ABSTRACT

Title of Document: BREAST CANCER TYPE 1 SUSCEPTIBILTY PROTEIN IS A CRITICAL REGULATOR OF SKELETAL MUSCLE LIPID METABOLISM.

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This dissertation research consists of three investigations in an effort to determine how circulating estrogens affect skeletal muscle lipid metabolism. Loss of circulating estrogens results in significant increases in visceral fat mass and intramuscular lipids (IMCL). These increases in lipid storage are strongly associated with an elevated risk of developing type 2 diabetes. The first investigation examined how the loss of circulating estrogens alters skeletal muscle metabolic function. Ovariectomy (OVX) resulted in significantly higher visceral fat mass and fatty acid sarcolemmal transporter content, which corresponded with elevated IMCL. Skeletal muscle in the OVX group exhibited lower acyl carnitine species suggesting impaired lipid flux through the mitochondria. Lastly, mitochondrial oxygen consumption rates were impaired in OVX skeletal muscle fibers. The results from this study gave rise to a search to identify an estrogen-sensitive mechanism that regulated lipid transport into the mitochondria. Study two determined for the first time that the BRCA1 protein, which is encoded by an

estrogen-sensitive gene, is present and functions as an integral regulator of lipid metabolism in skeletal muscle. Specifically, BRCA1 binds to acetyl CoA carboxylase in response to acute exercise. The *in vitro* induction of decreases in *BRCA1* expression resulted in higher IMCL content, reduced mitochondrial oxygen consumption rates, and elevated reactive oxygen species production. Surprisingly, no differences in BRCA1 content were detected between males and females. In the final study, an inducible, skeletal-muscle specific, BRCA1 KO mouse was developed. Ablation of BRCA1 in skeletal muscle resulted in exercise intolerance and the development of kyphosis. Contrary to our hypothesis, loss of functional BRCA1 in skeletal muscle attenuated the negative metabolic consequences of chronic high fat diet exposure. Collectively, these data provide strong rationale that BRCA1 is an important regulator of skeletal muscle metabolic function and further provide evidence that BRCA1 function is critical in multiple tissues across the body.

BREAST CANCER TYPE 1 SUSCEPTIBILTY PROTEIN IS A CRITICAL REGULATOR OF SKELETAL MUSCLE LIPID METABOLISM.

By

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CHAPTER 1: Introduction and Specific Aims

Obesity is currently at epidemic proportions in the United States and is associated with 300,000 deaths annually (58). Women constitute a substantial portion of the current obesity statistics with 62% of the female population being overweight (BMI≥25) or obese (BMI ≥ 30) (71, 74, 125). It is well established that there are differences in body fat distribution and body fat percentages between men and women with men partitioning fats to the abdominal region (android shape) and women apportioning and storing fats in the hip and thigh region (gynoid shape) (36). The underlying scientific rational as to why fat storage differs between sexes is largely undefined. In post-menopausal women, loss of circulating ovarian hormones, predominantly estrogens, results in a transition from the gynoid to the android shape as well as an overall percent increase in body fat. These results suggest that female sex hormones are a key regulator of fat partitioning, storage and utilization (9, 64). Therefore, it is no surprise that according to the Center for Disease Control (CDC) the highest percentage of obese women are post-menopausal (48%). Further, in response to treatments for estrogen positive cancers women are susceptible to visceral obesity due to use of anti-estrogen therapies (119). Since the loss of estrogen signaling, regardless of the mechanism, collectively results in visceral obesity it is evident that estrogens function as a potent lipid metabolism and fat storage regulator.

During low to moderate exercise intensities women oxidize lipids at a higher rate than men (198, 200). However, the additional lipids utilized by women during exercise are thought to be predominately derived from

intramuscular tricylglycerides (IMTG) as opposed to enhanced adipose tissue lipolysis (198, 200). Similar to the athletes' paradox, IMTG stores are higher in women than men (13, 21). However, loss of circulating estrogens results in further accumulation of IMTG, which is associated with insulin resistance and visceral adiposity (97, 117). Exercise training decreases IMTG in ovariectomized animals (OVX) but not to the level of sedentary ovary-intact animals, again implicating estrogens as a key regulator of muscle lipid metabolism (99, 117). Despite the considerable amount of evidence implicating the regulatory role of estrogens in lipid metabolism, no definitive estrogen sensitive targets have been identified in skeletal muscle. Further, the nation-wide clinical trial termed the 'Women's Health Initiative' (WHI) suggested that estrogen therapy in post-menopausal women may have some negative health consequences (224). As a result, there is a need to identify novel estrogen sensitive mechanisms to treat the metabolic conditions associated with a loss of circulating estrogens. **Therefore, the overall initial aim of my dissertation work was to identify a key mechanism responsible for the estrogen-associated differences in skeletal muscle lipid metabolism.** The initial investigation assessed the effects of loss of circulating estrogens on skeletal muscle lipid metabolism. Next, this work determined the importance of an estrogen sensitive target, breast cancer type 1 susceptibility protein (BRCA1) in skeletal muscle lipid metabolism and characterized the associated intracellular mechanism.

<u>Specific Aim 1</u>: To determine the impact of ovariectomy on skeletal muscle lipid metabolism.

This study determined the consequences of ovariectomy on skeletal muscle lipid metabolism. We found that loss of circulating estrogens in an OVX mouse resulted in a substantial increase in visceral fat mass and a significant increase in intramuscular lipids (IMCL) which was associated with an increase in fatty acid sarcolemmal transporters CD36/FAT and $FABP_{pm}$. Using a comprehensive metabolic profiling approach we identified differences in the skeletal muscle kreb's cycle intermediates, acyl-carnitines, and amino acid content in OVX animals compared to SHAM. Specifically, the metabolic profile revealed significant decreases in acyl carnitine species suggesting that lipid flux through the mitochondria was impaired in response to ovariectomy. Lastly, we detected differences in palmitate- and pyruvate-stimulated mitochondrial oxygen consumption rates in OVX single muscle fibers compared to SHAM. In conclusion, we determined that the increase in IMCLs is, in part, due to impaired fatty acid entry and utilization by the mitochondria (97). These results gave rise to a search to identify an estrogen sensitive mechanism responsible for regulating lipid transport into the mitochondria and, thus mediating the aforementioned skeletal muscle changes. Aims 2 and 3 of this proposal were therefore, derived from the results of this study.

<u>Specific Aim 2</u>: To determine if BRCA1 is present and functions as an integral regulator of lipid oxidation in female skeletal muscle through its interaction with phosphorylated acetyl CoA carboxylase.

Breast cancer type 1 susceptibility protein (BRCA1) (BRCA1:human protein; Brca1:mouse protein) is produced from the estrogen sensitive gene, breast cancer 1 early onset (*BRCA1*) (*BRCA1*:human gene; *Brca1*:mouse gene). *BRCA1* is commonly recognized as an estrogen sensitive human tumor suppressor gene because of the corresponding proteins ability to regulate the cell cycle in mammary and other reproductive cells (142, 211). More recently, BRCA1 was identified as a regulator of lipid metabolism in MCF7 breast cancer cells through its interaction and preservation of the phosphorylated form of acetyl CoA carboxylase (ACC) (126, 148). While phosphorylated, ACC is inactive resulting in reduced production of malonyl CoA (MaCoA), an allosteric inhibitor of carnitine palmitoyl transferase-1 (CPT-1) (213). By reducing MaCoA levels, CPT-1 activity increases, facilitating entry of lipids into the mitochondria where they undergo β-oxidation (140, 160, 213). It is well established that acute exercise induces phosphorylation of ACC and a reduction in malonyl-CoA levels in skeletal muscle, thus promoting lipid oxidation in response to the exercise bout (44, 140). Collectively, the data suggest that if BRCA1 is expressed in skeletal muscle, it could be an estrogen sensitive target that regulates lipid metabolism.

The purpose of this translational study was to 1) identify if *BRCA1* was expressed in skeletal muscle and if *BRCA1* is differentially expressed between males and females 2) to determine if the BRCA1 protein interacts with the phosphorylated form of ACC in both mice and humans, and 3) to determine if this potential interaction enhanced lipid utilization by the mitochondria. In this completed study BRCA1 levels were detected in skeletal muscle and BRCA1 was

assessed across different muscle groups from the mouse at both the mRNA and protein levels. Further, BRCA1 levels were compared across male, and female mouse skeletal muscle. In addition, the BRCA1 ACC-p native protein-protein interaction was measured in response to an acute bout of treadmill exercise in mice. These findings were also translated to human skeletal muscle where mRNA expression of all known isoforms of *BRCA1* (*BRCA1* total) was collectively measured and the 2 short isoforms (*BRCA1Δ11*, *BRCA1Δ11b*) were measured independently in sedentary male and female skeletal muscle. Similar to the mouse portion of this study, we measured total BRCA1 protein levels, and the BRCA1 ACC-p protein interaction in response to an acute bout of exercise in both male and female human skeletal muscle. Surprisingly, with the exception of BRCA1Δ11 protein in mouse skeletal muscle, no sex differences were detected in mRNA or protein levels or in the BRCA1 ACC-p protein interaction in either mouse or human skeletal muscle. However, there was a significant increase in BRCA1 bound to the ACC-p after the exercise bout in both mouse and human skeletal muscle. Despite no differences in sex, the results still demonstrate a protein-protein interaction between BRCA1 and ACC-p, implicating BRCA1 as a novel and likely integral regulator of lipid metabolism in skeletal muscle.

Lastly, reductions in *BRCA1* (*BRCA1total*, *BRCA1Δ11*, and *BRCA1Δ11b*) expression human primary myotubes using an shRNA approach resulted in elevated IMCL content in control and palmitate/oleate treated myotubes. In order to determine if this effect was due to reduced mitochondrial function, mitochondrial oxygen consumption rates (OCR) were measured in control and

BRCA1 reduced human myotubes. Here we determined that in human myotubes with reduced *BRCA1* content mitochondrial OCR were decreased in the basal, uncoupled, and palmitate stimulated states compared to control. To assess if the decreased OCR rates in human myotubes with reduced *BRCA1* expression was associated with a reduction in phosphorylation of ACC, ACC-p protein levels were measured in control and *BRCA1* shRNA human myotubes. Surprisingly, a decrease in *BRCA1* expression in human myotubes resulted in increased ACC-p levels in basal *BRCA1* shRNA transduced human myotubes. Elevated ACC-p levels in *BRCA1* shRNA were found to be the result of chronic elevation of AMPK-p due to increased ROS production by the mitochondria. These data collectively indicate that a decline in *BRCA1* levels results in a reduced ability to actively induce phosphorylation of ACC, coupled with a decline in human myotube mitochondrial function. Based on our observations to date **we conclude that i) BRCA1 is constitutively expressed in skeletal muscle regardless of sex or species, ii) an acute bout of exercise increases the BRCA1 p-ACC interaction, iii) reducing *BRCA1* levels decreases ACC phosphorylation likely impairing lipid entry into the mitochondria, iv) reducing *BRCA1* levels in human myotubes impairs mitochondrial function.**

<u>Specific Aim 3</u>: To determine if Brca1 is required to maintain optimal skeletal muscle lipid metabolism during exercise using an inducible, skeletal muscle specific, *in vivo*, loss of function approach.

Following the completion of study 2 in this proposal, the next phase of this investigation was to fully characterize the importance of Brca1 in skeletal muscle through an inducible, *in vivo* loss of functional Brca1 in skeletal muscle. In study 2, an *in vitro* model was used to examine the effects of reduced *BRCA1* expression in primary cultured human myotubes. Unfortunately, this *in vitro* approach does not allow full recapitulation of the physiological importance of BRCA1 to skeletal muscle. For example, it is not possible to measure force production in a cell culture model with reduced *BRCA1* levels, or assess the responses of the cells to acute exercise. To address these limitations, an animal model lacking *Brca1* in skeletal muscle is required. Due to the critical role of Brca1 as a cell cycle regulator, it is impossible to utilize a standard, whole body, knockout approach due to embryonic lethality. Therefore, a Cre-Lox breeding system was implemented to generate a mouse model of ablated *Brca1* expression (see methods for detailed description) (48, 56). To avoid any effects on developing skeletal muscle, my animal model utilizes an inducible approach to ablate *Brca1* expression in an age-controlled fashion. The following groups were used to address all three proposed hypotheses. The groups consisted of two control-injected groups (WT) and two tamoxifen treated groups (KO). Within those groups one group was fed a high fat diet (HFD) while the other was fed a control diet (CD). This animal model was employed to specifically investigate the following hypothesis.

Hypothesis I: Loss of a functional Brca1 protein will impair exercise tolerance and increase the onset of muscle fatigue.

Exercise Tolerance and Skeletal Muscle Function

Although *Brca1* is expressed in skeletal muscle its functional role remains poorly defined. Therefore, the following experiments were designed to assess the impact of Brca1 on overall exercise performance and skeletal muscle function. Specific experiments to determine *in vivo* running capacity and *in situ* skeletal muscle contractile function were conducted by subjecting animals from each group to an acute bout of treadmill exercise as previously described (177). The primary outcome was to determine whether or not a loss of Brca1 on skeletal muscle impairs exercise performance. In order to assess the effects of loss of Brca1 in skeletal muscle function *in situ* electrical stimulation of the gastrocnemius muscle group was performed as previously described (219). Here the gastrocnemius group of one limb was electrically stimulated and force production was measured while the opposite limb served as an internal control. Peak twitch force, peak tetanic force, force-frequency curve and fatigue resistance were assessed as previously described (219). Fatigued and the control contralateral gastrocnemius muscle groups were immediately removed, weighed, and frozen.

Hypothesis II: Loss of Brca1 in skeletal muscle will result in reduced phosphorylation of Acc in response to repetitive muscle contraction.

Skeletal muscle Acc-p protein content, Brca1 protein content, Brca1 Acc-p protein interaction & Acc activity:

The importance of Brca1 in maintaining phosphorylation of Acc in response to exercise and the resulting affect on Acc activity has yet to be clearly elucidated. Therefore, the following experiments were designed to evaluate the significance of Brca1 in Acc phosphorylation and activity in response to skeletal muscle contraction. To conduct these experiments, gastrocnemius muscles removed from the animal upon completion of *in situ* stimulation and were used for the aforementioned biochemical measures. The contralateral limb served as the control. Specifically, Acc-p was assessed via western blot analysis as described in methods. Similar to investigation 2 of this proposal the native protein-protein interaction between Acc-p and Brca1 was evaluated using immunoprecipitation and western blotting methods (for details see methods section of study 2 manuscript, Appendix 2). As a surrogate to measuring Acc activity, MaCoA levels will be measured in both the contralateral and repetitively stimulated skeletal muscle from all groups as previously described (219).

Hypothesis III. Loss of Brca1 in skeletal muscle will result in lower lipid utilization by the mitochondria, higher IMCL stores and reduced skeletal muscle insulin sensitivity.

Intramuscular Lipid Accumulation:

We anticipated that if a loss of Brca1 were impairing skeletal muscle lipid utilization by the mitochondria, skeletal muscle IMCL accumulation would be elevated compared to control animals. Therefore, IMCL was measured in WT and KO animals on CD or HFD, using the neutral lipid fluorescent stain,

BODIPY as previously described (97, 198). Specifically, cross sections from the tibialis anterior (TA) muscle was used to detect skeletal muscle lipid accumulation as a result of loss of Brca1 as previously described (97).

Glucose & Insulin tolerance testing:

Due to the established relationship between skeletal muscle lipid content and the development of peripheral insulin resistance, it is possible that KO animals could have exhibited an increased susceptibility to the development of insulin resistance. To address this issue, glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed on KO and WT animals after consumption of either CD or HFD for 10 weeks as previously described (98, 218).

Isolated Mitochondrial Respiration Measures:

Lastly, to analyze the effects of loss of Brca1 on skeletal muscle mitochondrial function basal, palmitate, FCCP (uncoupled) mitochondrial oxygen consumption rates (OCR) were recorded from isolated mitochondria. Mitochondria were isolated from the quadriceps muscles using a modified version of the previously described technique (59). These measures were recorded from both the HFD and CD fed animals with and without functional Brca1 as previously described using a oxytherm (65). Collectively these data were used to detect alterations in lipid utilization and metabolism within skeletal muscle due to loss of Brca1.

CHAPTER 2:

REVIEW OF LITERATURE

Obesity has surged to epidemic proportions in the United States of America as well as in several other countries throughout the world. Current obesity statistics provided by the U.S. Center for Disease Control (CDC) indicate that approximately 35% of adults in the U.S. or 78 million individuals are obese. Further, this epidemic also affects children and adolescents with approximately 15% being obese. It is well established that obesity contributes to multiple deleterious health consequences leading to shortened life span and increases in direct and indirect health care costs (39, 157). Specifically, direct costs are due to the cost of preventative, diagnostic and treatment services (57, 221, 222). Indirect costs include costs related to morbidity and mortality as a result of being overweight or obese (57, 221, 222). In total, direct and indirect costs of obesity in the U.S. were a staggering $147 billion dollars in 2008 (57). In addition to the economic impact, obesity directly contributes to a decline in life expectancy. For the first time since the onset of recognized "modern medicine" today's children are not expected to exceed the average lifespan of their parents, and the current obesity epidemic is likely responsible (111). According to the CDC obese individuals are more likely to develop metabolic dysfunction (type 2 diabetes, metabolic syndrome), various types of cancer, and are at an increased risk for coronary heart disease, stroke, hypertension, dyslipidemia, sleep apnea, respiratory problems, osteoarthritis, mental health conditions, and infertility.

Collectively, it is no surprise that given the current obesity statistics the predicted lifespan is decreased.

The obesity epidemic is mainly attributed to a combination of lifestyle and genetic factors, however sex hormone function is also a critical consideration. It is well established that differences in body fat distribution and body fat percentages exist between men and women. Men preferentially partition fat to the abdominal region (android shape) and women partition fat in the hip and thigh region (gynoid shape) (36). However, in women, a loss of circulating ovarian hormones, specifically estrogens, results in a transition from the gynoid appearance to the android appearance as well as an overall percent increase in body fat (9, 64). Current obesity statistics indicate that 62% of women are overweight (BMI≥25) or obese (BMI ≥ 30) (58), with the highest percentage of obese women being post-menopausal (48%). Consequences of menopause include the development of the metabolic syndrome which is commonly defined by a combination of disorders including increases in visceral fat mass, coupled with dyslipidemia, elevated fasting glucose, and/or high blood pressure (32). In addition, women who undergo therapy for estrogen positive cancers are also susceptible to visceral obesity due to use of anti-estrogen therapies (119). Surgically induced oophorectomy and premature ovarian failure also results in loss of circulating ovarian hormones corresponding with metabolic consequences identical to those resulting from age induced menopause. Therefore, the loss of ovarian hormones leading to reduced estrogen function results in a significant

increase in obesity risk indicating that estrogens are key regulators of fat partitioning in women.

WHOLE BODY METABOLIC FUNCTION: THE EFFECTS OF ESTROGENS AND OBESITY.

Metabolic flexibility is impaired in obese individuals. Metabolic flexibility is determined by two specific criteria: 1) insulin can stimulate glucose uptake and suppress lipid oxidation during a fed state, and 2) during a fasted state an individual can rely on lipids as a primary source of energy (63). Thus, metabolic flexibility describes the ability of an individual to switch between carbohydrates and lipid as an energy source. Metabolic flexibility is commonly assessed using respiratory quotient (RQ), which is derived as the ratio of CO_2 expired to O_2 consumed. Lower RQ values ($\leq$0.7) indicate more reliance on lipid metabolism whereas moderate values indicate a mix of lipid and carbohydrate (0.7-1.0) and high values suggest almost exclusive contribution from carbohydrate metabolism (1.0) (94, 95). Metabolic flexibility is impaired in obese individuals due to both a decline in insulin stimulated glucose uptake and a decrease in lipid oxidation in the fasted state (40, 63). The onset of metabolic inflexibility is a preceding factor to the development of insulin resistance and ultimately type 2 diabetes (40, 63).

Estrogens influence metabolic flexibility. Estrogens have a powerful influence on carbohydrate and lipid utilization by peripheral tissue, thereby significantly affecting metabolic flexibility in women 1(28). The flexibility effect

is the result of women maintaining both greater insulin sensitivity as well as having a greater capacity to oxidize lipid compared to men (90). As a result, women exhibit a greater difference in RQ from a fasted to a fed state compared to men (28). However, metabolic flexibility in women is impaired in response to loss of circulating estrogens, thus increasing the risk for the development of type 2 diabetes and the metabolic syndrome (28). The observed decline in metabolic flexibility in response to loss of circulating estrogens is attributed to a decline in skeletal muscle enzymes associated with insulin stimulated glucose uptake as well as a decrease in skeletal muscle lipid oxidation enzymes (28). Unfortunately, the mechanisms by which estrogens influence metabolic flexibility remain poorly defined and thus there remains a critical gap in our understanding of the regulation of metabolic function in women.

CELLULAR MECHANISMS THAT REGULATE SKELETAL MUSCLE LIPID METABOLISM:

Skeletal muscle possesses the unique ability to increase metabolic rate nearly 100-fold (112). The ability to increase metabolic rate to such an extent is attributed to the unique network of mitochondrial enzymes that allows skeletal muscle to utilize lipid as a substrate. In skeletal muscle, a complex network of both identified and unidentified pathways exist, working in a symbiotic relationship that allows for lipid uptake from circulation, intramyocellular lipid oxidation, and/or intramyocellular lipid storage. Management of these substrates is largely driven by intramyocellular energetic demand and substrate supply. The

following portion of this literature review will discuss the specific mechanisms associated with lipid transport from circulation into the intracellular region of skeletal muscle. Next, this literature review will discuss the intracellular lipid transport mechanisms through the cytosol, and into the mitochondria where lipids undergo β-oxidation. Lastly, this review will discuss how the aforementioned mechanisms are altered as a consequence of obesity and the presence of estrogens (see Figure 1&2). Also, mechanisms responsible for re-esterification of lipids will be addressed.

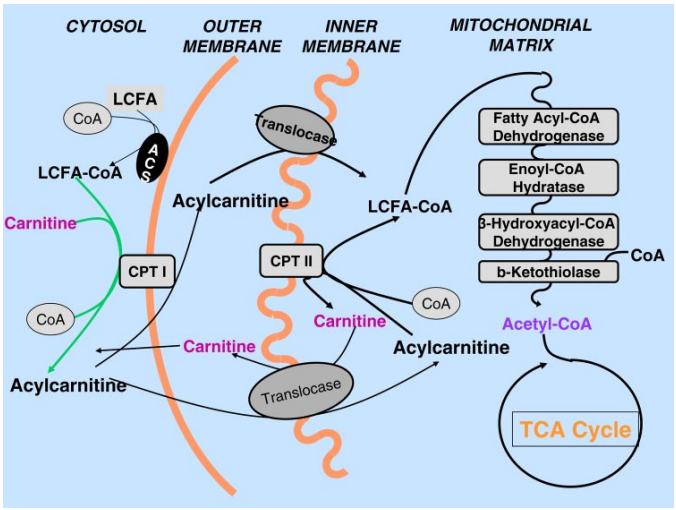

Figure 2.1. A schematic representation of the mechanisms associated with lipid transport from circulation across the sarcolemma and through the cytosol to the mitochondria. Circulating lipids and FFAs derived from the breakdown of VLDL-TG by LPL are transported across the capillary bed, interstitial space and the plasma membrane by multiple fatty acid transporters including FAT/CD36, $FABP_{PM}$, and FATP. Upon entering the cytosolic region FFA are acetylated by ACS resulting in formation of fatty acyl CoA. Fatty acyl CoAs are then transported through the cytosol while bound to ACBP and $FABP_C$ to the mitochondria (adapted from B. Kiens, Physiological Review, 2006)

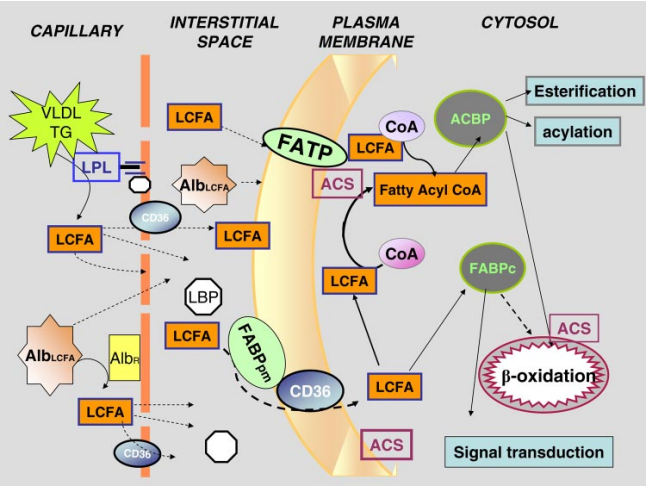

Figure 2.2. A schematic representation of the mechanisms underlying FFA transport into the mitochondria followed by β-oxidation. Initially, lipids in the form of FFA-CoA are converted to acylcarnitines by CPT-1. Acylcarnitines are they shuttled across the outer mitochondrial membrane followed by the inner mitochondrial membrane by a mitochondrial translocase. Once across the inner mitochondrial membrane acylcarnitine is converted back into FFA-CoA by CPT-1I and undergoes β-oxidation (adapted from B. Kiens, Physiological Review, 2006).

The delivery of circulating lipid to skeletal muscle occurs in the form of free fatty acids (FFA) derived from adipose tissue lipolysis or in the form of circulating triglycerides. Elevations in adipose tissue lipolysis are the result of increased β-adrenergic stimulation, which occurs as a result of exercise, fasting and/or stress. In addition to circulating lipids derived from adipose tissue stores, triglycerides are hydrolyzed from circulating lipoproteins by the enzyme lipoprotein lipase (LPL), providing additional available lipid to skeletal muscle (see figure 1). In contrast to adipose tissue hydrolysis, LPL activity is relatively steady with little to no changes associated with increased energetic demand or

stress (87, 223). However, LPL enzyme activity is impaired in response to a decrease in physical activity, such as in response to limb immobilization (76).

In obese individuals with large amounts of visceral fat, circulating lipids and VLDL-TG are chronically elevated (185). Chronic elevation of circulating lipids and VLDL-TG is the consequence of increased adipose tissue lipolysis. Although circulating VLDL-TG levels are elevated in response to obesity, LPL hydrolysis of VLDL-TG is not increased compared to lean individuals (180). However, chronically elevated circulating lipids and VLDL-TG corresponds with impaired skeletal muscle metabolic function, including a decrease in insulin sensitivity, indicating that this mechanism is a clinically important consideration in treating obesity.

Similar to obese individuals, loss of estrogens as a result of menopause, or oophorectomy corresponds with elevated visceral adipose tissue mass and an increase in circulating lipids (8, 42). Specifically, adipocytes isolated from adipose tissue of post-menopausal women exhibit enhanced lipolytic rate (43, 151). Enhanced lipolytic rate in adipose tissue has also been documented in ovariectomized rodent models (218). Previous research has demonstrated that women maintain greater levels of LPL compared to men (101). Further, loss of estrogens has been shown to increase skeletal muscle LPL content in ovariectomized rodents (43). These data collectively indicate that loss of estrogens results in elevated circulating adipose tissue derived lipids and FFA synthesized by LPL hydrolysis of VLDL-TG.

Specific protein transporters regulate transport of circulating lipids across the sarcolemma into skeletal muscle cells. Specifically, FFA must traverse the endothelium, the interstitial space, and finally the sarcolemma before entering the cytosol. Although specific mechanisms underlying transport of FFA through the endothelium and interstitial space are still under debate, three major transporters located on the sarcolemmal membrane have been identified. Fatty acid translocase (FAT/CD36), the plasma membrane bound form of fatty acid binding protein ($FABP_{PM}$) and fatty acid transport protein (FATP) are the critical regulators of FFA entry into skeletal muscle (17, 25, 26) (see Figure 1). Each of these aforementioned proteins function as integral components in FFA transport, however, the mechanism by which FFA transport occurs varies amongst each protein. In addition to the aforementioned protein transporters, recent evidence suggests that caveolins, a subclass of lipid rafts, are involved in skeletal muscle FFA uptake potentially through an interaction with FAT/CD36 (105). However, no specific mechanism has been elucidated in skeletal muscle.

FAT/CD36 is an integral plasma membrane protein with two predicted transmembrane domains that function to transport FFA from the interstitial space into the cytosol (4) (see Figure 1). The presence of FAT/CD36 has been confirmed in both rodent and human skeletal muscle (26, 91, 166). Further, both over expression models and FAT/CD36 null animals models validate the importance of FAT/CD36 (55). Over expression of FAT/CD36 resulted in reductions in circulating lipids and a corresponding elevation in FFA oxidation in the soleus muscle (91). In contrast, loss of FAT/CD36 expression resulted in

increased circulating lipids as well as reduced fasting blood glucose levels indicating impaired lipid oxidation and greater reliance on glucose as a primary source of energy (55). Collectively, these data implicate FAT/CD36 as an integral component required for normal FFA transport and metabolism in skeletal muscle.

$FABP_{PM}$ is located on the periphery of the plasma membrane and similar to FAT/CD36 also plays an integral role in FFA transport across the plasma membrane (see Figure 1). The function of $FABP_{PM}$ as a FFA transporter in skeletal muscle was made apparent when antibody treatment against $FABP_{PM}$ resulted in impaired skeletal muscle FFA entry in a dose dependent fashion (176, 186, 196, 209). Further, over expression of $FABP_{PM}$ in rat skeletal muscle resulted in elevated fatty acid transport in resting muscle (18). Although it is apparent that $FABP_{PM}$ plays an integral role in skeletal muscle FFA uptake and metabolism, characterizing the exact mechanism with regards to FFA transport across the plasma membrane and into the cytosol is problematic as $FABP_{PM}$ is identical to the mitochondrial isoform of the transporter (20, 197).

