carbohydrates to some degree resulted in greater fat loss. In addition, the meta-analysis found with weight loss, the diets higher in protein percentage were associated with better preservation of lean body mass (Krieger, et al., 2006). A recent systematic review (Hession, Rolland, Kulkarni, Wise, & Broom, 2009) concluded weight loss to be greater after 6 months and after 12 months on a VLCKD diet compared to a LF diet. Additionally, at the same time points, the low-VLCKD diet significantly improved subject cholesterol panels including triacylglycerols, and showed trends in improvements of blood pressure and glucose. Interestingly, there were higher attrition rates of those following the LF diet than those consuming the VLCKD.

Specifically looking at fat-mass loss, LCDs ensuing ketogenesis result in greater weight loss and fat-mass loss than LF diets because the body does not catabolize protein if proper ketoadaptation occurs. In a study involving overweight men and women on a ketogenic LCD or a LF diet, a 2-fold greater weight loss was observed in the LCD subjects despite similar caloric restrictions (J. Volek et al., 2004; J. S. Volek, Fernandez, Feinman, & Phinney, 2008). In normal weight men, LCDs significantly decreased fat-mass and significantly increased lean body mass after 6 weeks of being in ketosis. Johnston (Johnston et al., 2006) compared weight loss and other metabolic biomarkers in adults on a VLCKD and a LCD and found both groups decreased body weight (6.3 kg and 7.2 kg, respectively) and fat mass (3.4 kg and 5.5 kg,
respectively) with no significant differences between groups. Body composition changes seem favorable from a LCD in most all populations tested.

Severe energy restriction from hypocaloric diets can decrease nitrogen balance (Smith, Underwood, & Clemmons, 1995), and small decreases in dietary protein could potentially decrease lean body mass retention in the body. Therefore, ketogenic diets need to be well formulated with sufficient protein to maintain or potentially increase nitrogen balance and thus increase the amount of lean body mass retained. An early, well-controlled feeding study for 9 weeks improved body composition and nitrogen balance in men showing the effects of a well formulated LCD. An 1800 calorie, intentional weight-loss diet with 115 grams of protein (25% of energy) was sufficient for men to lose 12-18 kg of mostly fat mass, while maintaining nitrogen balance (Young, Scanlan, Im, & Lutwak, 1971). An 8-week hypocaloric study in women showed similar findings (Hoffer, Bistrian, Young, Blackburn, & Matthews, 1984). Women who were fed 1.5g/kg protein, and supplemented with vitamins and minerals following a LCD preserved more lean tissue mass than a cohort who consumed the same calories but only 0.8 g/kg protein. Results concluded the body is able to preserve lean tissue when adequate protein and micronutrients are consumed, despite being in a major calorie deficit.

Modest body weight loss on a LCD may be explained by a loss of fat mass but a gain of lean tissue. Research has shown it is possible to lose 100% of weight from fat mass on a VLCKD and still gain lean body mass (J. S. Volek, et al., 2002). Twenty-four hour urine collections with analysis of creatinine levels may help understand the gains of lean tissue seen in some individuals who consume LCDs. Research has found a high positive correlation between urinary creatinine concentrations and muscle mass (Edwards & Whyte, 1959), therefore, higher
urinary creatinine levels in are typically seen with men more than women (Bjornsson, 1979; Turner, 1975). Whole body muscle protein synthesis can be estimated from creatinine excretion and can be compared to whole body protein synthesis calculated by leucine flux (Nair, Halliday, & Griggs, 1988), a well-supplied nutrient on a LCD. Relating to resting energy expenditure (REE), loss of lean body mass from energy deficient diets is correlated to decreases in REE (Abraham & Wynn, 1987). However, when following a VLCKD or a LCD, REE has been seen to significantly increase (Johnston, et al., 2006), perhaps explained by sufficient leucine intake and lean tissue sparing, despite overall caloric deficit. Research has found resting metabolic rates (RMR) to be 10% higher in individuals consuming a LCD than a LF diet (Blundell, Cooling, & King, 2002) resulting in a considerable difference if calculated for calorie differences over a month or year.

In a controlled, randomized 3-way crossover study design, overweight and obese adults followed each different isocaloric macronutrient composition diet for a month. After achieving a 10-15% weight loss while consuming a ‘run-in’ diet, REE decreased the most following a month of the LF diet (comprised of 60% carbohydrates, 40% fat, 20% protein) and decreased the least with the VLCKD (comprised of 10% carbohydrate, 60% fat, 30% protein). Those on the LCD expended 300 calories more a day than those on the LF and 150 more calories than on the low-glycemic diet (comprised of 40% carbohydrate, 40% fat, 20% protein) (Ebbeling et al., 2012). These findings suggest the fewer carbohydrates consumed, the more energy expended by weight-reduced individuals.
Visceral adipose is highly active from a metabolic point of view. Visceral adiposity has been shown an independent predictor of insulin sensitivity (Cnop et al., 2002), glucose tolerance (Hayashi et al., 2003), elevated blood pressure (Bacha, Saad, Gungor, Janosky, & Arslanian, 2003) and dyslipidemia (Nieves et al., 2003). Research has found visceral adipose to be associated with higher production of pro-inflammatory cytokines including tumor necrosis factor-alpha (Bertin et al., 2000), plasminogen activator inhibitor-1 (Alessi et al., 1997), interleukin-6, and C-reactive protein (You et al., 2008), all of which are associated with adverse health effects. Visceral adiposity also leads to decreased secretion of growth hormone (GH), even though insulin-like growth factor-1 (IGF-1) levels remain normal and GH-binding protein levels are elevated (Maccario et al., 1999). Decreases in GH secretion have been associated specifically with body fat distribution regarding abdominal obesity and increased visceral fat (Johannsson et al., 1997).

In a cross-over, randomized study design comparing a LCD to a LF diet with similar caloric restrictions, the LCD intervention resulted in twice the amount of fat-loss, of which most was lost from the trunk region (J. Volek, et al., 2004). This study indicates that LCDs have the potential to preferentially lose fat in a region that carries a large associated health risk. Therefore, dietary interventions should focus on reducing visceral adiposity and decreasing the pro-inflammatory cytokines while maintaining lean body mass for maximal health benefits. In turn, this would reduce the incidence of MetS and the growing obesity epidemic.

Body mass loss from LCDs can be variable and should be assessed using body composition techniques allowing for discretion between lean body mass and fat mass, since a
scale is not reflective of actual changes occurring in body tissues. A study involving individuals with MetS used dual-energy x-ray absorptiometry (DEXA) analysis to compute body composition and was able to identify a grander weight loss from LCD than a LF diet, with a 1.6-fold greater fat loss specifically from abdominal adipose on the LCD (J. S. Volek et al., 2009). Although results continuously report weight loss from the trunk region, the mechanisms of which LCDs act upon body weight distribution in the mid-region are still unclear. A ‘metabolic advantage’ may help explain some of the weight changes on a VLCKD. This metabolic advantage may be explained by an increase in energy expenditure through the demand for protein turnover through gluconeogenesis (Bisschop et al., 2000), a greater thermogenic effect from protein and therefore a loss of energy as heat (Jequier, 2002), or simply excretion of ketones in urine or feces while on a VLCKD.

