

**The Role of Fasting Acylcarnitines in Metabolic Flexibility from
Short Term High Fat Feeding**

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Abstract

Metabolic flexibility plays a significant role in energy homeostasis by regulating fuel selection in correspondence to energy demand. Obese and type II diabetic populations have displayed a hindered ability to properly transition from fat oxidation while in a fasted state to carbohydrate oxidation once fed, leading to a buildup of mitochondrial metabolites such as acylcarnitines. Carnitine, essential for fatty acyl-CoA transport through the inner and outer mitochondrial membranes, can be an indicator of mitochondrial distress as elevated levels tend to spill over into plasma suggesting a disruption in oxidation. The current study was designed to examine the effect of short term, high fat feeding on plasma acylcarnitine species diversity and levels and if acylcarnitines are associated with metabolic flexibility. 13 healthy, non-obese, sedentary males, aged 18-40 years participated in this study. Following a 12-hour overnight fast a biopsy was taken from the quadriceps before and 4 hours after a high fat meal. Blood draws were obtained pre-biopsy while fasted and every hour for 4 hours post high fat meal consumption. Acylcarnitines from plasma were converted to their butyl esters and analyzed by electrospray ionization tandem mass spectrometry (MS/MS). Changes were observed in acetylcarnitine ($P=0.0125$), glucose oxidation ($P=0.0295$), C16:1/C16:0 desaturation index ($P=0.0397$), and C18:1/C18:0 desaturation index ($P=0.0012$). We did not find that individual changes in flexibility correlated with circulating acylcarnitine measurements in a fasted state.

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Chapter 1: Introduction

Introduction

Impaired skeletal muscle oxidative capacity places an over bearing stress on skeletal muscle mitochondria metabolism via inefficient or incomplete fatty acid β -oxidation¹. This demonstration in mitochondrial dysfunction may be the result of oxidative stress developed from fuel overload¹. The ability of skeletal muscle to adjust fuel oxidation to fuel availability is known as metabolic flexibility². Individuals that struggle to switch from carbohydrate oxidation to lipid oxidation, and vice versa, are deemed metabolically inflexible^{2,3}. Chronic positive energy balance can cause metabolic inflexibility, which in turn may lead to a cascade of health issues including the development of insulin resistance, type II diabetes mellitus, cardiovascular disease and ultimately metabolic syndrome^{3,4}.

Carnitine is an essential metabolite for fatty acid oxidation as it forms the backbone of acylcarnitine esters (acylcarnitines; AC), which act as carriers during the transfer of activated acyl and acetyl groups across intracellular membranes from the cytosol into the mitochondrial matrix where β -oxidation takes place^{5,6}. These ACs can provide insight into how well muscle mitochondria are handling fuel selection and metabolism.

Statement of the problem

Obesity trends in the United States have been on the rise since the 1960s⁷. Per the Centers for Disease Control (CDC), more than one third of the population is obese, with 36.5% of adults and 17% of youth falling into this category^{7,8}. Obesity occurs when the human body deposits excess calories in adipose tissue as a response to over nutrition and sedentary lifestyle^{3,8}. The traditional Western diet, which is rich in energy dense foods plays a huge role in overnutrition and ultimately obesity. Individuals living in western societies are more likely to have an “obesogenic” diet, characterized by increased consumption of energy-dense, processed foods along with a reduced consumption of nutrient-rich foods, such as fruits and vegetables thus promoting the development of dysregulated metabolic processes^{3,9}. A fundamentally important question is whether a sustained shift in substrate preference toward fat oxidation lowers disease risk, and whether food selection can protect against metabolic dysfunction¹⁰. Plasma AC profiles can approximately reflect the intramitochondrial acyl-CoA pattern, allowing us to obtain a better understanding of potential mechanisms underlying metabolic inflexibility¹¹.

Significance of the study

It had been thought that acylcarnitine concentrations increase in the blood plasma of lean, insulin sensitive subjects during long-term fasting and relatively healthy overweight subjects during caloric restriction¹². These observations suggest that the AC increase in obese populations may not only be due to an impairment of metabolism but also be a natural response to an excess supply of lipid¹². Thus, increased production of AC could result from excess fatty acid flux emanating from lipid stored either intracellularly or peripherally¹². Minimal research to date has been conducted using human subjects. Additional research on this metabolite can bring forth new

knowledge on mitochondrial stress via fatty acid oxidation. This study focuses on the effects of an acute high fat diet, the transition from a fasted to fed state on AC saturation in human blood plasma, and whether acylcarnitine profiles depict metabolic inflexibility.

Specific Aims

1. To determine the relationship between changes in fasting plasma ACs and skeletal muscle substrate metabolism in response to five days of high fat feeding.
 - a. **Hypothesis #1:** Plasma acetyl-carnitine will increase and be negatively correlated with skeletal muscle metabolic flexibility.
 - b. **Hypothesis #2:** Plasma acyl-carnitine desaturation indexes (16:1/16:0 and 18:1/18:0), markers of stearoyl-CoA desaturase 1(SCD1) activity, will decrease and be negatively correlated with changes in skeletal muscle fatty acid oxidation.
 - c. **Hypothesis #3:** Free plasma carnitine will be reduced and negatively correlated with skeletal muscle fatty acid oxidation.

Chapter 2: Literature Review

Carnitine Biosynthesis

Carnitine is primarily obtained from the diet; however, most mammals can synthesize it endogenously via de novo synthesis^{13,14}. Ultimately carnitine is synthesized from the essential amino acids lysine and methionine, where lysine provides the carbon backbone of the carnitine molecule and the 4-N-methyl groups derive from methionine¹⁵. 6-N-trimethyllysine (TML) is the substrate for carnitine biosynthesis and is a direct product of lysosomal or proteasomal degradation of proteins containing N-methylated lysines⁶. N-methylation is a post translational modification carried out by methyl-transferases that utilize S-adenosylmethionine as a methyl donor^{6,14}. In mammals, proteins such as calmodulin, myosin, actin, cytochrome c and histones undergo N-methylation to obtain 6-N-trimethyllysine¹⁵.

Several enzymes and cofactors are involved in the carnitine biosynthetic pathway (*Figure 1*) beginning with TML dioxygenase (TMLD), which hydroxylates TML to produce 3-hydroxy-TML (HTML)^{6,16}. TMLD is a nonheme ferrous-iron dioxygenase that relies on 2-oxoglutarate, Fe⁺² and oxygen to act as cofactors⁶. It is here that iron acts as a bridge between its associated oxygen molecule via the iron-bound oxygen to hydroxylate the substrate allowing the dioxygenases to react^{6,15}. In this class of enzymes, hydroxylation of the substrate is linked to the oxidative decarboxylation of 2-oxoglutarate to succinate and CO₂¹⁵. This allows oxygen to react at the active site of the enzyme to form an oxo-ferryl intermediate, recognized as an iron-bound oxygen atom, to hydroxylate the substrate¹⁵. The remaining oxygen atom is then incorporated into 2-oxoglutarate thus forming succinate and releasing CO₂ in the process^{6,15}. In order to maintain enzymic activity, TMLD requires ascorbate (vitamin C) to maintain iron in the ferrous state^{6,15}.

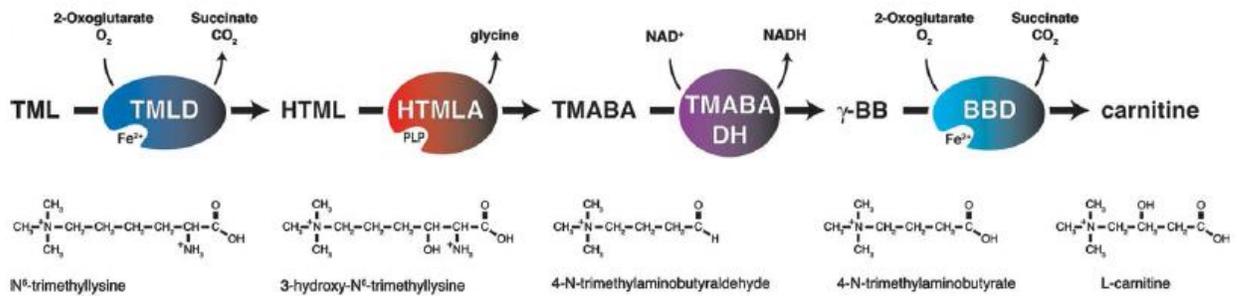


Figure 1. Enzymes incorporated with the carnitine biosynthetic pathway. TML: N⁶-trimethyllysine, TMLD: TML dioxygenase, HTML: 3-hydroxy TML, HTMLA: HTML aldolase, TMABA: 4-N-trimethylaminobutyraldehyde, TMABADH: TMABA dehydrogenase, c-BB: 4-N-trimethylaminobutyrate, BBD: c-BB dioxygenase, PLP: pyridoxal 5'-phosphate. (Strijbis, K. et al. IUBMB Life, 2010)

HTML aldolase (HTMLA) is the second enzyme in the carnitine biosynthetic pathway and is responsible for the pyridoxal 5'-phosphate adolytic cleavage of HTML, thus producing 4-trimethylaminobutyraldehyde (TMABA) and glycine⁶. Little is understood about HTMLA due to the lack of knowledge of the gene encoding HTMLA⁶. Evidence points to the possibility of the gene THA1P, a threonine aldolase ortholog, being a pseudogene of HTMLA encoding gene, but further research is required to fully comprehend this matter⁶. HTMLA is also thought to be identical to serine hydroxymethyltransferase (SHMT) as it has been shown previously that SHMT purified from rabbit liver acts upon HTML, yielding TMABA and glycine¹⁵. SHMTs are a class of PLP-dependent enzymes that perform interconversions between serine and glycine⁶. Scientists have hypothesized that in humans, an enzyme like SHMT, with low HTMLA activity, could catalyze the conversion of HTML to TMABA and glycine⁶.

The dehydration of TMABA to 4-N-trimethylaminobutyrate or γ-butyrobetaine (γ-BB) is catalyzed by the NAD⁺ dependent TMABA dehydrogenase (TMABA-DH)¹⁵. TMABA-DH requires NAD⁺ allowing its activity to be easily measured spectrophotometrically or

fluorimetrically by following the appearance of NADH¹⁵. Furthermore, cytosolic aldehyde dehydrogenase (ALDH) has been reported to act upon substrates that resemble TMABA including 4-aminobutyraldehyde and 2-trimethylaminoethanal¹⁵. ALDH9 is primarily expressed in areas where increased TMABA-DH levels exist including liver, kidney, heart, and muscle tissues¹⁵. Studies have shown that the recombinant protein had the highest activity with TMABA as a substrate, suggesting that the comparison of kinetic properties for a variety of substrates of rat TMABA-DH with heterologously expressed human ALDH9 showing that these enzymes have highly similar substrate specificities¹⁵. This leads to the belief that ALDH9 is the human TMABA-DH¹⁵.