The final, commonly recognized skeletal muscle FFA plasma membrane protein transporter, FATP1, is an integral membrane protein with six predicted transmembrane domains to mobilize circulating FFA across the sarcolemma (see Figure 1) (174). Evidence for the importance of FATP1 in FFA transport is largely derived from FATP1 knockout mouse models where loss of FATP1 in skeletal muscle resulted in decreased intramuscular triglycerides (IMTGs) and

diacylglyclerides (DAGs) storage in response to a high fat diet (103). However, the mechanisms of action of FATP1 in skeletal muscle remain largely understudied.

Elevated circulating lipids, as a consequence of obesity, results in increased levels of fatty acid plasma membrane transporters. Multiple studies have shown that humans consuming a high fat diet have higher circulating lipids, which corresponds with an increase in FAT/CD36, and $FABP_{PM}$ levels (26, 166). In rodents, exposure to a high fat diet results in elevated levels of all three of the aforementioned skeletal muscle plasma membrane transporters (101, 192). Furthermore, in obese men and women with type 2 diabetes, FAT/CD36 and $FABP_{PM}$ levels are greater compared to age matched, lean control subjects (18, 100). These data collectively demonstrate that elevated circulating FFA, due to high fat diet or in obese individuals, results in increased skeletal muscle FFA plasma membrane transporters to promote clearance of lipids from circulation.

Women express higher levels of plasma membrane FFA transporters than age-matched men (33, 34). Specifically, women maintain greater basal levels of FAT/CD36 protein and gene expression suggesting that women also maintain a greater basal capacity to uptake FFA from circulation compared to men (101, 192). In contrast, exercise training increases $FABP_{PM}$ content in men but not in women (101). The training induced increase in $FABP_{PM}$ is the consequence of adaptations in men that improve lipid transport capacity to the same extent as is observed in females (200). However, ovariectomy is associated

with increases in CD36/FAT and $FABP_{PM}$ protein content in female mice suggesting that ability of estrogen to regulate sarcolemmal FFA transport is complex and requires further research.

Carnitine palmitoyl transferase I (CPT-1) is a critical regulator of FFA metabolism in skeletal muscle. Transport of FFA into the mitochondria is an intricate process that requires FFA to cross two mitochondrial membranes (see Figure 2). Specifically, in order to enter the mitochondria, cytosolic FFA-CoA obtains a carnitine group via outer mitochondrial membrane (OMM) enzyme CPT-1, which then allows FFA-carnitine to cross the OMM and subsequently be transferred into the mitochondrial matrix (see Figure 2) (220). Carnitine availability is critical with limited amounts of free cytosolic carnitine reported to impair lipid oxidation (102). Transport of acyl-carnitine across the inner mitochondrial membrane (IMM) occurs via an IMM translocase (see Figure 2). Upon traversing the IMM acyl-carnitine is converted back to FFA-CoA by carnitine palmitoyl transferase-II (CPT-1I) and undergoes β-oxidation (220). Mitochondrial lipid transport and oxidation are primarily regulated by energetic demands. However, various forms of feedback inhibition including lack of free cytosolic carnitine or accumulation of mitochondrial β-oxidation or TCA cycle intermediates regulate this process as well.

In response to increased energetic demand in skeletal muscle CPT-1 mediated FFA mitochondrial transport is increased. In skeletal muscle the most potent driver of energetic demand is contraction. Therefore, the onset of

moderate or low intensity exercise increases FFA mitochondrial transport to accommodate elevated energetic demands (124). CPT-1 activity is critical to increasing FFA entry and subsequently oxidation of FFA by the mitochondria. In response to exercise, the inhibitory components regulating basal CPT-1 activity are reduced, resulting in elevated CPT-1 activity and subsequently, increased β-oxidation of FFAs (160).

The increase in CPT-1 activity is largely a response to the enhanced utilization of ATP in response to muscle contraction. Elevations in the intracellular AMP:ATP ratio triggering the activation of AMP activated protein kinase (AMPK) (31). Activation of AMPK stimulates lipid entry into the mitochondria through phosphorylation and thus inhibition of its substrate Acetyl CoA carboxylase (ACC). ACC when active, converts intracellular acetyl CoA to malonyl CoA (MaCoA) (160, 213). The production of MaCoA both inhibits FFA entry into the mitochondria through allosteric inhibition of CPT-1 and serves as a substrate in combination with acetyl CoA for lipogenesis in skeletal muscle via fatty acid synthase (FAS) (44, 169, 183). However, in response to AMPK activation, ACC phosphorylation results in deactivation of ACC and a subsequent decrease in intracellular MaCoA and increased FFA transport into the mitochondria (160, 213).

Obesity impairs skeletal muscle CPT-1 activity and increases ACC activity, subsequently decreasing FFA entry into the mitochondria. As an individual's BMI increases there is a corresponding decline in lipid oxidation in

skeletal muscle (104). Entry of FFA into the mitochondria has previously been shown to decrease in response to obesity. Specifically, skeletal muscle transport of FFA into the mitochondria via CPT-1 is impaired in obese compared to lean individuals (104). Further, other studies have demonstrated that skeletal muscle ACC activity and MaCoA content is elevated in obese individuals (11). A potential consequence of reduced FFA entry into the mitochondria is re-esterification of FFAs and subsequently accumulation of IMTG, which is a well-established corollary of obesity. Further, loss of ACC function in rodent skeletal muscle results in protection against high fat diet induced obesity and diabetes indicating the importance of FFA transport into the mitochondria (3).

In humans, it is well established that estrogens enhance mitochondrial FFA oxidation (27, 49, 127, 128, 171, 198, 200, 201). One mechanism by which estrogens enhance mitochondrial FFA oxidation is through increased FFA entry into the mitochondria through increased CPT-1 content and activity. Specifically, estrogens increases CPT-1 mRNA expression (61), while a decrease in circulating estrogens impairs CPT-1 activity (27). The decrease in CPT-1 activity found with a loss of circulating estrogens is associated with elevated intramuscular lipid accumulation, and evidence for decreased flux through β-oxidation (97). In conclusion, loss of circulating estrogens inhibits FFA oxidation through impaired FFA transport into the mitochondria via CPT-1 activity.

Mitochondrial transport of FFA results in increased β-oxidation. β-oxidation of FFA-CoA is a four-step process resulting in the formation of a two-carbon

acetyl CoA, which is utilized by the TCA cycle. The five critical enzymes involved in the β-oxidation are: acyl-CoA dehydrogenase, enoyl-CoA hydratase, β-Hydroxyacyl-Coa Dehydrogenase (β-HAD), and thiolase (see Figure 2). In response to elevated energetic demand, activity of the enzymes involved in β-oxidation is increased (199). Further, exercise training stimulates mitochondrial biogenesis, including increases in β-oxidation enzymes and subsequently enhanced FFA oxidation (84, 122, 171).

Multiple studies have demonstrated that obesity often corresponds with reduced capacity for skeletal muscle FFA mitochondrial oxidation (104). Previous research has demonstrated that a correlation exists between elevated adiposity and a decrease in fatty acid oxidation (104). Although several mechanisms are likely responsible for the decline in mitochondrial FFA oxidation, it is clear that it is partially attributed to a decrease in β-oxidation capacity and mitochondrial enzyme activity (104, 180). Specifically, impaired β-HAD and citrate synthase activity is associated with the onset of obesity (104). The decline in β-oxidation capacity and mitochondrial enzyme activity indicates that while obesity negatively impacts skeletal muscle oxidative capacity it may not be due to decreased β-oxidation enzyme content but instead a decline in function. A decrease in β-oxidation function can lead to incomplete oxidation of fatty acids, a primary contributor mitochondrial stress, and the development of insulin resistance (107).

Loss of estrogens decreases CPT-1 content and function. Previous research has reported that loss of circulating estrogens results in a decline in CPT-1 function (27). Other studies demonstrate that estradiol supplementation increases CPT-1 content in female rodents (29). These findings suggest that decreased mitochondrial transport of FFA associated with loss of circulating estrogens may be responsible for reduced lipid utilization. However, the underlying ability of estrogen to regulate FFA transport into the mitochondria is yet to be fully elucidated.

Estrogens enhance mitochondrial β-oxidation enzymes and activity in skeletal muscle. As previously discussed, women inherently maintain greater skeletal muscle oxidative capacity compared to men (198). Although the greater oxidative capacity observed in women is due to a variety of factors, mitochondrial FFA transport and β-oxidation capacity are likely key contributors to this effect. Previous research has demonstrated that increased estrogen exposure increases β-oxidation enzyme content in skeletal muscle (127, 201). Further, genetic ablation of the estrogen receptor α (ERα) results in impaired mitochondrial lipid oxidation (163). It is however, unclear if the decline in mitochondrial FFA oxidation associated with ablation of ERα is due to impairments in mitochondrial FFA transport or in the breakdown of FFA through β-oxidation. Further, the same study demonstrated that loss of estrogens decreased β-HAD activity by 20% compared to control rodents which corresponded with a significant decline in lipid oxidation (27). Other studies in post menopausal women and ovariectomized mouse models have demonstrated that loss of estrogenic effects

results in impaired mitochondrial function leading to decreases in FFA oxidation in skeletal muscle (1, 97). In women, estrogens directly impact β-oxidation enzyme content suggesting that elevated ability to oxidize FFA is partly due to influences of estrogens on mitochondrial enzyme content. In contrast, decreases in estrogens are associated with elevations in IMTG, and an overall increase in fat mass in post-menopausal women is, in part the result of impaired FFA oxidation (1, 43, 97). These data collectively demonstrate that estrogens function as mitochondrial β-oxidation agonists and that loss of estrogens impaired β-oxidation of FFA due to decreased mitochondrial enzyme content and activity.

Acetyl CoA, derived from β-oxidation and glycolysis, is shuttled through the tricarboxylic acid cycle (TCA cycle) to generate electron donors in the form of NADH and $FADH_2$. The TCA cycle is comprised of a series of chemical reactions generating energy through the oxidation of acetate (203). Specifically, the TCA cycle generates three NADH and one $FADH_2$ molecule for every cycle. Only a single GTP molecule is generated per TCA cycle. It is important to note that the significance of the TCA cycle is not to directly synthesize ATP but instead to produce large amounts of NADH and $FADH_2$, which are utilized by the electron transport chain (ETC) to synthesize ATP.

The electron transport chain utilizes electron donors in the electron transport chain to generate ATP through energy harnessed from the proton gradient. Specifically, NADH and $FADH_2$ serve as electron donors in the ETC where electrons are transferred across the IMM. The last complex in the ETC,

complex V, harnesses the energy derived from protons moving across the IMM to phosphorylate an ADP molecule in a process known as oxidative phosphorylation. Collectively, the ETC generates molecules of H_2O and ATP throughout the oxidative phosphorylation process.

Skeletal muscle electron transport chain activity is decreased as a consequence of obesity. Obesity impairs mitochondrial function, resulting in decreases in skeletal muscle citrate synthase, NADH oxidase, and succinate oxidase activity (139). The effects of obesity are even more apparent when comparing lean and obese individuals. Total activity of citrate synthase, NADH oxidase, NADH oxidase/citrate synthase and NADH oxidase/β-HAD ratios are reduced two to three fold in skeletal muscle biopsies obtained from obese individuals (38, 164). Impairments in these components of the mitochondrial function directly correspond with elevations in IMTG observed with elevated BMI (79, 104). Collectively, these marked decreases in activity levels of mitochondrial TCA cycle and ETC components correspond with a decline in skeletal muscle palmitate oxidation rates in obese compared to lean individuals (88, 104).

Loss of circulating estrogens decreases mitochondrial enzyme content and activity. Loss of estrogens in rodents results in decreased citrate synthase activity in skeletal muscle (12, 27). Previous research has demonstrated that loss of circulating estrogens in rodent models results in increased citrate and succinate content suggesting reduced flux through the TCA cycle (97). Further, the loss of

circulating estrogens also corresponded with a decrease in skeletal muscle free carnitine levels and acetyl-carnitine species, indicative of a decrease in FFA flux through the mitochondria (97). Collectively, the alterations in skeletal muscle phenotype associated with loss of circulating estrogens in the aforementioned study indicated that the decline in skeletal muscle oxidative capacity associated with loss of circulating estrogens is the result of mitochondrial FFA transport as opposed to mitochondrial oxidative capacity (97).

A loss of circulating estrogens impairs skeletal muscle mitochondrial function and potentially contributes to development of obesity and associated diseases. However, since the effects of obesity can result in a decline in activity of TCA cycle and ETC complex function it is difficult to determine whether the effects of loss of estrogens are primary or secondary effect to the associated alterations in mitochondrial function. The interpretation is further complicated by that fact that no true estrogen sensitive mechanism has been implicitly identified in the mitochondria of skeletal muscle.

CONSEQUENCES OF IMPAIRED MITOCHONDRIAL FFA OXIDATION IN SKELETAL MUSCLE

Chronic delivery of excessive FFA to skeletal muscle in obese individuals results in FFA being shuttled towards esterification rather than mitochondrial oxidation. Under normal circumstances skeletal muscle in lean healthy individuals maintains some degree of IMTG content. Upon entering the intracellular region the fate of FFAs is principally oxidation by the mitochondria

or esterification resulting in the generation of IMTGs. Oxidation of FFAs by the mitochondria is dependent upon energetic demand, mitochondrial content and the oxidative capacity of the mitochondria (62, 89, 210). Although commonly associated with metabolic impairment in obese individuals, IMTGs are imperative for metabolic function in skeletal muscle and serve as a critical source of energy in response to elevated energetic demand (88, 200).

Esterification of FFA is a multi step process attaching three single FFA to a glycerol backbone. Specifically, FFA-CoA is converted to lysophosphatidate by glycerol-3-phosphate acyl transferase (GPAT) which is the first committed step towards the formation of a triglyceride as FFA is added to a glycerol backbone (30). Lysophosphatidiate acyltransferase (LPAAT) then converts lysophosatidate to phosphatidate with the addition of another cytosolic FFA-CoA (118). Phosphatidate is then converted to a DAG by phosphatidate phosphohydrolase (PPH-1) and lastly, the final FFA-CoA is added to the DAG by diacylglycerol acyltransferase (DGAT) to form a single triacylglycerol (33). These IMTGs are stored intracellularly until substrate demand is elevated in response to an event such as prolonged endurance exercise. Accumulation of IMTG is observed in obese individuals where FFA delivery greatly exceeds energetic demand and mitochondrial capacity to oxidize lipids (88). In sedentary obese individuals, accumulation of IMTG often correlates with the onset of insulin resistance in skeletal muscle suggesting that excess IMTG storage can have negative consequences. However, endurance-trained athletes often exhibit elevated IMTG content coupled with normal insulin sensitive but an enhanced ability to oxidize

lipid. A prevailing thought is that the IMTG are an immediate FFA source for the mitochondria in response to the energetic demand of exercise and thus any stored IMTG has a high rate of turnover.

Elevated IMTG in obese individuals correlates with increased lipid intermediate species that are implicated as lipotoxic contributing to the negative association between elevated IMTG and insulin resistance (IR). Lipotoxicity is the result of excess FFAs being shuttled towards non-oxidative pathways resulting in the synthesis of reactive lipid species that promote "metabolically relevant cellular dysfunction" (110). Thus, chronic delivery of excessive amounts of lipid into skeletal muscle coupled with lower rates of fatty acid oxidation by the muscle potentially contributes to metabolic disease (100, 179). Lipotoxicity is typically defined as the accumulation of lipid intermediates such as DAG and ceramides (5, 53, 159). Specifically, Shulman et al. found that elevated skeletal muscle DAG resulted in activation of protein kinase C (PKC) θ subsequently inducing phosphorylation of serine residues on insulin receptor substrate 1 (IRS-1) and inhibiting stimulation of the insulin signaling pathway (108). Further, ceramides are also thought to inhibit insulin stimulated glucose uptake via inhibition of IRS-1 and Akt activity (5, 158, 159). However, not all findings support lipotoxicity as a mediator of insulin resistance in skeletal muscle.

Recent evidence suggests that elevated DAG and ceramides may not be directly responsible for impaired insulin resistance in skeletal muscle (207). For example, pharmaceutically induced, acute DAG elevations in rodent skeletal

muscle did not impair whole body glucose tolerance (207). Further, loss of malonyl CoA decarboxylase (MCD) which results in reduced mitochondrial fatty acid transport protects rodents from high fat diet induced glucose intolerance (107). Currently, the underlying mechanisms associated with lipotoxicity and insulin resistance in skeletal muscle are controversial suggesting more research is required.

Greater IMTG content in non-obese endurance athletes does not impair insulin stimulated glucose uptake. Although elevated IMTG is commonly associated with impaired metabolic function in skeletal muscle, a well-established exception is the elevated IMTGs observed in non-obese endurance trained athletes. The 'athletes paradox' is characterized by the inverse association between elevated IMTG and improved insulin sensitivity (14, 45, 46, 67). The underlying rational accounting for the difference in response to elevated IMTG in healthy athletes versus obese individuals is the difference in skeletal muscle oxidative capacity (7). Under circumstances where lipid is relied upon frequently as a source of energy, such as during endurance exercise, intracellular mechanisms have adapted to store large amounts of lipid and metabolize lipids efficiently (89).

Thus, a key difference between sedentary obese individuals and athletes is the frequent exposure to acute bouts of high energetic demand in the muscle. In sedentary obese individuals lipid overload results in enhanced IMTG storage and inefficient mitochondrial oxidation of FFA, while athletes store IMTG while maintaining effective FFA/IMTG turnover. Collectively, elevations in IMTG

content are not inherently debilitating to skeletal muscle metabolic function assuming complete oxidative function is maintained preventing mitochondrial overload and/or the generation of lipotoxic species that impair metabolic function (107).

Ovarian hormones are associated with greater IMTG content in healthy women without impaired insulin stimulated glucose uptake. In addition to non-obese endurance trained athletes, non-obese women present with greater IMTG content compared to age and fitness level matched men, yet maintain better insulin sensitivity (83, 90). Greater insulin sensitivity in women was also noted in response to lipid infusion, a known, relatively immediate inhibitor of insulin stimulated glucose uptake in skeletal muscle (90). In healthy women, elevated IMTG content that does not impair insulin function in skeletal muscle (83). However, IMTG accumulation as a consequence of obesity does impair skeletal muscle metabolic function in women. In contrast, estrogens clearly enhance oxidative capacity of skeletal muscle through a variety of mechanisms (127, 128, 198, 200, 201). Given the role of estrogens as a promoter of mitochondrial oxidative capacity in skeletal muscle, a loss of circulating estrogens blunts these estrogenic associated effects. As a result, loss of circulating estrogens results in elevated IMTG content corresponding with alterations in mitochondrial function and reduced insulin sensitivity in rodents as a consequence of lipotoxicity (97). Specifically, in ovariectomized mice insulin stimulated Akt phosphorylation is decreased compared to control mice and corresponds with a decrease in skeletal muscle glucose uptake (217). Further, previous research has indicated that this

effect may be the consequence of impaired mitochondrial FFA transport and/or function (98). It is however critical to mention that the mechanisms underlying the aforementioned estrogen regulated mechanisms are not well defined.

Mitochondrial overload may be responsible for impaired mitochondrial function in obese individuals. Recent evidence has demonstrated that chronic fatty acid exposure to the mitochondria results in deleterious metabolic consequences due to elevations in complete oxidation of fatty acids. Incomplete oxidation of fatty acids can lead to the overproduction of reactive oxygen species (ROS) production which is associated with accumulation of long acylcarnitine species (107, 116). Mitochondrial overload is a consequence of high FFA transport into the mitochondria coupled with a low energetic demand. This overload concept has given rise to a novel yet compelling argument discussing the advantages or disadvantages of promoting or inhibiting lipid entry into the mitochondria. Specifically, over expression of CPT-1 in skeletal muscle increases FFA oxidation and preventing insulin resistance in rodents (22), while loss of CPT-1 is associated with exercise intolerance in humans and a decreased ability to utilize lipids. Further, loss of ACC in rodents results in resistance to diet induced obesity due to increased CPT-1 activity (3). However, supplementation with carnitine, a limiting substrate for CPT-1 during times of high FFA flux, improves β-oxidation and improves insulin sensitivity (5, 7, 107, 158, 159). In contrast, more recent evidence suggests that inhibition of CPT-1, with the antagonist, etomoxir resulted in elevated IMTG and DAG content without effecting insulin sensitivity in human myotubes (207). Thus, when examining the evidence it is

clear that modulating CPT-1 activity can prevent diet induced insulin resistance and potentially obesity. Unfortunately, it is unclear why increasing or decreasing CPT-1 function can have both beneficial and negative effects on skeletal muscle insulin resistance. Additional research is required to better define the mechanisms that regulate FFA entry into the mitochondria of skeletal muscle.

CONCLUSIONS:

In conclusion, it is evident that lipid metabolism in skeletal muscle is a multifactorial process beginning with FFA transport across the plasma membrane and continuing through mitochondrial transport and oxidation or storage as IMTG. The intracellular lipid transport process is continuously in flux with changes in energetic demand or substrate availability altering intracellular lipid-handling dynamics. Given the current obesity status of the human population as well at the current trajectory of this disease, it is likely that elucidating novel mechanisms involved in skeletal muscle lipid metabolism will be critical to improving the human metabolic disease outlook.

Identifying estrogen-regulated mechanisms that modulate metabolic function is critical to for improving women's health. Specifically, defining mechanisms underlying mitochondrial FFA oxidation are essential to preventing metabolic based diseases. As previously discussed, a debate as to whether enhancing lipid transport into the mitochondria improves skeletal muscle metabolic function or impairs metabolic function is currently ongoing. Since pre-menopausal women are generally protected from developing metabolic disease, it

is hypothesized that elucidating the role of estrogens in improved metabolic health may provide novel insight into pathways and targets that could be used to treat the metabolic consequences of obesity. Further, due to reduction in the use of estrogen therapy in women as a consequence of the controversial results of the WHI it is critical to define mechanisms regulating lipid metabolism in women to attenuate and/or prevent the development of metabolic disease in post-menopausal women.

Chapter 3: Ectopic lipid deposition and the metabolic profile of skeletal muscle in ovariectomized mice.

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Ectopic lipid deposition and the metabolic profile of skeletal muscle in ovariectomized mice

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Abstract

Disruptions of ovarian function in women are associated with increased risk of metabolic disease due to dysregulation of peripheral glucose homeostasis in skeletal muscle. Our previous evidence suggests that alterations in skeletal muscle lipid metabolism coupled with altered mitochondrial function may also develop. The objective of this study was to use an integrative metabolic approach to identify potential areas of dysfunction that develop in skeletal muscle from ovariectomized (OVX) female mice compared to age-matched ovary intact adult female mice (SHAM). The OVX mice exhibited significant increases in body weight, visceral and inguinal fat mass compared to SHAM mice. OVX mice also had significant increases in skeletal muscle intramyocellular lipids (IMCL) compared to the SHAM animals, which corresponded to significant increases in the protein content of the fatty acid transporters CD36/FAT and FABPpm. A targeted metabolic profiling approach identified significantly lower levels of specific acyl carnitine species and various amino acids in skeletal muscle from OVX mice compared to the SHAM animals, suggesting a potential dysfunction in lipid and amino acid metabolism, respectively. Basal and maximal mitochondrial oxygen consumption rates were significantly impaired in skeletal muscle fibers from OVX mice compared to SHAM animals. Collectively, these data indicate that loss of ovarian function results in increased IMCL storage that is coupled

with alterations in mitochondrial function and changes in the skeletal muscle metabolic profile.

Introduction

It is well established that loss of ovarian function in women is associated with an increase in fat mass, primarily in the region of the visceral organs, without large changes in overall weight gain (71, 74, 125). This effect extends to female animal models of reduced ovarian function (69, 98, 163). Unlike diet-induced obesity, the accumulation of visceral fat mass after loss of ovarian function is not entirely due to alterations in activity level or eating behaviors, and can occur independent of age (74). The accumulation of visceral fat mass is strongly associated with multiple deleterious metabolic conditions, including peripheral insulin resistance, cardiovascular disease, and the metabolic syndrome in various models of menopause (32, 68, 156, 170).

We previously demonstrated that a decrease in circulating estrogens in ovariectomized female mice (OVX) results in an increased basal lipolytic rate of visceral fat, and subsequent increases in circulating non-esterified free fatty acids (NEFAs) (218). Under these conditions, increases in circulating NEFAs provoke an increase in demand for clearance of these moieties from circulation (15, 18, 71, 74, 125). Skeletal muscle has the capacity to oxidize or store substantial amounts of NEFA and therefore serves as a critical disposal site for circulating NEFAs. In skeletal muscle, NEFAs that do not undergo oxidization are stored as triglyceride creating a pool of intramyocellular lipid (IMCL), thus the fate of the NEFA is

largely dependent upon skeletal muscle energetic demands (69, 86, 98, 149, 163). Although IMCLs do not typically impair skeletal muscle metabolic function, excessive accumulation of IMCLs is associated with increased insulin resistance of skeletal muscle, due to increases in lipid intermediates and subsequent activation of specific inflammatory or stress signaling complexes (73, 74). Our data suggest that under conditions of reduced estrogen function, skeletal muscle would be challenged by excess circulating NEFAs, which would potentially affect vital metabolic processes within the muscle cell.

In women, circulating estrogens appear to play a critical role in defining the capacity to utilize lipids as a source of energy. For example, a number of publications have found that pre-menopausal women oxidize more lipid than men in response to an acute bout of exercise (32, 68, 156, 170, 201). Further, genetic ablation of the alpha form of the estrogen receptor (ERKO) results in a significant reduction in the ability of mitochondria to oxidize lipid (163, 218). These data support the theory that repetitive, but cyclic, estrogen exposure in women induces a metabolic profile that encourages lipid oxidation. Although largely untested, under conditions of reduced ovarian function, it would be predicted that skeletal muscle has increased IMCL content. Indeed, some investigations have suggested that reduced estrogen function leads to enhanced triacylglycerol (TAG) storage within whole muscle lysates (117, 163). However, it is unclear if the increased TAG is due to increases in extramuscular or intramuscular TAG (i.e. IMCL) storage.

The goal of this study was to utilize an integrative metabolic approach to identify potential areas of metabolic dysfunction in skeletal muscle under conditions of reduced ovarian function. We employed multiple physiological approaches coupled with a non-biased, comprehensive, metabolic profiling approach in skeletal muscle from aged match female mice with and without bilateral OVX to identify potential mechanisms. We hypothesized that a loss of ovarian function would result in a metabolic phenotype that would encourage increases in intracellular lipid storage in muscle. Currently, there is a poor understanding of changes induced by the removal of this critically important endocrine organ (i.e. ovary) in women. Therefore, it is important that we begin to address not only the effects of ovarian hormones on non-reproductive tissue, but also consider the effects of absence of ovarian hormone exposure on tissue function. The OVX model is critically important for women's health, in that a significant number of women undergo prophylactic removal of their ovaries (i.e. oophorectomy) for a variety of clinical reasons (141, 225). Since the ovary cyclically secretes numerous endocrine hormones in addition to estrogens, it is critical to recognize that the ovary may play a larger role in regulating peripheral tissue function beyond 17β-estradiol (i.e. the dominant form of estrogen in circulation). Further, current literature including 17β-estradiol supplementation provides 17β-estradiol at supra-physiological levels and does not mimic the cyclic nature of circulating 17β-estradiol. Some studies using 17β-estradiol supplementation also demonstrate that 17β-estradiol does not fully attenuate all consequences of the loss of functional ovaries (218). The experiments described

here will hopefully provide critical direction for investigators examining the role of female sex steroids in the regulation of metabolic function.

Methods

Animals: Prior to beginning this study, all aspects were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) Review Board. Eight-ten week old virgin female C57/BL6 mice were utilized in this study. The mice were divided into two groups: SHAM and OVX, where OVX mice underwent bilateral ovariectomy and the SHAM group was anaesthetized but ovaries were left intact. We have previously shown that OVX surgery results in an approximate ~70% reduction in circulating estrogens within 48 hours (182). Loss of circulating ovarian hormones due to ovariectomy was confirmed in OVX animals by a significant decrease in uterine weights compared to SHAM animals (Table 1). Utilizing uterine mass as indicator of reduced estrogen function is employed since most methods for quantifying murine derived estrogens are not considered accurate.

All mice were housed individually in a standard mouse cage in a temperature-controlled room with a 12 hour light/dark cycle and were provided with ad libitum access to standard rodent chow (Purina Laboratory Rodent Diet 5001: 23% protein, 4.5% fat, 6% fiber) and water. Unlike rats, mice do not become hyperphagic in response to ovariectomy and were therefore preferentially chosen as the animal model for this study (93, 133, 215). To confirm this finding, food intake was measured in a separate cohort of age-matched animals, as

previously described (206). All tissue was collected 8-10 weeks after the OVX surgery. All animals were age-matched and the food was removed 4-5 hrs prior to the tissue collection, with the food removed at 05:00 and tissue collected at ~10:00. We have previously observed that increases in visceral adiposity and loss of regulatory control of lipolytic function occurs at eight weeks post surgery (218).