Respiratory quotient (RQ) is consistently lower in people consuming a LCD in the fasting and postprandial state than those consuming a LF diet (Blundell, et al., 2002). In the fasted state, fat oxidation accounts for 53% of energy expenditure derived from fat oxidation in a LCD and only 39% from a LF diet. An elevated RQ and lowered RMR are considered risk factors for weight gain (Bouchard, 1996). Ketogenesis consumes oxygen but produces less carbon dioxide often retaining or excreting ketones in the blood or urine, resulting in an RQ less than 0.7. A lower RQ translates to increased oxidization of fat, increasing the flux of fat from adipocytes. Research has shown that a higher 24-hour RQ, indicating low rates of fat oxidation relative to carbohydrate oxidation, are associated with higher rates of subsequent weight gain (Zurlo et al., 1990). Additionally, researchers have found higher RQ values to be associated with weight fluctuations, ultimately leading to weight gain in subjects consuming a very low-calorie diet.
(Hainer et al., 2000). Therefore, conclusions suggest an elevated fasted RQ can be indicative of weight gain through the impaired ability to burn fat (Valtuena, Salas-Salvado, & Lorda, 1997).

Insulin Sensitivity

LCD have been shown to consistently decrease circulating triglycerides (Westman, et al., 2007) and stabilize blood glucose levels. Additionally, insulin secretion tends to be more controlled. Therefore, carbohydrate restrictive diets should be used for lifestyle modifications in diabetic individuals. With this in mind, assessing the homeostatic model of insulin resistance (HOMA-IR) (Matthews et al., 1985), the most common measure of insulin resistance, or a newer equation coined the insulin sensitivity index (ISI) (Venkataraman et al., 2013) would be useful in identifying individuals with insulin resistance for risk of diabetes or future cardio-metabolic events. The latter option takes into account triglycerides and waist-to-hip ratio, in addition to fasting glucose and insulin levels, allowing for a more sensitive prediction equation in calculating risk. Research shows following a VLCKD or a LCD for 6 weeks can significantly improve insulin sensitivity (Johnston, et al., 2006). Another study compared obese non-diabetic insulin sensitive women and obese non-diabetic insulin resistant women consuming hypocaloric LF and LCDs for 16 weeks. Although the LCD was 40% carbohydrates, the insulin resistant women lost significant more weight than those on the LF diet (13.4% vs. 8.5%, respectively). This suggests insulin sensitivity is associated with the macronutrient composition of hypocaloric diets (Cornier et al., 2005).
Theoretical Low-Carbohydrate Ideas

Nonequilibrium Thermodynamics

A thermodynamic-based theory has previously been used to argue issues of weight change stating ‘a calorie is a calorie’, but the basic ideas of nonequilibrium thermodynamics may provide a more advanced explanation. The emphasis of nonequilibrium thermodynamics is placed on the idea that systems may never attain equilibrium, and are constantly undergoing a flux of material, typically through a catalyst or other factors that affect the rate of reactions. Specifically with LCDs, adipocytes are continuously cycling between states of lipolysis or re-esterification, depending on the hormonal state, which is controlled by dietary macronutrient composition (Feinman & Fine, 2007). In other words, two calorically similar diets may affect fat-mass differently because of their carbohydrate content and their affect on insulin levels. Through glucose infusion testing, fat mobilization is decreased via the inhibition of lipolysis and stimulation of re-esterification resulting in an increase of TAGs in the adipose (Wolfe & Peters, 1987). Authors predicted if a diet were to be fed over time with increasing carbohydrate levels, the corresponding insulin levels would have a profound negative effect on lipase activity in adipose tissue (Feinman & Fine, 2007). A LCD that did not affect insulin levels would reduce lipogenesis and increase lipolysis, likely explaining some of the weight-loss accompanied with the diet (Cahill, 2006; Veldhorst, Westerterp-Plantenga, & Westerterp, 2009). One downfall of nonequilibrium thermodynamics is the inability for the equation to take into account de novo fatty acid synthesis or hepatic production of β-hydroxybuterate, which has been shown to increase twenty-fold while consuming a LCD. This level can blunt the effects of reduced insulin concentrations (Taggart et al., 2005) by increasing insulin secretion. More research is needed to
determine the effects of the innate compensatory processes involved with our complex biological systems to fully understand the nonequilibrium thermodynamics theory.

Bluher and Kahn were some of the first to demonstrate the significant effects of carbohydrate restriction on insulin stimulation of adipocytes. Using FIRKO mice (adipose insulin receptor knockout mice), studies found animals consuming the same amount of food were significantly thinner, had a reduced efficiency to store lipid and had increased longevity compared to their wild type (Bluher, Kahn, & Kahn, 2003; Bluher et al., 2002). These mice are potentially protected from obesity because of their lack of ability to be influenced by insulin.

Requirements of Metabolism

Substrate cycling refers to the dynamic process that must accompany the thermodynamic steady state, constantly using ATP and generating heat to meet the metabolic demands of the body. Similarly, the repeated breakdown and re-synthesis of proteins, lipids, and carbohydrates use ATP with no apparent net gain. These processes allow for tight regulation of metabolism and account for one of the many uses of ATP. More futile cycling of ATP occurs depending on the pathway and initial substrate of which ATP is eventually produced. Glucose directly oxidized to ATP is most efficient, where an average amino acid or protein oxidized to ATP is largely inefficient. Utilizing lipids as the substrate to create ATP can be just as inefficient (Fine & Feinman, 2004). In addition, the high costs of energy that occur with the formation and hydrolysis of a single peptide bond in protein turnover can reduce metabolic efficiency.
(Waterlow, 1984). The variations in metabolic efficiency may help to explain the utilization of ATP and energy expenditure, and argue the ideas of the first law of thermodynamics.

**Gluconeogenesis-stimulated Protein Turnover**

As glycogen stores are depleted while consuming a LCD, the requirements of glucose for energy are met through gluconeogenesis. Since one still consumes minimal carbohydrates while on a LCD (~20-50g), the brain needs to be supplied by the breakdown of protein or production of ketone bodies. Through studies involving protein tracers, gluconeogenesis has been shown to increased by day 11 on a LCD (Bisschop, et al., 2000). A sizeable amount of endogenous protein needs to be broken down for gluconeogenesis and re-synthesized at an energy cost of up to 400-600 kcals/day (Bisschop, et al., 2000). Gluconeogenesis can also be sourced by dietary protein and is thought to conserve metabolic energy; however, the protein source to fuel gluconeogenesis remains unclear (Bisschop, et al., 2000).

**Behavioral Effects**

Carbohydrate restrictive diets have been regarded a successful lifestyle change for controlling body weight because they have been accredited with a behavioral effect (Feinman & Fine, 2007). Several studies report when consuming an ad libitum LCD of 5-10% energy from carbohydrates, people tend to consume less calories (Nordmann et al., 2006). In addition to a behavioral effect, a metabolic effect or apparent reduction in energy efficiency has been seen in isocaloric diet studies. This effect is often referred to as a metabolic advantage; people gain less weight per calorie consumed (Feinman & Fine, 2007). One study using rats found those who
consumed a LCD consumed less food and actually gained less weight per calorie consumed than their counterparts who ate carbohydrates (Marsset-Baglieri et al., 2004). The metabolic advantage can be reflected in a lower respiratory quotient without effecting resting energy expenditure, metabolically expressed as an increased ability to efficiently oxidize TAGs as fuel (Paoli et al., 2012). The thermogenic effect of burning proteins and fats through gluconeogenesis may also affect the nonequilibrium equation favoring weight loss (Feinman & Fine, 2007; Fine & Feinman, 2004).

The initial weight loss associated with a LCD is one of the most contended issues when comparing LCDs to LF diets. Water is bonded with glycogen in muscle and adipose tissue in a 4:1 ratio. As glycogen stores deplete in the first days to weeks of a LCD, the initial weight loss is mostly attributed to water loss mainly from the lean tissue. Research has found a 2 kg weight loss to be associated with water and glycogen stores (Kreitzman, Coxon, & Szaz, 1992). Similarly, after the ketogenic phase of a VLCKD when carbohydrates are cautiously added back to the diet, one can expect a water weight gain as glycogen is being stored. This may help to explain some of the weight changes over the course of a diet progression and justify the need to analyze body composition via DEXA rather than a scale.