The fourth, and final step, of the carnitine biosynthesis pathway catalyzes the stereospecific hydroxylation of γ -BB via γ -butyrobetaine dioxygenase (BBD) to yield L-Carnitine⁶. Like TMLD, BBD is a dioxygenase that incorporates an oxygen molecule in its substrate and succinate allowing it to have the same cofactors, and have extensive general sequence homology⁶. It has been discovered that 2-oxoglutarate can stimulate BBD activity, and that the enzyme requires molecular oxygen, Fe²⁺ and ascorbate for activity⁶. Mammalian BBD can be found primarily in the cytosol; however, BBD activity has been reported in peroxisomes⁶. BBD activity is usually measured radiochemically using labeled butyrobetaine but can also be determined by measuring the butyrobetaine-dependent release of [¹⁴C]CO₂ that produces succinate from the decarboxylation 2-oxo-[1-¹⁴C]glutarate⁶. Lastly 3-(2,2,2-trimethylhydrazinium) propionate (mildronate), which has cardioprotective properties during ischemia, is a competitive inhibitor of BBD⁶. Thus, a cardioprotective effect is proposed to be based on a lowering of the carnitine levels in the heart, which results in inhibition of fatty acid oxidation, decreased levels of harmful long-chain ACs and conservation of ATP¹⁵.

Carnitine Enzymology

The carnitine acyltransferase family is comprised of four specific enzymes including carnitine palmitoyltransferases 1 and 2 (CPT-1 and CPT-2), carnitine octanoyltransferase (CrOT) and carnitine acetyltransferase (CrAT), all of which play key roles in the catabolism of fatty acids¹⁷. These enzymes are important for energy homeostasis and fat metabolism through modulation of the pools of acetyl-CoA and long-chain acyl-CoA in distinct cell compartments of animals and fungi¹⁸. The four enzymes belong to the family of carnitine/choline acyltransferases and have different properties with respect to intracellular location, substrate specificity, kinetics, and physiological function as depicted in *Figure 2*, provided by Ramsay, RR. et al. *Molecular Aspects of Medicine*, 2004^{18,19}.

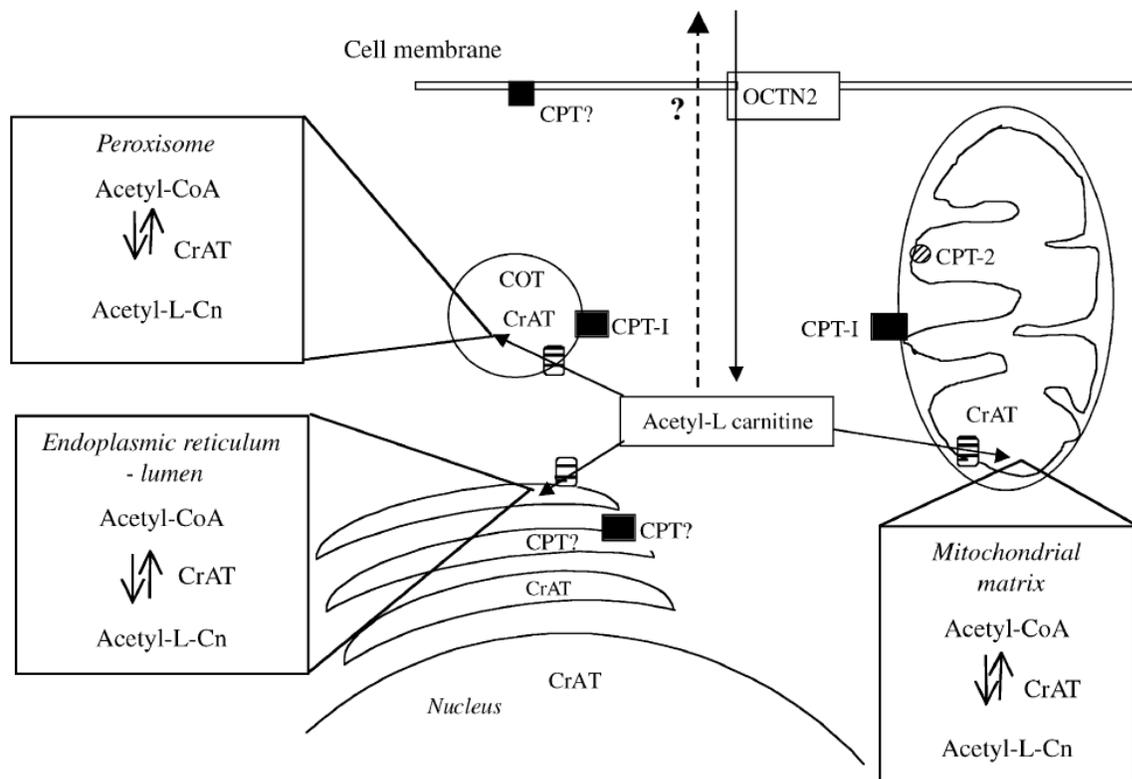


Figure 2: CPT-1/2 (black squares) act on cytosolic long-chain substrates. CrAT is only found inside the organelles using matrix acetyl-CoA and Acetyl-Carnitine. Carnitine acyl-carnitine translocase (CACT), depicted as the striped square, transfers carnitine and its esters across membranes. Sodium-dependent organic cation transporter (OCTN2) is the high affinity carnitine transporter for uptake of carnitine into the cell. (Ramsay, RR. et al. Molecular Aspects of Medicine, 2004)

CPT-1/ CPT-2

The CPT systems mediate the cellular transport of LCFAs by transesterification of long-chain acyl-CoAs into long-chain ACs in the cytosol, and vice versa, utilizing a reversible catalyzing reaction¹⁸. The long-chain specific CPT1 is the only transferase that has direct access to the cytosolic pool of acyl-CoA therefore permitting it advocate transesterification of long-chain acyl-CoAs¹⁹. Prior to mitochondrial β -oxidation, LCFAs need to be activated into CoA esters due to their large size preventing them from crossing into the inner mitochondrial membrane independently¹⁷. CPT-1, located within the outer mitochondrial membrane, converts these large acyl-CoAs into their respective carnitine esters thus allowing them to cross into the inner membrane¹⁷.

CPT-1 has three forms including CPT-1A for liver type carnitine palmitoyltransferase, CPT-1B for muscle type carnitine palmitoyltransferase, and CPT-1C for brain type carnitine palmitoyltransferase^{18,20}. CPT-1 is known to be the primary regulator of mitochondrial fatty acid oxidation²⁰. For example; in the liver, CPT-1 controls fatty acids flux via esterification and oxidative pathways due to its sensitivity to malonyl-CoA, an allosteric inhibitor, which also plays a role in the de novo synthesis of fatty acids^{20,21}. However, it should be noted that the muscle isoform of CPT-1 possess more sensitivity to the malonyl-CoA inhibition than the liver isoform¹⁸. In a fasting state, malonyl-CoA levels are reduced therefore allowing CPT-1 to

promote long-chain fatty acid oxidation²⁰. Malonyl-CoA levels begin to rise in the post absorptive state, leading to the inhibition of CPT-1 and the esterification of newly formed LCFAs²⁰. CPT-1 is established as an important element for maintenance of energy homeostasis in heart and skeletal muscle but remains unclear in hepatic tissue²⁰.

CPT-1 and CPT-2 work in tandem to transfer long-chain acyl moieties from the cytosol to the mitochondrial matrix, however transportation between the two enzymes is still necessary¹⁹. Carnitine/acyl-carnitine translocase (CACT) acts as an intermediate between CPT-1 and CPT-2 as it facilitates the transportation of the LCFA from CPT-1 in the outer membrane to CPT-2 in the inner membrane, with its catalytic site oriented towards the matrix^{18,19}. It is currently believed that only one isoform of CPT-2 exists and it is responsible for the conversion of ACs back into acyl-CoAs, an essential substrate for fatty acid β -oxidation^{20,22}. CPT-2 is thought to be more closely related, genetically, to CrOT and CrAT than to its CPT-1 counterpart¹⁸. It is responsible for the synthesis of medium- and long-chained acylcarnitines by producing a net flux in the reverse direction, therefore converting medium- and long-chain acyl-COAs into their respective acylcarnitine intermediates¹⁷. It has been found that CPT-2 activity increases with substrates ranging from C8 to C16-CoA²³.

CrOT/CrAT

A secondary enzyme responsible for the transesterification of medium- and long-chain acyl-CoAs is CrOT, which can be found in the peroxisomal matrix¹⁸. Although CrOT is not located within the mitochondria, it still plays a pivotal role in the β -oxidation of branch-chain fatty acids, including pristanic acid (2,6,10,14-tetrapentadecanoic acid)²⁴. β -oxidation of pristanic

acid is an early stage precursor to the peroxisomal derived acylcarnitines²⁴. Once it is oxidized efficiently for three cycles, pristanic acid yields 4,8-diethylnonanoyl-CoA (C11-CoA), a corresponding carnitine ester²⁴. C11-CoA is exported from the peroxisome to the mitochondria as depicted in *Figure 3*²⁴. Contrary to mitochondrial β -oxidation, the final product of peroxisomal oxidation is not acetyl-CoA but medium- and long-chain acyl-CoAs such as octanoyl-CoA²⁵.

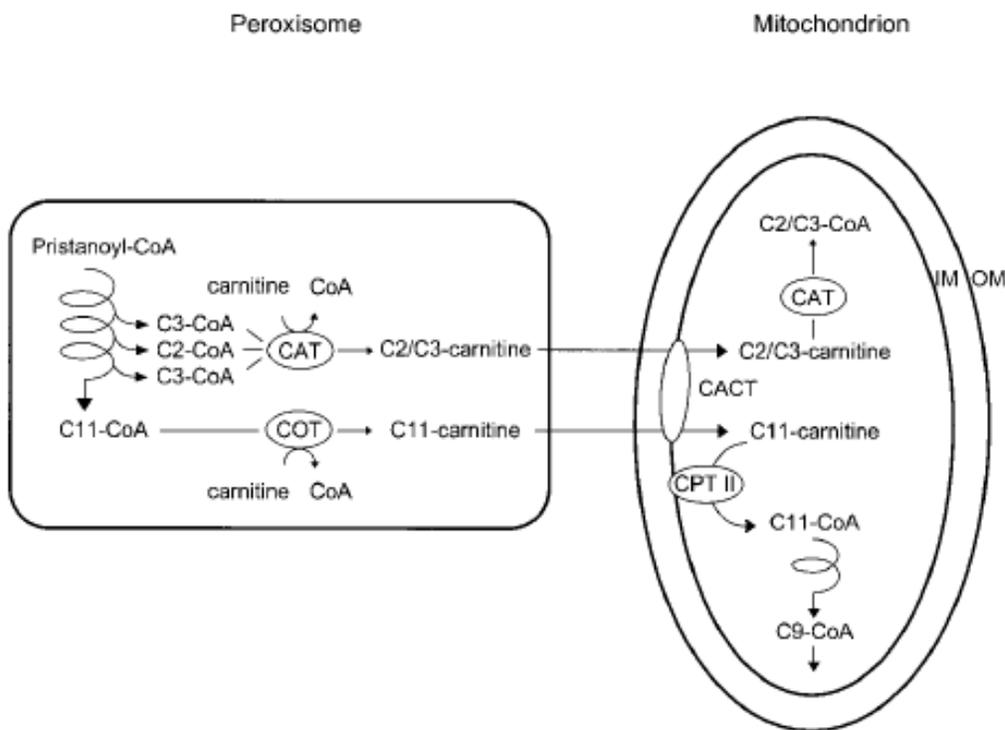


Figure 3: The role of peroxisomal CrOT (COT) and CrAT (CAT) β -Oxidation processes as depicted by; (Ferdinandusse S. et al. Biochemical and Biophysical Research Communications, 1999)

CrAT is an enzyme within the mitochondrial matrix that catalyzes the reversible conversion of acyl-CoAs into acylcarnitine esters^{17,26}. As previously mentioned, CrAT is active in the peroxisome as well, acting on acetyl-CoA and propionyl-CoA¹⁸. Peroxisomal CrAT acts

on short-chain acyl-CoAs converting them into carnitine esters^{17,27}. Mitochondrial CrAT regulates the maintenance of the acyl-CoA/CoA pool by facilitating the trafficking and efflux of carbon intermediates from the mitochondrial compartment to other cellular and extra cellular sites, thus acting as a modulator of whole body glucose^{26,27}. In addition to buffering the mitochondrial acetyl-CoA pool, CrAT regenerates free CoA by continuously converting acetyl-CoA to acetylcarnitine²⁶. Both outcomes influence the activity of several oxidative enzymes such as pyruvate dehydrogenase (PDH), an enzyme that promotes glucose oxidation, Furthermore, CrAT mitigates acetyl-CoA inhibition of PDH²⁶.