Intramyocellular lipid (IMCL) quantification: Lipid droplets (LD) were visualized and quantified using BODIPY (493/503) (Invitrogen, CA) as previously described by our group (189, 227). *Muscle cross-sections*: The soleus and plantaris muscles from SHAM and OVX mice were mounted using Tissue-Tek O.C.T. Compound (Andwin Scientific, Schaumburg, IL) and sectioned transversely through the mid-belly of the muscle. The cross-sections were mounted on glass slides and remained at room temperature for 10 mins prior to fixation in 10% paraformaldehyde for 5 mins. Sections were then rinsed 3 times using 1x PBS solution and were then exposed to BODIPY 493/503 (1 μg/mL) in 1xPBS for 30 mins. Sections were then rinsed 4 times for 10 mins using 1X PBS to remove excessive BODIPY stain. Vectashield (Vector Labs, Burlingame, CA) containing DAPI was then placed on each section followed by cover slips. Sections were imaged using a Nikon Eclipse 50i (Nikon Instruments Inc. Melville, NY) at 20x and 40x magnification. Pictures were taken with a Photometrics Coolsnap camera (Tucson, AZ) and processed using Image Pro-Express 6.3 software. Images were quantified using Image J (developed by National Institutes of Health) using the same criteria previously described for Oil Red O procedures (67). A total of 50 fibers were quantified per muscle section. *Single muscle fibers*: Intact single

skeletal muscle fibers were enzymatically isolated from the flexor digitorium brevis (FDB) muscle from SHAM and OVX animals. In brief, surgically excised FDB muscles were incubated in dissociation media (DM) containing DMEM (Invitrogen), gentamycin (50 μg/ml), FBS (2%, ATCC, #30-2020 Rockville, MD), and collagenase A (4 mg/ml, Roche, Indianapolis, IN,) in an incubator (37°C, 5% CO_2) for 1.5-2 hours. Following the dissociation, muscles were placed in a new 35 mm plate with warmed media containing gentamycin and FBS but without collagenase. FDB muscles were triturated with a small bore (~ 1mm) fire polished glass transfer pipette to yield single FDB myofibers. Following trituration, large debris (nerve, un-digested FDB muscle) was removed with forceps. The single fibers were then placed onto an ECM coated glass bottom plate (MatTek, Ashland, MA) according to previously described techniques (14). Fibers were allowed to adhere, rinsed with Ringer buffer to remove all media, and stained for 30 mins with BODIPY 493/503 and 4,6-diamidino-2-phenylindole (DAPI) to label myonuclei (Invitrogen, Cambridge, MA) (14). After 30 minutes, the dyes were removed by rinsing fibers 3X with fresh Ringer buffer. Fibers were imaged using a Zeiss AxioObserver Z1 fluorescent microscope (Carl Zeiss MicroImaging, Jena, Germany).

Immunoblot procedures: Plantaris muscles were used to determine the protein content of CD36/FAT and FATPpm in the SHAM and OVX mice according to previously described methods (16). The soleus muscle was not used because we were unable to extract a sufficient amount of total protein to reliably measure the CD36/FAT or FATPpm. Muscles were homogenized, proteins separated by SDS-

PAGE and the transporters were detected through immunoblotting using antibodies specific for CD36/FAT or FATPpm as previously described (16). Equal quantities of total protein were loaded (20 μg) on each gel and Ponceau S staining on the membranes was used to confirm equal loading.

Mitochondria Isolation and Immunoblot Procedure: Mitochondria were isolated from gastrocnemius muscles from SHAM and OVX muscles as previously described (59). Isolated mitochondria were used to determine the protein content of mitochondrial enzymes very long chain, long chain, and medium chain acyl CoA dehydrogenases (VLCAD, LCAD, MCAD) and mitochondrial complexes I-V as previously described (98, 113, 129, 162). Immunoblotting was conducted using antibodies specific for VLCAD, LCAD, and MCAD (kindly provided by Dr. Jerry Vockley, University of Pittsburgh) and aspects of mitochondrial complexes I-V were detected using an antibody cocktail (AbCam, Cambridge, MA.).

Metabolic Profiling: All procedures were performed as previously described (107). Briefly, the whole gastrocnemius muscle was isolated from SHAM and OVX animals and frozen in liquid nitrogen. The tissue was ground in a liquid nitrogen chilled mortar and pestle, and ~25 mg was suspended in water, homogenized on ice, sonicated, and then spun for 15 min at 4°C, 14,000 rpm. Data are normalized to the total protein content in each sample, as determined by BCA protein assay (Pierce Thermo Fisher Scientific, Austin, TX, USA). Measurement of free carnitine, acylcarnitines and amino acids in muscle was completed by direct-injection electrospray tandem mass spectrometry (MS/MS),

using a Micromass Quattro Micro LC-MS system (Waters-Micromass, Milford, MA, USA) equipped with a model HTS-PAL 2777 auto sampler (Leap Technologies, Carrboro, NC, USA), a model 1525 HPLC solvent delivery system (Agilent Technologies, Palo Alto, CA, USA) and a data system running MassLynx 4.0 software (Waters Corporation, Milford, MA) at the Sarah W. Stedman Nutrition and Metabolism Center Mass Spectrometry Lab. Organic acids in muscle were quantified using methods described previously employing Trace Ultra GC coupled to a Trace DSQ MS operating under Excalibur 1.4 (Thermo Fisher Scientific, Austin, TX, USA)

Skeletal Muscle Malonyl CoA Measures: Malonyl CoA measures were performed on the skeletal muscle from sedentary SHAM and OVX mice as previously described (138).

Single muscle fiber based microplate respirometry: Bioenergetic analyses of isolated FDB muscle fibers were performed using an XF24-3 Extracellular Flux Analyzer (Seahorse Bioscience) as previously described by our group with slight modifications (175). After euthanasia, both flexor digitorum brevis (FDB) muscles were removed from OVX mice and SHAM animals (n = 3/group). Individual fibers were isolated and plated on extracellular matrix (ECM; Sigma EW1270, St. Louis, MO) coated V7 microplate (Seahorse Bioscience, Billerica, MA) overnight according to our previously described methods (175). After calibration of the XF24-3 Extracellular Flux Analyzer, the microplate containing the SHAM and OVX single muscle fibers was placed in the analyzer. Basal

oxygen consumption rate (OCR, pmoles/min) were initially quantified across both groups in assay measurement buffer (MB) at ~37^{o}C contained 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl2, 0.4 mM KH2PO4, 1 mM MgCl2, 5 mM HEPES (pH 7.4) supplemented with 2.5 mM D-glucose (Sigma G7528) and 0.5 mM L-carnitine (Sigma CO158). Mitochondrial respiration was induced with either albumin (Roche, Indianapolis, IN, 03117405001) conjugated sodium palmitate (Sigma P9767; 50uM) or sodium pyruvate (10 mM, Sigma P8574) and OCR was measured. A second identical treatment of substrate was initiated after 20 mins and OCR was again recorded. Following the last OCR measure induced by the second exposure of substrate, 400nM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma C2920) was injected to induce maximal mitochondrial oxygen consumption and OCR was measured. At the conclusion of the last FCCP measure Antimycin A (Sigma A8674; 1 μM**)**, a known inhibitor of mitochondrial complex III, was injected to assess non-mitochondrial OCR measures. OCR measures presented are the average values detected after the OCR reaches a steady state following the introduction of the substrate or FCCP. Basal OCR values presented are taken immediately prior to the first injection of either pyruvate or palmitate. The mean derived for each group was determined by collecting the average OCR values from 7-10 different wells per muscle from each animal. This results in approximately 25-30 independent measures per group as we previously described (28). The single fibers were seeded so that each well is ~60% confluent.

Statistics: All data are expressed as means ± SE. Statistical significance was determined using a t-test for all measures. A *P* value of ≤ 0.05 was considered significant.

Results

Anatomical Characteristics: OVX mice have significantly greater body weight compared to age-matched SHAM animals (Table 1). The OVX mice also exhibited significant increases in visceral (omental/mesenteric) and subcutaneous (inguinal) fat mass compared to the SHAM mice (Table 3.1). As expected, uterine mass was significantly decreased in OVX mice compared to SHAM mice indicating a successful reduction in circulating estrogens (Table 3.1). OVX mice also ate significantly less food per day over the 8 weeks, even though they continued to gain body weight (Table 3.2). When comparing feed efficiency as calculated body weight per kcal consumed, the OVX animals had increased ability to store energy as body weight (Table 3.2).

IMCL Accumulation: Based on our previous studies demonstrating that OVX animals exhibit significant increases in basal lipolytic rates of visceral fat and elevations in circulating NEFA, we sought to determine if OVX mice have increased IMCL in their muscle compared to SHAM animals. IMCL levels were significantly elevated in both the soleus and plantaris muscles from the OVX mice compared to SHAM mice (Figures 3.1A-B and 3.2A). In addition, we observed IMCL within the single muscle fibers to confirm that the IMCL elevations in the muscle from the OVX were due to increased LD frequency within the myoplasm

and not between the muscle fibers (Fig 3.2B). In the SHAM animals, we found very few LD within the isolated single muscle fibers (Fig 3.2B).

Fatty Acid Transporter protein content: Since IMCL content was elevated in the OVX groups compared to the SHAM animals, we measured the protein content of the two primary sarcolemmal fatty acid transporters in skeletal muscle. We found that both FAT/CD36 (Figure 3.3A) and FABPpm (Figure 3.3B) were significantly increased in OVX mice compared to SHAM mice.

Metabolic Profiling: Due to the metabolic complexity of skeletal muscle, we assessed the metabolic profile of skeletal muscle from OVX animals compared to SHAM animals using a targeted metabolomics approach. The purpose of these experiments was to identify potential pathways or targets that could account for the metabolic dysfunction that appears in the OVX animals. These metabolites are byproducts of fuel degradation that reflect shifts in substrate availability and/or flux limitations at specific catabolic enzymes.

Acylcarnitine Intermediate Profile: Acylcarnitine species levels were quantified in gastrocnemius muscles from the SHAM and OVX groups to assess specific steps of β-oxidation. This approach provides the investigator a snapshot of substrate flux and can provide the investigator specific experimental direction for identifying potential metabolic limitations. For example, high levels of long chain acylcarnitines (LC) might suggest that movement of fatty acids via mitochondrial Carnitine palmitoyltransferase I (CPT-1) exceeds flux through β-oxidation enzymes, such as long-chain (LC) acyl-CoA dehydrogenase and/or β -OH-acyl-

CoA dehydrogenase (205). Based on previous results in other models of obesity and due to the accumulation of IMCL in the OVX muscles, we hypothesized that LC in the muscle from OVX muscles would be elevated compared to the SHAM animals (107, 194, 205). Surprisingly, we found total LC species to be reduced in the OVX group compared to the SHAM group (Figure 3.4A). No differences in medium chain (MC) acylcarnitines were identified (Figure 3.4B), but we did detect significantly lower levels of short chain (SC) acylcarnitines in the OVX group compared to the SHAM group (Figure 3.4C). These data suggest a reduced flux through the β-oxidation pathway in the muscle from the OVX group compared to the SHAM group. Coupled with the reduction in acylcarnitines, we determined that skeletal muscle free carnitine levels were significantly lower ($P \leq 0.05$) in OVX mice compared to SHAM mice (Figure 3.4D). A number of previous publications have found that a reduction in free carnitine levels correlates with decreases in skeletal muscle lipid metabolism (102, 152, 210). Collectively, these data imply that reductions in flux through β-oxidation could contribute to a decline in skeletal muscle lipid catabolism under conditions of reduced estrogenic function.

Skeletal Muscle Malonyl CoA and CPT-1 Content: The reduced levels of acetyl-carnitine coupled with the reduced free carnitine content might suggest an impaired flux through β-oxidation as a result of reduced fatty acid transport through CPT-1. We measured CPT-1 mRNA levels and detected no differences between SHAM and OVX animals (CPT-1/18S: SHAM = 0.881 ± 0.03 vs. OVX = 0.0822 ± 0.02). CPT-1 activity is negatively regulated by allosteric interactions

with malonyl CoA, therefore the observed reductions in acylcarnitines in the OVX animals could be the result of increased malonyl CoA content (19, 161, 169). However, no differences were detected in skeletal muscle malonyl CoA content between sedentary SHAM and OVX animals (Figure 3.5).

Glycolytic and TCA cycle metabolites: Previous data in other models of obesity have shown that accumulation of LC-acylcarnitines species was associated with a significant reduction in Krebs cycle intermediates, reflecting a form of mitochondrial dysfunction. We found no differences in either pyruvate or lactate levels between OVX and SHAM animals (Figure 3.6A). However, citrate and succinate levels were significantly ($P \leq 0.05$) higher in skeletal muscle from OVX compared to SHAM (Figure 3.6B,D). Finally, no significant differences were detected in TCA cycle intermediates, α-ketogluterate (αKG), fumarate, and malate (Figure 3.6C,E).

Amino acid profile: It is well established that amino acids play an integral role in skeletal muscle metabolism by serving as substrates for catabolic and anabolic processes that directly contribute to overall function of the muscle. In addition, previous work has shown that the branched chain amino acid (BCAA) profile is a potential contributor to insulin resistance in the obese state (150). Therefore, we evaluated the skeletal muscle amino acid profile in the OVX mice compared to the SHAM mice (Figure 3.7A-D). In OVX mice, we identified significant decreases in the BCAAs, leucine and isoleucine, however there was no significant difference in valine as compared to SHAM mice ($P \leq 0.05$) (Figure 3.7B,C). We

also found significant decreases in the concentrations of alanine, glutamine/glutamic acid, proline, serine, and histidine in OVX mice compared to SHAM (Figure 3.7A,B,C).

Odd Chain Acyl-Carnitine Species: Odd chain acyl-carnitine species propionylcarnitine (C3), isovalerylcarnitine (C5), and tiglyl carnitine (C5:1) are products of BCAA catabolism (130, 144). Due to the observed decline in skeletal muscle amino acids in OVX mice, we measured C3, C5, and C5:1 species in SHAM and OVX mice. We detected a reduction in C3 species in OVX mice compared to SHAM ($P \leq 0.05$) and no difference in C5 species (Figure 3.8). However, we observed a reduction in the unsaturated C5:1 acyl-carnitine species ($P \leq 0.05$) (Figure 3.8). No significant differences in hydroxylated odd-chain acyl carnitine species ratio C5-OH/C3-OH were detected. Collectively, these data indicate a decline in catabolism of BCAAs and likely a reduced pool of available amino acids to the muscle in the OVX condition (144).

Mitochondrial Oxygen Consumption: To determine if any of these alterations in metabolite levels was associated with compromised mitochondrial function, we assessed mitochondrial oxygen consumption induced by either palmitate or pyruvate in intact single FDB muscle fibers isolated from SHAM and OVX mice. The advantage to this approach is that we can assess mitochondrial function with no disruption to the integrity of the muscle fiber or the organelle, thereby allowing measures to be taken in the most native form of the mitochondria. Basal OCR was significantly lower in fibers isolated from the OVX animals compared to the

SHAM animals (Figure 3.9A). To determine the response to substrate exposure we normalized the OCR to the baseline OCR values, since the basal OCR measures were different between the SHAM and OVX prior to substrate exposure. Palmitate exposure stimulated significant increases in both normalized OCR from baseline in SHAM and OVX fibers, with no significant differences between groups (Figure 9B). Pyruvate stimulated normalized OCR was increased from baseline in both OVX and SHAM, however, there was no significant difference between the OVX and SHAM groups (Figure 3.9C). To assess substrate specific maximal ETC activity, we added the uncoupling agent, FCCP, and found significant increases in normalized OCR in the OVX and SHAM compared to baseline OCR with both palmitate and pyruvate substrates (Figure 3.9B and C). However, regardless of substrate, FCCP stimulated normalized OCR were significantly lower in the fibers from the OVX group compared to the SHAM indicating a potential impairment in electron transport chain function (Figure 3.9B,C). Calculation of the spare respiratory capacity (SRC), as previously described by our group (175), suggests a reduced ability of mitochondria from the OVX fibers compared to SHAM fibers to respond to stimuli that would activate mitochondria (Figure 3.9 D,E).

Mitochondrial Enzyme Content: To determine if our observations of decreased long-chain acylcarnitines and diminished FCCP stimulated respiration were due to differences in mitochondrial enzyme content, we measured mitochondrial enzyme proteins in skeletal muscle from SHAM and OVX mice. No differences

in VLCAD, LCAD, MCAD protein content or ETC mitochondrial protein content were observed between SHAM and OVX animals (Figure 3.10).

Discussion

Reductions in circulating estrogens in women, due to either the onset of age-induced menopause or menopause as a result of ovariectomy, correlate with a robust increase in visceral fat mass (71, 74, 125). Our data demonstrate that compared to the SHAM group, OVX mice exhibit increased adiposity that is coupled with significant accumulation of IMCL, CD36/FAT and FABPpm protein content within the skeletal muscle. To identify potential metabolic mechanisms, we employed a metabolic profiling approach in the skeletal muscle from these animals. When comparing data from OVX and SHAM animals, the results suggest that reduced substrate flux through β-oxidation in the OVX animals may contribute to increased IMCL content. Finally, we determined that there are specific deficits in basal and maximal stimulated mitochondrial oxygen consumption in single muscle fibers from the OVX animals compared to the SHAM that are not explained by reductions in mitochondrial protein content. Collectively the data indicate that loss of ovarian function leads to visceral adiposity and IMCL accumulation, and our experiments document the metabolic profile of skeletal muscle under this condition.

Consistent with other studies, we found that ovariectomy resulted in significant increases in both visceral and subcutaneous fat mass compared to the SHAM mice (69, 98). We previously reported that increases in visceral fat mass

in the OVX model are associated with an enhanced lipolytic rate, yielding an increase in circulating NEFAs (218) that suggests skeletal muscle in the OVX animals is exposed to higher levels of NEFA. Based on experiments in cultured skeletal muscle cells, exposure of the cells to exogenous NEFA results in increased IMCL content (114). OVX mice exhibited significantly more IMCL than SHAM animals, which was associated with markedly higher protein content of two key fatty acid sarcolemmal transporters FABPpm and FAT/CD36. Although other models of obesity have demonstrated similar findings (18, 86), to our knowledge this is the first time these increases have been documented in the OVX model. The observed increase in FAT/CD36 and FABPpm in the OVX group is likely the result of an enhanced demand for clearance of NEFAs from circulation and corresponds with an increase in IMCL deposition into skeletal muscle.

Due to the metabolic complexity of skeletal muscle, we employed a targeted metabolic profiling approach to generate a comprehensive non-bias analysis of skeletal muscle in the SHAM and OVX groups (107). Since the OVX group exhibited significant increases in adiposity coupled with high IMCL, we hypothesized that, in a similar fashion to other obesity models (29,33), we would find substantial increases in LC in the OVX group compared to the SHAM group. Surprisingly, we found relative decreases in the LC levels in the OVX, which could suggest impaired transport of LCFA into the mitochondria. The enzyme CPT-1 combines cytosolic free carnitine with long chain fatty acids, producing an acyl-carnitine that is then transported into the mitochondria (22). CPT-1 activity

is reduced through allosteric inhibition by malonyl CoA and a lack of free carnitine (81, 102, 210). Therefore, because we detected no differences in malonyl CoA levels or CPT-1 mRNA levels, but did detect a reduction in free carnitine levels in the OVX group, we suspect that transport of LCFA into the mitochondria via CPT-1 is a potential point of limitation for lipid oxidation under conditions of reduced ovarian function. In agreement with this conclusion, others have shown in the OVX rat model that CPT-1 activity in skeletal muscle is significantly lower than in SHAM controls (27).

The observed decline in SC in response to ovariectomy might suggest reduced flux of fatty acids through β-oxidation. This theory is further supported by evidence in humans that suggests numerous points in the β-oxidation and other lipid metabolism pathways are sensitive to fluctuations in estrogens (61, 127, 201). However, we found no differences in the mitochondrial protein content of VLCAD, LCAD, or MCAD in the muscle from the SHAM and OVX animals. Based on our data it seems critical to measure SCAD as well, however we were unable to obtain an antibody specific to murine tissue. Collectively, these data indicate that under conditions of reduced estrogen function, there are critical changes that may be affecting flux through β-oxidation and contributing to alterations in lipid metabolism.

Upon entry into the cell, NEFAs are routed either towards re-esterification and subsequently synthesized into IMCL, or if energetic demand is elevated, shuttled into the mitochondria for oxidation (86). Our data demonstrate that skeletal muscle IMCL is robustly increased in both the plantaris and soleus

muscles in the OVX group; suggesting NEFA flux into the muscle is elevated. To determine if alterations in mitochondrial function are contributing to the increased IMCL, we measured mitochondrial oxygen consumption (OCR) of intact single muscle fibers from SHAM and OVX animals. Under basal conditions (i.e. low glucose), mitochondrial OCR was significantly reduced in the muscle fibers from the OVX fibers compared to SHAM fibers. However, when we added substrate (i.e. Pyr or PA) the OCR increased by the same percent in both groups. These findings suggest that under conditions of low energetic demand, skeletal muscle mitochondria in the OVX animals respond in similar fashion to SHAM animals to increased substrate delivery. When stimulated with FCCP to induce mitochondrial uncoupling, the fibers from the OVX animals demonstrated a reduced ability to respond to increased oxygen consumption compared to the SHAM animals. Further, the lower SRC capacity of the fibers from OVX animals suggests that mitochondria have a reduced ability to respond to a maximal stimulus that would enhance oxidation of metabolic substrates such as lipid or glucose. However, since mitochondria rarely operate in these maximal ranges, it seems unlikely that reduced SRC capacity is a limiting factor that explains the IMCL accumulation in the OVX model. Since, the fibers from the OVX animals were able to increase their OCR to the same magnitude as the fibers from the SHAM animals in response to the PA exposure, it also seems unlikely that the mitochondria are the limiting factor to explain the IMCL buildup. We also detected no differences in the protein content of various mitochondrial proteins, making it unlikely that a loss of mitochondrial content is a contributing factor.

Using FCCP to drive mitochondrial oxygen consumption is a common experimental procedure, however it is unclear if lower FCCP-driven OCR rates would translate to a measurable physiological deficiency (i.e. reduced exercise capacity) in skeletal muscle of the OVX mice. For example, we have found little evidence indicating enhanced *in vitro* or *in situ* fatigue development in skeletal muscle from OVX animals (219), however it is well documented that OVX animals often exhibit poor exercise performance. Specifically, we and others previously reported a decline in voluntary wheel running in OVX animals (69, 98). In agreement with our findings, Rogers et al. found a decline in nocturnal ambulatory activity levels in OVX mice as well as decreased oxygen consumption compared to SHAM (167). Therefore, the enhanced deposition of IMCL in skeletal muscle is not solely a response of reduced mitochondrial function in the OVX fibers. Considering the number of *in vitro* and *in vivo* studies showing that estrogens influence mitochondrial biogenesis and function in tissues other than skeletal muscle (132, 195, 230-232), it remains plausible that optimal mitochondrial function in skeletal muscle is influenced by estrogen function however it is likely that other unknown factors are contributing to the increased IMCL.

Skeletal muscle amino acid metabolism is not well characterized with regards to estrogenic influences. Recent evidence in other obesity models has suggested that elevated concentrations of BCAA are a significant contributor to the development of insulin resistance (150). Surprisingly, we found that concentrations of the majority of amino acids were significantly reduced in the

OVX group compared to the SHAM groups, with even the non-significant differences tending to decrease in the OVX group. The decreases in amino acid levels may suggest enhanced metabolic amino acid catabolism or a loss of the available amino acid pool. The former is a perplexing finding, since we detected decreases in odd-chain acetylcarnitine species and our previous research has not identified losses in skeletal muscle mass of the OVX animals compared to the SHAM animals (218). However, an accelerated decline in skeletal muscle mass has been observed in postmenopausal women (6), which may suggest that if OVX animals were allowed to remain in the reduced estrogen condition longer, reductions in muscle mass become apparent. Our data may also suggest there is a reduction in the amino acid pool within the muscle of the OVX animals, which leads to a reduction in the availability of amino acids for protein translation. This finding may explain previous results in which we and others have shown that OVX animals have reduced mTOR signaling response coupled with lower muscle growth with loading of the skeletal muscle compared to SHAM animals (136, 182). However, this hypothesis would need to be further tested.

Potential Limitations: It should be noted that using the metabolic profiling approach does not elucidate the mechanism that may be disrupted in the experimental model, but provides direction and insight into potential mechanisms that would require further in-depth studies. The data collected in these studies resulted in a number of unexpected findings that we are currently following up on. We chose to employ the OVX model as a means to define metabolic function under conditions of reduced female sex steroids concentrations. Although it is

likely that decreases in estrogens are contributing to a number of these effects, we cannot rule out the possibility that other ovarian hormones are playing a critical role in defining these phenotypes. Since estrogens affect multiple tissues, it is difficult to assess which effects are a direct result of reduced estrogen levels and which are secondary effects due to changes in other non-skeletal muscle tissues. It is critical to develop experimental models to specifically isolate estrogen signaling in skeletal muscle to determine the primary role for estrogens in skeletal muscle metabolism.

Overall, utilization of the metabolic profiling approach in the OVX model demonstrates that metabolic disturbances in the OVX animal do not recapitulate the same metabolic profile observed in other rodent models of obesity. Two frequently observed results include an accumulation of LC (107) and increase in skeletal muscle BCAA levels in both human and animal models of obesity (80). However, the OVX mice do not exhibit this metabolic profile, suggesting that more studies are needed to critically examine the mechanistic role of female sex steroids in the regulation of peripheral metabolism. In summary, our data suggest that reduced ovarian function results in an increase in IMCL content that is not entirely explained by a reduction in the capacity of the mitochondria to utilize fatty acids. Due to the critical relationship between insulin signaling and IMCL content, it will be important to examine alternative explanations for the accumulation of lipid in the skeletal muscle of the OVX animals.

Perspectives and Significance: The results from this study provide a critical base of knowledge in an understudied area of women's health and we hope the data will provide critical direction for additional mechanistic studies to be conducted. The OVX model is the most frequently used murine model to study menopause in women, however is likely most representative of women undergoing surgical-induced menopause prior to the onset of age-induced menopause. Thus, the resulting data are also likely most relevant to women experiencing pre-mature ovarian failure or being treated for estrogen positive cancers. Previous publications have shown that the surgical removal of ovaries or the onset of ovarian failure in women leads to adiposity, particularly in the visceral region, which corresponds with glucose intolerance and insulin resistance (32). In addition, there are indications of altered responses to muscle loading and reduced force output by the muscle (52, 72). We and others have observed many of the same effects in the OVX model, indicating the importance of the OVX model for issues relevant to women's health (98, 147, 167, 182). The novel aspect of our study is that we now demonstrate a more comprehensive metabolic phenotype of skeletal muscle in the OVX model, which will help to elucidate the underlying mechanisms responsible for changes induced under conditions of estrogen signaling disruption. In conclusion, the data demonstrate the importance of considering each model of obesity independently and highlight the need for more research in order to understand the role of female sex steroids in the regulation of metabolic function. Specific metabolic profiles likely exist across multiple models of metabolic disease and each model should be evaluated

individually when assessing the mechanisms behind the development of metabolic dysfunction.

Acknowledgements

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TABLES:

Table 3.1. Anatomical characteristics of SHAM and OVX animals

	Age	BM (g)	SEM	VF (g)	SEM	IF (g)	SEM	Uterus (g)	SEM
SHAM	4 mos	23.46	0.526	0.262	0.03	0.175	0.009	0.070	0.009
OVX	4 mos	28.37*	0.998	1.296*	0.18	0.575*	0.134	0.019*	0.005

BM = body mass; VF = visceral fat; IF = inguinal fat; * Indicates statistically different from SHAM $P \leq 0.05$

Table 3.2. Daily Food Consumption

	Food consumption (g/day)	SEM	Feed Efficiency Body mass (g)/Food Intake (Kcal*day)	SEM
SHAM	4.943	0.096	1.139	0.007
OVX	4.228*	0.33	1.457*	0.008

FIGURES:

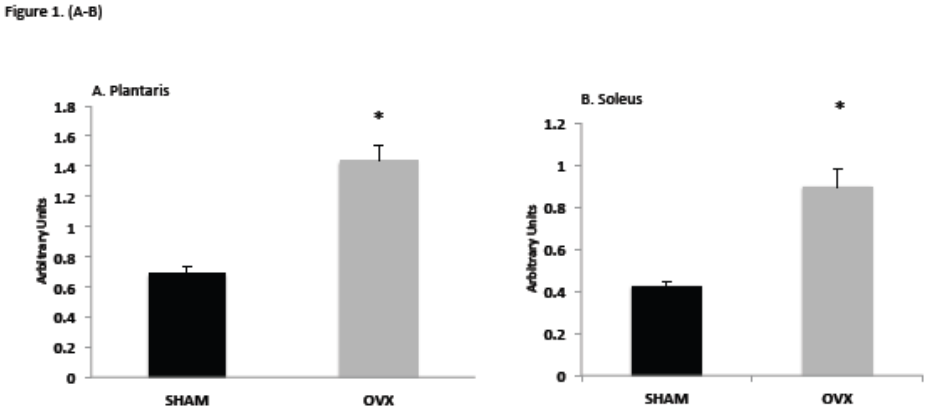

Figure 3.1 (A-B): IMCLs quantified using direct fluorescent visualization were significantly elevated in ovariectomized (OVX) female mice in both the **(A)** plantaris and **(B)** soleus muscle groups compared to age matched sham surgery (SHAM) female mice. N=3 animals/group and 50 fibers/muscle quantified. * indicates significant difference from SHAM ($P \leq 0.05$)

Figure 2. (A-B)

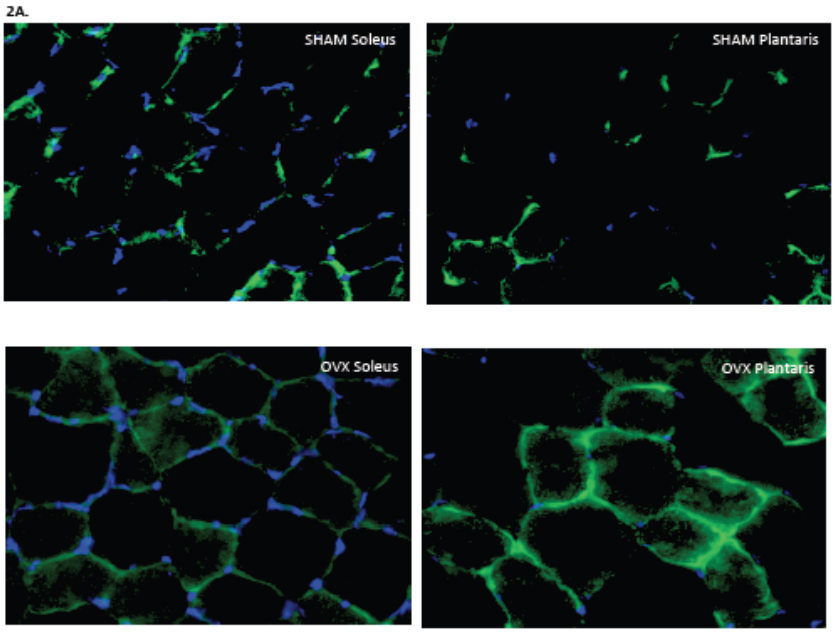

Figure 2 (A-B)

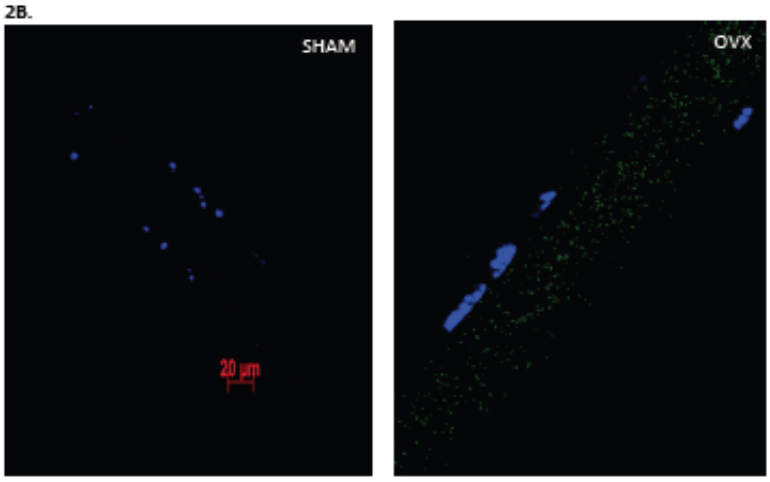

Figure 3.2 (A-B): Representative images of IMCL through BODIPY (493/503) staining of neutral lipid droplets and nuclei (DAPI-blue) within **(A)** skeletal muscle cross-sections of soleus and plantaris muscle and **(B)** single muscle fibers from SHAM and OVX age matched female mice.