LCDs are often coupled with the absence of hunger (Weigle et al., 2005) and less energy swings, as seen with people who consume carbohydrates regularly. There are a few theories proposed to explain why those in a ketogenic state do not complain of the symptoms of starvation: hunger, fatigue, or irritability like those who are purposefully avoiding calories for weight loss reasons. Ketone bodies are thought to be partly responsible for the appetite suppression (Johnstone, Horgan, Murison, Bremner, & Lobley, 2008). In addition, LCDs promote consuming satiating fats and proteins that digest slower and keep one satiated longer.
(Westerterp-Plantenga, Nieuwenhuizen, Tome, Soenen, & Westerterp, 2009), and do not elevate blood glucose, blood insulin levels or other appetite related hormones (Sumithran et al., 2013). In a highly controlled study, subjects consuming a LCD reported hunger levels similar to those on a LF diet, despite consuming 1000 kcals less each day (Boden, Sargrad, Homko, Mozzoli, & Stein, 2005).

Low-Carbohydrate Diet Research Limitations

A recent meta-analysis including 23 trials compared LCDs (defined as 4-45% of energy from carbohydrate) with LF diets (defined as ≤30% energy from fat) on metabolic risk factors. The main findings included an improved cholesterol panel with those in the LCD group, but no significant differences between body weight and waist circumference (Hu et al., 2012). Perhaps the wide range of carbohydrates in the LCDs grouped for analysis is the reason no differences found for body composition measure, when typically the VLCKD or LCD shows improvements. Ketogenesis does not occur unless carbohydrates are restricted to a more stringent degree, which is necessary to liberate fatty acids from the adipose and utilize for energy.

Often LCDs are researched without an ample induction phase into ketosis. As mentioned, carbohydrates need to be restricted for at least 3-4 weeks to see optimal benefits. Additionally, individual subject’s starting lean body mass may dictate the degree to which lean body mass is gained or lost. Although adipose tissue is 85% fat, overweight individuals, usually males, require more muscle to carry the weight. When body weight is decreased, obligatory lean body mass is lost since it is not required to carry a smaller load. In addition, there will always be
people who respond to a diet intervention and those who do not, and these varied responses to diet outcomes can likely be explained by genetics.

Understanding the theory of nonequilibrium thermodynamics may help to comprehend the complexity of weight changes on diets that are similar in caloric content but different in macronutrient composition. This idea really challenges the notion that ‘a calorie is a calorie’ in respect to weight loss regimens and ultimately changing body composition. Hypocaloric, low-fat diets are known to decrease body weight, typically comprising of weight from both fat mass and lean body mass, but these results are undesirable for health outcomes. However, a diet able to preserve lean body mass and efficiently decrease fat mass would be apt to decrease metabolic syndrome in adults, thereby reducing risk for diabetes and cardiovascular events. Insulin plays a significant role is regulating fat metabolism and specifically fat oxidation, primarily regulated by carbohydrate level in the diet. Therefore, studying the role of carbohydrate content in a diet and its’ effects on body composition may help elucidate a preferred diet for improved health outcomes revolving around body weight. The primary aim of this study was to examine the effects of dietary carbohydrate and fat manipulation in sequential diet phases of an isocaloric, isonitrogenic, moderately calorie deficient controlled feeding study on body composition, fat oxidation, respiratory quotient, and insulin changes.
INTRODUCTION

Obesity is most accurately described as an excess storage of fat in adipose tissue. Ultimately driving the storage of fat is the hormone insulin, which is under the influence of carbohydrate consumption. Since storage of carbohydrate in the body is limited, carbohydrates are metabolized to sugar and preferentially burned as fuel while simultaneously decreasing the oxidation of fat for fuel. Carbohydrate intake above what can be stored or burned as fuel is diverted to fat via de novo lipogenesis. With the increases in obesity rates and relatively high rates of processed carbohydrates in the typical Western diet, understanding nutrient utilization associated with dietary macronutrient compositions may help to understand the often dramatic inter-individual differences in fat loss responses to diets varying macronutrient composition.

The oxidation of carbohydrates, proteins, and fats must occur in proportions matching the composition of their nutrients in the diet, otherwise respective accumulation and/or depletion occurs (Flatt, Ravussin, Acheson, & Jequier, 1985). Thus, in order to reduce fat mass a negative state of fat balance must be achieved. A negative fat balance may be obtained by altering fat intake or expenditure such that fat oxidation exceeds fat intake (Melanson, MacLean, & Hill, 2009), which has been shown to be most efficient while consuming a low-fat (LF) diet, also resulting in increased energy expenditure (Hurni, Burnand, Pittet, & Jequier, 1982). These data support the current American Dietary Guidelines (Services, December, 2010) to limit dietary fat, but paradoxically the obesity epidemic has occurred during a time when dietary fat has been replaced with an excess of carbohydrate among adults. Nutrient oxidation rates have been shown to proportionally match dietary macronutrient composition percentages (Hurni, et al., 1982) in subjects in weight maintenance when higher in carbohydrates for studies completing 24-hour analysis in a respiratory chamber. However, it remains unclear whether fat oxidation
rates rise concomitantly with an increase in fat consumption (Schutz, Flatt, & Jequier, 1989) indicating the potential for substantial imbalances between consumption and utilization when dietary fat is substantial increased. When consuming an excess of carbohydrates, the body is able to regulate carbohydrate oxidation after glycogen stores are saturated (Jequier & Schutz, 1983) but how the body regulates dietary fat is less clear. One of the most cited studies compared breakfasts of similar carbohydrate contents with altered fat compositions and found no increases in postprandial fat oxidation (Flatt, et al., 1985), suggesting carbohydrate content of the diet is influencing oxidation rates. The fact that dietary carbohydrate is a primary stimulator of insulin, and insulin potently inhibits fat oxidation suggests that carbohydrate, or more specifically the metabolic tolerance to carbohydrate, is a primary control element in determining fat balance. While consuming a well formulated very low-carbohydrate diet (LCD), dietary fat intake is greatly increased compared to current dietary guidelines (Lichtenstein, et al., 2006), and presumably fat oxidation increases as well, but this has not been measured directly under controlled conditions.

Despite much research performed on nutrient oxidation rates in exercise, very few studies exist on nutrient oxidation rates in adults with metabolic syndrome (insulin resistance syndrome or pre-diabetes). This population is known to have a disorder in glucose metabolism (i.e., carbohydrate intolerance) and generally impaired oxidative capacity of both glucose and fatty acids (Westman, et al., 2007) potentially influencing insulin sensitivity at the skeletal muscle cells (Horowitz, 2007) and exacerbating weight gain (Zurlo, et al., 1990).

It is increasingly recognized that individual variation is an important consideration in interventions for weight loss and other health conditions, such as cardiovascular disease. Even in highly controlled metabolic ward studies where dietary intake and exercise are tightly controlled,
there is wide variability in weight loss and weight gain (Bouchard & Tremblay, 1997). Therefore, different interventions may be better suited for specific individuals based on behavior and biological factors, such as level of insulin resistance (McClain, Otten, Hekler, & Gardner, 2013; J. S. Volek, et al., 2009) or genetic markers (J. Gardner et al., 2012; Qi et al., 2011; Seip et al., 2008; Zhang, Wu, Liu, & Klaassen, 2012). Insulin resistance is known to vary across a spectrum in individuals with metabolic syndrome, and this may manifest in variable fat loss to increasing carbohydrate intake. For example, it has been shown that for individuals with insulin resistance, a low carbohydrate diet dramatically outperforms a low fat diet as a weight loss intervention (C. D. Gardner et al., 2007; J. S. Volek & Feinman, 2005; J. S. Volek, et al., 2009).