In humans, it is understood that as exercise intensity increases, fat oxidation and carnitine concentrations decrease, leading to an increase of carbohydrate oxidation for production of ATP²¹. Evidence has shown that changes in the glucose supply, regulate the concentration of malonyl-CoA via CrAT which is believed to have the ability to affect the pyruvate dehydrogenase flux¹⁷. The Acetyl-CoA/CoA ratio is known to regulate the PDH complex when acetyl-CoA acts on the PDH inactivating kinase PDK4, thus having an influence on substrate fuel preference ultimately influencing whole body glucose homeostasis^{17,18,21}. This in turn can be considered a precursor to developing insulin resistance.

Acylcarnitines & Metabolism

L-Carnitine is a conditionally essential nutrient that serves as a substrate for a family of acyltransferase enzymes that catalyze the interconversion of acyl-CoAs and ACs²⁶. In recent years, research has demonstrated the significance of carnitine's role in regulating skeletal muscle fuel selection as over 95% of the body's total carnitine supply being present in skeletal muscle tissue¹⁶. It is essential to fully understand carnitine's influence on skeletal muscle metabolism.

Over the past several years interests in the differing metabolic roles carnitine possesses has led to the theory that the muscle carnitine pool can be manipulated and have a significant impact on physiological functions¹⁶.

Starting in the mid-1950s Irving Fritz & colleagues had established that mitochondria are impermeable to fatty acyl-CoA¹². Long-chain fatty acids in tissues primarily exist as components of triglycerides or phospholipids where triglycerides stores are the primary source of fatty acids used for fatty acid oxidation during fasting conditions²⁸. Lipases act on triglycerides to release free fatty acids and are then transported to tissues via the blood stream²⁸. These free fatty acids are then acquired by hepatocytes and muscle, where they are subsequently activated to their CoA esters²⁸. Transport of the long chain acyl-CoAs into mitochondria for β -oxidation requires a mitochondrial membrane transporter that utilizes AC instead of acyl-CoAs²⁸.

Initial conversion of acyl-CoA to an AC ester, followed by transport of the AC across the inner mitochondrial membrane into the mitochondrial matrix and subsequent reformation of acyl-CoA, constitutes a “carnitine shuttle” that requires the intensive action of 3 proteins: CPT-1, carnitine AC translocase (CACT), and CPT-2²⁸. CPT-1 is associated with the outer membrane of the mitochondria and furthermore it catalyzes the formation of the O-acylcarnitine, which is then transported across the inner membrane by a translocase as depicted in *Figure 4*²⁹. The acylcarnitine is then passed to CPT-2 on the matrix side of the inner membrane which transfers the fatty acyl group back to CoA to reform the fatty acyl-CoA, leaving free carnitine to return across the membrane via the translocase²⁹.

Additional information to consider is that, although oxygen esters usually have lower group-transfer potentials than thiol esters, the O-acyl bonds in ACs have high group-transfer potentials, and the transesterification reactions mediated by the acyltransferases have an equilibrium

constants close to 1²⁹. It is also important to note that eukaryotic cells maintain separate pools of CoA in the mitochondria and the cytosol²⁹. The cytosolic pool is utilized principally in fatty acid biosynthesis and the mitochondrial pool is important in the oxidation of fatty acids, pyruvate and some amino acids²⁹.

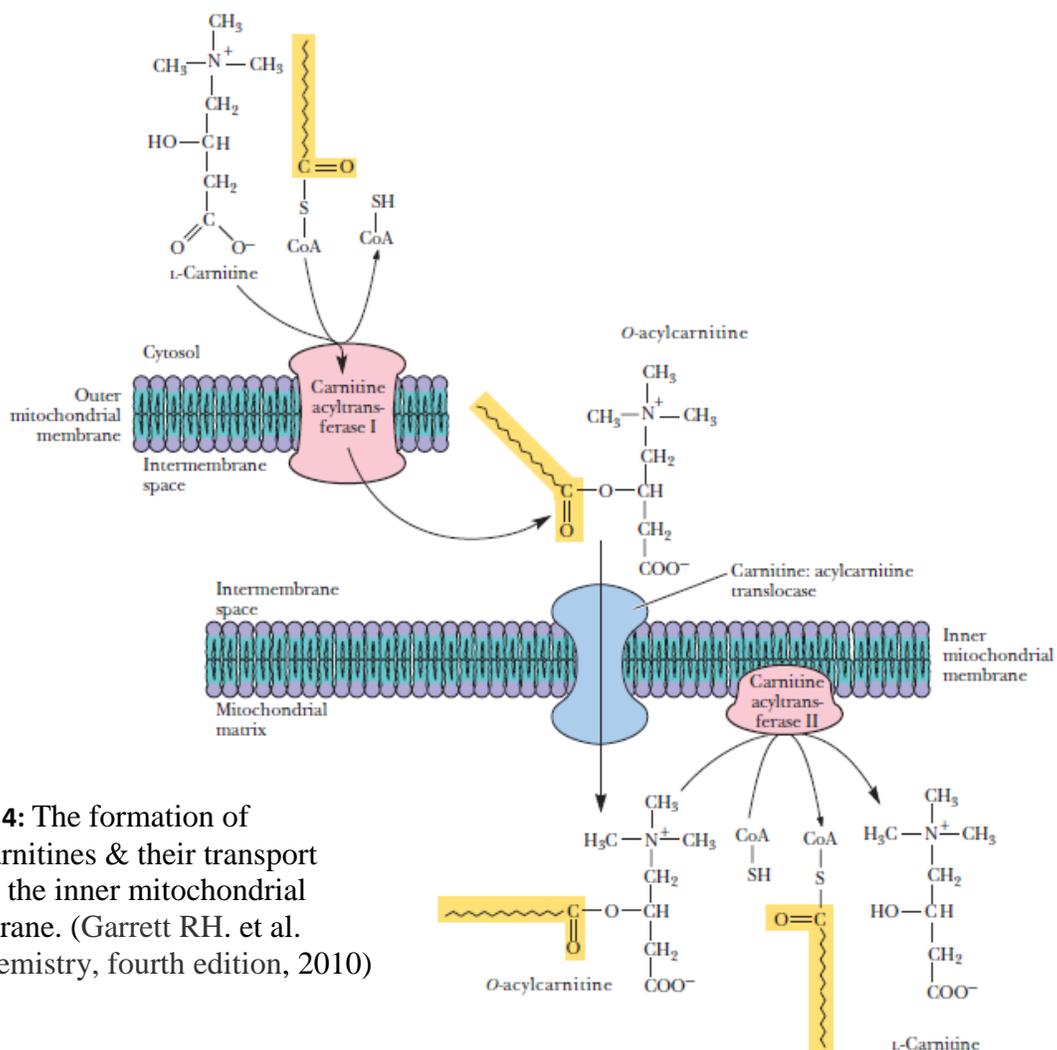


Figure 4: The formation of acylcarnitines & their transport across the inner mitochondrial membrane. (Garrett RH. et al. Biochemistry, fourth edition, 2010)

Fasting Acylcarnitines

Short term fasting can be described as the first 72 hours of starvation in which progressive alterations in lipid and glucose metabolism occur³⁰. Furthermore, the adaption to short term fasting is characterized by an increase of lipolysis with associated increases in plasma free fatty acids and FAO combined with a decrease in peripheral insulin sensitivity and carbohydrate oxidation³⁰. In a fasted state, long-chain acylcarnitines can be found in blood plasma, however little is known about whether muscle derived long-chain acylcarnitines increase during short term fasting³⁰.

During fasted conditions, hepatic fatty acid mobilization is enhanced and fatty acids reaching the liver from fat stores are rapidly oxidized, accompanied by accelerated production of ketone bodies¹¹. Carnitine not only enables the transport of long-chain acyl-CoA esters across the inner mitochondrial membrane, but it also acts as an acyl sink to maintain adequate cellular levels of free CoA¹¹. As fasting or a fat load lead to an increase in mitochondrial fatty acid β -oxidation, there is an associated intramitochondrial flux of acyl-CoA esters¹¹. The formation of ACs may be important in maintaining the size of the mitochondrial acyl-CoA pool or to remove poorly metabolized acyl-CoAs¹¹. The ACs formed are transported out of the mitochondria and may leave the cell¹¹. The plasma AC profile will approximately reflect the intramitochondrial acyl-CoA pattern¹¹.

Postprandial Acylcarnitines

The transition from the fasted to fed state is characterized by changes in the metabolism of long-chain fatty acids that are determined by a balance between adipose tissue derived fatty

acid release, availability of tissue lipid stores, mitochondrial fatty acid transfer, release of dietary fatty acids by intravascular lipolysis, tissue fatty acid uptake, and mitochondrial FAO rates¹². In using nonsmoking, sedentary, and overweight or obese subjects Ramos-Roman et al. had demonstrated that there is a negative correlation between elevated levels of dietary derived fatty acids in the free fatty acid pool and decreased levels of long chained ACs along with an increase in AC concentrations postprandially. These observations were believed to be due to a lack of suppressed adipose fatty acid release caused by adipose insulin resistance¹². In obese subjects, plasma long-chain ACs have shown decreases while in the postprandial state, however the extent of the decrease has been reported to reflect the idea that individuals with higher levels of insulin sensitivity are more capable of metabolizing fatty acids³¹. To date it is still not certain whether endogenous fatty acids, dietary fatty acids, or both, have a more substantial impact on AC concentrations¹². With minimal research committed to postprandial plasma ACs concentrations, extensive information regarding them is limited¹² thus hinting towards the need for further investigation.