Figure 3. (A-B)

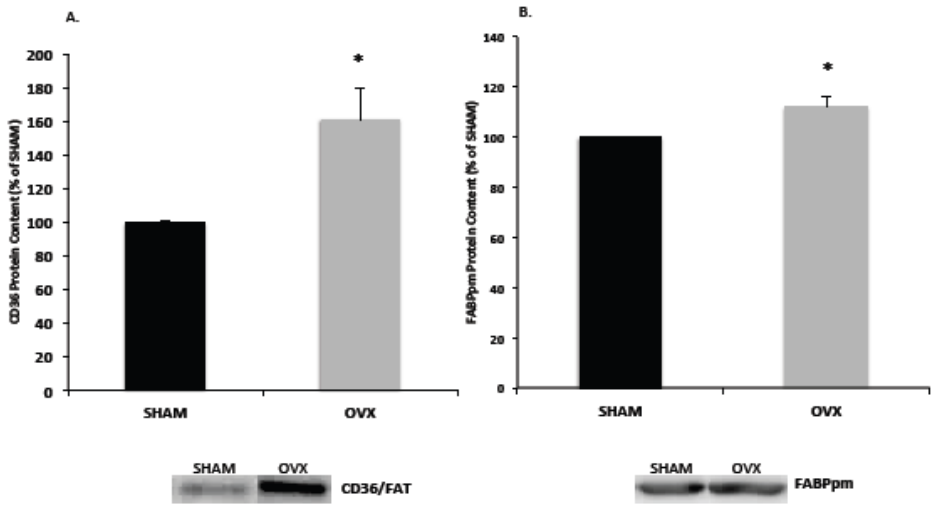

Figure 3.3 (A-B): OVX female mice demonstrated significant elevations in **(A)** CD36/FAT protein and **(B)** FABPpm in the plantaris muscle compared to age matched SHAM animals. Example western blots are presented for each target. N=5 animals/group. * indicates significant difference from SHAM ($P \leq 0.05$)

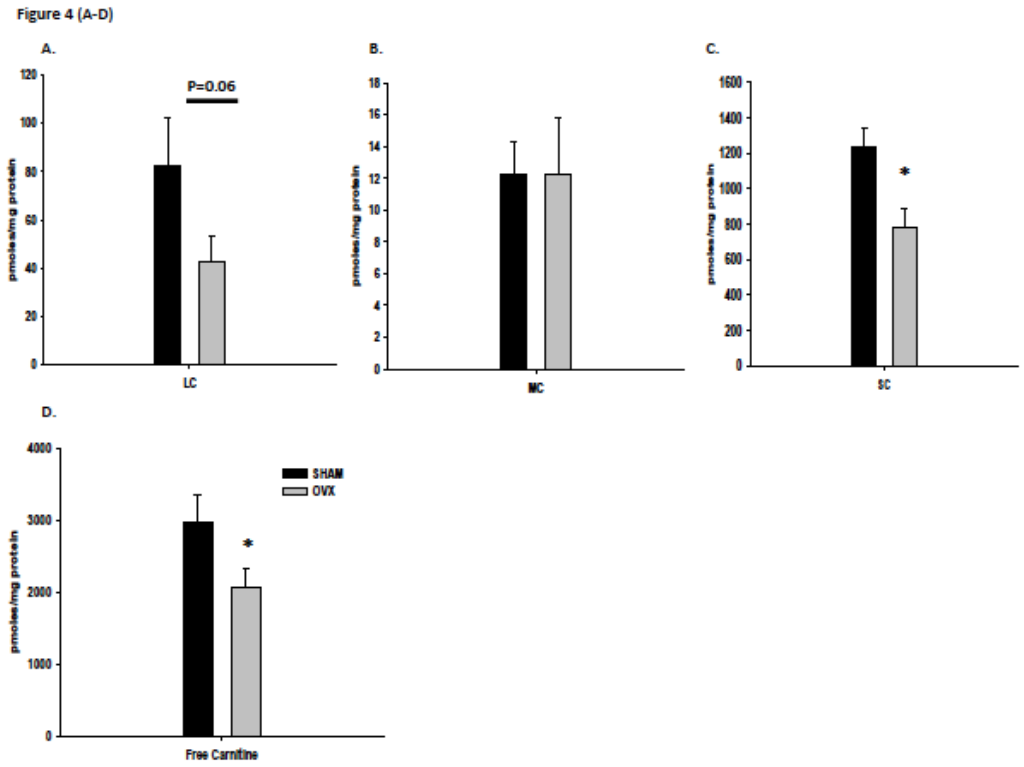

Figure 3.4 (A-D): OVX animals exhibited significant reductions in various acylcarnitine species coupled with lower carnitine levels in the whole gastrocnemius muscle compared to age-matched SHAM animals. **(A)** OVX female mice demonstrated a trend (p=0.06) for lower levels of long chain (LC; C16-C18) acylcarnitine species compared to age matched SHAM animals. **(B)** No differences between OVX and age-matched SHAM mice were detected for medium chain (MC, C10-12) acylcarnitine species. **(C)** OVX female mice had significantly lower levels of short chain (SC; C2-C5) acylcarnitine species compared to age matched SHAM animals. **(D)** OVX female mice had significantly lower levels of free carnitine compared to age matched SHAM animals. N=6 animals/group. * indicates significantly different from SHAM ($P \leq 0.05$).

Figure 5

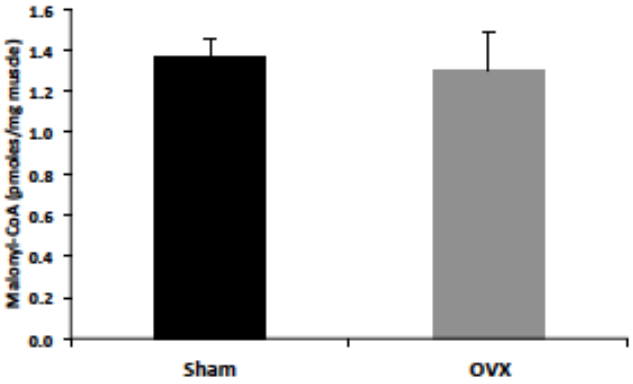

Figure 3.5: No differences in the whole gastrocnemius skeletal muscle malonyl CoA content was detected between SHAM and OVX age matched female mice. N=5 animals/group.

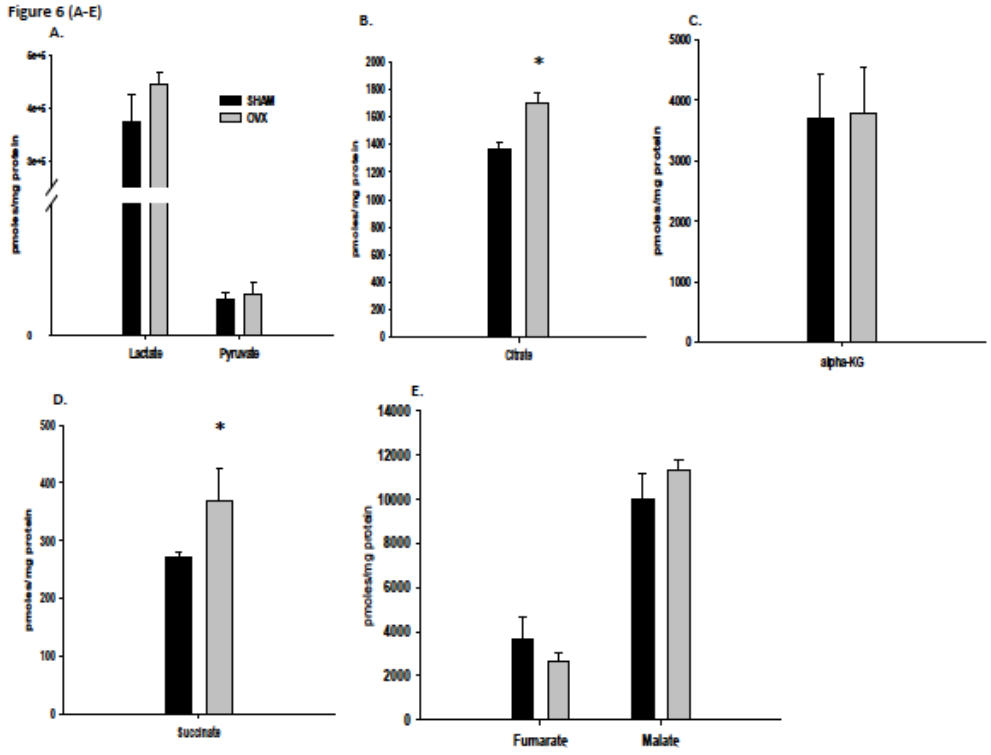

Figure 3.6 (A-E): OVX mice exhibit no changes in whole gastrocnemius muscle pyruvate or lactate levels, but exhibit selective differences in Krebs cycle intermediates compared to age-matched SHAM mice. **(A)** OVX mice exhibited no differences in skeletal muscle lactate and pyruvate levels compared to age matched SHAM animals. **(B)** OVX animals had significantly higher levels of citrate levels compared to age matched SHAM animals. **(C)** No significant differences in alpha ketogluterate were detected in the gastrocnemius muscle between age matched OVX and SHAM female mice. **(D)** OVX mice demonstrated significant elevations in skeletal muscle succinate levels compared to age matched SHAM female mice. **(E)** No significant differences were detected in skeletal muscle fumarate or malate levels between OVX and SHAM groups. N=6 animals/group. * indicates significantly different from SHAM ($P \leq 0.05$).

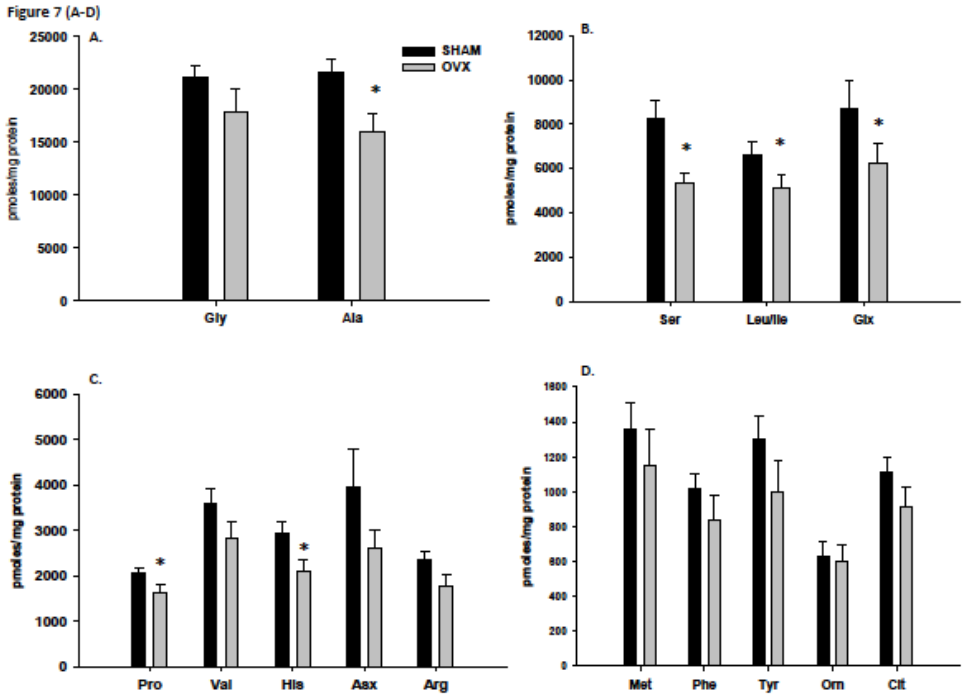

Figure 3.7 (A-D): OVX animals exhibited significantly lower levels of various amino acid species in the gastrocnemius muscle compared to age matched SHAM animals. **(A)** OVX mice had significantly lower levels of alanine, and no difference in glycine levels compared to SHAM animals. **(B)** OVX mice had significantly lower levels of serine, leucine/isoleucine, glutamate in skeletal muscle compared to SHAM animals. **(C)** OVX mice had significantly lower levels of proline and histidine, and no differences in valine, aspartate, or arginine levels compared to SHAM animals. **(D)** No differences were detected methionine, phenylalanine, tyrosine, ornithine, or citrulline in the skeletal muscle between the OVX and SHAM groups. Amino acid abbreviations are as follows: alanine (Ala), serine (Ser), leucine/isoleucine (Leu/Ile), glutamate (Glx), proline (Pro), histidine (His), glycine (Gly), valine (Val), aspartate (Asx), arginine (Arg), methionine (Met), phenylalanine (Phe), tyrosine (Tyr), ornithine (Orn), and Citrulline (Cit). N=6 animals/group. * indicates significantly differs from SHAM ($P \leq 0.05$).

Figure 8

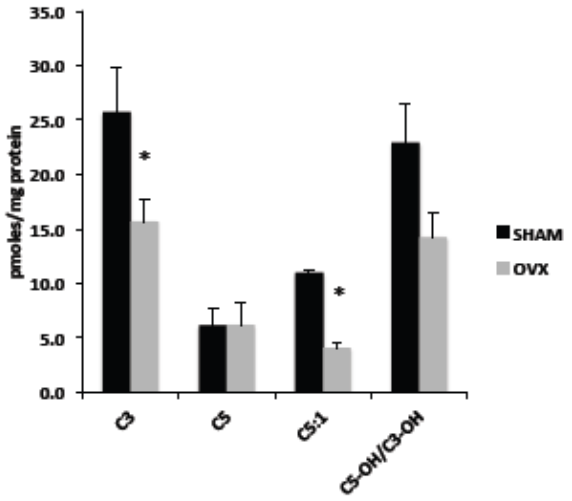

Figure 3.8: Significant decreases in odd chain acylcarnitine species propionylcarnitine (C3) and unsaturated isovalerylcarnitine (C5:1) in the whole gastrocnemius muscle from the OVX compared to age matched SHAM mice. No significant differences were detected in isovalerylcarnitine (C5) acylcarnitine species or in the hydroxylated species ratio C5-OH/C3-OH. N=6 animals/group. * indicates significantly differs from SHAM ($P \leq 0.05$).

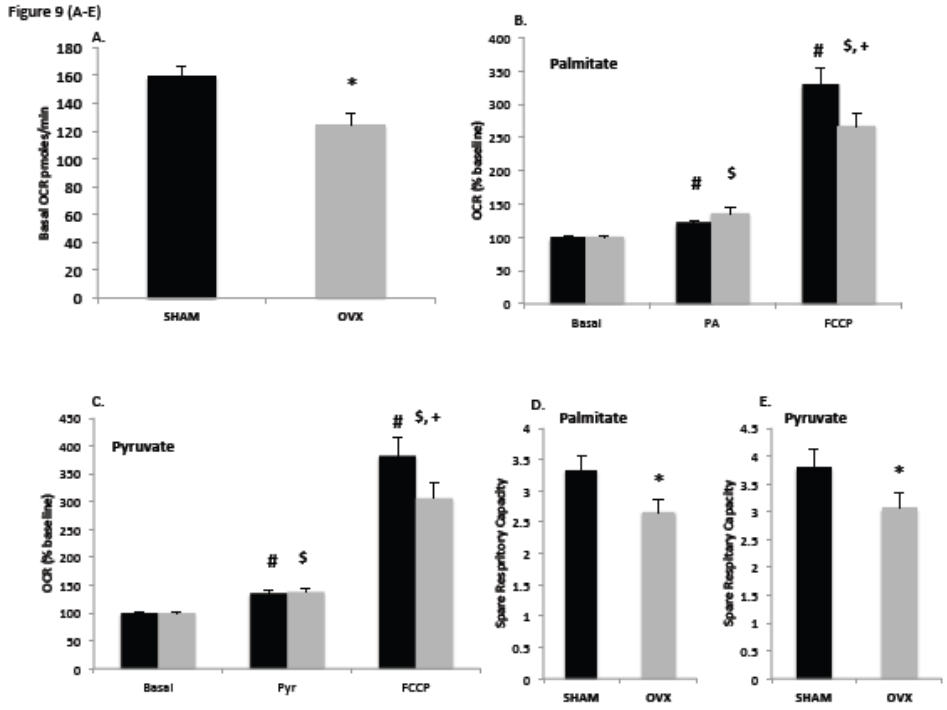

Figure 3.9 (A-E):
Basal and stimulated oxygen consumption rates (OCR) of cultured single skeletal muscle fibers (isolated from the flexor digitorum brevis) from OVX and age matched SHAM mice. **(A)** Basal OCR was significantly lower in OVX compared to SHAM animals. * indicates significant difference from SHAM ($P \leq 0.05$). **(B, C)** Maximal OCR rates induced by FCCP (calculated as percent change in OCR from baseline) in the presence of either palmitate (PA, 50μM) or pyruvate (Pyr, 10mM) were significantly reduced in skeletal muscle fibers from OVX compared to SHAM animals. However, no differences were detected in OCR responses to PA stimulation or Pyr stimulation alone. # indicates significantly different from SHAM basal ($P \leq 0.05$), $ indicates significantly different from OVX basal ($P \leq 0.05$), + indicates significantly different from SHAM FCCP ($P \leq 0.05$). **(D, E)** In response to PA or Pyr stimulation spare respiratory capacity (absolute OCR_{FCCP}/absolute OCR_{basal}) was significantly reduced in OVX single muscle fibers compared to age matched SHAM single muscle fibers. N=5 animals/group. * indicates significantly different from SHAM ($P \leq 0.05$)

Figure 10 (A-B)

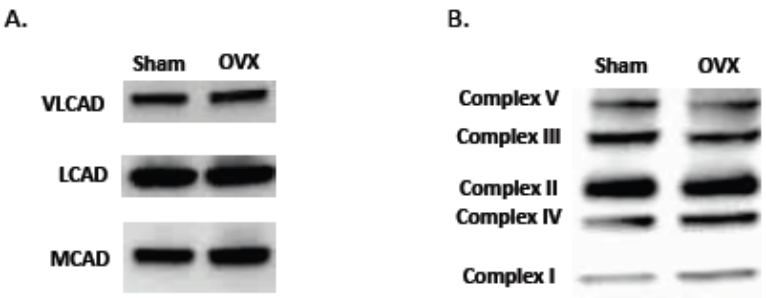

Figure 3.10 (A-B): Mitochondrial enzyme content did not differ in the whole gastrocnemius muscle between SHAM and OVX mice. **(A)** Mitochondrial enzymes responsible for the initial steps of β-oxidation of fatty acids VLCAD, LCAD, and MCAD did not differ between SHAM and OVX animals. **(B)** Mitochondrial respiratory chain complexes I-V did not differ between SHAM and OVX animals. N=5 animals/group.

Chapter 4: BRCA1 is a Novel Regulator of Lipid Metabolism in Skeletal Muscle

The following manuscript is currently in review at Nature Communications.

BRCA1 is a Novel Regulator of Metabolic Function in Skeletal Muscle.

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ABSTRACT: (250 word max)

Breast cancer type 1 susceptibility protein (BRCA1) was recently identified as a regulator of lipid metabolism in breast tissue through an interaction with the phosphorylated form of Acetyl CoA Carboxylase (ACC-p). In skeletal muscle, ACC influences lipid dynamics through enzymatic production of malonyl CoA (MaCoA), a known regulator of mitochondrial fatty acid transport and fatty acid synthesis. The purpose of this investigation was to define a functional role for BRCA1 in skeletal muscle using a translational approach. In both mice and humans, we identified the presence of multiple isoforms of BRCA1 in skeletal muscle. In response to an acute bout of exercise, we found increases in the interaction between the native forms of BRCA1 and ACC-p. Decreasing *BRCA1* expression using an shRNA approach in cultured primary human myotubes resulted in increased storage of intracellular lipid, and reduced insulin-induced Akt signaling which corresponded to impaired mitochondrial function. Collectively, these data reveal BRCA1 as a regulator of metabolic function in skeletal muscle and provides a novel target to consider in our understanding of skeletal muscle metabolic function.

INTRODUCTION:

Breast cancer 1 early onset (*BRCA1*) is recognized as an estrogen sensitive human tumor suppressor gene (142, 211). Genetic variation in the *BRCA1* gene is associated with increased risk for the development of breast cancer and/or tumorigenesis in reproductive tissues (60). The *BRCA1* gene produces either a full length breast cancer type 1 susceptibility protein (BRCA1) or through alternatively splicing two documented variants, BRCA1Δ11 or BRCA1Δ11b, both of which lack a nuclear localization signal (212). Recently, BRCA1 was identified as a regulator of lipid metabolism in human breast cancer cells (MCF7) as a result of direct interaction with the phosphorylated form of acetyl CoA carboxylase (ACC-p) at the BRCA1 C-terminal BRCT domains (54). The interaction encourages the maintenance of the phosphorylated state of ACC thereby altering lipid metabolism in the MCF7 cell line (126, 148).

ACC has two isoforms, ACC1 or ACC2, with ACC2 containing an extra 146 amino acids in the NH_2-terminus region (2, 193). ACC activity is negatively regulated by phosphorylation of residue Ser^{79} on ACC1 and Ser^{221} on ACC2. In the active form (i.e. dephosphorylated), ACC catalyzes the carboxylation of acetyl CoA into malonyl CoA (MaCoA). Changes in cellular MaCoA content can alter intracellular lipid dynamics in two specific manners (140, 160, 213). MaCoA directly contributes to *de novo* synthesis of palmitic acid via fatty acid synthase (FAS) and MaCoA also allosterically inhibits carnitine palmitoyltransferase-I (CPT-1) a mitochondrial long chain fatty acid transporter (140). Thus, in

mammary tissue the ability of BRCA1 to affect ACC activity alters cellular lipid concentrations by indirectly regulating rates of fatty acid synthesis and/or the flux of fatty acids into the mitochondria.

In response to increased energetic demand, such as during exercise, skeletal muscle increases fatty acid entry into the mitochondria through CPT-1 mediated transport (85). During acute exercise, this rate is enhanced in muscle by a reduction in MaCoA content due to reduced ACC activity (160) with the inhibition of ACC mediated by AMP activated protein kinase (AMPK) (213). Since regular exercise is known to encourage metabolic health, multiple investigations have targeted this metabolic mechanism in an effort to treat metabolic disease. ACC function is critical to the regulation of lipid metabolism in skeletal muscle since genetic ablation of ACC resulted in higher basal and insulin stimulated palmitate oxidation rates (3). Collectively, these results indicate that ACC plays an important role in skeletal muscle metabolism however key regulators of ACC remain unidentified.

A previous investigation has shown BRCA1 mRNA is expressed in C2C12 myoblasts, which would suggest a possible role in skeletal muscle (109). However, to our knowledge no studies have mechanistically examined the role of BRCA1 in skeletal muscle. Thus, the purpose of this investigation was to establish if *BRCA1* was expressed in skeletal muscle and determine whether it serves as a regulator of metabolic function. Using an integrative and translational approach, we have identified that BRCA1 is expressed in both mouse and human

skeletal muscle and plays a critical metabolic function in the skeletal muscle cell. These results indicate that BRCA1 function extends beyond reproductive tissues suggesting that broader roles across multiple tissues should be evaluated.

MATERIALS AND METHODS:

Animals: The University of Maryland Institutional Animal Care and Use Committee (IACUC) Review Board approved all aspects involving animal research. Male and female C57Bl/6 mice ranging from 8-10 weeks were utilized in this study. Two groups of C57Bl/6 mice were divided into an exercise (Male n=6; Female n=7) or sedentary (Male n=6; Female n=7) group. All animals were treadmill acclimated and then only the exercise group was subjected to an acute bout of treadmill exercise (Male=21.92±0.57m/min; 40.1±2.75min; 5% incline) (Female=26.57±0.30 m/min; 36.5±4.3 min; 5% incline) while the sedentary animals were placed on the treadmill in a stationary position for an equivalent time. The males were run at a lower speed to maintain similar relative intensities between the males and females. At the conclusion of exercise, animals were euthanized and skeletal muscle was harvested, snap frozen in liquid nitrogen, and stored at -80^{o}C.

Human Subjects: The Ethics Committee of the Karolinska Institutet approved all aspects of this study. Twenty-four healthy subjects were included in the study, 13 males and 11 females, all of who gave their informed consent to participate. The mean (range) age, height, and weight were 26 (21–30) yrs, 177 (158–190) cm, and 75 (58–90) kg, respectively. The mean (range) maximal oxygen consumption

(VO_2max) was 48 (43–64) $ml \cdot kg^{-1} \cdot min^{-1}$. Well-trained subjects (VO_2max > 65 $ml \cdot kg^{-1} \cdot min^{-1}$) were excluded to maximize the subjects' exercise responses (173). Skeletal muscle biopsies from m. vastus lateralis (VL) were obtained using the percutaneous needle biopsy technique at rest (prior to the exercise bout) and at 30 min after the exercise bout, alternating between the legs. All biopsy samples were frozen in liquid nitrogen and stored at -80° C until further analysis.

Mouse mRNA Analysis: Isolation of RNA and subsequent cDNA synthesis from the gastrocnemius muscles and mouse testes (positive control) was performed according to the previously described techniques (98, 188). Specific primers for mouse *Brca1* were as follows: forward 5'-CAC AGC GTA TGC CAG AGA AA-3' and reverse 5'ATC CTG GGA GTT TGC ATT TG-3'.

Human mRNA Analysis: Total RNA from the skeletal muscle biopsies was isolated using standard methods (34) and real-time RT-PCR was used to measure two short *BRCA1* splice variants (*BRCA1 Δ11, BRCA1 Δ11b*) and total *BRCA1* (*BRCA1* total). Amplicons were synthesized using previously described sequences (212). GAPDH was used as an internal control (4352934E, Applied Biosystems Inc.). Primer efficiency was tested with standard titration curves and did not differ between the primer pairs. The expression of each target was determined by 2^{-DDCT} (DeltaDelta Ct method) (214).

Homogenization. Mouse skeletal muscle was mechanically homogenized according to previously described techniques (98, 216). Human skeletal muscle biopsies were homogenized on ice using glass-on-glass homogenizers in RIPA

buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.5 % Na Deoxycholate, 0.1 % SDS, 1 % Triton X-100, Protein inhibitor cocktail complete mini (Roche Diagnostics)). Total protein was determined in each sample using the Pierce BCA protein assay (mouse) or Bradford protein assay (human) as previously described (97, 153).

BRCA1 Immunoprecipitation. Immunoprecipitation of endogenous BRCA1 protein in mouse (500μg total protein) or human (150μg total protein) skeletal muscle homogenate was performed with 2μg BRCA1 antibody (I-20, sc-646, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated, rocking, overnight at 4 ^{o}C. The antigen-antibody complex was combined with protein A affinity and then washed through repeated centrifugation steps. After the final wash the pellet was suspended in sample buffer and heated to 100 ^{o}C for 5 min. The sample was then cooled and the eluted protein was loaded onto an SDS-PAGE gel for western blot analysis.

Immunoblotting analysis. Immunoblotting for either BRCA1 immunoprecipitation solution or whole muscle homogenate protein was performed as previously described (97, 98, 219). Membranes were probed with an antibody specific for BRCA1 (I-20; or D-20)(1:200; Santa Cruz Biotechnology, Santa Cruz, CA), ACC-p or ACC total (1:1000; Cell Signal; Boston, MA), Akt-p or Akt total (1:1000; Cell Signal; Boston, MA), α-actinin (1:5000; Sigma-Aldrich; Saint Louis, MO), RFP (1:2000; Thermo Scientific, Waltham, MA). MCF7 cell lysates or mammary gland tissue from BRCA1 knockout mice (BRCA1 MG KO)

were used as a positive or negative control, respectively. BRCA1 MG KO lysates also served as a positive control to detect the BRCA1 protein short splice variants (*BRCA1Δ11, BRCA1Δ11b*) as previously described (10, 41, 145, 202).

Nuclear and Cytoplasmic fractions and BRCA1 Immunoblotting. Nuclear and cytoplasmic fractions were prepared using a Nuclear and Cytoplasmic Extraction Kit (NE-PER® Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific 78833) as previously described (120). Purity and enrichment of nuclear fractions was demonstrated by measuring nuclear protein Lamin A/C and the cytosolic protein β-tubulin (T5201; Sigma-Aldrich; Saint Louis, MO) as previously described (75).

Mouse Malonyl CoA Measures. Malonyl CoA measures were performed on the gastrocnemius muscles from sedentary and acute exercise female and male mice as previously described (97).