The unique feeding study design of 3-week continuous incremental increase in carbohydrate and decrease in dietary fat from this study may help to shed light on the importance of macronutrient distribution on substrate oxidation rates and fat mass changes in the context of a moderate energy deficit. Using data obtained from indirect calorimetry (i.e., substrate oxidation and respiratory exchange ratios) we will determine for the first time how incremental decreases in fat and increases in carbohydrate affect substrate oxidation patterns and changes in body composition within the same person while keeping caloric intake constant. A primary objective of this research is to examine the role of varying carbohydrate and fat on the composition of weight loss and the factors potentially modulating those effects. The specific aims of this study were to: 1.) determine variability in fat loss velocity while consuming hypocaloric, isonitrogenic diets that progressively alter the ratio of fat and carbohydrate in adults with metabolic syndrome and 2.) examine the possible contribution of respiratory quotient (RQ), resting energy expenditure, and insulin in explaining differences in fat loss over time. We hypothesized that lower RQs or higher rates of fat oxidation would be associated with greater loss in body fat.
Additionally, an increase in dietary fat consumption, and commensurate decrease in carbohydrate, would increase whole body postabsorptive fat oxidation rates. Since body weight and body composition are complex traits influenced by a large number of metabolic pathways/networks, we expect there will be wide variability between people in their response to highly controlled diets varying carbohydrate and fat. It is also expected that a higher post-absorptive fat oxidation will show greater fat loss in the context of increasing dietary carbohydrates. This may be related to the level of insulin resistance.
METHODS

Screening

The University of Connecticut Institutional Review Board approved the study protocol. Subjects were recruited through email list-serves, word of mouth, and posted flyers around the local area. Subjects initially completed screening questionnaires for background information on their medical, exercise and diet history and given written and oral details of the study individually at a screening meeting. Women completed a menstrual history questionnaire regarding information about hormonal supplements and symptoms of menopause to ensure no confounding variables. Waist circumference and blood pressure were measured and a fasting blood sample was obtained for determination of METs risk characteristics waist circumference > 35 in for women, >40 in for men; a systolic or diastolic blood pressure > 130/85 mmHg; a fasting blood glucose >100 mg/dL; HDL-Cholesterol < 50 mg/dL for females, < 40 mg/dL for males; triglycerides >150 mg/dL. Three of the five METs characteristics qualified a subject for study enrollment. Sixty-three subjects were interested, attended a screening meeting, however, only twenty-one subjects qualified and were enrolled. Exclusion criteria included individuals who were lactose intolerant/ any food allergies, a medical history of Type 1 or Type 2 Diabetes Mellitus, kidney, liver or any other metabolic or endocrine disorder, or currently using tobacco products. Subjects needed to be between 21 and 70 years old and weight stable for the last 3 months, not taking any anti-inflammatory medications, not highly trained or currently exceptionally active, not taking any other lipid or cholesterol medications, and either not taking or on a stable medication for controlled blood pressure. Subjects agreed to follow the diet they were given for the first three weeks and then only consume foods they were provided for the
reminder of the study. Subjects agreed to not leave the state for extended periods of time during the study protocol.

**Study Design**

This was a 21-week nutrition intervention consisting of 7 sequential 3-week phases. The first three weeks of the study, described as a run-in “free-living phase”, 16 subjects consumed a low-carbohydrate diet. They were educated and instructed by a registered dietitian to consume minimal carbohydrates and replace calories with dietary fats. The following 6 phases (18 weeks) all food was provided to subjects. Testing occurred before, and then after each phase as outlined in Figure 1.

![Study Design Schematic](image)

**Figure 1.** The study design schematic for sixteen subjects who started the study starting on a low-carbohydrate diet.

**Testing**

Subjects arrived to the laboratory following a minimum 12-hour fast and 24-hour abstinence from exercise, caffeine, and alcohol. Testing included a 24-hour urine collection for
urine nitrogen and ketone concentration prior to an indirect calorimetry test. On site, subjects provided a small urine sample to assess specific gravity as a measure of hydration. Subjects with a USG >1.025 were asked to consume water and wait 30 minute before another urine sample was measured. Resting energy expenditure and substrate oxidation was measured by indirect calorimetry (Parvomedics TrueOne 2400 metabolic cart) in a thermal neutral room. The metabolic carts were calibrated with a standard gas mixture each morning. Subjects relaxed quietly for approximately 30 min and oxygen consumption (VO\textsubscript{2}) and carbohydrate expiration (VCO\textsubscript{2}) were averaged for 15 min to determine respiratory quotient (VCO\textsubscript{2}/VO\textsubscript{2}), relative rates of carbohydrate and fat oxidation and metabolic rate (Weir, 1990). Body weight was measured and recorded to the nearest 0.1 kg on a digital scale (OHAUS Corp., Parsippany, NJ) for each subject. Height was recorded at the baseline via tape measurer and recorded to the nearest 0.1cm and waist circumference was measured using a standard tape measurer performed by the same person. Body composition was measured by dual-energy X-ray absorptiometry (DEXA) (Prodigy, Lunar Corporation, Madison, WI) and analyzed by the same technician. All females were given a pregnancy test (Quidel Corporation, San Diego, CA) through urine sample prior to each DEXA scan.

**Blood Draws and Analysis**

Blood samples were obtained from an arm vein after subjects rested quietly for 15 min in the supine position. Whole blood was collected into tubes with a serum separator and ethylenediaminetetraacetic acid (EDTA). Tubes with serum separator remained at room temperature for 15 min before spinning to allow clotting to occur. Whole blood was centrifuged at 1500 x g for 15 min and 4°C, aliquoted into storage tubes, and stored in ultra-low freezers for batch analysis. Frozen samples were thawed only once before analysis. Serum glucose was
determined by Quest Diagnostics (Wallingford, CT). Serum insulin was analyzed in duplicate
by ELISA (ALPCO, Salem NH) with a sensitivity of 5 mmol/L. Intra- and inter-assay
coefficients of variation (CV) were 5.3 and 7.2% respectively. Glucose and insulin values were
used to calculate an index of insulin resistance [HOMA-IR; calculated as Glucose
(mmol/L)·Insulin (µIU/mL /22.5)] (Matthews, et al., 1985). Total ketones were determined by a
cyclic enzymatic method that measures both acetoacetate (AcAc) and 3-hydroxybutyrate (3-HB)
(Wako Chemicals USA Inc, Richmond, VA) with a sensitivity of 1.2 µmol/L, and intra- and
inter-assay CV 7.7 and 20.3% respectively.

Dietary Intervention

Six diets were developed that spanned a range of carbohydrate from approximately 50 to
350 g/day (Feeding Phase 1→FP2→FP3→FP4→FP5→FP6) using nutrient analysis software
(Nutritionist Pro, Axxya Systems, Stafford, TX). The highest carbohydrate phase (FP6) was
designed to model national dietary recommendations. While carbohydrate was adjusted every 3
weeks, total fat decreased proportionately so that total energy remained constant. Saturated fat
was 40% of total fat for all phases. Protein was constant at 1.8 g/kg reference body weight
determined from midpoint of Metropolitan Height-Weight Tables. Based on individual resting
metabolic needs and activity factors (1.2-1.5) obtained at baseline, the diets were designed to
provide a 300 kcal/day energy deficit to induce moderate weight loss and motivation for subjects
to continue participation in the 21-week experiment. Thus, total caloric and protein intake for
each individual did not change throughout the study. Estimated nutrient composition of select
diets showed high concordance with chemical analysis (Exova, Portland, OR).
For each diet phase 7-day rotational menus were developed that included a wide range of whole foods. Beef, eggs, and dairy were used throughout all diet phases as primary sources of saturated fat. For the low carbohydrate diet phases, higher-fat beef and meats, whole eggs, and full-fat dairy products (e.g., cheese, whole milk yogurt, cream, butter) were emphasized. For the higher carbohydrate diet phases with lower saturated fat, leaner versions of beef, egg substitutes, and low-fat dairy (e.g., reduced-fat dairy, skim milk, low-fat/non-fat yogurt) were used instead. Whole grain and relatively low glycemic index carbohydrate sources were emphasized.