Metabolic Disorders

Plasma ACs have been proposed as biomarkers of insulin resistance and metabolic inflexibility in adults¹². Research has demonstrated dietary changes, as brief as seven days, can result in similarities between the dietary fatty acid composition and muscle triglyceride and AC, suggesting that endogenous and dietary fatty acids have the ability to impact the composition of AC species¹². Obese individuals with either impaired glucose tolerance or diabetes have shown elevated fasted AC concentrations suggesting incomplete fatty acid oxidation¹². These observations indicate that the increase in ACs may not only be due to an impairment of

metabolism but also be a natural response to excess lipids, highlighting the importance of understanding how ACs reflect metabolic transitions from fasting to fed¹².

The accrument of lipids and lipid metabolites alike within the blood stream and tissues, leading to metabolic instability, is known as lipotoxicity³². Since the 1960s fatty acid inhibition of glucose oxidation has been a focal point of metabolic feedback regarding how substrate competition influences lipotoxicity¹¹. In human plasma, free fatty acid concentrations in healthy adults stay within 350 to 550 $\mu\text{mol/L}$ ³². However, an increase of up to 700 $\mu\text{mol/L}$ has demonstrated a disruption in insulin signaling by inhibiting insulin receptor tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and AKT serine phosphorylation. This supports the notion that short term exposure to a lipid rich environment causes skeletal muscle to shift to fatty acid utilization combined with induced skeletal muscle insulin resistance and a reduction of glucose uptake³².

Lipid-Induced Insulin Resistance

Insulin resistance is recognized as when peripheral tissues become unresponsive to the glucose-lowering, antilipolytic, and anabolic properties of the hormone and is thought to be associated with reduced FAO combined with cytosolic lipid accumulation³³. Skeletal muscle, being highly insulin responsive, is a favorable tissue for glucose metabolic regulations as it accounts for the majority of insulin-mediated glucose disposal throughout the body³². Insulin resistance is associated with weight gain, a sedentary lifestyle and systemic hyperlipidemia, indicating that on the molecular level insulin resistance is driven by an increase in lipid accumulations³³.

Traditionally, insulin binds to the insulin receptor therefore stimulating autophosphorylation of tyrosine residues combined with subsequent activation of the tyrosine kinase receptor³². Tyrosine kinase is responsible for the phosphorylation of intracellular substrates, such as insulin receptor substrate (IRS) 1 and 2 which, play key roles in the insulin response that is regulated by IRS activation of phosphatidylinositol 3-kinase, a known component of insulin signaling³². Studies have looked at how long-chain acyl-CoAs derived from circulating lipids, or intramuscular triacylglycerol, become diverted away from CPT1 and redirected toward the synthesis of signaling intermediates, such as diacylglycerol and ceramide, where accumulation of bioactive molecules is believed to affect stress-activated serine kinases that interfere with insulin signaling transduction³³, further suggesting a correlation between insulin resistance and elevated intramyocellular lipids³². When examining the disease in humans, insulin resistance displays characteristics of diminished mitochondrial function, reduced expression of oxidative metabolism genes, decreased mitochondrial size, and reduced mitochondrial density³² indicating that the mitochondria is in distress.

Mitochondrial dysfunction has been a topic of interest as it is unknown whether it has a cause or effect relationship with insulin resistance. In sedentary and overweight or obese subjects, individuals with lower concentrations of long chained ACs in the fed and fasted states also showed higher rates of glucose oxidation at the same time¹² giving acknowledgment to the existence of a positive correlation between insulin sensitivity and skeletal muscle oxidation¹⁰. This in turn can provide evidence that insulin resistance has a negative impact on mitochondrial ability to function properly. To further support this, Muoio et al. discusses how an insulin resistant state can lead to defective muscle mitochondria combined with deficiencies in oxidative metabolism and impinge on insulin signaling by pushing fatty acids towards toxic lipid species

instead of oxidation¹⁰ leading to the eventual breakdown of regular metabolic pathways within the organelle. However, Muoio's counterargument challenges this by referring to the principles of bioenergetics and stating that supply must adjust to demand and not the other way around.¹⁰

Regarding ACs, elevated levels of long-chain plasma ACs have been apparent in insulin-resistant patients³¹. In addition, muscle insulin resistance is associated with accumulated plasma and skeletal muscle ACs¹. Another suggestion refers to ACs interfering with insulin signaling via activation of proinflammatory mechanisms reflecting an inverse relationship between efficient muscle FAO and reactive oxygen species (ROS) signifying that more reducing agents, produced from β -oxidation, are entering the electron transport chain from the electron transfer flavoprotein, thus skipping complex one where ROS production occurs¹. To date, the question of whether ACs impose or reflect insulin resistance has remained unanswered and we seek to shed more light on these mechanisms.

Disorders of Fatty Acid Oxidation

Mitochondrial β -oxidation has many biochemical steps that result in the oxidation of fatty acids resulting in the cleavage of two carbon units from fatty acids^{28,34}. However, more than twenty FAO disorders are known to exist, causing defects in the mitochondrial FAO pathway effecting approximately 1 in every 10,000, due to an autosomal recessive inheritance^{28,35}. Patients with FAO disorders possess symptoms when their impaired oxidative capacity is overwhelmed in times of fasting, stress, or prolonged exercise²⁸. Currently, little is known of additional genetic and environmental influences³⁴.

Traditionally FAO supplies carbon substrates for gluconeogenesis while fasted, and provides electrons to the respiratory chain for energy production²⁸. However, if one of these

steps becomes dysfunctional, the entire process is susceptible to FAO impairment resulting in onsets of clinical disorders including hypoglycemic seizures, muscle damage, cardiomyopathy, metabolic acidosis, and liver dysfunction²⁸. When defects occur in fatty acid degradation, AC intermediates begin to accumulate within the tissues²⁸. ACs that spill into the blood stream provide biomarkers for diagnosis, which is commonly used in newborn screenings²⁸.

Existing FAO disorders have a variety of flaws including defects in fatty acids and carnitine transport (CPT-I, CPT-II & CACT), acyl-CoA dehydrogenase(s) deficiencies, a variety of enzymatic defects in mitochondrial β -oxidation, and defects of ketone body production³⁴. Using tandem mass spectrometry to screen newborns, successful identification of these metabolic defects has proven to be a valid method of preventive medicine in targeting the disease while it remains pre-symptomatic³⁴. When considering adults, chronic over nutrition leading to increased cytosolic lipid accumulation is thought to stress FAO pushing it past its capacity³¹. Understanding adult induced FAO disorders is not an easy achievement because many of the mechanisms are still unknown, however what is evident is that fatty acid oxidation seems to outpace the tricarboxylic (TCA) cycle and the respiratory chain leading to an accumulation of intramitochondrial FAO intermediates, including ACs³¹.

Metabolic Flexibility

Traditionally tissues express flexibility in choosing which substrate that will be utilized for energy homeostasis, the process is commonly known as the Randle cycle³⁶. During a “normal” fed state glucose is the preferred substrate for oxidation, while in a fasted state fatty acids and ketone bodies become an alternative fuel source to glucose³⁶. The purpose of the substrate switch is to leave enough glucose reserves for the brain when nutritional glucose is

limited,³⁶ such as in a prolonged fasted state. Metabolic flexibility is the ability to adjust fuel oxidation depending on fuel availability. In recent years the prevalence of obesity and type II diabetes has highlighted the importance of understanding nutrient-induced substrate switching, as these populations are more likely to succumb to irregular metabolic flexibility^{2,10}.

In healthy adults a switch in fuel oxidation depends on the quality and quantity of nutrients available for oxidation at the cellular level where fuel availability is regulated by fuel sensors that either activate or inhibit metabolic pathways². In the occurrence of excess fuel supply, anabolic pathways become activated pushing nutrients into reserve stores, and when fuel supply is limited hydrolytic and lipolytic pathways become more active allowing the liberation of nutrients from reserves². “Metabolic inflexibility” is a term used to represent impairments in substrate selection and was first utilized in a report showing the shift from fat oxidation in a fasted state to higher rates of glucose oxidation in response to a hyperinsulinemic-euglycemic clamp in health adults, while obese and type II diabetic subjects still favored fat oxidation post clamp¹⁰.

Fuel oxidation variability is heavily dependent on energy balance and macronutrient dietary composition². When carbohydrates become readily available, a non-diseased state response would prompt a rapid increase in carbohydrate oxidation accompanied by suppression of lipid oxidation, though when dietary fat intake increases a slow shift favoring fat oxidation takes effect². New evidence suggests that muscle oxidative abilities make the shift from carbohydrates to lipids in the presence of acute high fat consumption, suggesting fatty acid catabolism surpasses the need for ATP production, in part due to the mitochondria becoming flooded with a heavy carbon load¹⁰. The idea of mitochondrial carbon flooding has been supported with evidence of accumulated mitochondrial-derived fatty AC intermediates and

reduced TCA intermediates where the ACs represent byproducts of mitochondrial metabolism that reflect fluctuations in substrate supply and flux limitations of catabolic enzymes¹⁰. Currently the molecular and pathophysiology detailing metabolic inflexibility remains vague¹⁰, limiting our understanding of its role in metabolic diseases.

Conclusion

As evidence continues to mount, the existence of metabolic inflexibility is becoming more apparent. Chronic over nutrition seems to be at the forefront of where to point the finger, given the high quantity of metabolic dysfunctions, such as; fatty acid oxidation disorders, impaired glucose tolerance, and improper substrate selection, all in which seem to place an overload on the mitochondria. Mutually, what appears to be a warning sign that global mitochondria are in distress is the presence of AC species present in the blood stream in elevated measures. The questions we ask is whether ACs are a reflection of metabolic distress and if they are, are they capable of diagnosing metabolic inflexibility?

Chapter 3: Methods

Participants

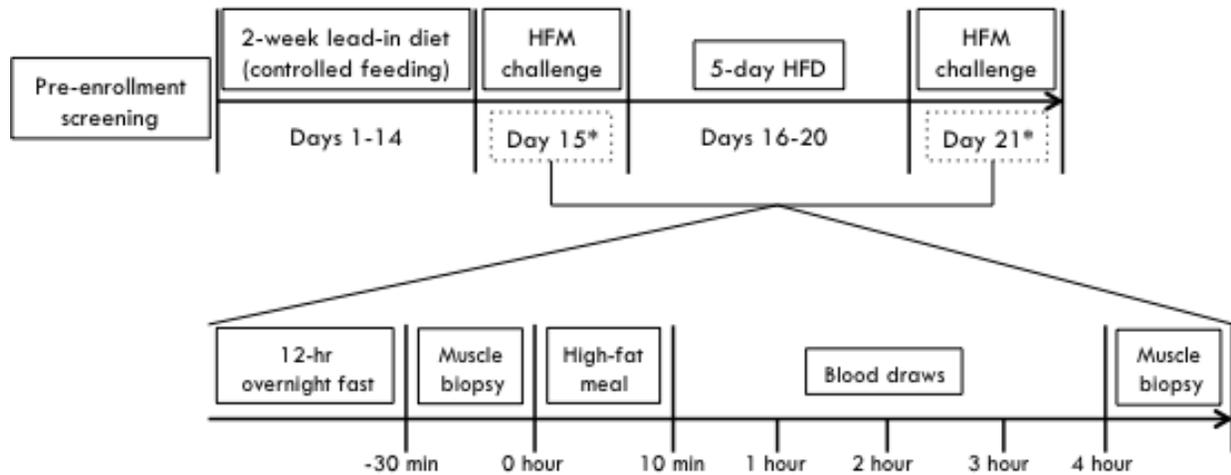
Thirteen healthy, non-obese, sedentary males (≤ 2 days, 20 min/day of low-intensity physical activity), aged 18-40 years participated in the study. Participants were weight stable ($< \pm 2.5$ kg) for 6 months prior to initiation of study with a BMI > 18 or < 30 kg/m² and were not under the influence of any medications known to affect study measures. All participants were required to have the following measurements; blood pressure $< 140/90$ mmHg, fasting glucose < 100 mg/dL, LDL cholesterol < 130 mg/dL, total cholesterol < 200 mg/dL, and triglycerides < 250 mg/dL. Their percentage of habitual calorie intake was composed of $< 40\%$ fat and 15% saturated fat. Participants were non-smokers with no history or family history of cardiometabolic disease. The Virginia Polytechnic Institute and State University Institutional Review Board approved all study procedures. Participants were informed of all procedures, benefits and any potential risks associated with the study before written consent is obtained.