Human Myoblast Cell Culture. Human skeletal muscle myoblasts and medias were purchased from Zen-bio (RTP, Research Triangle Park, N.C). Human skeletal myoblasts were derived from the vastus lateralis muscle biopsy from a healthy, lean (normal BMI), twenty four year old female and only used at low passage number (<7). Myoblasts were induced to differentiate to myotubes upon reaching ~90% confluency. All plates were visually examined to ensure myotubes covered ~90% of the well prior to any experimental utilization.

Adenovirus shRNA-hBRCA1 Myotube Infection. To reduce BRCA1 content in the human myotubes, the cells were transduced with either scrambled shRNA

adenovirus (scrambled-shRNA) or adenovirus containing an shRNA sequence targeting the coding region of *BRCA1* (nt.530-550_NM_007294)(shRNA-hBRCA1) and containing a red fluorescent protein tag overnight (Vector Biolabs, Philadelphia, PA). Myotubes were then returned to regular growth media for 48hrs and equivalent adenovirus infection was confirmed via imaging detection of RFP. *BRCA1* mRNA was isolated from adenovirus infected human myotubes as previously described (123). Reduction in *BRCA1* mRNA expression was confirmed in human myotubes 72hrs post adenovirus infection using the same primers as above for *BRCA1 total*, *BRCA1 Δ11, BRCA1 Δ11b.*

Mitochondrial oxygen consumption. Mitochondrial oxygen consumption rates were measured in shRNA-hBRCA1 or scrambled-shRNA treated human myotubes similar to our previously described technique (97, 175). In parallel plates, ACC phosphorylation was assessed after AICAR treatment in the same myotubes with either normal or reduced *BRCA1* content.

Human Myotube Insulin Signaling. Scrambled-shRNA or shRNA-hBRCA1 treated human myotubes were serum starved for 4hrs in DMEM. Myotubes were then either control treated or treated with 50nM insulin for 30 minutes as previously described (187). Protein was then isolated from control treated or insulin treated scrambled-shRNA or shRNA-hBRCA1 human myotubes as previously described (190).

Human Myotube Palmitate/Oleate Incubation and Bodipy Imaging. shRNA-hBRCA1 or scrambled-shRNA treated human myotubes were incubated in 30μM

BSA-conjugated palmitate/oleate mixture in DMEM for 4hrs as previously described (207). Myotubes were then stained with BODIPY (Molecular Probes, Carlsbad Calif, USA) as previously described (189). Myotubes were imaged with a Zeiss Axiovision 4 (Zeiss, Oberkochen, Germany) as previously described (106, 207).

Human Myotube ROS measures and imaging. Scrambled-shRNA or shRNA-hBRCA1 treated human myotubes were placed in H2-DCF supplemented KRB buffer for 30 mins at 37° and then washed 3 times. H2-DCF signed was quantified using a fluorescent plate reader (H2, Biotek, Burlington, VT) as previously described. The myotubes were also imaged Zeiss Axiovision 4 (Zeiss, Oberkochen, Germany).

Statistical Analysis

All data are represented as the mean ± SEM. Statistical analysis was conducted using ANOVA or t-test approaches with post-hoc tests (Tukey) employed when appropriate. A *P*-value of ≤ 0.05 was considered significant.

RESULTS:

Brca1 Expression in Mouse Skeletal Muscle. In mouse skeletal muscle, *Brca1* mRNA and protein were detectable (Figure 4.1A-B). In addition, using standard immunoblotting or an immunoprecipitation approach with different antibodies specific to either the N- or C-terminus region we consistently detected Brca1 (220 kDa) in whole muscle homogenates (Figure 4.1 B-C). The resulting protein mass

is consistent with previously published data concerning BRCA1 in other tissues (126, 146, 207, 211).

In reproductive tissue, *BRCA1* is an estrogen sensitive gene (154), however in the gastrocnemius muscle of mice we detected greater levels of both the full-length and short (Brca1Δ11, 85kDa as previously defined (10)) Brca1 splice variants in males compared to females (Figure 4.1D). Full-length Brca1 protein levels did not differ between gastrocnemius and tibialis anterior (TA) muscles within female or male mice, respectively (Figure 4.1E,F). Further, Brca1 protein was undetectable in the soleus muscle (Figure 4.1E,F). In female mice, no differences were detected in Brca1Δ11 protein content in the soleus, TA, or gastrocnemius muscle (Figure 4.1E). In contrast, Brca1Δ11 was significantly higher in TA and soleus compared to the gastrocnemius muscle in male mice ($P \leq 0.05$) (Figure 4.1F).

Mouse Brca1 Acc-p Acute Exercise Induced Protein Interaction. In order to assess the potential interaction between the endogenous forms of Acc-p and Brca1, mice were subjected to an acute bout of treadmill exercise. Exercise induced significant elevations in the ratio of Acc-p/Acc-total in both male and female gastrocnemius muscle compared to sedentary animals ($P \leq 0.05$) (Figure 4.2A,B). As expected, MaCoA content significantly decreased in response to the exercise bout in the female animals, but surprisingly the increase in Acc-p levels did not correspond to a decline in MaCoA levels in the male mice (Figure 4.2C). Further, MaCoA levels in sedentary female skeletal muscle were significantly higher

compared to sedentary males ($P \leq 0.05$) (Figure 4.2C). In response to the exercise bout there was a significant increase in Brca1-Acc-p protein-protein interaction in both female and male gastrocnemius muscle compared to sedentary animals ($P \leq 0.05$) (Figure 4.2D). However, in females, the exercise induced Brca1-Acc-p interaction tended to be greater than males ($P = 0.09$) (Figure 4.2D).

BRCA1 mRNA and Protein Expression in Human Skeletal Muscle. In an effort to translate the observations found in a murine model, similar experiments were performed in humans. *BRCA1* mRNA (full length and splice variants) was detected in human skeletal muscle, with no differences between sedentary women and men (Figure 4.3A). The two well-recognized short variants in human tissue, *BRCA1Δ11* and *BRCA1Δ11b* mRNA, were readily expressed in human skeletal muscle with no sex differences (Figure 4.3B,C). Using antibodies specific to the C-and N-terminal regions, the presence of BRCA1 protein in human skeletal muscle was verified (Figure 4.3D), with no detectable sex differences (Figure 4.3E). Previous research has suggested that BRCA1 isoforms exhibit specific intracellular localization (146, 212). Thus, BRCA1 protein content was measured in nuclear and cytosolic fractions from male and female skeletal muscle biopsies. The short splice variants (lacking nuclear localization signal, BRCA1Δ11 and BRCA1Δ11b) of BRCA1 were almost exclusively detected in the cytosolic fraction whereas the full length BRCA1 protein was predominantly detected in the nuclear fraction (Figure 4.3F). Although mRNA for *BRCA1Δ11* and *BRCA1Δ11b* were individually detected, it was not possible to distinguish the protein products due to similar molecular weights.

Acute Exercise in Humans Induced ACC-p and BRCA1 Protein Interaction. To assess the role of BRCA1 in skeletal muscle, women and men performed an acute bout of exercise. The exercise bout resulted in a significant increase in ACC-p/ACC compared to pre exercise values ($P \leq 0.05$) (Figure 4.4A-B). There was considerable individual variability in the magnitude of interaction between ACC-p and BRCA1 in both male and females prior to and after the completion of the exercise bout (Figure 4.4C). When comparing the average group response, males demonstrated a significant increase in endogenous ACC-p and BRCA1 interaction ($P \leq 0.05$), while women presented with a trend for increased endogenous protein interaction in response to the acute exercise bout ($P = 0.07$) (Figure 4.4C).

Reductions in *BRCA1* expression results in accumulation of intramyocellular lipid and reduced insulin signaling. Infection of human myotubes with shRNA-hBRCA1 resulted in nearly undetectable *BRCA1* mRNA expression compared to infection with scrambled shRNA (Figure 4.5A). Reduced *BRCA1* expression (shRNA-hBRCA1) in human myotubes resulted in accumulation of intracellular neutral lipid storage compared to control myotubes (scrambled-shRNA) (Figure 4.5B). When myotubes were treated with conjugated-FFA, we observed an additional increase in lipid storage in myotubes with reduced *BRCA1* expression compared to treated control myotubes (Figure 4.5B). Myotubes with reduced BRCA1 expression exhibited reduced Akt phosphorylation in response to insulin treatment (50nM for 30mins) compared to the insulin response in the control myotubes (Figure 4.5C).

Reductions in *BRCA1* expression results in decreased mitochondrial oxygen consumption in human myotubes. Human myotubes with reduced *BRCA1* expression exhibited lower basal oxygen consumption rates (OCR) compared to scrambled-shRNA myotubes ($P \leq 0.05$) (Figure 4.5D). OCR was lower in shRNA-hBRCA1 treated myotubes after mitochondrial uncoupling induction compared to scrambled-shRNA myotubes (Figure 4.5E). Similar reductions in OCR were seen between groups after the addition of palmitate (Figure 4.5F).

Reductions in *BRCA1* expression results in reduced AICAR-induced phosphorylation of ACC in human myotubes. AICAR can induce-phosphorylation of ACC via activation of AMPK, thus we determined the ability of AICAR to induce phosphorylation of ACC under reduced BRCA1 conditions. ACC-p content did not increase after AICAR treatment of the human myotubes infected with shRNA-hBRCA1 compared to scrambled-shRNA treated myotubes (Figure 4.5G-H). However, we were surprised to find that reducing BRCA1 content resulted in elevated phosphorylation of AMPK and thus increased phosphorylation of ACC suggesting that BRCA1 is not required for phosphorylation of ACC.

Reduced *BRCA1* expression increases ROS production leading to AMPK phosphorylation. In order to determine why ablation of BRCA1 in the human myotubes led to hyper-activation of AMPK, we assessed global ROS content in myotubes with or without BRCA1. Using a global ROS indicator (H2-DCF) (Figure 4.6A-B) we found higher ROS content in myotubes transduced with

shRNA-hBRCA1 compared to scrambled-shRNA treated myotubes (Figure 4.6A-B).

DISCUSSION:

BRCA1 is a large, polyfunctional protein (220kDa) that regulates a variety of intracellular functions through multiple mechanisms including protein-protein interactions (48). To date, BRCA1 has been predominantly recognized as a cell cycle regulator and a DNA damage repair protein in reproductive tissues (48). However, the data presented here provide evidence for a novel functional role for BRCA1 as a regulator of metabolic function in skeletal muscle of both mice and humans (Figure 7). Specifically, in response to an energetic stress (i.e. acute exercise) there is a significant increase in the interaction of BRCA1 with the phosphorylated form of ACC. Reducing BRCA1 content in human myotubes resulted in increased lipid storage, decreased insulin signaling, reduced mitochondrial function, and enhanced ROS production. Overall, our observations provide evidence for a previously un-described role for BRCA1 and lend support for more detailed examinations of the role of BRCA1 as a metabolic regulator in skeletal muscle.

Genetic alteration in the BRCA1 sequence and/or alterations in BRCA1 expression are strongly correlated with risk of tumorigenesis in reproductive tissues (134). However, a functional role involving BRCA1 in skeletal muscle has gone unrecognized even though BRCA1 mRNA content is known to increase in differentiating C2C12 myoblasts (109). Our data significantly extend this

initial observation by demonstrating that both the long and short BRCA1 isoforms are present at the mRNA and protein level in adult mouse skeletal muscle and in human skeletal muscle. Consistent with previous literature, the full-length BRCA1 proteins, which have an intact NLS (202, 212), were detected predominantly in the nuclear fraction of human skeletal muscle while the short splice variants, which lack an NLS (212), were detected in the cytosolic fraction. It is well established that *BRCA1* is an estrogen-sensitive gene (35), thus it was surprising to find higher Brca1 and Brca1Δ11 content in male than in female mouse gastrocnemius muscle. However, this sex difference did not translate to human skeletal muscle, as neither *BRCA1* mRNA nor protein content differed across sexes. Finally, few differences were detected across skeletal muscle groups in mouse Brca1 expression and protein content. In fact, only the male mice presented with significant differences in BRCA1Δ11 across muscle groups with the TA and the soleus muscles having greater levels of BRCA1Δ11 compared to the gastrocnemius muscle. In male mice, including the soleus muscle, most muscles are composed of a mixed phenotype thus it is unclear why TA and/or soleus muscle would have significant differences in only BRCA1Δ11 (24). In conclusion, our data demonstrate that multiple recognized forms of BRCA1 are detectable at both the mRNA and protein level in skeletal muscle from both mice and humans.

In a series of elegant experiments, BRCA1 was previously shown to directly bind to the phosphorylated form of ACC in mammary cells (126, 148). ACC is a critical regulator of lipid metabolism as it catalyzes the production of

MaCoA in skeletal muscle (3, 160, 213). MaCoA can act as a potent allosteric inhibitor of CPT-1 or can serve as a precursor for lipid synthesis (137, 213), with the former being the likely mechanism for ACC regulation of lipid metabolism in skeletal muscle (160, 191). Using an acute bout of exercise to energetically challenge the muscle resulted in increased ACC-p content in skeletal muscle. The increase in ACC-p also resulted in increased interaction between BRCA1 and ACC-p. Further, our data in the mouse indicate differential Brca1/Acc-p interactions in females and males both at rest and in response to an acute bout of exercise, which was also associated with differing content of MaCoA in the same muscle. To the best of our knowledge, this is the first time a sex-based difference in skeletal muscle MaCoA content has been demonstrated. Re-analysis of data from a previous publication (204) where male and female mice were treated with AICAR demonstrated a similar finding. Specifically, female mice presented with higher levels of resting MaCoA and a greater response to AICAR treatment than age-matched male mice. Unfortunately, due to limited sample amounts we were not able to directly measure MaCoA content in the human samples to compare the BRCA1-ACC-p interaction in a quantitative fashion, thus it is currently unclear if there was also a differential response in our human samples. Regardless, the data clearly indicate that in response to an acute bout of exercise there is a resulting increase in the interaction of BRCA1-ACC-p in skeletal muscle in both mice and humans.

To determine if BRCA1 plays a critical role in regulation of skeletal muscle metabolic function we reduced *BRCA1* expression through shRNA

technology. Reducing *BRCA1* content in human myotubes resulted in increased neutral lipid storage and resulted in a concurrent reduction in mitochondrial function in the myotubes. Thus, our results in skeletal muscle are in agreement with previous findings in MCF7 cells that reductions in *BRCA1* expression results in increased lipid storage (148). In muscles cells, ACC2 localizes to the mitochondria while in non-muscle cells, BRCA1 can localize to the mitochondria (2, 37) which when coupled with our results suggests a likely role for BRCA1 regulating mitochondrial function. Our data would indicate that BRCA1 is at least in part influencing mitochondrial function via interaction with ACC, however alternative mechanisms are likely contributing. Specifically, our data indicate ACC phosphorylation can occur independent of BRCA1 since loss of BRCA1 induced an increase in AMPK phosphorylation by inducing ROS production. Others have found in breast cancer cells, that BRCA1 reduced ROS production and protected macromolecules against oxidative damage (172). The increased production of ROS in the absence of BRCA1 likely explains the hyper-activation of AMPK and thus downstream phosphorylation of ACC. Specifically, in cultured skeletal muscle cells ROS exposure induces substantial increases in AMPK phosphorylation (92). These data suggest that phosphorylation of ACC is not dependent upon BRCA1 expression, but would suggest that BRCA1 encourages the maintenance of ACC phosphorylation (148).

Loss of BRCA1 expression also resulted in a lower Akt phosphorylation response to acute insulin exposure suggesting that optimal insulin signaling requires BRCA1. This is in contrast to results in breast cancer cells where

BRCA1 is thought to negatively regulate the oncogenic function of Akt (226). At this time, BRCA1 expression appears to be necessary for optimal metabolic function in skeletal muscle, however further examination of the role of BRCA1 is necessary. In addition, since our data suggest that decreases in BRCA1 could contribute to metabolic disease susceptibility, further and more detailed exploration of interventions that alter BRCA1 content are warranted.

Due to the large size of the BRCA1 gene there is a substantial amount of documented genetic variation, which results in altered protein function and/or expression (143). Given our data, such variation may affect metabolic function and could be linked to a form of metabolic disease (i.e. obesity, type 2 diabetes, etc). However, to our knowledge no single nucleotide polymorphisms have been identified in the BRCA1 gene in genome-wide association studies relating such polymorphisms to metabolic dysfunction. The phosphorylated form of ACC interacts with BRCA1 in the C-terminal region, specifically in the BRCT domains (66). Although the BRCT domains are conserved across all confirmed BRCA1 isoforms (126) substantial genetic variation is often present in this region (115). Since the phosphorylated form of ACC binds to the BRCT domain within BRCA1, it is possible that specific sequence variation in this domain may contribute to variations in the degree of BRCA1 function in humans. Indeed, in response to the acute bout of exercise our subjects had a substantial amount of variation in the magnitude of BRCA1-ACC-p interaction. Further examination of this hypothesis will be necessary to determine if documented SNPs within the BRCT domains of BRCA1 are associated with risk development of metabolic disease in humans.

In summary, we have identified that BRCA1 expression is an important regulator of metabolism in both mouse and human skeletal muscle. These findings significantly extend our understanding of BRCA1 physiology by considering mechanistic aspects outside of its classically known role as a regulator of DNA repair and cell cycle in reproductive tissues. We propose that our data collectively identifies BRCA1 as a regulator of ACC dynamics and overall metabolic function of skeletal muscle. Future studies will be necessary to examine the therapeutic potential of manipulating BRCA1 expression and/or function in skeletal muscle as a means to prevent the development of metabolic diseases.

Acknowledgements

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FIGURES:

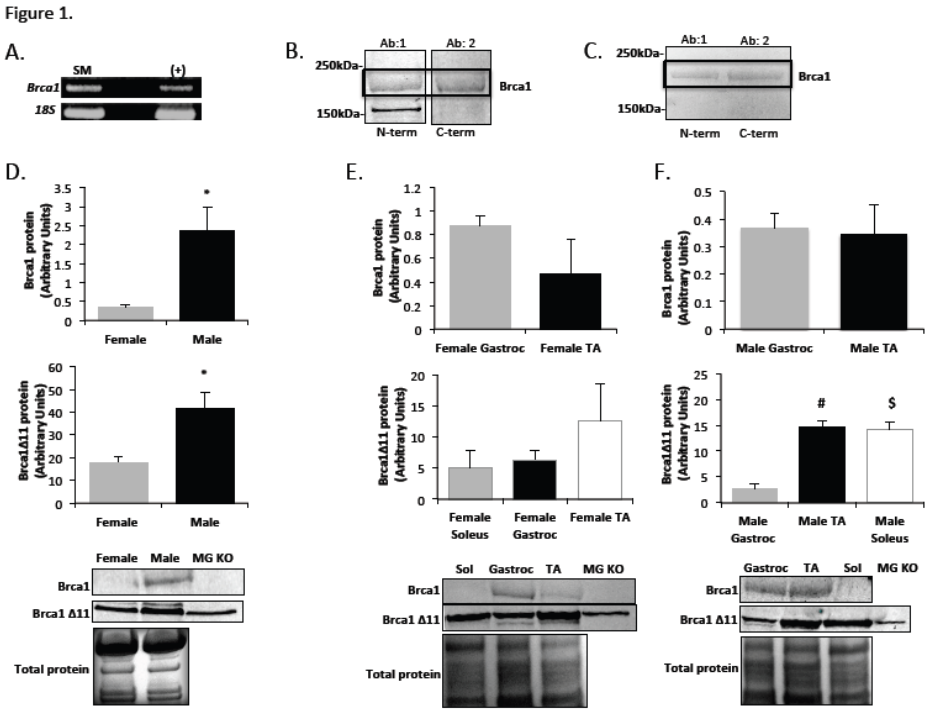

Figure 4.1: Identification of Brca1 in male and female mouse skeletal muscle. (A) *Brca1* mRNA detection identified in female mouse gastrocnemius muscle (SM) with mouse testes serving as a positive control (B) Protein identification and verification of Brca1 (220kDa) using an antibody comparison Ab1: (SC-I:20) Brca1 N-terminal specific antibody Ab:2 (SC-D:20) Brca1 C-terminal specific antibody. (C) Immunoprecipitation Brca1 in female mouse skeletal muscle using Ab1 and Ab2. (D) Brca1 protein was greater in males compared to female mouse gastrocnemius muscle ($P \leq 0.05$). No significant differences were detected between male and female mice for Brca1Δ11 (78kDa). Mammary gland from BRCA1 knockout mouse (MG KO) served as a negative control for full length Brca1 and a positive control for the Brca1 splice variant (Brca1Δ11). (E) No differences in Brca1 and Brca1Δ11 protein content in soleus, gastrocnemius, and tibialis anterior (TA) muscles from adult female mice were detected. (F) In skeletal muscle from adult male mice, no differences across muscle groups were detected in Brca1 protein content, while Brca1Δ11 content as significantly higher in the TA and soleus compared to the gastrocnemius muscle. Total protein staining gels were used to ensure equal loading of protein across samples. Data are presented as mean + SEM (n=3 mice per group for western blotting measures) $^{*}P \leq 0.05$ female vs. male; $^{\#}P \leq 0.05$ TA vs. gastroc; $^{\$}P \leq 0.05$ TA vs. soleus.

Figure 2

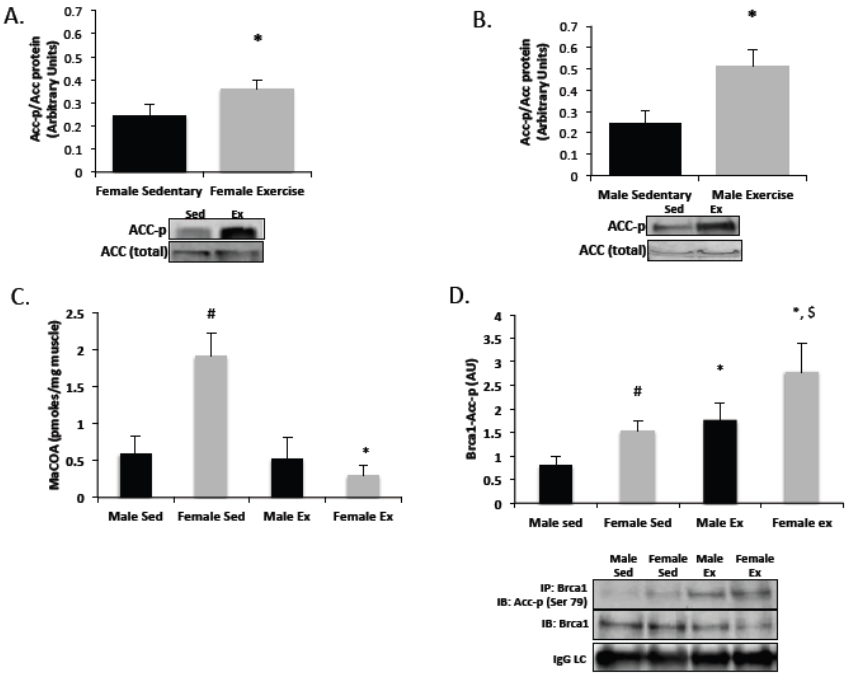

Figure 4.2: An acute bout of exercise increases endogenous Acc-p and Brca1 interaction in skeletal muscle in adult male and female mice. (A-B) Acc-p levels were significantly higher in response to an acute bout of exercise in the gastrocnemius muscle from adult female and male mice compared to sedentary mice. (C) MaCoA levels were significantly higher in gastrocnemius muscles from sedentary female mice compared to sedentary male mice. Adult female mice exposed to an acute bout of exercise had significantly lower MaCoA levels compared to the female sedentary mice and no significant differences were apparent in gastrocnemius muscle from male mice. (D) Interaction between Brca1 and Acc-p was greater in gastrocnemius muscle from sedentary females compared to sedentary males. Brca1 and Acc-p interaction was significantly higher after an acute bout of exercise in gastrocnemius muscles from both male and female mice when compared to their sedentary counterparts. Data are presented as mean + SEM (n=6-7 per group) $^{*}P \leq 0.05$ sedentary vs. exercise; $^{\#}P \leq 0.05$ male sedentary vs. female sedentary; $^{\$}P=0.09$ male exercise vs. female exercise.

Figure 3.

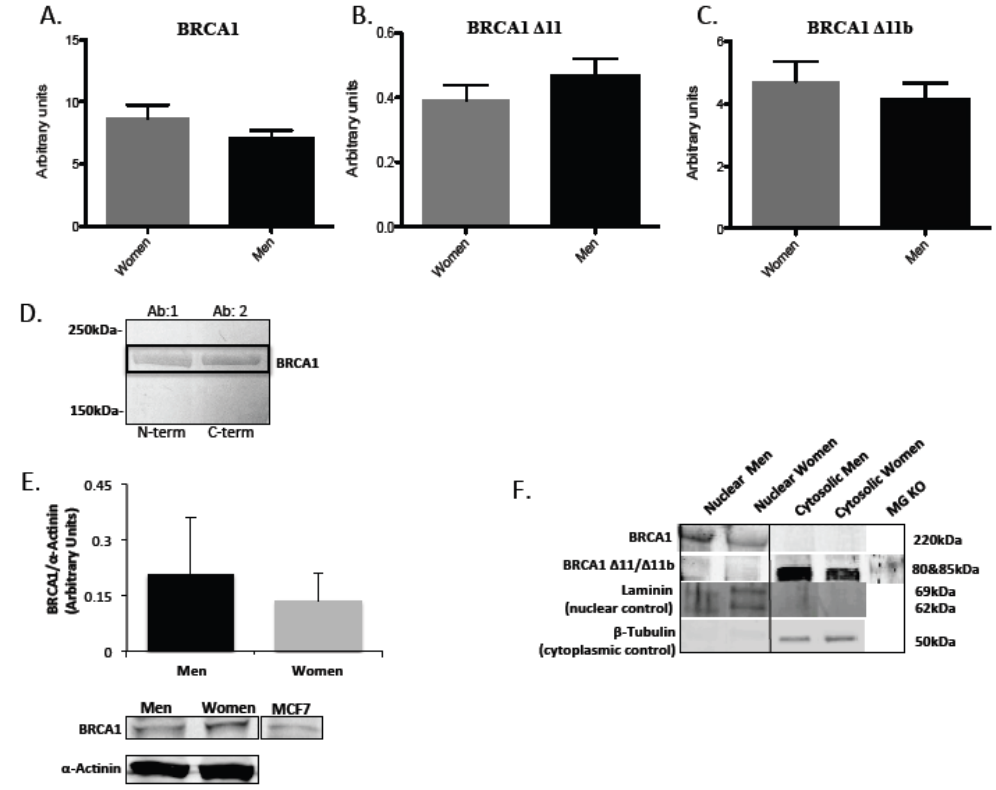

Figure 4.3. BRCA1 expression is detectable in biopsies taken from human vastus lateralis (VL). (A-C) No significant differences were detected in mRNA expression of all recognized human *BRCA1* variants in skeletal muscle biopsies taken from males and females. All data were normalized to GAPDH (D). The presence of BRCA1 protein in human skeletal muscle was verified using antibodies specific to the N-terminus (Ab:1) or C-terminus (Ab:2). (E) No significant differences in BRCA1 protein were detected between men and women in biopsies from the VL. (F) Full length BRCA1 was detected predominantly in the nuclear fraction isolated from the VL from men and women whereas the BRCA1Δ11/ Δ11b splice variant was detected in the cytoplasmic fraction. Cell lysates isolated from the mammary gland of BRCA1 knockout animals as a positive control for the BRCA1Δ11/ Δ11b splice variants. Laminin was used as a nuclear control protein and β-tubulin as the cytoplasmic control protein. Data are presented as mean + SEM (n=13 per group for mRNA analysis; n=5-6 per group for BRCA1 western blot analysis).

Figure 4.

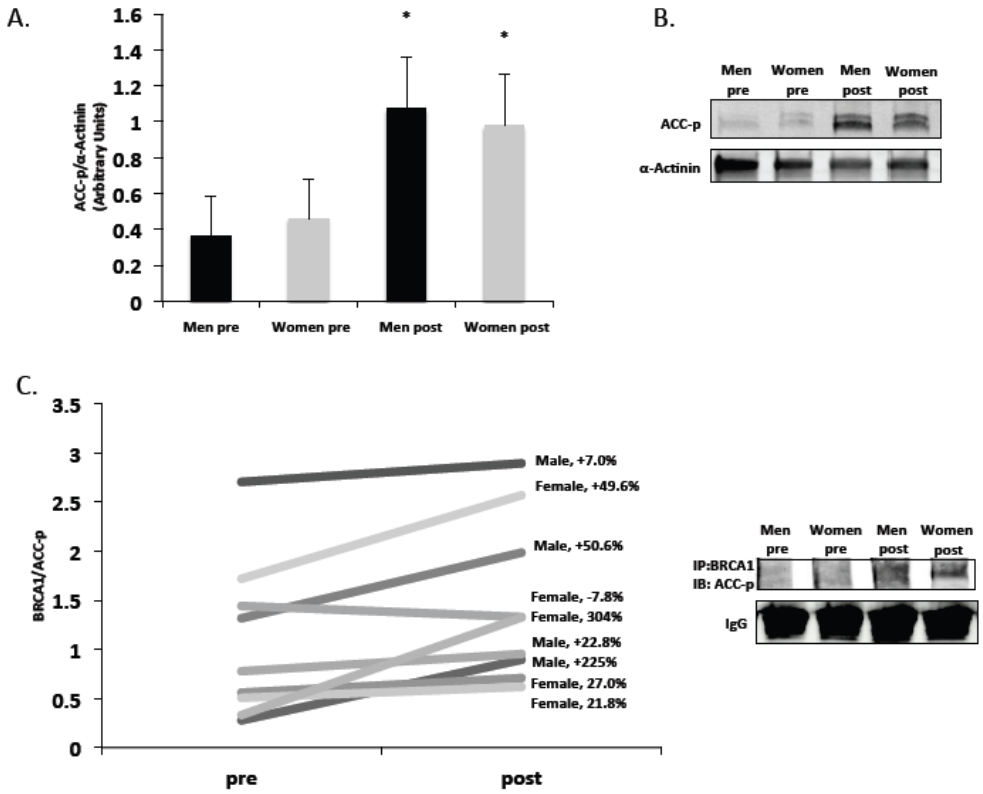

Figure 4.4. An acute bout of exercise in men and women resulted in a greater interaction between the phosphorylated form of ACC and BRCA1 in muscle biopsies taken from the VL. (A-B) Significant increases were found in ACC-p content compared to pre exercise in both male and female subjects. (C) In response to an acute bout of exercise in men and women, the majority of subjects had increased BRCA1-ACC-p interaction compared to pre exercise. Each line represents an individual subject and is marked with the sex and magnitude of response. Data are presented as mean + SEM (n=5-6 per group for BRCA1 western blot analysis); *$P \leq 0.05$ sedentary vs. exercise within female or male subjects.