Subjects were counseled to consume a 3-week run-in diet that mirrored the first feeding phase. Thus, subjects consumed a diet similar to the lowest carbohydrate phase (Feeding Phase 1, ~50 g carbohydrate/day) in order to initiate metabolic adaptations to carbohydrate restriction. Subjects complete three-day diet records at baseline to determine habitual nutrient intake and during the run-in diet.

Food was prepared in our research kitchen, packaged and labeled per individual serving sizes based on individual caloric and macronutrient needs and was picked up by the subjects 3-4 times/wk. All ingredients of meals were weighed to the nearest 0.1g on digital kitchen scales. If unable to travel to obtain the food, arrangements were made to ensure the subject received his/her food as planned. No other foods or beverages were allowed except very-low/non-caloric products (e.g., coffee, tea, water, diet soda). All food containers were returned unwashed and inspected to document that all food was consumed.
Statistical Analyses

One subject dropped after completing FP4 due to a rise in his blood pressure. His FP5 and FP6 data were interpolated based on mean percent changes for the group. Unless otherwise stated, a paired samples t-test was used to examine the effects of 6-week of very low carbohydrate intake (BL verse FP1). Similarly, a repeated measures analysis of variance (ANOVA) was used to assess changes across the six diet phases (FP1→FP2→FP3→FP4→FP5→FP6) and Fisher’s LSD post hoc was used to examine pairwise comparisons when significant main effects were observed. Correlations were done in multiple variables using the Pearson’s r correlation coefficient with absolute numbers and/or change in variable from the previous phase. Additionally, a multivariate linear regression analysis was completed using the enter method. The alpha level for significance was set at $p \leq 0.05$. 
RESULTS

Subjects

The descriptive characteristics of the 16 subjects are shown in Table 2. All but two subjects qualified for this study with an increased waist circumference as a metabolic syndrome characteristic. Twelve of sixteen subjects qualified with an elevated blood pressure (systolic or diastolic >135/85 mmHg), nine subjects started with a low HDL (< 40 mg/dL for males, < 50 mg/dL for females), six subjects with a fasted triglyceride level >150 mg/dL, and ten subjects with fasted blood glucose >100 mg/dL. All subjects qualified with at least 3 MetS characteristics; five subjects qualified with four MetS characteristics, and two subjects started with all five MetS characteristics.

Table 2. Baseline subject characteristics1.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>12/4</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>44.9 ± 9.9</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>108.4 ± 15.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>37.9 ± 6.3</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>116.8 ± 10.5</td>
</tr>
<tr>
<td>Body Fat (%)2</td>
<td>40.0 ± 3.2</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>191 ± 34</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>123 ± 27</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>134 ± 54</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>93 ± 70</td>
</tr>
<tr>
<td>Insulin Resistance (HOMA)3</td>
<td>3.3 ± 2.0</td>
</tr>
<tr>
<td>Ketones (mmol/L)</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>122 ± 10</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>83 ± 11</td>
</tr>
</tbody>
</table>

1Values are mean ± SD
2Determined by dual-energy X-ray absorptiometry
3HOMA = homeostatic model assessment = [fasting glucose (mmol/L) x insulin (mU/L)]/22.5
Table 3. Average days subjects consumed each diet in the controlled feeding phases.

<table>
<thead>
<tr>
<th></th>
<th>Days in FL</th>
<th>Days in FP1</th>
<th>Days in FP2</th>
<th>Days in FP3</th>
<th>Days in FP4</th>
<th>Days in FP5</th>
<th>Days in FP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-HC</td>
<td>30.4 ± 3.5</td>
<td>27.6 ± 3.9</td>
<td>27.8 ± 3.7</td>
<td>25.6 ± 3.8</td>
<td>24.3 ± 4.2</td>
<td>24.2 ± 2.6</td>
<td>21.8 ± 4.1</td>
</tr>
</tbody>
</table>

Values are mean ± SD

Dietary Intake

There were no significant differences in total days spent in each phase (Table 3). All diets were well tolerated and compliance was high based on verbal communication and inspection of returned, unwashed food containers. Total caloric intake at baseline showed an average consumption of 2,969 kcal/day (Table 4). The controlled feeding diets averaged ~2,550 kcal/day. The diets provided were consistent with the goal to create a moderate calorie deficit (~300 kcal/day) calculated from baseline REE and activity constant factor based on self-reported activity. As designed, energy and protein intakes across the six controlled diet phases were constant within each person. Habitual carbohydrate intake was 44% of total energy, slightly lower than the average dietary recommendations. From FP1 to FP6, carbohydrate increased from 47 to 346 g/day corresponding to 7% and 55% of total energy, respectively.

Fat consumption was 128 ± 45g (39%en) at baseline. Fat consumption increased to 152 ± 46g in the FL phase, and was then controlled by researchers for FP1 through FP6 at 74%, 69%, 61%, 54%, 43%, and 28% total energy as grams of carbohydrate increased, respectively. Dietary saturated fat was consumed at a constant 40% of fat calories across all phases. Protein was provided at a consistent 1.8 g/kg reference body weight for each phase.
Weight Loss, Resting Energy Expenditure

Relative to BL, cumulative mean weight loss after the free-living, FP1, FP2, FP3, FP4, FP5 and FP6 phases was -3.7 ± 1.4, -6.4 ± 2.8, -7.9 ± 3.0, -8.4 ± 3.4, -8.5 ± 3.8, -9.4 ± 3.9, and -9.0 ± 4.5 kg, respectively. Relative to BL, cumulative body fat loss after FL through FP6 was -2.3 ± 1.5, -4.4 ± 2.1, -6.1 ± 3.1, -7.0 ± 3.3, -7.7 ± 3.5, -8.1 ± 4.1, and -8.3 ± 4.5 kg, respectively. The variability in fat loss increased over time as carbohydrates were re-introduced, as evidenced by the progressive increase in standard deviation from FP1 to FP6. Individual body fat mass loss is represented as percent of body mass in Figure 2.

![Individual Fat Mass Percent Change](image)

**Figure 2.** Individual change in fat mass percent over phases.
For participant motivation, diets were designed in about a 300-calorie/day deficit calculated from BL RMR analysis to promote a slight weight loss of about 0.3 kg/week or 1.0 kg/phase (about 0.6 lb/week or 1.8 lb/phase). After multiplying the actual calorie deficit (calculated from REE delta change across phase, divided by two) multiplied by days in each phase of the study, subject total caloric deficit can be translated into predicted fat mass deficit. By this calculation, subjects were expected to lose \(-12.74 \pm 15.81\) kg in total over the 21-weeks. Table 5 shows the average actual fat mass loss (kg) per phase and the predicted fat mass loss (kg) per phase based on the calorie restriction. Actual fat mass loss was significantly different after FL, but was not significantly less than predicted from FP2 through FP6 (Figure 3).

**Figure 3.** Cumulative predicted fat mass changes from controlled diet caloric deficit and actual fat mass changes over weeks enrolled in the study, relative to baseline fat mass.\(^1\)

\(^1\)Values are mean ± SE.