Experimental design

Following successful completion of screening procedures, participants began a two-week lead-in controlled feeding period (control diet). The prepared meals were isocaloric to their habitual diet and consisted of 55% CHO, 30% fat, and 15% protein. Following the control diet, participants consumed a 5-day high-fat diet (HFD) consisting of 50% fat (45% of which was saturated fat), 35% CHO, and 15% protein. Both diets were isocaloric to each participant's daily energy requirements. Participants completed a high-fat meal (HFM) challenge [820 kcal ($\sim 30\%$ kcal/d), 52 g CHO (25%), 24 g protein (12%), 58 g fat (63% , $\sim 26\%$ saturated fat)], before and after the 5-day HFD. After an overnight fast, muscle biopsies were taken immediately prior to, and 4

hours after the HFM for assessment of skeletal muscle metabolic response and adaptation (see **Figure 6**).

Figure 6: Schematic of research design



Controlled Feeding Procedures

Four-day food intake records was used to confirm that habitual diets contained less than 40% of total calories from fat. After being trained on proper reporting techniques (using food models and measurement devices) by a research dietitian, participants will record food intake for 3 weekdays and 1 weekend day. The research dietitian using the 3-pass method reviewed habitual diet records with the participant. The food intake was analyzed using Nutrition Data System for Research (NDS-R) software version 2012 (University of Minnesota) by a trained diet technician. In order to estimate appropriate energy requirements for each participant, the Institute of Medicine equation will be used based on height, weight, age, and activity level³⁷. Both the control diet and HFD operated on a 7-day cycle of menus consisting of meals and snacks with

two optional snack modules (± 250 kcals). Diets were planned by a registered dietitian using NDS-R software. Each diet was isocaloric to the participants' daily energy requirements. All meals were prepared in the Department of Human, Nutrition, Foods and Exercise Metabolic Kitchen. Participants ate breakfast in the Dining Laboratory for Eating Behavior and Weight Management every day and took the remaining food for the day with them. Participants weighed in each day at the lab prior to breakfast to ensure they remained weight stable. A trend of > 1.0 kg weight loss or gain was offset by adding or subtracting 250 kcal food modules with the same macronutrient composition as the overall diet. All uneaten items and unwashed containers were returned to the metabolic kitchen where trained research staff monitored compliance. Participants were not permitted to consume any additional food, caffeine or alcohol for the duration of the study. They were instructed to report consumption of all non-study foods.

Measurements and Procedures

Muscle biopsies

Biopsies were taken from the vastus lateralis muscle using a suction-modified Bergström-type needle (Cadence, Staunton, VA) technique^{38,39}. An area of skin in the region of the vastus lateralis was shaven and cleansed with a povidine-iodine solution. The skin, adipose tissue and skeletal muscle fascia was anesthetized using 10mL lidocaine (1%). An incision (0.75 cm) was made in the skin with a #10 scalpel, and the fascia fibers were separated with the blunt edge of the scalpel. The Bergstrom needle (5 mm) was inserted into the vastus lateralis and suction was applied. The muscle tissue was pulled into the needle, snipped and extracted. Tissue samples

were immediately placed in ice cold PBS to remove blood and connective tissue. Muscle tissue used to assess substrate oxidation was immediately placed in 200uL of SET buffer (0.25 M Sucrose, 1 mM EDTA, 0.01 M Tris-HCl and 2 mM ATP) and stored on ice until homogenization (~25 min). Muscle tissue used to assess mitochondrial function was immediately placed in ice cold buffer 1 for mitochondrial isolation (IBM1) (67 mM sucrose, 50 mM Tris/HCl, 50 mM KCl, 10 mM EDT/Tris and 0.2% BSA) and stored until isolation (~25 min). Muscle tissue used for western blotting was placed in ice-cold cell lysis buffer (50 mM Tris-HCl, EDTA 1 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, igepal Ca 630 1%, pH 7.5) with halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Pittsburgh, PA), then snap-frozen in liquid nitrogen. Samples collected for western blotting were stored at -80°C for later analysis.

Muscle homogenization

Muscle samples for substrate oxidation (~ 75mg) were collected and minced with scissors followed by the addition of SET Buffer to produce a final 20-fold dilution (wt:vol). The samples were then homogenized in a Potter-Elvehjem glass homogenizer (Thomas Scientific, Swedesboro, NJ) at 10 passes across 30 seconds at 150 RPM with a motor-driven Teflon pestle.

Metabolic Flexibility

Metabolic flexibility in vastus lateralis muscle was assessed by measuring $^{14}\text{CO}_2$ production from [1- ^{14}C]- pyruvate (American Radiolabeled Chemicals, St. Louis Mo.) in the presence and absence of non-labeled BSA (0.5%) bound-palmitic acid. Specifically, samples were incubated in 0.35 $\mu\text{Ci/mL}$ of [1- ^{14}C]-pyruvate for 1 hour after which the media was

acidified with 200 μL 45% perchloric acid for 1 hour to liberate $^{14}\text{CO}_2$. The $^{14}\text{CO}_2$ was trapped in a tube containing 1 M NaOH, and the sample was placed into a scintillation vial with 5 mL scintillation fluid. The vial's ^{14}C concentrations were measured on a 4500 Beckman Coulter scintillation counter (Indianapolis, IN). Metabolic flexibility was denoted by the percentage decrease in pyruvate oxidation in the presence of free fatty acids.

Plasma Acylcarnitine Analysis

Stock solutions of standards were prepared by reconstituting VU Medical Center [Dr. H.J. ten Brink. Amsterdam] standards with 50mL of methanol ($\sim 1\mu\text{M}$) and unlabeled & labeled (D3) standards were prepared individually. Mixed internal standard solution was prepared by 1:500 dilution of each individual internal standard ($\sim 2\text{nM}$ Table 1). The derivatization solutions were prepared fresh daily using 9.5 mL of butanol [VWR. Radnor, PA] mixed with 0.5 mL of acetyl chloride (5% v/v) [Sigma. St. Louis, MO] and 250 mL of acetonitrile (ACN) [VWR] mixed with Milli-Q water at a ratio of 80:20 (v/v).

Plasma samples were thawed at room temperature, and 50 μL plasma was combined with 100 μL of methanol and 5 μL of IS solution at in a 1.5-mL microcentrifuge tube. The mixture was frozen at $-20\text{ }^\circ\text{C}$ for 10 min and then samples were centrifuged for 3 minutes at 17000g at room temperature in a accuSpin Micro 17 [Fisher Scientific. Waltham, MA] to remove the precipitated proteins. 100 μL of supernatant was transferred to glass tubes and evaporated using a Savant SpeedVac Concentrator SPD1010 [Thermo Electron Corporation, Waltham MA.] set at 45°C for 20 minutes.

Derivatization of ACs was accomplished by adding 100 μL of fresh butanol solution containing 5% v/v acetyl chloride to the evaporated samples. The mixture was then heated at

60°C for 20 minutes in a water bath followed by a second round in the speed vac set at 45°C for 20 minutes. Samples were then reconstituted in 50 µL of acetonitrile/water (80:20 v/v), vortexed for one minute and sonicated. Samples were then filtered using 4mL PTFE syringe filters (0.2 µm) [VWR] into Waters [Milford, MA] certified LCMS vials with 150 µL deactivated glass inserts.

Samples were then injected into a Waters UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 150 mm column with a Waters ACQUITY BEH C18 VanGuard Pre-column, 1.7 µm, 2.1 x 5 mm attached to it.

Samples were introduced into the UPLC ethylene bridge hybrid C₁₈ column in 7 µL increments. Chromatographic runs were performed at a rate of 400 µL/Min with a gradient elution of a mobile phase consisting of solution A (H₂O with 0.1 % TFA, v/v) and solution B (acetonitrile with 0.1 % TFA, v/v). Column temperature was set at 50°C and the sample temperature was set to 5°C. The gradient began with 99% solution A, then program as follows: 0–0.1 min, gradient to 83 % solution A; 0.1–0.28 min, gradient to 76 % solution A; 0.28–0.35 min, gradient to 74 % solution A; 0.35-0.80 min, gradient to 71% solution A; 0.80-1.71 min, gradient to 69 % solution A; 1.71-2.96 min, gradient to 66 % solution A; 2.96-4.50 min, gradient to 64% solution A; 4.50-5.44 min, gradient to 44 % solution A; 5.44-6.37 min, gradient to 30 % solution A; 6.37-8.01 min, gradient to 18 % solution A; 8.01-11.30 min, gradient to 7 % solution A; 11.30-12.50 min, gradient to 5 % solution A; 12.50-13.50 min, gradient back to 99 % solution A; 13.50–15 min, hold at 99 % solution A to reequilibrate the column. MS-MS data were acquired in multiple reaction monitoring (MRM) mode with the source temperature set to 90°C, with a desolvation temperature set to 200°, capillary voltage was set to 3.5 kV and dissolving gas flow was set to 400 L/min. Cone voltage and collision energy were determined for each AC (**Table 1**).

Free Carnitine Analysis

Fasting plasma concentrations of L-carnitine betaine were quantified by isocratic UPLC-MS/MS using the stable isotope dilution method against internal standards (IS). The L-carnitine hydrochloride standards were obtained from Sigma (St Louis, MO, USA). The UPLC solvents (ACN and water) were liquid chromatography–mass spectrometry grade (VWR, Randor, PA, USA).

For analysis of L-carnitine, a stock solution of the IS (29.4 $\mu\text{mol/L}$ L-carnitine-d9) was prepared in water and stored at -20°C . Immediately before sample preparation, the IS stock solution was diluted 25-fold with ACN (4 mL IS stock + 96 mL ACN) for final IS concentrations of 1.18 $\mu\text{mol/L}$ L-carnitine-d9. Plasma samples were thawed at room temperature, and 50 μL plasma was combined with 600 μL of the ACN/IS solution in a 1.5-mL microcentrifuge tube. The addition of ACN resulted in protein precipitation. Analytes were extracted by vigorous vortexing for 30 seconds. The samples were centrifuged for 3 minutes at 17000g at room temperature. Samples were filtered using 0.2- μm Philic PTFE Millex Smplicity vacuum filters (Millipore, Billerica, MA, USA) into Waters (Milford, MA, USA) Total Recovery LCMS Certified high-performance liquid chromatography vials and analyzed immediately by UPLC-MS/MS. The UPLC-MS/MS analyses were carried out using a Waters Acquity UPLC system coupled to a Waters TQD triple quadrupole mass spectrometer. The system software was Mass Lynx (Waters).