Figure 5.

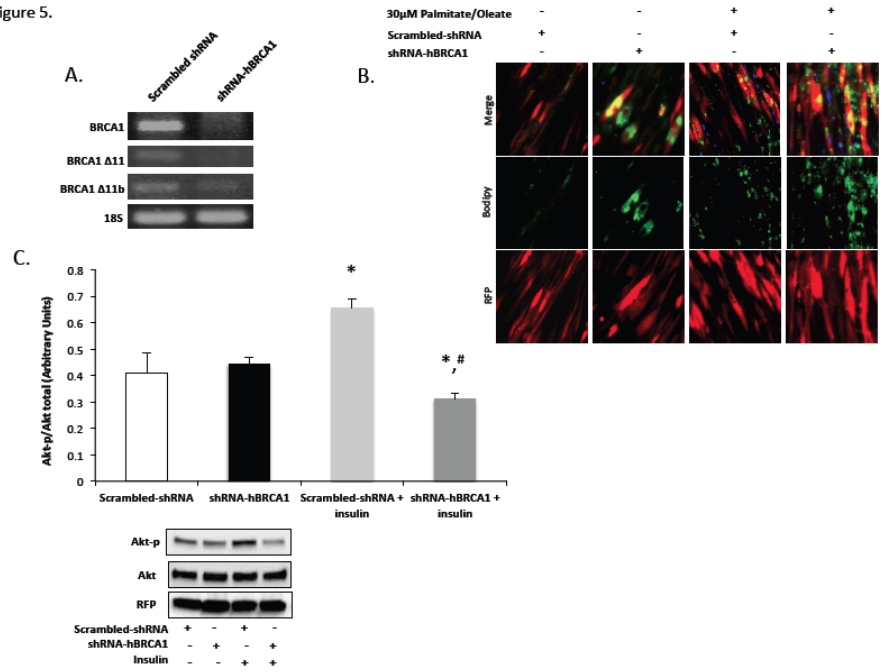
A.
Scrambled shRNA
shRNA-hBRCA1
BRCA1
BRCA1 Δ11
BRCA1 Δ11b
18S
B.
30μM Palmitate/Oleate
Scrambled-shRNA
shRNA-hBRCA1
Merge
Bodipy
RFP
C.
Akt-p/Akt total (Arbitrary Units)
Scrambled-shRNA
shRNA-hBRCA1
Scrambled-shRNA + insulin
shRNA-hBRCA1 + insulin
Akt-p
Akt
RFP
Insulin

Figure 5. (cont)

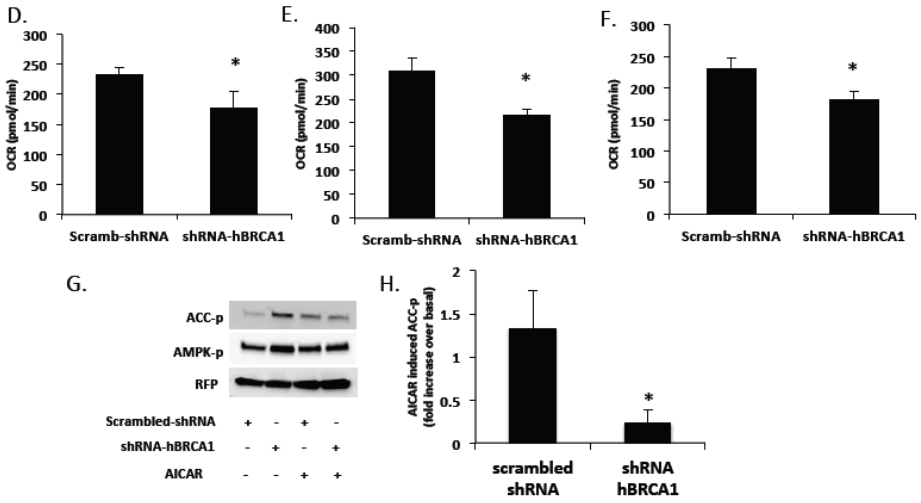

Figure 4.5. Reduction in *BRCA1* content enhances neutral lipid storage and decreases mitochondrial function in primary human myotubes. (A) Human myotubes transduced with shRNA for human BRCA1 (shRNA-hBRCA1) presented with reduced BRCA1 total, BRCA1Δ11 and BRCA1Δ11b compared to cells infected with scrambled-shRNA. (B) Myotubes with reduced *BRCA1* expression exposed to either BSA or 30uM palmitate/oleate conjugated BSA exhibited increased neutral lipid accumulation in myotubes compared to myotubes infected with scrambled shRNA. (C). Insulin-induced phosphorylation of Akt was reduced in myotubes with reduced BRCA1 expression compared to control myotubes. Myotubes were treated with 50nM insulin for 30 mins. (D) Basal OCR was reduced in shRNA-hBRCA1human myotubes compared to Scrambled-shRNA myotubes. (E) Uncoupling the mitochondria with FCCP resulted in a reduced OCR in shRNA-hBRCA1 myotubes compared to Scrambled-shRNA. (F) Palmitate stimulated OCR was significantly reduced in shRNA-hBRCA1 myotubes compared to Scrambled-shRNA myotubes. (G) No difference in mitochondrial protein ATP 5A was detected in scrambled-shRNA and shRNA-hBRCA1 myotubes. Equal loading was confirmed through Red Fluorescent Protein (RFP). (H) ACC-p response was attenuated in oligomycin treated shRNA-hBRCA1 compared to scrambled-shRNA. Data are presented as mean + SEM (n=3-5 per group for all analyses); *$P \leq 0.05$ scrambled-shRNA vs. shRNA-hBRCA1 myotubes.

Figure 6

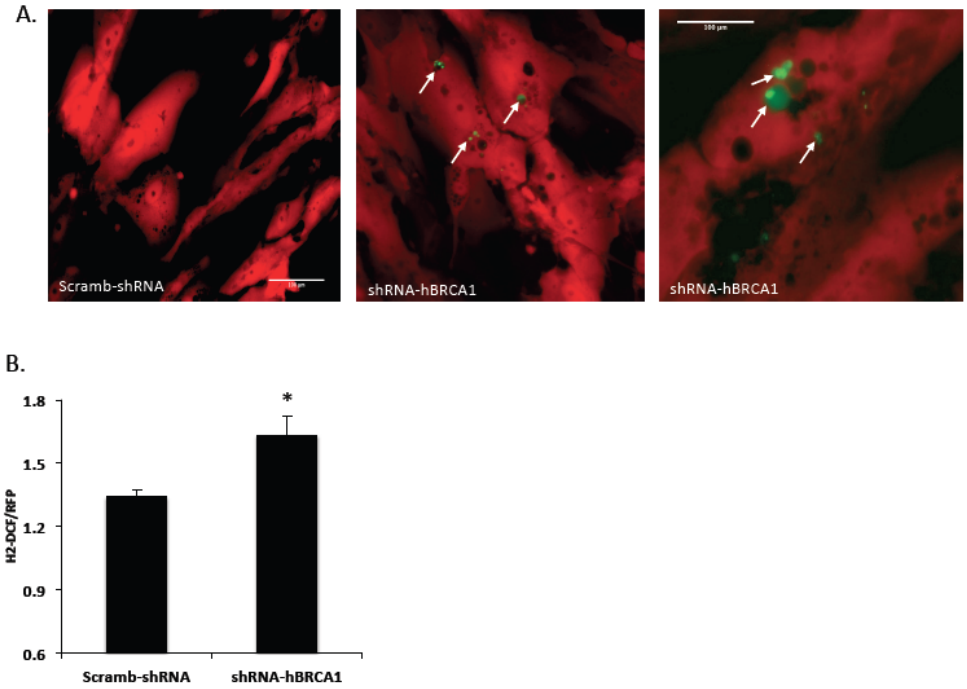

Figure 4.6. Reduction in *BRCA1* content enhances ROS production in primary human myotubes. (A) Human myotubes transduced shRNA-BRCA1 exhibit visual increases in ROS signal (green-marked with arrows) compared to cells infected with scrambled-shRNA. (B) Myotubes with reduced *BRCA1* (shRNA-BRCA1) expression exhibited increased basal ROS production in myotubes compared to myotubes infected with scrambled shRNA. Data are presented as mean + SEM (n=3-5 per group for all analyses); **P*≤0.05 scrambled-shRNA vs. shRNA-hBRCA1 myotubes.

Figure 7.

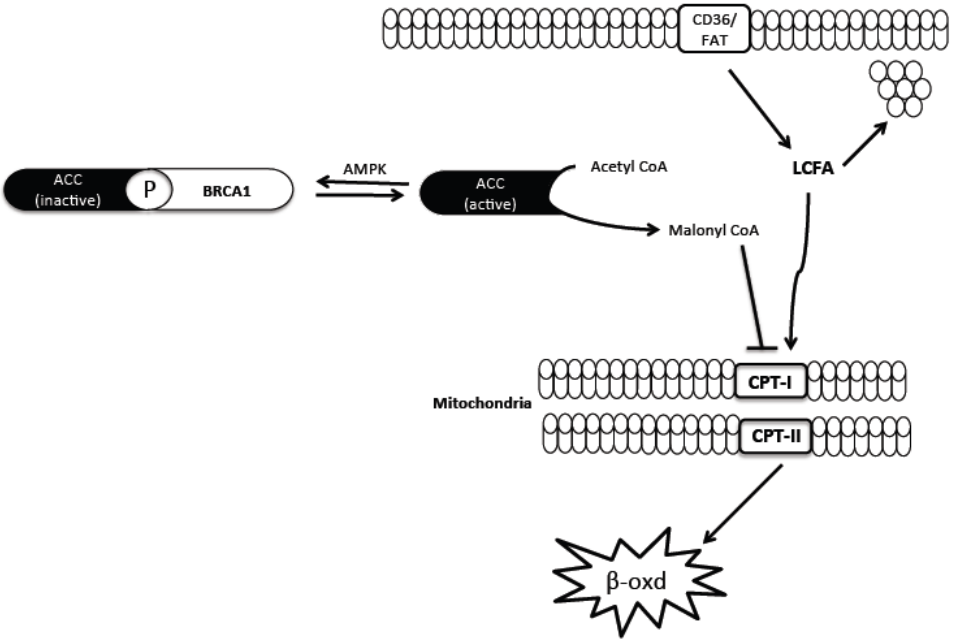

Figure 4.7: Schematic representation of the proposed mechanism for BRCA1 in skeletal muscle.

Chapter 5: Deletion of Brca1 in adult skeletal muscle results in altered skeletal muscle function and protection from high fat diet-induced metabolic insults

The following is a manuscript in preparation based on my final dissertation work.

Deletion of Brca1 in adult skeletal muscle results in altered skeletal muscle function and protection from high fat diet-induced metabolic insults

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ABSTRACT:

Breast cancer type 1 susceptibility protein (BRCA1) is a large polyfunctional protein commonly associated with breast cell carcinoma in humans due to its established role as a cell cycle regulator. Recently, we identified BRCA1 as a critical regulator of lipid metabolism in skeletal muscle. However, the role of BRCA1 in an *in* vivo, adult skeletal muscle model has yet to be determined. Here we employed an inducible, skeletal muscle-specific, Brca1 knockout mouse model to further elucidate the role of Brca1 in skeletal muscle. At 10 weeks of age male and female mice were injected with a tamoxifen solution inducing flox-mediated recombination of the Brca1 gene (KO) or vehicle-based solution in age-matched wildtype (WT) mice. The mice were then placed on a control (CD) or high fat diet (HFD). Loss of Brca1 in skeletal muscle attenuated HFD-induced gains in body weight, visceral fat mass, and/or liver mass when compared to WT+HFD. Female KO mice demonstrated an onset of kyphosis that was surprisingly attenuated by the HFD with no other groups exhibiting this effect. Regardless of diet, KO mice exhibited lower maximal treadmill speed compared to the WT+CD group. In addition, KO mice demonstrated protection from HFD-induced glucose intolerance when compared to the WT-HFD group. Similarly, Brca1 KO mice had lower IMCL content in response to HFD compared to WT mice despite lower skeletal muscle mitochondrial function measures. Lastly, loss of functional Brca1 resulted in less contraction-induced phosphorylation of Ampk and Acc in both KO+CD and KO+HFD mice. However, basal Acc-p levels were higher in the sedentary muscle from KO+CD mice compared to WT+CD mice.

Collectively, these data indicate that loss of functional Brca1 in skeletal muscle attenuates the metabolic consequences of HFD despite impaired mitochondrial function and alterations in Ampk/Acc phosphorylation.

INTRODUCTION:

Whole body metabolic function is largely influenced by changes in skeletal muscle lipid and/or carbohydrate metabolism. In particular, it is well documented that disruptions in the ability to either oxidize or store lipid is associated with a number of different chronic health conditions. Thus, the identification of mechanisms that regulate lipid metabolism in skeletal muscle is critical to elucidating approaches for attenuating the onset of various metabolic-based diseases.

Breast cancer 1 early onset gene (*Brca1*) is a caretaker gene that is highly susceptible to mutations and predominantly associated with breast cancer in women (48, 50, 142, 184). The corresponding protein to *Brca1,* breast cancer type 1 susceptibility protein (Brca1), is a large, polyfunctional protein that regulates a variety of intracellular mechanisms (23, 48, 50, 60, 148, 172). Historically, Brca1 is associated with mechanisms that regulate DNA repair or the cell cycle (48, 109, 184). However, recent evidence has also implicated BRCA1 as critical regulator of metabolic function in breast cancer cells (23, 126, 148).

In MCF7 cells, Brca1 regulates lipid metabolism dynamics through direct interactions with the phosphorylated form of acetyl CoA carboxylase (Acc) (126, 148). When active, Acc inhibits lipid entry into the mitochondria through

production of malonyl CoA, a potent allosteric inhibitor of the mitochondria lipid transport enzyme, carnitine palmitoyl transferase-I (CPT-1) (140, 160, 213). In addition, malonyl CoA directly contributes to *de novo* intracellular synthesis of palmitic acid through the enzymatic reaction catalyzed by fatty acid synthase (FAS) (140). The interaction between Brca1 and Acc-p could maintain phosphorylation of Acc, thereby inactivating the enzyme and subsequently promoting CPT-1 activity (160, 161). In addition, Brca1 has been found to localize to the mitochondria, suggesting a potential role in the regulation of mitochondrial function (37, 131). Therefore, although Brca1 plays a critical role in regulation of metabolism in breast cancer cells, the role of Brca1 in other metabolically active tissues remains poorly defined.

Although Brca1 is expressed in skeletal muscle, very little is known about the function of this protein in skeletal muscle. Original descriptions of Brca1 in skeletal muscle were limited results that described increases levels of expression during the differentiation process of immortalized skeletal muscle myoblasts (109). We have previously demonstrated in both murine and human, male and female skeletal muscle models that BRCA1 is present and, similar to human breast cancer cells, binds directly to the phosphorylated form of ACC (96). Further, we also demonstrated that loss of BRCA1 in human myotubes results in impaired mitochondrial function, leading to intramyocellular lipid accumulation (IMCL), impaired insulin stimulation, and increased reactive oxygen species (ROS) production (96). Also, Brca1 expression appears to be critical to the regulation of metabolic function in cardiac muscle (178). Although the *in vitro*

evidence is compelling, it is unclear how critical Brca1 function is to skeletal muscle *in vivo*.

Therefore, the purpose of this study was to assess the *in vivo* functional role of Brca1 using a novel inducible skeletal muscle-specific knockout mouse. Here we demonstrate that *in vivo* ablation of Brca1in skeletal muscle results in physiological alterations, including metabolic changes that implicate Brca1 as a critical regulator of skeletal muscle function.

MATERIALS AND METHODS:

Generation of HSA-Cre(+) Brca1 Flox (+/+) Mouse: Animals from a C57/BL6 background with flanked flox sites surrounding exon 11 of the Brca1 gene (referred to as Brca1 Flox +/+) were bred with a skeletal muscle tissue-specific inducible CreER male mouse model (C57/BL6; HSA-Cre(+)) to generate HSA-Cre (+) Brca1 Flox (+/+) animals, which were used for this study (47, 135) (Figure 1A,B,C).

Animal Genotyping: All animals were genotyped for the presence of HSA-Cre and homozygote Brca1 Flox expression using genomic DNA. Primer sets F-5'-GGCATGGTGGAGATCTTTGA-3' and R-5'-CGACCGGCAAACGGACAGAAGC-3' were used to screen for HSA-Cre (+) animals as previously described (135). Amplification was performed with an initial denaturing step of 95^{o}C for 5min; followed by 32 cycles of the following program: 94^{o}C 30sec, 60^{o}C 1min, 72^{o}C 1min; the final cycle ended with 72^{o}C for 10min. Concurrently, all animals were also genotyped for Brca1 flox using primer

sets 5'-CTGGGTAGTTTGTAAGCATGC-3' and 5'-CAATAAACTGCTGGTCTCAGG-3' where the Brca1 Flox amplicon is ~500bp and the wild type is ~450bp. Amplification was performed with an initial denaturing step of 94^{o}C for 3min; followed by 35 cycles of the following program: 94^{o}C 1min, 60^{o}C 2min, 72^{o}C 1min; the final cycle ended with 72^{o}C for 3min.

***Brca1* recombination DNA/mRNA analysis:**

Cre-mediated recombination was confirmed through PCR analysis of genomic DNA isolated from the plantaris muscle using a DNA isolation kit (Qiagen, Valencia, Ca.). Specifically primer Brca1 Flox-A-5'CTGGGTAGTTTGTAAGCATGC-3' and primer Brca1 Flox-E-CTGCGAGCAGTCTTCAGAAAG-3' were used to generate an amplicon that is approximately 621bp in length indicating the removal of the flox region of Brca1 as previously described (47). Amplification was performed with an initial denaturing step of 95^{o}C for 5min; followed by 30 cycles of the following program: 95^{o}C 30sec, 54^{o}C 1min, 72^{o}C 30sec; the final cycle ended with 72^{o}C for 5min.

To determine tissue specificity, total RNA was isolated from the heart, lung, liver, kidney, soleus, tibialis anterior (TA) and gastrocnemius muscle as previously described and RT/PCR was performed using Reverse Transcription and HotStarTaq PCR kits (Qiagen, Valencia, Ca.) (98). PCR was performed using primer set B004-5'-CTGGGTAGTTTGTAAGCATGC-3' and B006-5'-

CTGCGAGCAGTCTTCAGAAAG-3' which if recombination had occurred in the flox region of *Brca1* an mRNA amplicon of ~600bp would be produced (98). Amplification was performed with an initial denaturing step of 94^{o}C for 3min; followed by 35 cycles of the following program: 94^{o}C 1min, 60^{o}C 2min, 72^{o}C 1min; the final cycle ended with 72^{o}C for 3min.

Experimental Approach:

Ten-week-old HSA-Cre (+) Brca1 Flox (+/+) male and female mice were injected with 12.75mg/mL tamoxifen (KO) or control (WT) solution for 5 consecutive days. Upon completing injections mice underwent a 14-day washout period and then were assigned to either a control diet (CD; 10%kcal fat; D12450K) (KO + CD; WT+ CD) or high fat diet (HFD; 45% kcal fat; D12451) (WT+HFD; KO+HFD) (Research Diets Inc. New Brunswick, N.J.) for a duration of 10 weeks. Food intake and body weight were recorded for the duration of 10 weeks of feeding. At the conclusion of 10 weeks mice were subjected to either a muscle fatigue protocol (n=6/group; paired males and females) as previously described (219) or underwent glucose tolerance testing, insulin tolerance testing, endurance treadmill testing, and isolated skeletal muscle mitochondrial measures (n=6-10/group). Upon completing these measures mice were humanely euthanized and tissue was flash frozen and stored at -80^{o}C.

Exercise Tolerance:

An endurance exercise test was administered for each of the four groups of mice. The endurance test consisted of a three-day treadmill acclimation period

where animals were placed on the treadmill with the electrical shock grid in the rear turned on for 3 minutes and then the treadmill speed was increased to 11m/min for 5 minutes each day as previously described (177). Mice rested for 1 day prior to the exercise test. On the day of the test mice began running at 11m/min and 5% incline and speed was increased 2m/min every 5 min to maximal speed. Maximal speed was determined by each animal's ability to remain on the treadmill for a minimum of 20sec bouts. Failure was determined when animals remained on the shock grid for more than 15 continuous seconds. Exercise distance and exercise duration were recorded for each animal to assess exercise tolerance in each group of animals.

***In Situ* Muscle Fatigue:**

In situ muscle fatigue was measured on the right gastrocnemius muscle group from KO and WT, CD or HFD treated animals. The contralateral limb served as a sedentary control limb as previously described (219). Specifically, animals were anesthetized with isoflurane. While the animal was lying prone the hindlimb was stabilized and the achilles tendon was released. The proximal portion of the tendon was secured in a custom clamp and attached to a load cell (Grass Instruments FT03). Contractions of the gastrocnemius group were induced by direct stimulation and single twitches were applied at different muscle lengths to determine the optimal length (resting length L_o). With muscles set at L_o maximal tetanic force (P_o) was measured and fatigue was induced through 90 Hz stimulation for 200 ms duration every 2 sec for 5 mins. Skeletal muscle fatigue

was defined as an 80% loss of initial force. Maximal tetanic tension was measured after fatigue development and expressed as a percentage of P_o, to provide an index of fatigue. The fatigued and contralateral gastrocnemius muscles were immediately removed, weighed, and frozen in liquid nitrogen.

Immunoprecipitations and Immunoblotting Analysis:

All protein content analysis was performed using the gastrocnemius muscle from either control or *in situ* fatigued limbs in both WT and KO animals consuming CD or HFD. For detailed methods regarding Brca1 immunoprecipiation from skeletal muscle homogenate, Acc-p, and all western blotting conditions please refer to Study 2 located in Chapter 4.

Glucose/Insulin Tolerance Testing:

Animals were fasted overnight in cages containing hardwood bedding prior to GTT, while animals retained access to their food for insulin tolerance tests (ITT). Before injecting glucose/insulin on the day of the test, fasted (GTT) and non-fasted (ITT) basal blood glucose levels were measured. Animals were then IP-injected with 0.001g glucose per gram body weight or, for insulin tolerance testing, 1.0 units of insulin per 1kg body weight of insulin solution dissolved 1:1000 in sterile 1xPBS. Blood glucose levels were measured with a glucometer (AlphaTrak from Abbott) at pre-injection (0) and 30, 60, 90 and 120 minutes post glucose or insulin injection. A 72-hour washout period occurred between GTT and ITT procedures.

Sectioning, IMCL Staining, and Quantification:

Skeletal muscle sectioning and IMCL staining was performed on the TA muscles from KO or WT animals that were fed either HFD or CD (n=4; 2 male/2 female). Sectioning of the TA muscle, IMCL staining with the neutral lipid stain BODIPY, and quantification were performed as previously described (97, 189).

Skeletal Muscle Mitochondrial Isolation:

Mitochondria were isolated according to previously described methods with slight modifications (59). Quadriceps muscles were collected and immediately placed in ice-cold PBS supplemented with 10mM EDTA. Connective tissue was removed and muscles were minced with scissors while incubating in PBS/EDTA 0.05% trypsin for 30 min. Muscles were then homogenized using a 30ml glass/Teflon potter Elvehjem tissue grinder in isolation buffer 1 (IBm1) containing 67mM mannitol, 50mM Tris/HCl, 1mM EDTA, and 1mg/ml BSA (pH 7.4). The homogenate was transferred to a conical tube and centrifuged at 700g for 10 minutes at 4°C. The supernatant was transferred and centrifuged at 8,000g for 10 minutes at 4°C. The pellet was re-suspended in a second isolation buffer (IBm2) containing 300mM mannitol, 10mM Tris/HCl, and 3mM EGTA/Tris, then centrifuged at 8,000g for 10 minutes at 4°C. The resulting supernatant was discarded. Then the pellet was resuspended in 50 µl of respiration buffer (125mM KCl, 2mM K_2PO_4, 10mM HEPES, 1mM MgCl, 0.1mM EGTA, 1%BSA) for mitochondrial respiration measures. The protein concentration of the pellet was determined using the Pierce BCA protein assay.

Mitochondrial Oxygen Consumption Measures:

Oxygen consumption was measured polarographically using an Oxytherm (Hansatech) as previously described (65). Freshly isolated mitochondria (~200μg) from each group were placed in the calibrated electrode chamber containing 500μl respiration buffer for basal respiration, 30μM palmitoyl-Coa supplemented with 2mM carnitine for state 2 respiration, 300μM ADP for state 3 respiration, 0.5mM glutamate/0.025mM malate for state 3b respiration, 40μM oligomycin (Calbiochem) to induce state 4 respiration, and finally 200nM FCCP to induce state 5 respiration. This was repeated up to six times for each sample. Oxygen consumption rates were then normalized to mitochondrial protein content.

Statistical Analysis:

The experimental design outlined for this study consisted of two independent variables, each with two levels. The independent variables were tamoxifen induced Cre mediated recombination of Brca1 (WT or KO) and diet (CD or HFD). For GTTs, ITTs, endurance exercise test, IMCL, and mitochondrial function measures statistical analysis was performed using a two-way ANOVA for non-repeated measures. If a significant F ratio was found, Student-Newman-Keuls post-hoc analysis was used (see statistical outputs; Appendix B). Due to an un-balanced design (a result of unexplained loss of animals) Acc kinetics, Ampk-p values and *in situ* fatigue were statistically analyzed using either a one-way, paired T-test (in situ muscle fatigued gastrocnemius vs. sedentary limb) or a one-way, non-paired T-test (comparing

across treatments). Statistical significance was accepted at an α level of $P \leq 0.05$. All analysis was completed with Sigma Stat statistical software (Systat software, Inc. San Jose, CA.)

RESULTS:

Anatomical Characteristics: Kyphosis was apparent to varying degrees only in the female KO+CD mice compared to WT+CD (Figure 5.2A,B) with the HFD appearing to attenuate this effect (Figure 5.2C). Kyphosis was not readily apparent in male KO mice (Figure 5.2D,E,F). Liver and visceral fat (VF) mass was elevated in WT+HFD female mice compared to WT+CD and KO+HFD (Table 5.1). Heart mass/BW was greater in KO+CD and KO+HFD compared to WT+CD and WT+HFD female mice (Table 5.1). In male mice, VF and liver mass were elevated in WT+HFD male mice compared to WT+CD (Table 5.2). In addition, gastrocnemius muscle mass was elevated in KO+CD male mice compared to WT+CD (Table 5.2). Male WT+HFD mice exhibited lower heart mass/BW compared to WT+CD mice (Table 5.2). Further, Male KO+HFD mice presented with lower liver mass/BW and VF mass/BW compared to WT+HFD male mice (Table 5.2). However, liver mass/BW was lower in KO+CD compared to KO+HFD male mice (Table 5.2). In WT+HFD mice VF mass/BW was elevated compared to WT+CD male mice (Table 5.2). VF mass/BW was lower in KO+CD mice compared to KO+HFD male mice (Table 5.2). Body weight change from baseline was greater in WT+HFD mice beginning after 6 weeks of HFD compared to all other groups (Figure 5.3A). The increase in body weight

effect was not due to greater food intake in WT+HFD mice with KO mice consuming more calories per day regardless of diet (Figure 5.3B).

Skeletal muscle functional capacity: To determine if loss of Brca1 in skeletal muscle resulted in adverse effects in overall functional capacity we performed treadmill testing. A main effect was detected for KO mice in total distance run compared to WT mice (Figure 5.4A). Maximal speed was significantly lower in WT+HFD and KO+CD mice compared to WT+CD mice (Figure 5.4B). In response to repetitive in situ contractions, WT+CD mice exhibited a significantly higher rate of decline at 1 min compared to KO, however no differences were detected after 5-mins of stimulation across all groups (Figure 5.4C). No significant differences in absolute tetanic force or specific force were detected across groups (Figure 5.4D).

Glucose Tolerance Testing: To determine if loss of functional Brca1 in skeletal muscle altered glucose tolerance, overnight fasted GTTs were performed in WT and KO groups. No differences in basal glucose levels were detected across groups, however WT+HFD mice presented with significantly higher blood glucose at all times points post injection compared to WT+CD mice and KO+HFD mice (Figure 5.5A). In addition, the area under the curve (AUC) was also significantly elevated in the WT+HFD mice compared to all groups (Figure 5.5B).

Insulin Tolerance Testing: ITTs were utilized as a means to assess if loss of Brca1 resulted in altered peripheral insulin sensitivity. Baseline blood glucose

levels in non-fasted mice prior to the insulin tolerance test were significantly higher in KO+HFD mice compared to KO+CD mice (Figure 5.6A). In response to the insulin injection there was a main effect of genotype at 120min post insulin injection where blood glucose levels were elevated in WT mice compared to KO mice (Figure 5.6B). At 30 and 120min post insulin injection blood glucose levels for both WT+CD and KO+HFD mice were lower compared to WT+HFD mice (Figure 5.6B).

Intramuscular Lipid Content: IMCL content was assessed in cross sections of the TA muscle using the neutral lipid stain, BODIPY. Significant main effects on IMCL content were detected for both diet and genotype (Figure 5.7A,B). Specifically, animals on a HFD exhibited elevated IMCL content, while the KO mice had significantly less IMCL content. Within groups, IMCL content was greater in WT+HFD mice compared to WT+CD and KO+HFD mice (Figure 5.7A,B). Similarly, IMCL in KO+HFD mice was greater than KO+CD (Figure 5.7A,B).