** Significant change in body fat mass from FL via one-way ANOVA \((p<0.01)\).

**** Significant change in body fat mass from FL via one-way ANOVA \((p<0.0001)\).

# Significantly less actual fat mass loss verse predicted fat mass loss via t-test \((p<0.0001)\).
Baseline resting energy expenditure (REE) was 2126 ± 276 kcals/day (Table 5).

Absolute REE was significantly lower at FP1 (1955 ± 270 kcals/day) than BL through a paired t-test (Figure 4), however the one-way ANOVA analysis comparing FP1 over the following six phases found no significant differences. When expressed in relative terms (kcals/kg/day) to account for body weight change over the hypocaloric diet feeding phases, REE did not significantly change at any phase compared to BL (Figure 5).

---

Figure 4. Absolute resting energy expenditure and body weight changes across weeks in the study. 1

1 Values are mean ± SE

*** Significant body mass change from BL via paired t-test (p < 0.0001).

* Significant body mass change from FP1 via one-way ANOVA (p < 0.0001).

# Significant resting energy expenditure change from BL via paired t-test (p<0.01).
Respiratory Quotient

Respiratory quotient (RQ) at baseline was 0.80 ± 0.05, which complements baseline dietary records where subjects reported consuming a diet containing a moderate to high amount of carbohydrates (44%en). RQ was significantly lower at FP1 when carbohydrate was lowest and fat intake was the highest (0.75 ± 0.04) compared to BL via paired t-test. RQ increased linearly as carbohydrates increased from FP1 through FP6: 0.75 ± 0.04, 0.77 ± 0.03, 0.79 ± 0.04, 0.80 ±0.04, 0.82 ± 0.03, 0.84 ±0.05, respectively (Figure 6). ANOVA comparisons result RQ was significantly higher than FP1 at FP3 through FP6 (p<0.001). RQ was negatively correlated to fat consumption (r= -0.303, p<0.001), and positively correlated to carbohydrate consumption (r= 0.453, p<0.0001).
RQ was significantly correlated to fat mass loss ($r = 0.258$, $p<0.01$) as shown in Figure 7. Similarly, RQ was also correlated specifically to trunk fat loss ($r = 0.224$, $p<0.005$). The relationship showed less fat mass loss with an increase in RQ.

![Respiratory Quotient Across Phases](image)

Figure 6. Respiratory quotient values for all subjects (n=16) measured after consumption of the diet for about 3 weeks.\(^1\)

\(^1\)Values are mean ± SE

* Significant change in RQ from baseline via paired t-test ($p < 0.001$).

** Significant change in RQ from FP1 via one-way ANOVA ($p < 0.001$).
Figure 7. Correlation of the change in fat mass from the free-living phase through FP6 with respective respiratory quotient.\(^1\)
\(^1\)Correlation is significant (p<0.01)

Fasted Fat Oxidation

Fat oxidation rates were obtained by morning indirect calorimetry when subjects were rested and fasted. At baseline, fat oxidation was 146 ± 37 g/day. With increases of dietary fat and decreases of carbohydrate intake during the FL phase, fat oxidation significantly increased to 184 ± 55 g/day (p< 0.01).

As carbohydrate was re-introduced from FP1 through FP6, fasted fat oxidation rates steadily declined (173 ± 34, 165 ± 36, 150 ± 33, 136 ± 35, 127 ± 26, and 110 ± 33 g/day, respectively). Fasted fat oxidation rates were significantly higher with the consumption of the highest dietary fat (FP1: 173 ± 34 g/day), compared to dietary fat at BL (146 ± 37 g/day) (p<
0.05), and significantly lower at the highest dietary carbohydrate phase (FP6: 110 ± 33 g/day), compared to BL (p< 0.01) (Figure 8).

![Fat Oxidation and Consumption](image)

**Figure 8.** Fasted fat oxidation and dietary fat consumption over weeks enrolled in the study.\(^1\)

\(^1\)Values are mean ± SE

* Significantly different fat oxidation compared to BL (p< 0.05).

** Significantly different fat oxidation compared to BL (p< 0.01).

# Significantly greater fat oxidation compared to fat consumption via t-test (p< 0.05).

Overall fasted fat oxidation was significantly correlated to fat consumption (r=0.438, p<0.0001) when including baseline and free-living diets. When excluding these two phases that were not controlled by the research team, fat oxidation and fat consumption were correlated to a greater extent (r=0.629, p<0.0001). These results are shown in Figure 9. Similarly, fat oxidation was negatively correlated to carbohydrate consumption (r= -0.397, p= <0.0001), and to a greater extent when excluding BL and FL phases(r= -0.534, p= <0.0001) (Figure 10).
Figure 9. Fasted fat oxidation correlation with fat consumption in all phases of the study (left) and with just the controlled feeding phases (right) (p<0.0001).

Figure 10. Fat oxidation correlation with carbohydrate consumption in all phases of the study (left) and with just the controlled feeding phases (right) (p<0.0001).
**Insulin, Ketones, and HOMA**

Serum ketones increased approximately 5-fold during FP1, 3-fold in FP2, and decreased across phases until returning to baseline values by FP5. Ketones were significantly higher at FP1 from BL, and significantly lower at each phase from FP1 (Figure 11). Serum insulin and HOMA (not shown) were significantly lower at FP1 than BL, and significantly higher at each controlled feeding phase compared to FP1. Ketone levels were inversely correlated to trunk fat mass change \((r= -0.274, p<0.001)\), RQ \((r= -0.419, p<0.0001)\), insulin levels \((r= -0.319, p<0.001)\), and carbohydrate consumption \((r= -0.571, p<0.0001)\). Conversely, ketone levels were positively correlated with fat consumption \((r= 0.314, p<0.001)\).

![Figure 11](image-url)  
**Relative Serum Ketones and Insulin**

*Figure 11. Serum ketones and insulin expressed relative to baseline values.\(^1,a\)*  
\(^1\text{Values are mean } \pm \text{SE}\)  
\(a\) Insulin and ketones were both significantly higher at FP1 than BL via paired t-test. All following ketone and insulin values were significantly different from FP1 via one-way ANOVA \((p<0.0001)\).
Dietary Correlations

Correlations of diet with body fat mass, trunk fat mass, and REE help explain some of the change in rates of body fat loss. Table 6 is a correlation matrix of significant correlations between dietary and metabolic variables analyzed as change from previous phase (FL-FP6) and/or absolute numbers from the feeding phases (FP1-FP6 only). Through regression analysis, 37% of fat mass loss could be predicted by absolute RQ, fat consumption, carbohydrate consumption, insulin, ketones, and fat oxidation.
DISCUSSION

Dietary guidelines currently recommend low-fat diets to be the most ideal for weight loss, suggesting the difference between fat intake and oxidation is ideal for being in a negative fat balance. Nevertheless, obesity rates have continued to rise over the past few decades. In this study, we sought to shed light on the individual variability of changes in weight loss, respiratory quotient, resting energy expenditure, and fat oxidation when consuming different controlled hypocaloric diets, varying in fat and carbohydrate content. Subjects were studied over 21 weeks while consuming diets that were progressively higher in carbohydrates and lower in dietary fat in a dose-response manner. The results show consistent body mass and fat mass loss while consuming a low-carbohydrate diet and a lower rate of fat mass loss while consuming a high-carbohydrate diet. Actual fat loss was less than predicted fat loss in each phase, with wide variation within subjects reflected by a large standard deviation (and range). Additionally, the lowest RQ and highest fat oxidation rates with diets containing the most dietary fat and least amount of carbohydrates. As dietary carbohydrates increased, RQ increased and fat oxidation decreased. Our results suggest dietary carbohydrates influence RQ, insulin levels, and fat oxidation, slow the rate of weight loss. Therefore, quantitative information on carbohydrate consumption can be useful with making predictions of weight change and assist with making weight management recommendations to adults with metabolic syndrome.