The samples were separated on a Waters BEH HILIC analytical column (2.1 \times 100 mm; particle size, 1.7 μm) with a Waters BEH HILIC VanGuard precolumn (2.1 \times 5 mm; 1.7 μm). The column temperature was at 30°C , and the sample compartment was maintained at 10°C . The

mobile phases were 15 mmol/L ammonium formate, pH 3.5 (phase A) and ACN (phase B). The system flow rate was 0.65 mL/min, and isocratic elution was achieved using 20% A/80% B over 3 minutes. Following UPLC separation, the target analytes and their respective IS were identified and quantified using electrospray ionization in (+)-mode. The source and capillary temperatures were 150°C and 400°C, respectively. The capillary voltage was 0.60 kV, and the desolvation and cone gas (both N₂) flow rates were 800 and 20 L/h, respectively.

The compounds were quantified using multi reaction monitoring (MRM) functions optimized by Intellistart, as shown in Table 1. The MRM functions used the Auto dwell function to optimize the number of points per peak (12 points for a 10-second peak). The detection span was ± 0.2 amu for the mass.

Quantification was completed using Quan Lynx v. 4.1 (Waters). Quantification was performed using the ratio of the target analyte and respective IS peak areas based on external standard curves prepared using a wide range of target analyte concentrations (bracketing the peak areas observed in the plasma samples) and the same IS concentrations used to prepare the plasma samples.

Table 1 - MRM Functions for target analytes and IS. Molecular weight (MW) represents individual compounds while parent ions represent derivatives of compound.

Compound	Retention Time	MW(g/Mol)	Parent [M+H] ⁺	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)
D3 Acetyl	1.89	242.7	263.2	85.04	40.00	20.00
D3 Butyryl	3.15	270.8	291.7	85.04	42.00	20.00
D3 Hexanoyl	5.80	298.8	319.33	85.04	40.00	24.00
D3 Octanoyl	6.61	326.9	347.33	85.10	42.00	24.00
D3 Decanoyl	7.10	354.9	375.39	85.10	64.00	26.00
D3 Dodecanoyl	7.56	383	403.39	85.09	50.00	28.00
D3 Tetradecanoyl	8.06	411	431.39	85.10	50.00	28.00
D3 Hexadecanoyl	8.57	439.1	459.46	85.10	58.00	28.00
D3 Octadecanoyl	9.04	467.1	487.46	85.10	58.00	28.00
L-Carnitine-d9	2.08	170.25	171.28	84.99	34.00	20.00
L-Carnitine	2.09	161.20	162.26	84.99	34.00	20.00
Acetyl	1.89	239.7	260.14	85.04	40.00	30.00
Propionyl	2.36	253.7	274.2	85.04	120.00	22.00
Butyryl	3.15	267.8	288.2	85.04	40.00	22.00
Isobutyryl	3.04	267.8	288.2	85.04	40.00	22.00
Valeryl	4.02 or 4.18	281.8	302.27	85.03	42.00	20.00
Isovaleryl	4.02 or 4.18	281.8	302.27	85.10	42.00	22.00
2-Methylbutyryl	4.02 or 4.18	281.8	302.27	85.03	40.00	22.00
Pivaloyl	4.02 or 4.18	281.8	302.27	85.10	42.00	22.00
Hexanoyl	5.80	295.8	316.27	85.04	42.00	22.00
Octanoyl	6.61	323.9	344.33	85.04	44.00	24.00
Decanoyl	7.10	351.9	372.33	85.03	64.00	28.00
10:1	6.88	349.9	370.44	85.03	60.00	25.00
Dodecanoyl	7.56	380	400.39	85.04	46.00	24.00
12:1	7.35	378	398.44	85.04	50.00	25.00
Tetradecanoyl	8.06	407.9	428.39	85.04	66.00	26.00
14:1	7.73	405.9	426.75	85.10	60.00	25.00
Hexadecanoyl	8.57	436.1	456.46	85.03	66.00	28.00
16:1	8.17	415.9	454.53	85.10	58.00	25.00
Octadecanoyl	9.04	464.1	484.46	85.10	56.00	28.00
18:1	8.64	462.1	482.53	85.10	58.00	25.00

Statistical analysis

All statistical analyses were performed using GraphPad Prism v.7 (La Jolla, CA, USA).

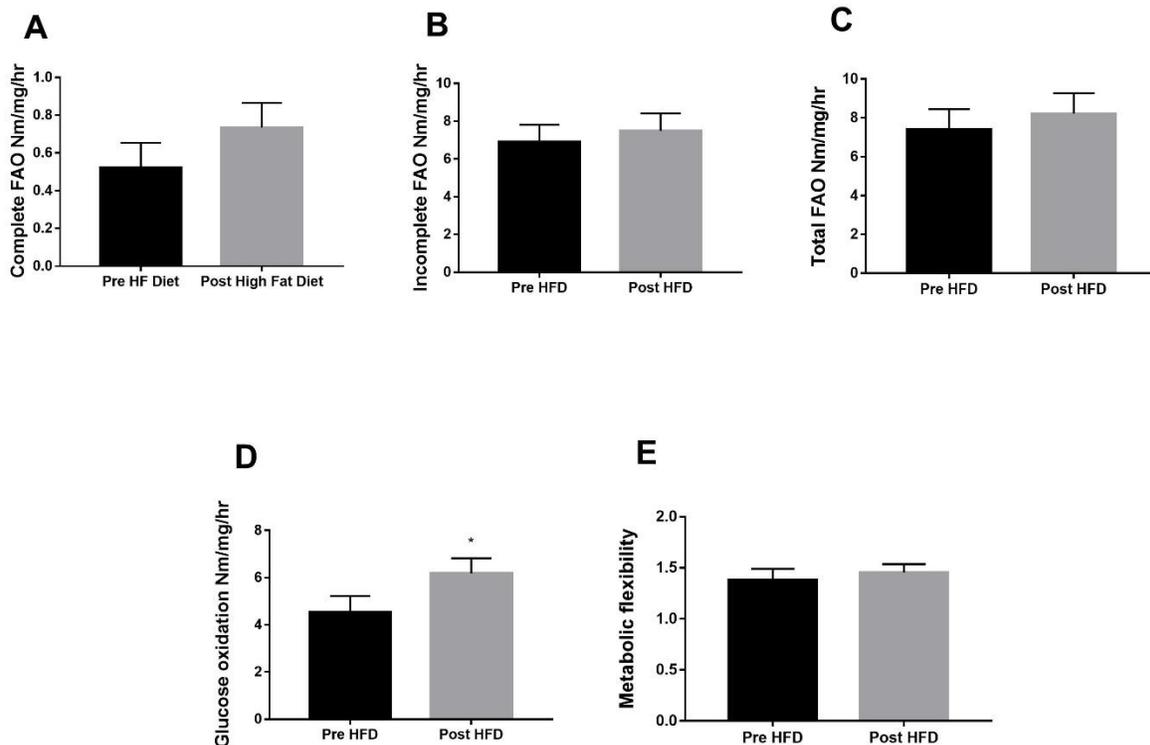
Differences between individual parameters before and after high-fat feeding were determined using paired t-tests. Pearson's correlation was performed to determine the relationships between acylcarnitines and substrate metabolism. Data will be presented as mean +/- SEM, and statistical significance was defined as $p < 0.05$.

Chapter 4: Results

Substrate metabolism

No significant changes were observed in fasting complete *Figure 7A*, incomplete FAO *Figure 7B*, Total FAO *Figure 7C* and metabolic flexibility *Figure 7E* from pre HFD to post HFD. However, glucose oxidation increased ($P=0.0295$); *Figure 7D*.

Figure 7: Changes in fasting substrate metabolism after five days of high fat feeding using a two-tailed paired t-test ($n=13$, mean \pm sem).

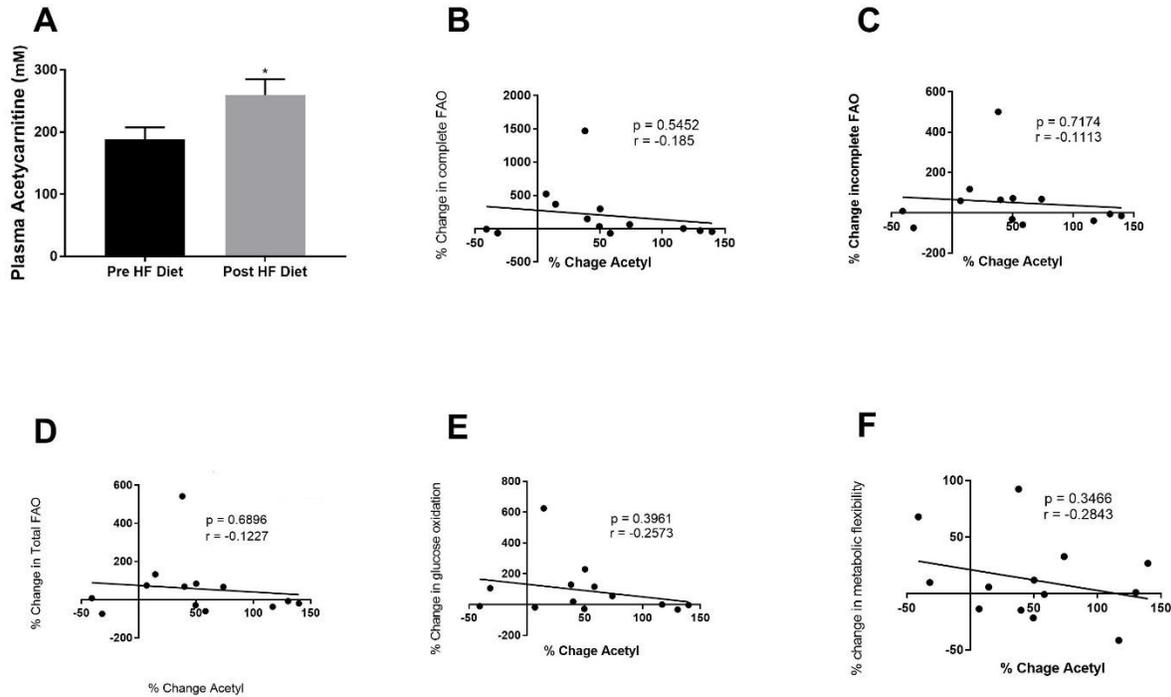


Acetylcarnitine/ Acylcarnitines

Fasting plasma acetylcarnitine increased from pre HFD to post HFD ($P=0.0125$); *Figure 8A*. We found that changes in plasma acetyl carnitine did not significantly correlate with changes in either complete fatty acid oxidation ($P=0.5452$); *Figure 8B*, incomplete fatty acid oxidation

($P=0.7174$); **Figure 8C**, total complete fatty acid oxidation ($P=0.6896$); **Figure 7D**, glucose oxidation ($P=0.3961$); **Figure 8E**, or metabolic flexibility ($P=0.3466$); **Figure 8F**.