Mitochondrial Function: In order to determine if the detected metabolic alterations were due to changes in mitochondrial function we measured mitochondrial OCR in isolated mitochondria. A main effect was detected in mitochondrial OCR during basal and state 3 conditions with mitochondria isolated from KO mice having lower OCR compared to WT (Figure 5.8). Further, under basal conditions a trend (P=0.14) was detected within the WT group where HFD resulted in elevated mitochondrial OCR (Figure 5.8). In addition, a trend

(P=0.08) was detected with WT+HFD mice exhibiting greater mitochondrial OCR under basal conditions compared to KO+HFD (Figure 5.8). No significant differences were detected in mitochondrial OCR during state 3b or 4 respiration (Figure 5.8). During state 5 respiration, a main effect of diet was detected with HFD presenting with greater mitochondrial OCR compared to CD mice (Figure 5.8). Further, under state 5 respiration conditions WT+HFD mice exhibited significantly greater OCR compared to WT+CD mice. (Figure 5.8).

Contraction Induced Acc phosphorylation: We previously found that BRCA1 directly bound to ACC in response to an acute bout of exercise (96). Thus, we sought to determine if loss of Brca1 altered the phosphorylation status of Acc in response to muscle contraction. Contraction resulted in increases in gastrocnemius Acc-p in WT+CD and WT+HFD mice (Figure 5.9A). No significant differences were detected in gastrocnemius Acc-p levels in KO+CD or KO+HFD mice in response to contraction (Figure 5.9A). In KO+CD gastrocnemius muscles basal Acc-p levels were higher compared to WT+CD levels (Figure 5.9A). However, Acc-p percent increase in response to contraction was significantly lower in KO+CD mice compared to WT+CD (Figure 5.9B).

Contraction-Induced Ampk phosphorylation: ACC is a known substrate of AMPK, thus AMPK phosphorylation was assessed as a surrogate of upstream regulation of ACC. Similar to Acc-p, Ampk-p levels were significantly greater in WT+CD and WT+HFD mice in response to contraction (Figure 5.10A). However, contraction did not result in increased Ampk-p levels in KO mice (Figure 5.10A).

The percent increase in response to skeletal muscle contraction was significantly lower in KO+CD mice compared to WT+CD mice (Figure 5.10B).

DISCUSSION:

Previously, we identified BRCA1 as a novel regulator of lipid metabolism in adult skeletal muscle (96). Here, we determined that loss of Brca1 in skeletal muscle of adult mice results in lower exercise tolerance coupled with lower mitochondrial function. Surprisingly, deletion of Brca1 in skeletal muscle of adult mice prevented HFD-induced gains in BW and VF mass. In contrast to our previous results in cultured myotubes, *in vivo* loss of functional Brca1 in skeletal muscle attenuated the onset of HFD-induced insulin resistance and glucose intolerance. Lastly, loss of Brca1 resulted in greater phosphorylation of Acc, but attenuated the ability of fatiguing contractions to induce increases in the phosphorylation content of Acc or Ampk. Overall, these data clearly demonstrate that loss of Brca1 specifically in skeletal muscle leads to a complex and multi-faceted phenotype in mice.

Brca1 has been described as a cell cycle regulator in multiple tissues, including murine immortalized skeletal muscle myoblasts (C2C12) (109). The importance of Brca1 as a critical cell cycle regulator is well established, as overexpression of Brca1 in non-muscle cells results in embryonic lethality in mice due to cell cycle arrest (208). In C2C12 myoblasts, Brca1 expression increases during differentiation suggesting that Brca1 plays an inhibitory role in the cell cycle of proliferating myoblasts (109). Beyond this one correlative finding, the

role of Brca1 in skeletal muscle has been largely unexplored. Based on these studies that demonstrate Brca1 is expressed in adult skeletal muscle, we hypothesize that Brca1 plays a major functional role in the regulation of muscle physiology (96, 109).

Our data demonstrate that Brca1 expression is critical for maintenance of exercise tolerance. Specifically, during exercise testing Brca1 KO mice ran shorter distances and were unable to achieve similar maximal exercise speeds compared to WT mice on a normal chow diet. However, the Brca1 KO mice appear to exhibit protection from fatiguing *in situ* contractions during the early phases of stimulation with the protection lost over time. Thus, loss of Brca1 specifically in skeletal muscle appears to compromise overall function of the animal, however the exercise intolerance may not be due to increased muscle fatigue susceptibility. Previous publications have shown that ablation of gene targets specifically in skeletal muscle results in similar phenotypes. Interestingly, these genes often directly contribute to mitochondrial function and/or substrate metabolism. For example, loss of PGC1α in skeletal muscle, a critical regulator of mitochondrial function, results in impaired exercise tolerance (78). Further, rendering Ampk inactive also results in exercise intolerance in mice due to significant alterations in metabolic function (155). In similar fashion, the loss of functional Brca1 in skeletal muscle resulted in exercise intolerance that was coupled with altered metabolic and/or mitochondrial function compared to WT mice.

Brca1 KO mice exhibit lower skeletal muscle mitochondrial function compared to the WT mice regardless of diet. These metabolic alterations suggest that Brca1 expression impacts the overall phenotype of the skeletal muscle by affecting mitochondrial function. Brca1 is known to localize to the mitochondria in various cell types, including MCF7 breast carcinoma cells (37, 131). In the mitochondria Brca1 functions as a mtDNA repair protein, thereby playing a critical role in mitochondrial function since mtDNA encodes for a significant portion of mitochondrial proteins (131). Previous results have shown that increases in mtDNA mutations and/or damage corresponds with impaired mitochondrial function in skeletal muscle (168, 228). Therefore, it is logical to hypothesize that loss of functional Brca1 in skeletal muscle may impair mitochondrial function due to a decreased ability to repair damaged mtDNA. Similar effects occur with the loss of Brca1 in cardiac tissue, which results in decreased mitochondrial biogenesis and function (181). Collectively, it is evident that Brca1 is required for optimal mitochondrial function in skeletal muscle.

Chronic HFD consumption results in elevated IMCL accumulation that often corresponds with glucose intolerance and insulin resistance (121, 229). In Brca1 KO mice IMCL content associated with chronic HFD feeding was lower compared to WT+HFD mice. Brca1 KO+HFD mice also exhibited greater glucose tolerance and insulin sensitivity compared to WT+HFD mice. In fact, Brca1 KO+HFD mice exhibited glucose and insulin tolerance testing responses similar to WT and KO+CD mice. These data suggest that loss of Brca1

attenuated the negative metabolic responses commonly associated with HFD consumption. Although the protection of the KO mice from a HFD is surprising when considering the compromised mitochondrial function there is precedence for similar results in the literature. For example, in other models where lipid oxidation mechanisms are impaired within the mitochondria, glucose reliance becomes elevated resulting in an increase in insulin sensitivity (107). Therefore, it is possible that chronic *in vivo* deletion of Brca1 induces an adaptation resulting in enhanced dependence on glucose as a substrate and as a result improved insulin sensitivity.

Brca1 KO mice exhibited lower IMCL content in response to HFD compared to WT mice despite KO mice consuming more calories per day independent of diet. Typically exposure of rodents to a HFD is associated with higher IMCL content with reduced mitochondrial function, however the KO animals exhibited lower IMCL and mitochondrial dysfunction. It is plausible that the KO mice exposed to a HFD are shunting the excess lipid away from the muscle towards the liver, as there were elevations in the normalized liver mass. However, this effect is specific to the male mice, thus further experiments will be necessary to truly elucidate this effect. A similar effect has been observed in mice lacking the critical plasma membrane lipid translocase, CD36/FAT (70). Specifically, the CD36-/- mice exhibit lower skeletal muscle uptake of circulating lipids, improved glucose tolerance and insulin sensitivity as well as reduced IMCL content (70). Therefore, one possible explanation for the lower IMCL

content in Brca1 KO mice fed a HFD is that lipid delivery into skeletal muscle was reduced in Brca1 KO mice.

Acetyl CoA carboxylase functions as an important intracellular regulator of lipid metabolism in skeletal muscle (160, 213). Our previous investigation reported an interaction between Brca1 and Acc-p which was thought to increase LCFA entry into the mitochondria by maintaining Acc-p levels (96). However, this same study also demonstrated that acute decreases in *BRCA1* expression resulted in elevated ROS production which was associated with increased phosphorylation levels of AMPK and ACC (96). In agreement with our previous results, basal Acc-p levels were elevated in basal KO+CD mice compared to WT, however no significant activation of Ampk (upstream regulator) was seen in the Brca1 KO mice. Previous research has demonstrated that phosphorylation of Acc, in some cases, can occur independent of Ampk activation (51, 77). In response to *in situ* muscle contractions there was no increase in phosphorylation of either Ampk or Acc in the KO mice suggesting that further increases in phosphorylation content is dependent upon Brca1. However, it is possible that the lack of a contraction response is because phosphorylation status is at a maximal level under basal conditions in KO mice. These data collectively suggest that the mitochondria in the KO mice are permissive to LCFA entry, however, the reduced mitochondrial function suggests that complete fatty acid oxidation is likely not occurring.

The *in vivo* effects of loss of functional Brca1 in skeletal muscle contrast some of our previous findings where we induced acute decreases in *BRCA1* expression in cultured human myotubes (96). We hypothesize that our data indicate differential consequences of acute versus chronic loss of Brca1. As a result, the contrast in results may be due to an adaptation response from prolonged *in vivo* loss of Brca1 in the mouse model. It is not unreasonable to speculate that our data indicate that prolonged loss of Brca1 in skeletal muscle induces a fiber type switching effect towards a more glycolytic and less oxidative muscle type. Similar responses have been seen with deletion of the α1 subunit of Ampk which significantly altered the muscle fiber type composition (165). However, in preliminary analyses when assessing fiber type by myosin heavy chain expression we did not observe a clear shift in fiber type of the TA muscle (data not shown). It is possible that the observed effect is specific to the metabolic aspects of the muscle and the loss of Brca1 has little effect on the contractile phenotype of the muscle. Therefore, the observed shift in metabolic phenotype associated with Brca1 KO is a plausible explination underlying the elevated glycolytic phenotype observed in Brca1 KO mice.

BRCA1 mutations resulting in partial or complete deletion of BRCA1 in humans are known to exist (82, 142), with little to no information regarding the peripheral consequences of the whole body *BRCA1* mutations in humans being reported. Although whole body *BRCA1* mutations in women are frequently associated with an increased risk of tumorigenesis our data suggests skeletal muscle is another likely target for alterations in function due to the mutation. For

example, women with *BRCA1* mutations may have reduced exercise capacity and/or alterations in skeletal muscle metabolic function resulting in a more glycolytic skeletal muscle phenotype similar to what we observed in our mouse model. Life-long loss of functional BRCA1 in women may induce lipid metabolism impairments in skeletal muscle resulting in elevated lipid storage in other organ systems within the body, including the liver and visceral fat pad. Consequences such as these may unknowingly be altering overall health in women with BRCA1 mutations.

Collectively, these data demonstrate for the first time that Brca1 is as an important regulator of skeletal muscle function. Surprisingly, deletion of Brca1 protected mice from developing the metabolic consequences commonly associated with a HFD. These results are intriguing on multiple levels and may suggest that Brca1 is a target to treat metabolic diseases, however deletion of Brca1 did result in negative functional consequences suggesting that is endeavor is unlikely. Thus, it is clear that more research will be required to fully elucidate the underlying mechanisms responsible for the phenotype associated with loss of functional Brca1 in skeletal muscle.

TABLES:

Table 5.1. *Anatomical characteristics of female WT and KO mice fed CD and HFD.*

Female											
		Whole tissue mass (mg)					Tissue mass/BW (mg/g)				
	BW	Heart	Liver	VF	Gastroc	TA	Heart/BW	Liver/BW	VF/BW	Gastroc/BW	TA/BW
WT+CD	22.8±0.9	143.2±7.2	1132±77	238.4±31	75±12	33.3±3	6.3±0.1	49.9±3.0	10.4±1.3	3.3±0.5	1.4±0.1
WT+HFD	26.5±2.7	152.0±8.0	1526±142*,#	738.7±43*,#	87±3.5	39.0±2	5.9±0.4	58.3±4.2	22.5±10	3.4±0.2	1.5±0.5
KO+CD	21.2±1.5	151±8.7	1053±113	267±54	83±8.9	34±2	7.2±0.3$	49.0±2.3	12.1±1.6	3.9±0.15	1.6±0.4
KO+HFD	22.0±0.4	156±4.0	950±48	171±51	84±2.0	34±2	7.1±0.9$	43.2±2.4	7.7±.2.2	3.8±0.2	1.6±0.9

Values are mean ± SEM.
*$P \leq 0.05$ vs. WT CD
#$P \leq 0.05$ vs. KO HFD
$$P \leq 0.05$ vs. WT mice with corresponding diet

Table 5.2. *Anatomical characteristics of male WT and KO mice fed CD and HFD.*

Male											
		Whole tissue mass (mg)					Tissue mass/BW (mg/g)				
	BW(g)	Heart	Liver	VF	Gastroc	TA	Heart/BW	Liver/BW	VF/BW	Gastroc/BW	TA/BW
WT+CD	27.9±0.9	190±11	1239±210	306±69	108±5	37±2	6.9±0.5	43.1±7	10.5±2.2	3.9±0.2	1.4±0.1
WT+HFD	32.9±2.6	179±1.2	1653±146*	698±15.3*	121±3	44±1*	5.4±0.1*	55.7±3	19.7±2.8*	3.8±0.2	1.3±0.1
KO+CD	29.4±1.7	197±0.9	1441±88	243±34	126±0.8*	40±2	6.7±0.2	49.13±2#	8.26±1.1#	4.3±0.2	1.4±0.8
KO+HFD	33.04±2.01	199±0.8	1728±99	577±143	124±0.3	43±2	6.1±0.3	52.3±2$	16.5±3.1	3.8±0.2	1.3±0.5

Values are mean ± SEM.
*$P \leq 0.05$ vs. WT CD
#$P \leq 0.05$ vs. KO HFD
$$P \leq 0.05$ vs. WT mice with corresponding diet

FIGURES:

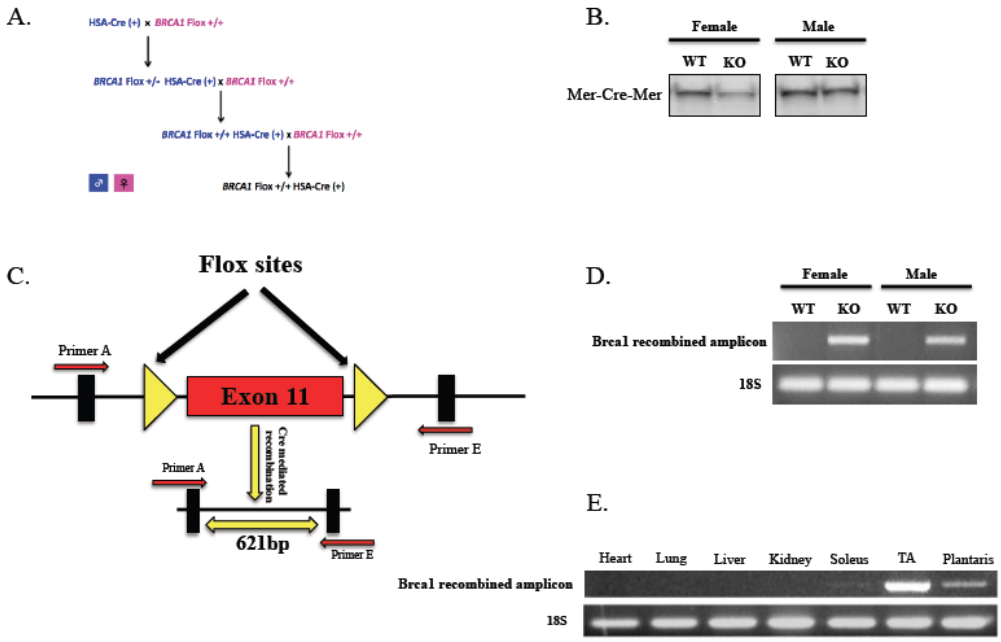


Figure 5.1 (A-E): Development of an inducible skeletal muscle BRCA1 knockout (HSA-Brca1-KO) mouse. (**A**) HSA-Cre-Brca1 Flox breeding scheme (**B**) Western blot verifying the presence of Mer-Cre-Mer (112kDa) in the gastrocnemius muscle of the HSA-Brca1-KO mice (**C**) schematic representation of the Flox site recombination after injection (**D**) DNA analysis of recombined amplicon in injected male and female mice (621bp band) compared to control (vehicle only) injected mice (no band). (**E**) *Brca1* recombined amplicon in injected mice was present exclusively in skeletal muscle

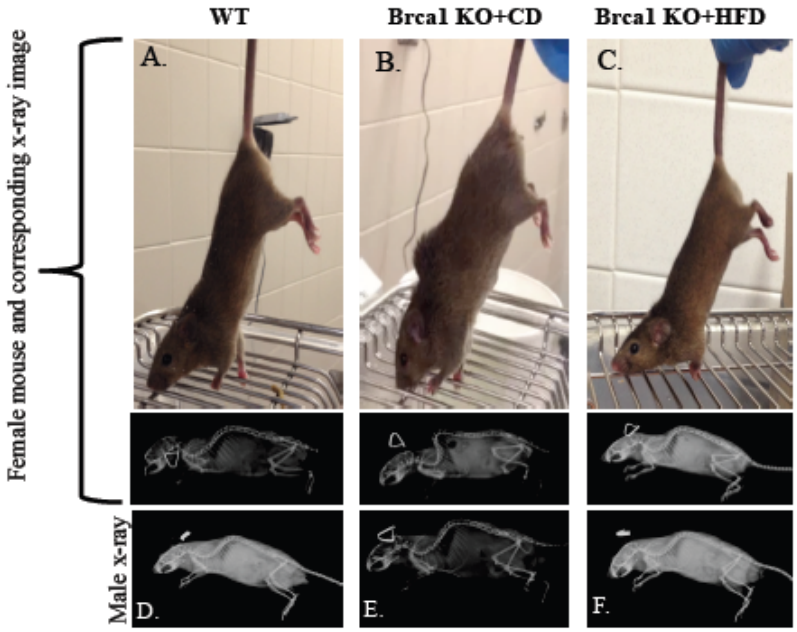
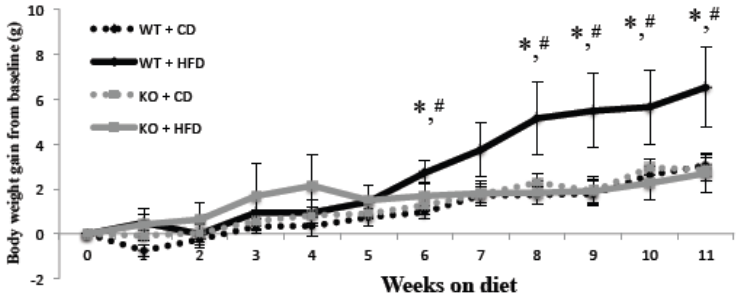


Figure 5.2 (A-F). Brca1 KO+CD mice exhibit kyphosis which is partially attenuated in response to HFD. Images of female (**A**) wild type (WT), (**B**) Brca1 knockout (KO) control diet (CD), and (**C**) KO high fat diet (HFD) mice and a matching x-ray (below). Loss of functional Brca1 in skeletal muscle results in the appearance of kyphosis in female mice consuming CD. Kyphosis was not as apparent in female KO mice consuming HFD. (**D,E,F**) Kyphosis was not consistently evident in male mice regardless of group.

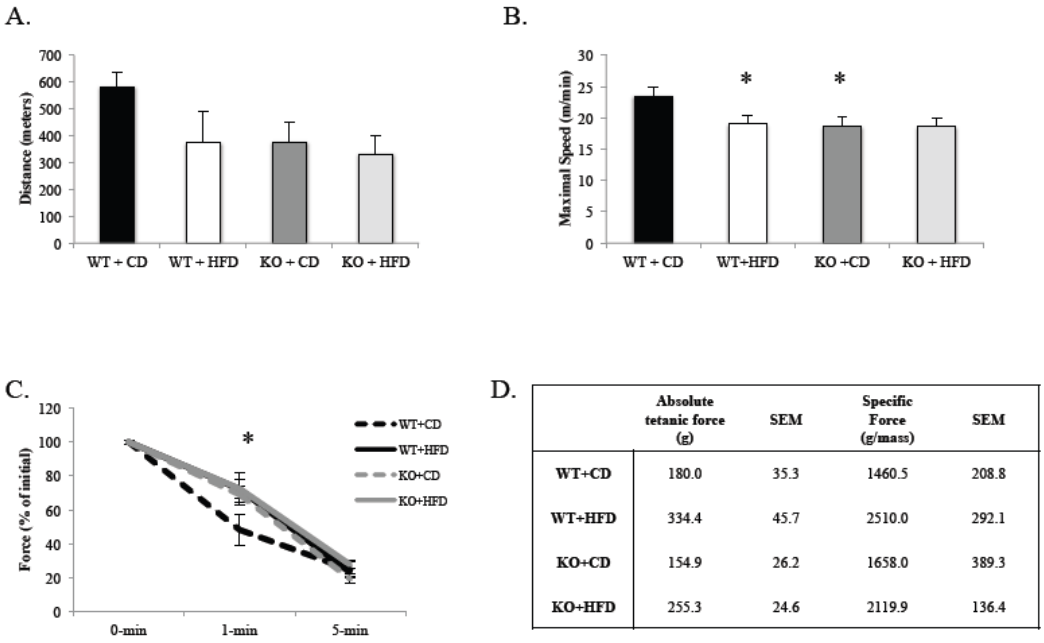
A.



B.

	Food consumption (g)/day	SEM	Food consumption (kcal)/day	SEM
WT+CD	3.4	0.2	13.0	0.7
WT+HFD	3.1	0.2	14.9	0.8
KO+CD	3.9	0.3	15.2	1.3
KO+HFD	3.5	0.1	16.5	0.7

Figure 5.3 (A-B). Loss of functional Brca1 in skeletal muscle prevents HFD-induced increases in body weight. (**A**) WT and KO mice were placed on CD or HFD for 10 weeks. After 6 weeks of feeding WT+HFD mice had significantly greater body weight compared to WT+CD (*$P \leq 0.05$) and KO+HFD ($^{\#}P \leq 0.05$) mice. (n=7-10mice/group) (**B**) Average daily feed intake did not differ across groups. A main effect was detected between WT and KO animals where KO animals consumed more kcal/day ($P \leq 0.05$) (n=6-10/group).



	Absolute tetanic force (g)	SEM	Specific Force (g/mass)	SEM
WT+CD	180.0	35.3	1460.5	208.8
WT+HFD	334.4	45.7	2510.0	292.1
KO+CD	154.9	26.2	1658.0	389.3
KO+HFD	255.3	24.6	2119.9	136.4

Figure 5.4 (A-D). Deletion of Brca1 alters functional capacity of the skeletal muscle (A) A main effect in endurance exercise capacity was detected with KO mice exhibiting shorter distance compared to WT ($P \leq 0.05$). (B) Maximal exercise speed was lower in WT+HFD mice and in CD+KO mice compared to WT+CD mice (*$P \leq 0.05$). (C) Force output over time during fatiguing muscle contractions. After 1 minute of stimulated contractions the gastrocnemius muscle force production was lower in WT+CD mice compared to KO+CD mice (*$P \leq 0.05$) when normalized to baseline force production. At 5 minutes of contraction no significant differences between groups were detected. (D) No differences in gastrocnemius force production were detected across groups at 100 Hz.

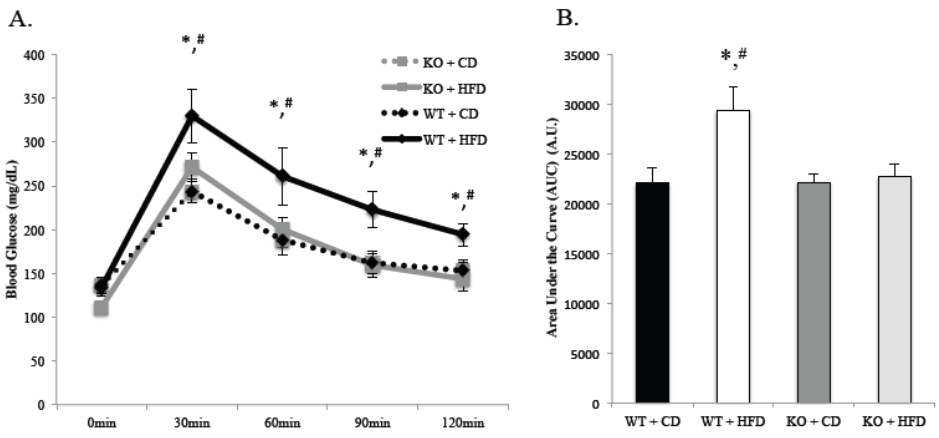


Figure 5.5 (A-B). Deletion of Brca1 prevents HFD-induced glucose intolerance. (A) No significant differences in blood glucose levels were detected across groups at baseline. After glucose injection blood glucose levels were higher in WT+HFD mice compared to WT+CD mice at 30, 60, 90 and 120min after glucose injection (*$P \leq 0.05$). WT+HFD blood glucose levels were greater than KO+HFD at 30, 60, 90, and 120min post glucose injection ($^{\#}P \leq 0.05$) (B) Total AUC was greater in WT+HFD mice compared to WT+CD mice (*$P \leq 0.05$), and KO+HFD mice ($^{\#}P \leq 0.05$). No significant differences were detected between WT CD and KO+CD mice. (n=6-10mice/group)

A.

	WT+CD	WT+HFD	KO+CD	KO+HFD
Baseline blood glucose (mg/dL)	191.2±5.2	201.4±11.7	181.9±11.2	214.3±9.5$

$P≤0.05 vs. KO CD

B.