Variability in fat loss in response to increasing carbohydrate intake in the context of moderately hypocaloric feeding was remarkable. Based on calculations of predicted fat mass deficit from the energy restricted diets and change in RMR, subjects should have lost 15 kg of fat mass while in the six controlled feeding phases, however actual fat loss was 6.0 kg (range: 1.0–13.9 kg), with a total of 8.34 kg including the run-in free living phase. Weight loss, specifically
fat mass loss and trunk fat loss, occurred to a greater extent while consuming the low-carbohydrate/high fat diet and slowly tapered to a minimal loss while consuming a high carbohydrate diet. Perhaps the most intriguing finding is the variation in weight change at each phase. This finding suggests it is difficult to estimate how much weight any individual may lose even with a constant caloric deficit since dietary macronutrient composition clearly regulates metabolism. The variation in weight change is highly correlated to the presence of insulin, elevated in the presence of dietary carbohydrates, while simultaneously decreasing fasted fat oxidation (Table 6), promoting the storage of excess carbohydrates.

Actual fat mass loss at FL was statistically different from predicted fat loss; however, there were no significant differences between actual and predicted fat mass loss during the feeding phases. It may be assumed the weight loss in the early phases likely occurred from the caloric deficit, however, within the same caloric restriction confinements, by FP6 there was a range of -3 to -41% cumulative fat mass loss within adults with metabolic syndrome. Our study found a weight-loss induced non-significant decrease in absolute REE across phases, where in FP6 the REE was significantly lower than baseline. The maintained relative REE (kcals/kg/day) rationalizes the unnecessary need to reassess provided calories as subjects lost weight, indicating metabolism did not slow as subjects lost weight, similar to previous findings (J. Volek, et al., 2004).

In regards to REE, it has been previously shown that the body has the ability to maintain REE when following a low-carbohydrate diet (J. Volek, et al., 2004). Researchers believe consuming the low-carbohydrate diet first did not provide an order effect. Similar results of weight changes have been observed in obese subjects participating in a crossover study consuming hypocaloric macronutrient-different diets (Rabast, Vornberger, & Ehl, 1981),
implying less fat mass loss while consuming a high carbohydrate diet would have occurred even if the diets were to have been reversed. Additionally, if the caloric deficit is able to explain the initial weight loss, feeding the diets in reverse order (high carbohydrate to low-carbohydrate) may have maintained the rate of fat mass loss through a lower RQ as carbohydrate consumption decreases. This addresses the ongoing debate as to the ideal initiation of a low-carbohydrate diet: eliminate all dietary carbohydrate sources to rapidly deplete glycogen stores and initiate high efficiency fat-oxidizing capacity, or gradually reduce dietary carbohydrates allowing equilibration of nutrient oxidation at incremental decreases. Research specifically aimed at this question in terms of weight loss, substrate oxidation, and RQ has yet to be published.

Respiratory quotient tracked consistently with the pattern of progressively increasing carbohydrates across phases. Despite variability between subjects, RQ was significantly different from baseline (0.80) values at the lowest carbohydrate-feeding phase (0.75) where subjects had the greatest rate of weight loss. Not surprisingly, RQ significantly increased as dietary carbohydrate percentage increased. Researchers suspect if RQ were to have been monitored in a respiratory chamber over 24 hours, the postprandial RQ in FP5 and FP6 would have exceeded 1.0 in some subjects, indicating elevated carbohydrate oxidation and de novo lipogenesis (Acheson et al., 1988). Our study found increases in RQ to be correlated with carbohydrate intake ($r^2 = 0.20, p<0.0001$), and with a decreased rate of weight loss ($r^2 = 0.24, p<0.0001$), consistent with previous findings correlating higher RQs to weight gain and weight fluctuations (Hainer, et al., 2000; Valtuena, et al., 1997; Zurlo, et al., 1990). Despite our results demonstrating RQ predicting only 8% of fat mass change, other metabolic variables (insulin, ketones, fat oxidation) and dietary components (fat and carbohydrate consumption) correlate highly with each other. Therefore, theoretically an individual may be able to use a fasted RQ as
a function of current fat and carbohydrate intake to predict subsequent weight change while consuming diets of different macronutrient proportions. Moreover, an individual who can maintain a lower RQ (meaning a higher fat to carbohydrate oxidation ratio) with increasing consumption of carbohydrates may possess an enhanced ability for weight maintenance and/or weight loss.

Increases in carbohydrate oxidation have been previously shown to increase parallel to intake levels (Acheson, Flatt, & Jequier, 1982; Acheson, Thelin, Ravussin, Arnaud, & Jequier, 1985), demonstrating the flexible capacity of the body to use carbohydrates for energy. When consumed in excess (above storage capacity as glycogen), de novo lipogenesis occurs, resulting in adipose mass gains with simultaneous decreases in fat oxidation or increases in carbohydrate oxidation (Acheson, et al., 1988). It has been proposed that adiposity expansion also occurs with high fat diets because oxidation rates have not been shown to match the increases (Westerterp, 1993). The current results provide intriguing data showing fasted and resting fat oxidation rates can increase in the absence of carbohydrates (7\%en), ultimately demonstrating carbohydrates to be regulating nutrient metabolism. We found fat consumption to predict 40\% of fat oxidation and carbohydrate consumption to predict 29\% of fat oxidation (Figure 9, Figure 10) suggesting other dietary or metabolic variables account for only a small percentage of fat metabolism.

Additionally, fasted fat oxidation linearly decreased as dietary carbohydrate content incrementally increased. Fat oxidation rates were significantly lower than baseline when consuming 346 g/day of carbohydrates (FP6), even after subjects had experienced significant weight loss. This finding was interesting and unexpected, as we would have expected improvement in the ability to oxidize glucose and fatty acids in adults with metabolic syndrome as they became less obese (Westman, et al., 2007) and were consuming a similar amount of
carbohydrates prior to starting the study. Subjects may have reached their ‘threshold’ of carbohydrate tolerance in FP6 where the body can no longer utilize or oxidize what was consumed. This suggests de novo lipogenesis was occurring at FP6 and may further explain the decline in weight loss.

In terms of protein oxidation, we assume net protein balance in this study secondary to the ability of the body to preserve lean body mass when consuming sufficient dietary protein with a hypocaloric diet (Krieger, et al., 2006; J. S. Volek, et al., 2002). In this case, we purposefully designed the diets to contain 1.8 g protein per kg reference body weight across all controlled feeding phases to lessen the likelihood of adverse loss of lean body mass in the presence of body weight loss. Our results found fat mass to account for 93% of total body weight loss over the 21 weeks when consuming protein at this adequate level.

The relatively short feeding phases of the study can be considered a design limitation. These were intended to keep menus changing and subjects interested in continuing the study. Subjects reported feeling full, satisfied, and able to maintain energy levels throughout the day while consuming the small volume, calorically dense low-carbohydrate diets, despite being in a moderate hypocaloric state. However, while consuming the high-carbohydrate diets, that were much greater in food volume and were not as calorically dense in weight, subjects complained of bloating, tiredness, and intermittent hunger. Paradoxically, the highest carbohydrate diet consisted of 55%en carbohydrates, which may not fully represent the high carbohydrate diets consumed by adults with metabolic syndrome. Furthermore, oxidation rates were taken following an overnight fast, therefore, do not account for daily activity or postprandial changes. Otherwise, this unique 18-week feeding study with an additional 3-week run-in diet is the first to
analyze adults with metabolic syndrome over a series of controlled hypocaloric diets, ranging from 7-55% carbohydrates, using DEXA, indirect calorimetry and related blood marker analysis.