Figure 8: Changes in Fasting plasma acetylcarnitine concentrations using a two-tailed paired t-test (mean \pm sem) and percent change in fasting plasma acetylcarnitine correlations with percent change in fasting substrate metabolism using Pearson correlation ($n=13$).



Individual acylcarnitine species are shown in **Table 2**. Decanoyl ($P < 0.0001$), Dodecanoyl ($P = 0.0049$) Tetradecanoyl ($P = 0.0004$), Hexadecanoyl ($P = 0.0009$), Octadecanoyl ($P < 0.0001$) and C14:1 ($P = 0.0446$) increased from pre HFD to post HFD were found in. There were no significant changes Propionoyl, Hexanoyl, Octanoyl, C10:1, C12:1, C16:1 and C18 with the HFD.

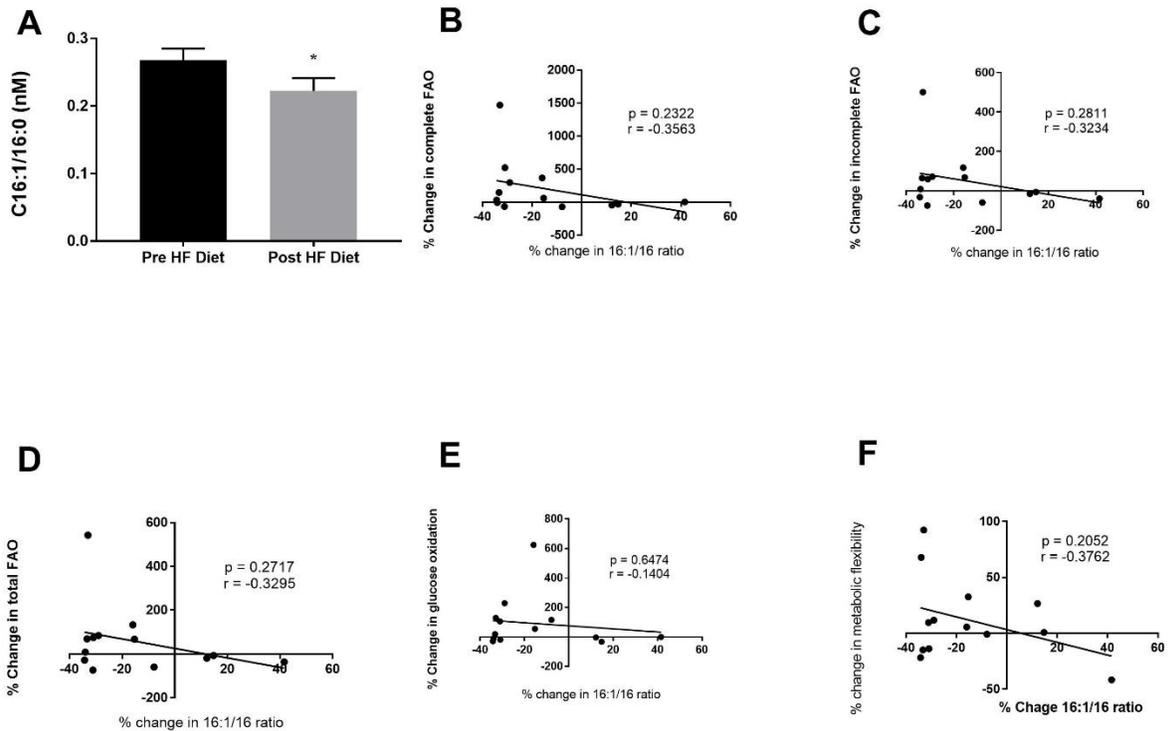
Table 2: Fasting concentrations of acylcarnitines in human blood plasma after five days of high fat feeding (n=13, *= α 0.05, **= α 0.01, ***= α 0.001, ****= α 0.0001).

Compound	Short name	Pre HFD	Mean Post HFD	P value
		Mean(\pm SD) (nM)	Mean(\pm SD) (nM)	
Acetyl	C2	188.4 (\pm 69.03)	259.7 (\pm 91.55)	0.0125*
Propionyl	C3	0.128 (\pm 0.059)	0.1461 (\pm 0.07)	0.1941
Hexanoyl	C6	0.07042 (\pm 0.0268)	0.0976 (\pm 0.0505)	0.0684
Octanoyl	C8	0.0675 (0.04302)	0.0760 (\pm 0.0245)	0.2566
Decanoyl	C10	0.0541 (\pm 0.0126)	0.1118 (\pm 0.0374)	<0.0001****
Decenoyl	C10:1	0.0821 (\pm 0.0564)	0.0904 (\pm 0.0438)	0.3850
Dodecanoyl	C12	0.0541 (\pm 0.0126)	0.0704 (\pm 0.0209)	0.0049**
Dodecenoyl	C12:1	0.0602 (\pm 0.0175)	0.0691 (\pm 0.0194)	0.1275
Tetradecanoyl	C14	0.0423 (\pm 0.0061)	0.0542 (\pm 0.0095)	0.0004***
Tetradecenoyl	C14:1	0.0370 (\pm 0.0066)	0.0415 (\pm 0.0098)	0.0446*
Hexadecanoyl	C16	0.2894 (\pm 0.0685)	0.3766 (\pm 0.0578)	0.0009***
Palmitoleyl	C16:1	0.0808 (\pm 0.030)	0.0869 (\pm 0.0278)	0.5553
Octadecanoyl	C18	0.4529 (\pm 0.1239)	0.7444 (\pm 0.1353)	<0.0001****
Oleyl	C18:1	0.8693 (\pm 0.3125)	1.062 (\pm 0.2889)	0.0647

C16:1/C16:0 and C18:1/C18:0 desaturation index

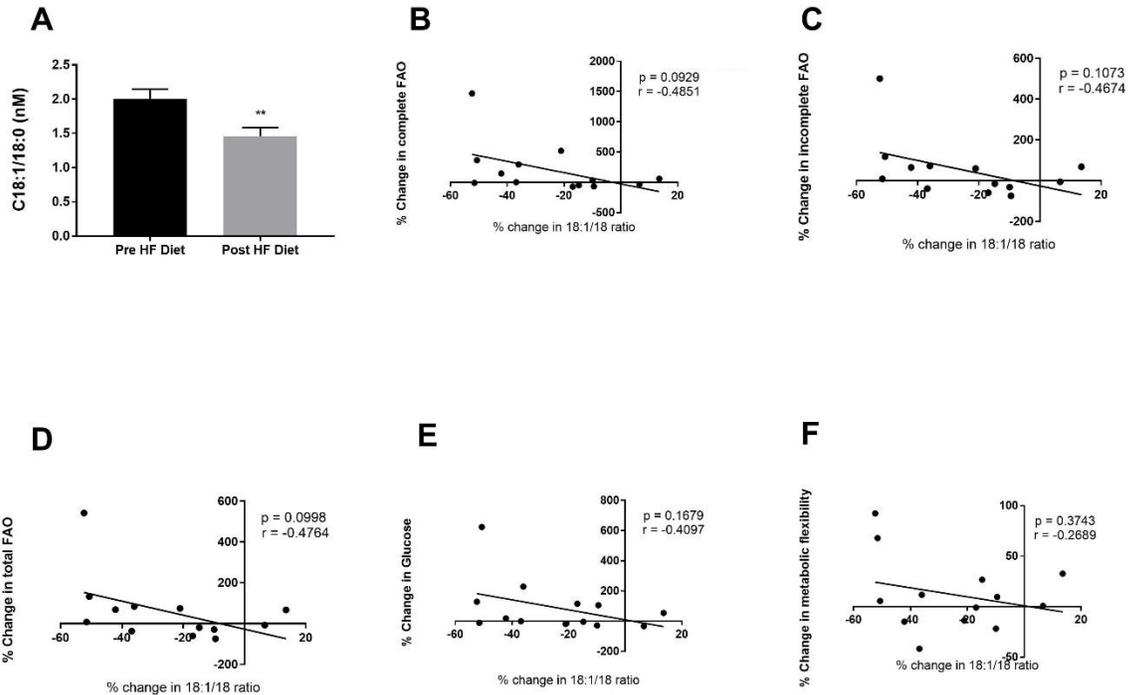
16:1/16:0 ratio decreased significantly from pre HFD to post HFD (P= 0.0397) **Figure 9A**. No significant correlations were observed between changes in 16:1/16:0 and changes in complete fatty acid oxidation **Figure 9B**, incomplete fatty acid oxidation **Figure 8C**, total fatty acid oxidation **Figure 9D**, glucose oxidation **Figure 8E**, or metabolic flexibility **Figure 9F**.

Figure 9: Changes in fasting 16:1/16:0 desaturation index using a two-tailed paired t-test (mean \pm sem) and percent change in 16:1/16:0 correlations with percent change in substrate metabolism using Pearson correlation (n=13).



C18:1/18:0 ratio decreased significantly from pre HFD to post HFD ($P=0.0012$) **Figure 10A**. No significant correlations were observed between changes in 18:1/18:0 and changes in complete fatty acid oxidation **Figure 10B**, incomplete fatty acid oxidation **Figure 10C**, total fatty acid oxidation **Figure 9D**, glucose oxidation **Figure 10E**, or metabolic flexibility **Figure 10F**.

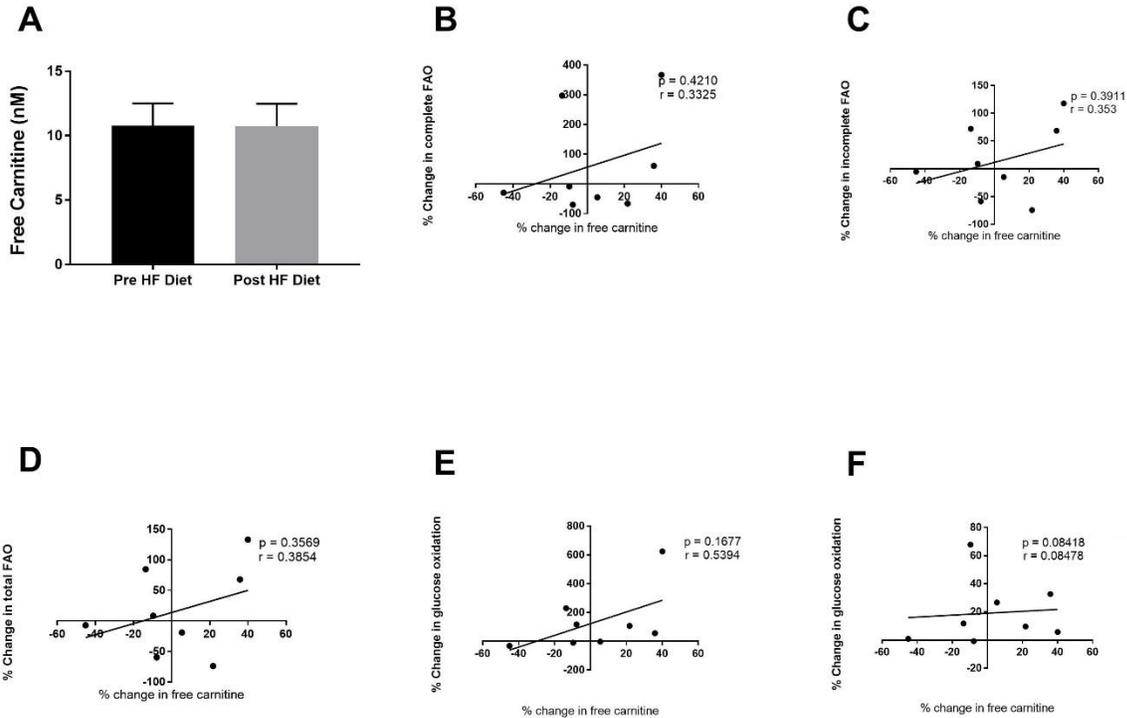
Figure 10: Changes in fasting 18:1/18:0 desaturation index using a two-tailed paired t-test (mean \pm sem) and percent change in 18:1/18:0 correlations with percent change in substrate metabolism using Pearson correlation (n=13).



Free plasma carnitine

Plasma free carnitine did not change in response to HFD *Figure 11A*. No significant correlations were observed between free plasma carnitine and complete fatty acid oxidation *Figure 11B*, incomplete fatty acid oxidation *Figure 11C*, total fatty acid oxidation *Figure 11D*, glucose oxidation *Figure 11E*, or metabolic flexibility *Figure 11F*.

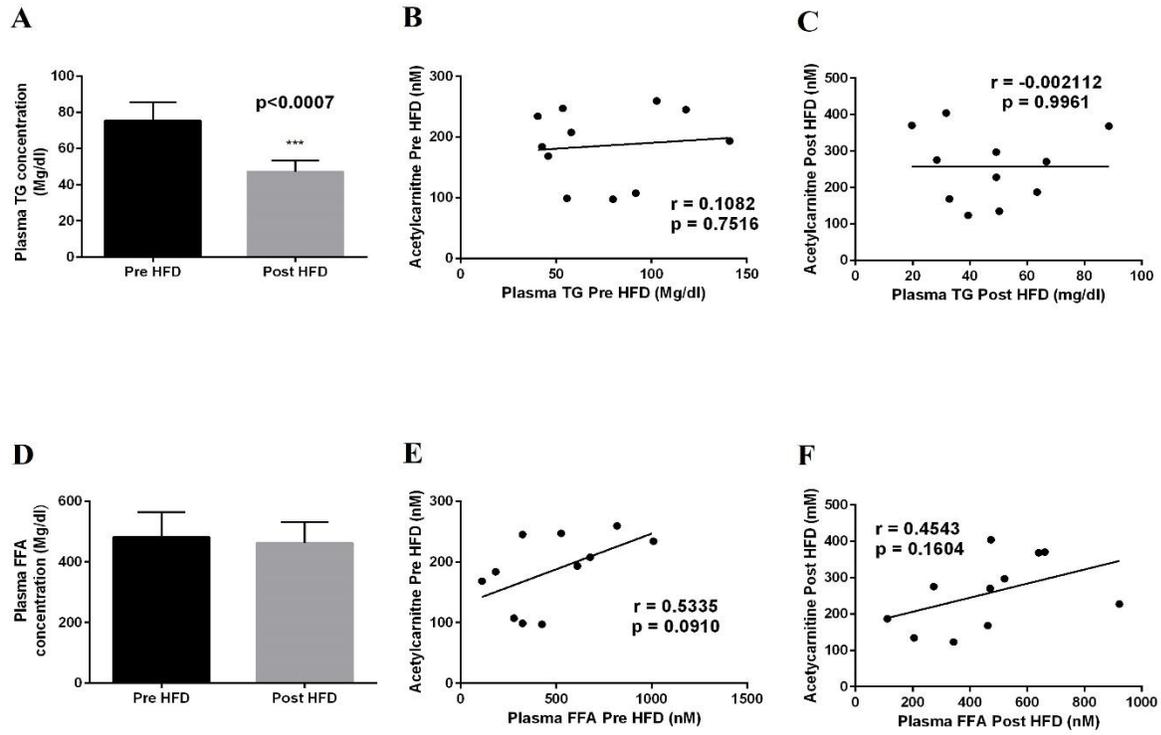
Figure 11: Changes in fasting plasma free carnitine concentrations using a two-tailed paired t-test (mean \pm sem) and percent change of plasma free carnitine correlations with percent change in substrate metabolism using Pearson correlation (n=13).



Plasma free fatty acids and triglycerides

Plasma free fatty acids (FFA) showed a decrease **Figure 12A**; ($P=0.0007$) and triglycerides (TG) did not change in response to HFD **Figure 12D**. No significant correlations were observed between plasma TG and acetylcarnitine pre HFD **Figure 11B**, or post HFD **Figure 11D**. No significant correlations were observed between plasma FFA and acetylcarnitine pre HFD **Figure 11E**, or post HFD **Figure 11F**.

Figure 12: Changes in fasting plasma TG and FFA concentrations using a two-tailed paired t-test (mean \pm sem) and plasma TG and FFA correlations with plasma acetylcarnitine pre and post HFD using Pearson correlation (n=11).



Chapter 5: Discussion

In the current study we quantified plasma acylcarnitines in clinically healthy, non-obese, sedentary males before and after providing them with a HFD for five days. To our knowledge, this was the first time fasting acylcarnitines were studied using these specific parameters. We examined a change in substrate preference by providing a lead in diet high in carbohydrates and switching to a HFD in efforts measure how flexible human metabolism can be. Our secondary goal was to determine how changes in fasting plasma acylcarnitines relate to adaptations in skeletal muscle substrate oxidation. The major new findings of the present study were an increase in plasma acetylcarnitine, a decrease in C18:1/C18 desaturation, and no changes to plasma free carnitine.

The results agree with our first hypothesis by demonstrating an increase in acetylcarnitine. This is an indication that CrAT activity was not hindered by the HFD, therefore not allowing a buildup of acetyl-CoA to occur. Acetylcarnitine is the most abundant of the acylcarnitine species as it is an end product CrAT produces from fatty acid, glucose, and amino acid catabolism⁴⁰. This explains why acetylcarnitine levels are much higher than the remaining acylcarnitine species. Furthermore, Lindeboom et al. explains that a decrease in acetyl-CoA turnover can impede PDH and limit oxidative degradation of glucose⁴¹ and these claims can be supported by Muoio et al. where CrAT knockout mice had difficulty regulating proper substrate selection resulting in increased fat mass and weight gain when fed a HFD²⁷.

In obese and diabetic rodent models, CrAT activity has been reported to be reduced leading to an accumulation of acetyl-CoA²⁶. This buildup of acetyl-CoA in turn inhibits PDH through a feedback mechanism and the mice experienced a drop off in glucose oxidation²⁶. Translating this to our human subjects, we observed an increase in glucose oxidation and acetylcarnitine production further indicating that CrAT activity was able to adapt.

SCD1 is an enzyme that has a role in *de novo* lipogenesis and is associated with metabolic diseases like leptin-resistance induced obesity, hepatic steatosis, and insulin resistance⁴². SCD1's ability to catalyze the Δ^9 -cis desaturation of C16 & C18 to produce C16:1 and C18:1, which are substrates for triacylglycerol synthesis⁴³. When measuring acylcarnitine desaturation we observed a decrease in both C16:1/C16:0 and C18:1/C18:0 desaturation which may be indicative to SCD1 activity being limited. Studies using SCD1 knockout rodent models have shown lower TAG levels in their tissues⁴³. This can be interpreted as, maintaining metabolic flexibility. Our results may not directly prove this theory to be true, however we are able to provide supporting evidence to make this claim but only through further research will we find an endpoint answer.

Regarding our third hypothesis there were no observed changes in free carnitine with HFD, nor any significant correlations with substrate metabolism. This would indicate carnitine production was able to match the demand of acylcarnitines transport and maintain the free carnitine pool. It seems likely that having an energy balanced diet didn't cause a shift in free carnitine levels. With no change in plasma free carnitine after short term high fat feeding, we are led to believe that carnitine production was able to adapt to the HFD. Whether or not the maintenance of the carnitine pool was due to endogenous synthesis or from dietary sources is not known.

Limitations

Limitations of the current study include acylcarnitine measurements were only obtained in blood plasma. When quantifying acylcarnitines in plasma there is no way of differentiating the origin of the carnitine esters. They can be derived from skeletal muscle, hepatic tissue or any

tissue that possess mitochondria. For more accurate analyses of acylcarnitine quantification, it would be ideal to obtain the sample directly from the desired tissue.

The plasma acetylcarnitine measurements obtained were indicative of CrAT's ability to maintain the acyl-CoA/ CoA pool, however direct analysis of CrAT activity would have provided additional evidence of this. At the time this study was conducted there were no viable CrAT antibodies to conduct such measurements.

The sample size for our study was small and consisted of strictly male participants. Small sample groups tend to carry less statistical power when analyzing data. By utilizing only male subjects we limited ourselves to the gender specific results as we don't know if females would display similar responses as their male counterparts.

Future Directions

Knowledge of acylcarnitine's role in human metabolism has progressed, however is still in its infancy. Studies have focused on acylcarnitines in both healthy and diseased (metabolic syndrome) states but we have had difficulty in pinpointing an accurate threshold when a transition between the two states occurs. This grey area of information could provide answers to whether acylcarnitines influence metabolic disorders or merely reflect them.

Schoonman et al. suggested that the plasma pool does not provide insight into the sources of acylcarnitines due to the origin of the acylcarnitine species emanating from various tissues⁴⁴. Therefore considerations for future studies should include measurements of acylcarnitines directly from tissues. Quantification of tissue acylcarnitines have not been extensively measured in human subjects due to how invasive nature of sample collection.

Lastly, it would be important to quantify and compare acylcarnitines in a similar design using various fatty acid compositions to gain insight into metabolic flexibility in differing diets. To further address this issue, it could be insightful to collect data regarding dietary values of each meal consumed and look for correlations with acylcarnitine species. The current literature poorly reflects information regarding specific nutrients and their impact on human acylcarnitine quantification.

Conclusion

In conclusion, our data demonstrates that a five-day HFD is associated with a significant increase in fasting acetylcarnitine levels, C16:1/16:0 and C18:1/18:0 desaturation indexes, and glucose oxidation in healthy sedentary males. We also observed a significant increase in C10, C12, C14, C16, and C18. We interpret this as a result of the increase in fat consumption via HFD diet and may also contribute to the abundance of acetylcarnitine production post diet. However, it is difficult to pinpoint the increase of these long chain acylcarnitine species directly to the HFD as we have no knowledge of the fatty acid makeup of the meals provided. This information accompanied by no observed significant changes in free carnitine, FAO measurements and metabolic flexibility provides evidence of an adaption to the change in diet composition. If this is to be true it would appear our subjects made an adjustment of fuel oxidation to the most abundant fuel source. Individual changes in flexibility did not correlate with fasting circulating acylcarnitine measurements.

Chapter 6: References

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