In summary, in the context of a low-carbohydrate diet, high intakes of dietary fat (including 40% saturated fats) can still promote substantial weight loss (and fat mass loss), greater than a low-fat diet indicating that fat oxidation is particularly responsive to dietary carbohydrate. Specifically, this study has shown the ability of the body to preferentially lose trunk fat mass while maintaining lean body mass under weight loss conditions. Although we report significant correlations between elevated respiratory quotient, insulin, and carbohydrate consumption with the decreased ability to lose weight, there remains a large unexplained variability. These results are consistent with the notion that individuals vary in their tolerance to carbohydrate and therefore may need to personalize their intake of carbohydrate in order to promote continued fat loss. Monitoring of metabolic parameters may be useful to determine individual responses to graduated increases in carbohydrate.
Table 4. Daily nutrient intakes at baseline (habitual diet) and during each dietary phase.

<table>
<thead>
<tr>
<th></th>
<th>Baseline(^2)</th>
<th>FP1</th>
<th>FP2</th>
<th>FP3</th>
<th>FP4</th>
<th>FP5</th>
<th>FP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2969 ± 1058</td>
<td>2553 ± 327</td>
<td>2527 ± 339</td>
<td>2585 ± 286</td>
<td>2506 ± 332</td>
<td>2517 ± 339</td>
<td>2509 ± 336</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>130 ± 45</td>
<td>129 ± 7</td>
<td>125 ± 6</td>
<td>125 ± 8</td>
<td>123 ± 9</td>
<td>123 ± 10</td>
<td>123 ± 11</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>324 ± 150</td>
<td>47 ± 2</td>
<td>83 ± 4</td>
<td>131 ± 3</td>
<td>179 ± 2</td>
<td>251 ± 12</td>
<td>346 ± 28</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>128 ± 45</td>
<td>209 ± 34</td>
<td>193 ± 35</td>
<td>179 ± 29</td>
<td>152 ± 34</td>
<td>121 ± 32</td>
<td>80 ± 27</td>
</tr>
<tr>
<td>SFA (g)</td>
<td>45 ± 19</td>
<td>84 ± 14</td>
<td>76 ± 15</td>
<td>71 ± 11</td>
<td>61 ± 15</td>
<td>49 ± 14</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>33 ± 13</td>
<td>77 ± 14</td>
<td>64 ± 11</td>
<td>57 ± 8</td>
<td>48 ± 11</td>
<td>36 ± 10</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>20 ± 10</td>
<td>31 ± 5</td>
<td>35 ± 8</td>
<td>35 ± 9</td>
<td>27 ± 7</td>
<td>24 ± 6</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>534 ± 225</td>
<td>844 ± 96</td>
<td>878 ± 91</td>
<td>824 ± 68</td>
<td>583 ± 131</td>
<td>448 ± 136</td>
<td>334 ± 154</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>26 ± 15</td>
<td>15 ± 1</td>
<td>19 ± 1</td>
<td>23 ± 4</td>
<td>27 ± 3</td>
<td>29 ± 2</td>
<td>35 ± 5</td>
</tr>
</tbody>
</table>

\(^1\)Values are mean ± SD from all 16 subjects.

\(^2\)Determined from 3-day diet records
Table 5. Actual and predicted fat mass loss per feeding phase.\(^1\)

<table>
<thead>
<tr>
<th>Low-Carbohydrate Free-Living</th>
<th>FP1</th>
<th>FP2</th>
<th>FP3</th>
<th>FP4</th>
<th>FP5</th>
<th>FP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calorie Deficit (kcals/phase)(^3)</td>
<td>20998 ± 19135</td>
<td>5737 ± 9222</td>
<td>4046 ± 7589</td>
<td>3676 ± 6039</td>
<td>3648 ± 4268</td>
<td>3811 ± 4239</td>
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<tr>
<td>Predicted FM Loss (kg)</td>
<td>-6.00 ± 5.47</td>
<td>-1.64 ± 2.63</td>
<td>-1.16 ± 2.17</td>
<td>-1.05 ± 1.73</td>
<td>-1.04 ± 1.22</td>
<td>-1.09 ± 1.21</td>
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<tr>
<td>Actual FM Loss (kg)</td>
<td>-2.32 ± 1.53(^a)</td>
<td>-2.10 ± 1.52</td>
<td>-1.63 ± 1.41</td>
<td>-0.97 ± 0.77</td>
<td>-0.65 ± 1.01</td>
<td>-0.40 ± 1.12</td>
</tr>
</tbody>
</table>

\(^1\)Values are mean ± SD from all 16 subjects.
\(^2\)Resting energy expenditure
\(^3\)Calculated by: Calorie deficit = calories consumed – (REE, determined by change in needs each day through calculation of change between phases, divided by days in each phase) * IPAQ activity factor * average days in each phase (Table 3).
\(^a\)Actual fat mass loss is significantly less than predicted fat mass loss (p<0.0001)
<table>
<thead>
<tr>
<th>Correlation Variable</th>
<th>Pearson's r</th>
<th>n size</th>
<th>$r^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Body Mass</td>
<td>Change in Insulin</td>
<td>0.34</td>
<td>112</td>
<td>0.116</td>
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<tr>
<td></td>
<td>Change in Ketones</td>
<td>-0.46</td>
<td>112</td>
<td>0.212</td>
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<tr>
<td></td>
<td>Change in Carbohydrate Consumption</td>
<td>0.49</td>
<td>112</td>
<td>0.240</td>
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<tr>
<td></td>
<td>Change in Fat Consumption</td>
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<td>112</td>
<td>0.240</td>
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<tr>
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<td>Change in Ketones</td>
<td>-0.32</td>
<td>112</td>
<td>0.102</td>
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<tr>
<td></td>
<td>Change in Carbohydrate Consumption</td>
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<td>112</td>
<td>0.176</td>
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<tr>
<td></td>
<td>Change in Fat Consumption</td>
<td>-0.38</td>
<td>112</td>
<td>0.144</td>
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<tr>
<td>Change in Trunk Fat Mass</td>
<td>Change in Carbohydrate Consumption</td>
<td>0.33</td>
<td>112</td>
<td>0.109</td>
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<tr>
<td>Change in Fat Oxidation</td>
<td>Change in RQ</td>
<td>-0.87</td>
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<td>0.757</td>
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<tr>
<td>Absolute Trunk Fat Mass</td>
<td>Absolute Insulin</td>
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<td>96</td>
<td>0.260</td>
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<tr>
<td>Absolute Fat Mass</td>
<td>Absolute Insulin</td>
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<td>96</td>
<td>0.281</td>
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<tr>
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<td>Absolute REE</td>
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<td>96</td>
<td>0.397</td>
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<tr>
<td>Absolute Ketones</td>
<td>Absolute RQ</td>
<td>-0.42</td>
<td>96</td>
<td>0.176</td>
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<td></td>
<td>Absolute Fat Consumption</td>
<td>-0.57</td>
<td>96</td>
<td>0.325</td>
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<tr>
<td>Absolute Carbohydrate Consumption</td>
<td>Absolute RQ</td>
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<td>96</td>
<td>0.203</td>
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<tr>
<td></td>
<td>Absolute Ketones</td>
<td>-0.51</td>
<td>96</td>
<td>0.260</td>
</tr>
</tbody>
</table>

$^1$Change from previous phase (FL-FP6)  
$^2$Absolute is actual number from feeding phases (FP1-FP6)
REFERENCES