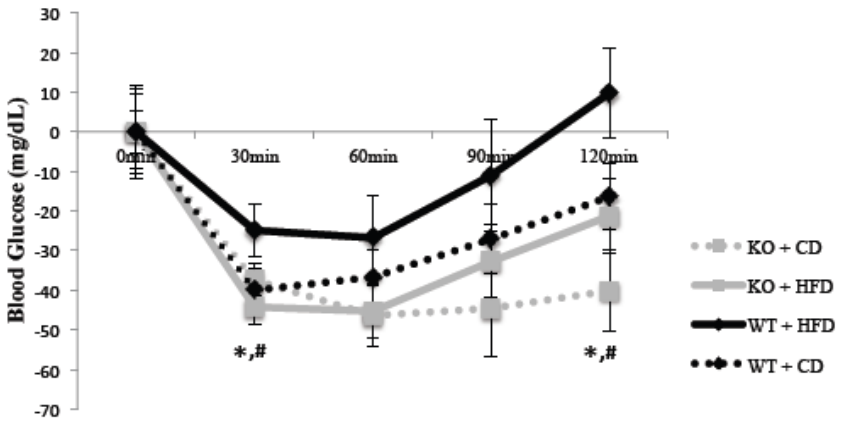


Figure 5.6. (A-B) Loss of Brca1 enhances insulin sensitivity in HFD mice and in control mice. (A) Non-fasted, baseline blood glucose levels a main effect was detected for diet with HFD mice presenting with greater baseline blood glucose values. Baseline blood glucose levels were also greater in KO+HFD diet mice compared to KO+CD ($^{\$}P≤0.05$). (B) A main effect of genotype was detected at 120min after insulin injection was detected with lower blood glucose in KO mice compared to WT mice ($P≤ 0.05$). At 30 and 120min after insulin injection blood glucose was higher in WT HFD compared to WT CD ($^{*}P≤0.05$) and KO HFD mice ($^{\#}P≤0.05$) (n=7-10mice/group)

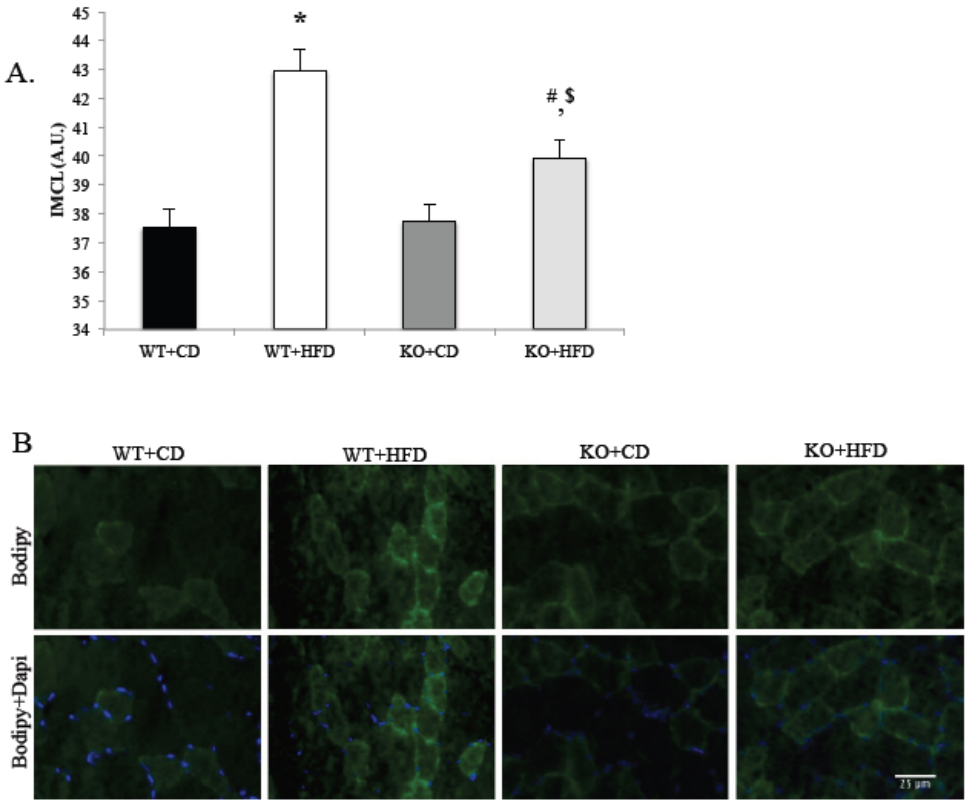


Figure 5.7 (A-B). HFD-induced IMCL accumulation is attenuated in response to loss of Brca1 in skeletal muscle (**A**) IMCL was significantly elevated in the tibialis anterior (TA) muscle in both the WT and KO+HFD treated mice compared to the WT and KO+CD mice. KO+HFD IMCL accumulation was lower compared to WT+HFD (*$P \leq 0.05$ vs. WT+CD; $^{\#}P \leq 0.05$ vs. KO+CD; $^{\$}P \leq 0.05$ WT+HFD vs. KO+HFD) (**B**) Representative images of IMCL through BODIPY (green-493/503) staining of neutral lipid droplets and nuclei (DAPI-blue) within skeletal muscle cross sections of the TA. (n=4mice/group; 50-60 fibers/muscle quantified)

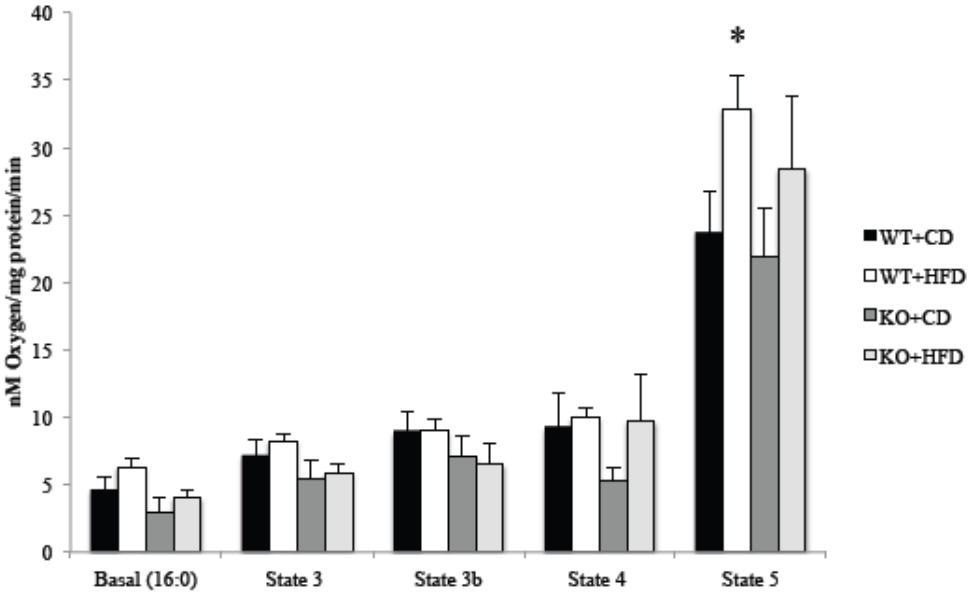


Figure 5.8. Loss of functional Brca1 impairs maximal skeletal muscle mitochondrial function. A main effect was detected in mitochondrial oxygen consumption rate during basal and state 3 conditions with mitochondria isolated from KO mice were lower compared to WT ($P \leq 0.05$). No significant differences were detected in mitochondrial oxygen consumption rates during state 3b or 4 respiration. During state 5 respiration a main effect of diet was detected with HFD presenting with greater mitochondrial oxygen uptake rates compared to CD mice ($P \leq 0.05$). A significant difference was also detected between WT+CD and HFD mice with a greater oxygen consumption rate in mitochondria isolated from HFD mice ($*P \leq 0.05$) (n=6-10 mice/group)

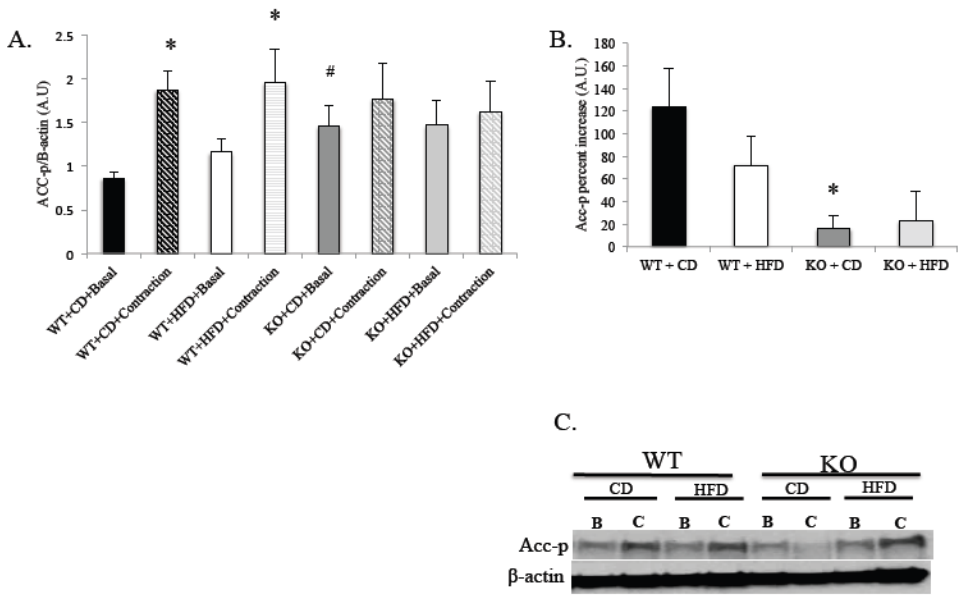


Figure 5.9 (A-C). Loss of functional Brca1 in skeletal muscle increases basal Acc-p levels and prevents an increase in Acc-p levels in response to fatiguing muscle contractions. (B=basal; C=contraction) (**A**) In response to muscle contractions, Acc-p levels in WT+CD and HFD mice were increased (*$P \leq 0.05$) in the gastrocnemius muscle compared to the non-contracting gastrocnemius muscle from the same animal. No increase in Acc-p levels was detected in KO mice in response to gastrocnemius muscle contractions. KO+CD+Basal Acc-p levels were greater than WT+CD+Basal ($^{\#}P \leq 0.05$) (**B**) Contraction-induced percent change in Acc-p was lower in KO + CD compared to WT + CD (*$P \leq$ 0.05). (n=5-6 mice/group). (**C**) Representative immunoblot is depicted for each condition.

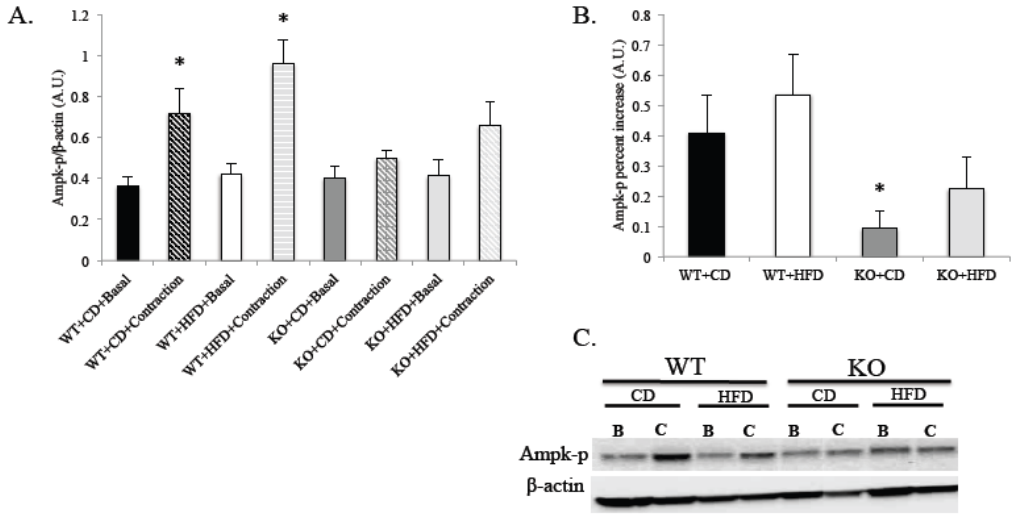


Figure 5.10 (A-C). Loss of functional Brca1 in skeletal muscle prevents an increase in Ampk-p levels in response to fatiguing muscle contractions. (B=basal; C=contraction) (**A**) In response to muscle contractions, Ampk-p levels in WT+CD and HFD mice were increased (*$P \leq 0.05$) in the gastrocnemius muscle compared to the non contracting gastrocnemius muscle from the same animal. No increase in Ampk-p levels was detected in KO mice in response to gastrocnemius muscle contractions. (**B**) Contraction-induced percent change in Ampk-p was lower in KO+CD compared to WT+CD (*$P \leq 0.05$). (n=5-6 mice/group). (**C**) Representative immunoblot is depicted for each condition.

Chapter 6: LIMITATIONS, SUMMARY AND FUTURE DIRECTIONS

LIMITATIONS:

Due to the magnitude of this study multiple limitations were encountered with regards to the experimental approach and as a result of assumptions made *a priori* during the study design. This section will address some of the limitations encountered and list them from most impactful to least.

Sex differences: Both males and females were included in this study in an attempt to fully elucidate the effects of Brca1 in skeletal muscle. Surprisingly, ablation of Brca1 resulted in some unique sex-specific responses. For example, skeletal muscle force production was lower in the male KO mice, but higher in the female KO mice when compared to WT mice. Unfortunately, due to a low number of animals it was not possible to explore these results in greater depth in this dissertation. In addition, the female KO mice exhibited the onset of kyphosis, which was not apparent in the males. Also, sex differences were seen in tissue mass (i.e. visceral fat), however in this case it was possible to present the data separately (Table 1&2). To address these initial observations of sex-related differences, it will be necessary to repeat these experiments adding more males and females to these groups to allow for presentation of the sexes separately.

Brca1 antibody performance: Although these methods had previously been optimized, we were forced to purchase new reagents (i.e. new lots) and we have been unable to achieve optimal antibody results utilizing these antibodies. Specifically, the most recently purchased antibody was derived from a different lot number and failed to meet our previously established criteria for quality

control. Unfortunately, the majority of antibodies for Brca1 are human-specific and very few options are available for mouse tissue (four total). Thus, we were not confident enough in these results to present them as a portion of this research study but they are included in Appendix 1.

SUMMARY:

The studies conducted in this dissertation work have resulted in the identification of a novel regulatory protein, Brca1, in skeletal muscle. The compilation of results from these studies demonstrates that Brca1 is a critical metabolic regulator in skeletal muscle. The initial purpose of study one in this dissertation was to identify specific mechanisms responsible for the metabolic alterations in skeletal muscle that occur in response to loss of circulating estrogens. In this study we demonstrated that loss of circulating estrogens resulted in elevated IMCL content which corresponded with alterations in acyl carnitine species and mitochondrial dysfunction. The results from study one suggested that lipid mitochondrial transport was altered in response to loss of circulating estrogens, pinpointing a potential estrogen-sensitive mechanism underlying these results. Therefore, we began searching for an estrogen-sensitive gene that was also a known regulator of some aspect of lipid metabolism.

Breast cancer 1, early onset (*BRCA1*) is a well-established estrogen-sensitive, human caretaker gene that is the blueprint for the protein breast cancer type 1 susceptibility protein (BRCA1/Brca1). Recent evidence had suggested that Brca1 regulated lipid metabolism in breast carcinoma cells through an interaction with the widely recognized skeletal muscle lipid mediator, acetyl CoA

carboxylase (Acc). Thus, we sought to determine in study two if BRCA1 was present in skeletal muscle in an estrogen sensitive manner and if the interaction between BRCA1 and the phosphorylated form of Acc (Acc-p) was underlying the altered metabolic responses from loss of circulating estrogens. In study two we determined that BRCA1 was expressed in both mouse and human skeletal muscle and to our surprise BRCA1 expression did not differ between males and females. Further, we determined that BRCA1 interacted with ACC-p in both mouse and human skeletal muscle suggesting that the protein-protein interaction promoted the phosphorylation status of ACC, thereby improving lipid metabolism during exercise. In addition, we demonstrated that loss of BRCA1 in human myotubes resulted in impaired mitochondrial function, elevated IMCL content, decreased insulin sensitivity and elevated ROS production. Collectively, the data in study two indicated that while BRCA1 was unlikely to explain our results in the OVX model, the data did suggest that Brca1 is a critical regulator of skeletal muscle metabolic function. Therefore, the data in study two made a compelling argument for a research study to be performed *in vivo* in an effort to more thoroughly analyze BRCA1 as a physiological and metabolic regulator of skeletal muscle. Therefore, in study three, we developed a skeletal muscle-specific, Brca1 inducible knockout mouse model to assess the physiological importance of Brca1 in skeletal muscle. We determined that chronic *in vivo*, as opposed to acute *in vitro*, loss of Brca1 in skeletal muscle resulted in multiple physiological and metabolic alterations. Specifically, loss of functional Brca1 in skeletal muscle resulted in impaired endurance exercise tolerance compared to wild type mice.

Considerable differences in metabolic function were also detected in response to loss of Brca1 in skeletal muscle including reduced IMCL content and improved glucose tolerance and insulin sensitivity in response to HFD. However, loss of Brca1 resulted in impaired mitochondrial function, elevated basal Acc-p levels and attenuated the skeletal muscle contraction-induced increase in phosphorylation of Acc and Ampk. Collectively, these studies, specifically study two and three, have provided insight into the previously undocumented role of Brca1 in skeletal muscle. These findings may serve as the foundation for a considerable amount of future research endeavors to further elucidate the role of Brca1 in skeletal muscle.

FUTURE DIRECTIONS:

As discussed in the limitations section of this dissertation, sex-specific differences were detected in a number of measures and most notably in our force production measures. Therefore, additional male and female animals have been injected with tamoxifen or control drug and placed on CD or HFD to be used for further force production measures. Additional male and female animals used in this portion of the study will allow for males and females to be assessed individually to further characterize sex-specific differences associated with Brca1 and force production in skeletal muscle.

Given the variety of alterations detected in our mouse models as a consequence of the loss of functional Brca1 in skeletal muscle, one of the next goals of this study will be to perform microarray analysis. Theoretically, we

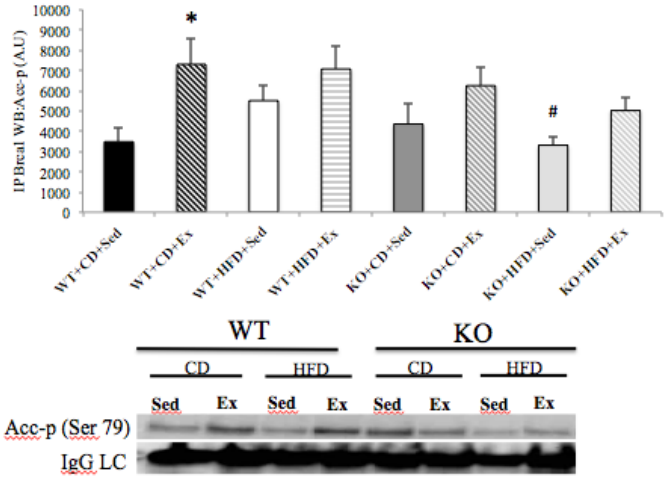
anticipate that microarray analyses will help to identify specific targets underlying the documented physiological changes associated with loss of Brca1 in skeletal muscle and provide more direction to our results as we pursue the mechanisms in greater detail.

Finally, our initial investigation regarding the functional role of BRCA1 in human myotubes demonstrated that an acute decrease in BRCA1 expression resulted in elevated ROS production leading to further metabolic alterations in skeletal muscle. Therefore, we attribute a significant portion of our findings in Brca1 KO mice to adaptations that resulted from chronic elevations in ROS production due to mitochondrial dysfunction, however this hypothesis will need to be addressed with more mechanistic-based experiments.

Appendices

Appendix A:

Brca1 Immunoprecipitation Data



Appendices figure 1: Loss of functional Brca1 did not reduce Brca1 immunoprecipitated Acc-p content in sedentary skeletal muscle or in response to muscle contractions. (A) In response to muscle contractions Acc-p was increased in WT CD (*$P \leq 0.05$). No differences were detected in response to exercise in the WT HFD group or the KO CD and HFD groups. Acc-p was lower in KO HFD Sed compared to WT HFD Sed mice ($^{\#}P \leq 0.05$). Loss of the functional Brca1 long isoform did not result in decreased Brca1 immunoprecipitated Acc-p.

Appendix B:

Statistical Outputs

Two Way Analysis of Variance Monday, August 05, 2013, 10:47:56 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.136)

Source of Variation	DF	SS	MS	F	P
Genotype	1	49.068	49.068	2.301	0.145
Diet	1	27.052	27.052	1.269	0.273
Genotype x Diet	1	10.228	10.228	0.480	0.497
Residual	20	426.416	21.321		
Total	23	535.986	23.304		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.145).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.273).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.497)

Power of performed test with alpha = 0.0500: for Genotype : 0.181
Power of performed test with alpha = 0.0500: for Diet : 0.0744
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	24.660	1.234
KO	21.603	1.593

Least square means for Diet :

Group	Mean	SEM
CD	21.996	1.234
HFD	24.266	1.593

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	22.827	1.745
WT x HFD	26.493	1.745
KO x CD	21.166	1.745
KO x HFD	22.040	2.666

Two Way Analysis of Variance Monday, August 05, 2013, 10:48:33 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: Heart wt

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.589)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.000203	0.000203	0.475	0.498
Diet	1	0.000245	0.000245	0.574	0.458
Genotype x Diet	1	0.0000123	0.0000123	0.0289	0.867
Residual	20	0.00856	0.000428		
Total	23	0.00901	0.000392		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.498).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.458).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.867)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.147	0.00553
KO	0.154	0.00714

Least square means for Diet :

Group	Mean	SEM
CD	0.147	0.00553
HFD	0.154	0.00714

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.143	0.00782
WT x HFD	0.152	0.00782
KO x CD	0.151	0.00782
KO x HFD	0.156	0.0119

Two Way Analysis of Variance Monday, August 05, 2013, 10:49:31 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: Liver

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.409)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.563	0.563	6.535	0.019
Diet	1	0.111	0.111	1.289	0.270
Genotype x Diet	1	0.324	0.324	3.760	0.067
Residual	20	1.722	0.0861		
Total	23	2.835	0.123		

The difference in the mean values among the different levels of Genotype is greater than would be expected by chance after allowing for effects of differences in Diet. There is a statistically significant difference (P = 0.019). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.270).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.067)

Power of performed test with alpha = 0.0500: for Genotype : 0.609
Power of performed test with alpha = 0.0500: for Diet : 0.0764
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.339

Least square means for Genotype :

Group	Mean	SEM
WT	1.329	0.0784
KO	1.002	0.101

Least square means for Diet :

Group	Mean	SEM
CD	1.093	0.0784
HFD	1.238	0.101

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	1.132	0.111
WT x HFD	1.526	0.111
KO x CD	1.053	0.111
KO x HFD	0.950	0.169

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	0.327	2	3.615	0.019	Yes

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.145	2	1.606	0.270	No

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.394	2	3.550	0.021	Yes

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	0.103	2	0.719	0.617	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.0790	2	0.713	0.620	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.576	2	4.021	0.010	Yes

Two Way Analysis of Variance Monday, August 05, 2013, 10:50:18 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: VF

Normality Test: Failed (P = <0.001)

Equal Variance Test: Passed (P = 0.516)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.381	0.381	0.920	0.349
Diet	1	0.214	0.214	0.517	0.481
Genotype x Diet	1	0.467	0.467	1.127	0.301
Residual	20	8.289	0.414		
Total	23	9.549	0.415		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.349).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.481).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.301)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0604

Least square means for Genotype :

Group	Mean	SEM
WT	0.489	0.172
KO	0.219	0.222

Least square means for Diet :

Group	Mean	SEM
CD	0.253	0.172
HFD	0.455	0.222

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.238	0.243
WT x HFD	0.739	0.243
KO x CD	0.267	0.243
KO x HFD	0.171	0.372

Two Way Analysis of Variance Monday, August 05, 2013, 10:51:09 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: Gastroc

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.492)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0000357	0.0000357	0.0708	0.793
Diet	1	0.000242	0.000242	0.481	0.496
Genotype x Diet	1	0.000182	0.000182	0.362	0.554
Residual	20	0.0101	0.000504		
Total	23	0.0107	0.000464		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.793).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.496).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.554)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.0811	0.00600
KO	0.0838	0.00775

Least square means for Diet :

Group	Mean	SEM
CD	0.0791	0.00600
HFD	0.0858	0.00775

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.0748	0.00849
WT x HFD	0.0875	0.00849
KO x CD	0.0833	0.00849
KO x HFD	0.0842	0.0130

Two Way Analysis of Variance Monday, August 05, 2013, 10:51:54 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: TA

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.674)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0000179	0.0000179	0.378	0.546
Diet	1	0.0000480	0.0000480	1.013	0.326
Genotype x Diet	1	0.0000272	0.0000272	0.574	0.458
Residual	20	0.000948	0.0000474		
Total	23	0.00107	0.0000466		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.546).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.326).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.458)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.0359	0.00184
KO	0.0341	0.00238

Least square means for Diet :

Group	Mean	SEM
CD	0.0335	0.00184
HFD	0.0365	0.00238

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.0333	0.00260
WT x HFD	0.0386	0.00260
KO x CD	0.0337	0.00260
KO x HFD	0.0344	0.00398

Two Way Analysis of Variance Monday, August 05, 2013, 10:48:52 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: Heart/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.206)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0579	0.0579	8.957	0.007
Diet	1	0.00263	0.00263	0.408	0.530
Genotype x Diet	1	0.000638	0.000638	0.0988	0.757
Residual	20	0.129	0.00646		
Total	23	0.201	0.00872		

The difference in the mean values among the different levels of Genotype is greater than would be expected by chance after allowing for effects of differences in Diet. There is a statistically significant difference (P = 0.007). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.530).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.757)

Power of performed test with alpha = 0.0500: for Genotype : 0.776
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.609	0.0215
KO	0.714	0.0277

Least square means for Diet :

Group	Mean	SEM
CD	0.673	0.0215
HFD	0.651	0.0277

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.626	0.0304
WT x HFD	0.593	0.0304
KO x CD	0.720	0.0304
KO x HFD	0.709	0.0464

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	Diff of Means	p	q	P	P<0.050
KO vs. WT	0.105	2	4.232	0.007	Yes

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
CD vs. HFD	0.0224	2	0.903	0.530	No

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	0.0334	2	1.100	0.446	No

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	0.0114	2	0.290	0.840	No

Comparisons for factor: **Genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.0940	2	3.093	0.041	Yes

Comparisons for factor: **Genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.116	2	2.958	0.050	Yes

Two Way Analysis of Variance Monday, August 05, 2013, 10:49:51 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: Liver/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.023)

Source of Variation	DF	SS	MS	F	P
Genotype	1	3.395	3.395	4.337	0.050
Diet	1	0.0822	0.0822	0.105	0.749
Genotype x Diet	1	2.617	2.617	3.343	0.082
Residual	20	15.655	0.783		
Total	23	21.543	0.937		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.050).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.749).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.082)

Power of performed test with alpha = 0.0500: for Genotype : 0.400
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.294

Least square means for Genotype :

Group	Mean	SEM
WT	5.410	0.236
KO	4.606	0.305

Least square means for Diet :

Group	Mean	SEM
CD	4.945	0.236
HFD	5.070	0.305

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	4.994	0.334
WT x HFD	5.825	0.334
KO x CD	4.896	0.334
KO x HFD	4.315	0.511

Two Way Analysis of Variance Monday, August 05, 2013, 10:50:40 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: VF/BW

Normality Test: Failed (P = <0.001)

Equal Variance Test: Passed (P = 0.450)

Source of Variation	DF	SS	MS	F	P
Genotype	1	2.244	2.244	0.988	0.332
Diet	1	0.792	0.792	0.349	0.562
Genotype x Diet	1	3.612	3.612	1.590	0.222
Residual	20	45.429	2.271		
Total	23	52.894	2.300		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.332).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.562).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.222)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.107

Least square means for Genotype :

Group	Mean	SEM
WT	1.645	0.403
KO	0.992	0.520

Least square means for Diet :

Group	Mean	SEM
CD	1.124	0.403
HFD	1.513	0.520

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	1.037	0.570
WT x HFD	2.254	0.570
KO x CD	1.212	0.570
KO x HFD	0.771	0.870

Two Way Analysis of Variance Monday, August 05, 2013, 10:51:34 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: Gastroc/BW

Normality Test: Failed (P = <0.001)

Equal Variance Test: Passed (P = 0.718)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0122	0.0122	1.760	0.200
Diet	1	0.0000773	0.0000773	0.0111	0.917
Genotype x Diet	1	0.000460	0.000460	0.0663	0.799
Residual	20	0.139	0.00694		
Total	23	0.154	0.00668		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.200).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.917).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.799)

Power of performed test with alpha = 0.0500: for Genotype : 0.124
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.337	0.0223
KO	0.385	0.0288

Least square means for Diet :

Group	Mean	SEM
CD	0.359	0.0223
HFD	0.363	0.0288

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.330	0.0315
WT x HFD	0.343	0.0315
KO x CD	0.388	0.0315
KO x HFD	0.382	0.0481

Two Way Analysis of Variance Monday, August 05, 2013, 10:52:16 AM

Data source: Female tissue weights in KCJ_Tissue wts_female_7-22-13

General Linear Model

Dependent Variable: TA/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.401)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.000516	0.000516	0.841	0.370
Diet	1	0.0000235	0.0000235	0.0384	0.847
Genotype x Diet	1	0.000136	0.000136	0.222	0.643
Residual	20	0.0123	0.000614		
Total	23	0.0131	0.000570		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.370).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.847).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.643)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.148	0.00662
KO	0.158	0.00855

Least square means for Diet :

Group	Mean	SEM
CD	0.152	0.00662
HFD	0.154	0.00855

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.144	0.00936
WT x HFD	0.151	0.00936
KO x CD	0.159	0.00936
KO x HFD	0.156	0.0143

Two Way Analysis of Variance Monday, August 05, 2013, 10:32:59 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.291)

Source of Variation	DF	SS	MS	F	P
Genotype	1	4.507	4.507	0.155	0.697
Diet	1	140.181	140.181	4.830	0.037
Genotype x Diet	1	2.925	2.925	0.101	0.753
Residual	27	783.684	29.025		
Total	30	939.371	31.312		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.697).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.037). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.753)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.460
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	30.430	1.347
KO	31.201	1.420

Least square means for Diet :

Group	Mean	SEM
CD	28.665	1.455
HFD	32.966	1.309

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	27.969	1.905
WT x HFD	32.891	1.905
KO x CD	29.362	2.199
KO x HFD	33.041	1.796

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	Diff of Means	p	q	P	P<0.050
KO vs. WT	0.771	2	0.557	0.697	No

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
HFD vs. CD	4.301	2	3.108	0.037	Yes

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	4.922	2	2.584	0.079	No

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	3.679	2	1.833	0.206	No

Comparisons for factor: **Genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	1.392	2	0.677	0.636	No

Comparisons for factor: **Genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.150	2	0.0810	0.955	No

Two Way Analysis of Variance Monday, August 05, 2013, 10:33:44 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: Heart wt

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.896)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.00131	0.00131	1.592	0.218
Diet	1	0.000129	0.000129	0.157	0.695
Genotype x Diet	1	0.000346	0.000346	0.421	0.522
Residual	27	0.0222	0.000821		
Total	30	0.0241	0.000802		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.218).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.695).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.522)

Power of performed test with alpha = 0.0500: for Genotype : 0.108
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.185	0.00716
KO	0.198	0.00755

Least square means for Diet :

Group	Mean	SEM
CD	0.194	0.00774
HFD	0.190	0.00696

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.190	0.0101
WT x HFD	0.180	0.0101
KO x CD	0.197	0.0117
KO x HFD	0.199	0.00955

Two Way Analysis of Variance Monday, August 05, 2013, 10:36:30 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: Liver

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.543)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.146	0.146	0.857	0.363
Diet	1	0.933	0.933	5.486	0.027
Genotype x Diet	1	0.0299	0.0299	0.176	0.678
Residual	27	4.590	0.170		
Total	30	5.790	0.193		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.363).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.027). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.678)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.526
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	1.446	0.103
KO	1.585	0.109

Least square means for Diet :

Group	Mean	SEM
CD	1.340	0.111
HFD	1.691	0.100

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	1.239	0.146
WT x HFD	1.653	0.146
KO x CD	1.441	0.168
KO x HFD	1.729	0.137

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
KO vs. WT	0.139	2	1.309	0.363	No

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.351	2	3.312	0.027	Yes

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.414	2	2.837	0.055	No

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.288	2	1.874	0.196	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.201	2	1.279	0.374	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.0759	2	0.535	0.708	No

Two Way Analysis of Variance Monday, August 05, 2013, 10:39:39 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: VF

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.086)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0641	0.0641	0.558	0.462
Diet	1	1.002	1.002	8.723	0.006
Genotype x Diet	1	0.00639	0.00639	0.0556	0.815
Residual	27	3.102	0.115		
Total	30	4.149	0.138		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.462).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.006). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.815)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.775
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.502	0.0847
KO	0.410	0.0893

Least square means for Diet :

Group	Mean	SEM
CD	0.274	0.0915
HFD	0.638	0.0824

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.306	0.120
WT x HFD	0.698	0.120
KO x CD	0.243	0.138
KO x HFD	0.577	0.113

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	0.0919	2	1.056	0.462	No

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.364	2	4.177	0.007	Yes

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.393	2	3.277	0.028	Yes

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.335	2	2.649	0.072	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.0629	2	0.486	0.734	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.121	2	1.039	0.469	No

Two Way Analysis of Variance Monday, August 05, 2013, 10:41:13 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: Gastroc

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.626)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.000822	0.000822	4.431	0.045
Diet	1	0.000211	0.000211	1.139	0.295
Genotype x Diet	1	0.000465	0.000465	2.509	0.125
Residual	27	0.00501	0.000185		
Total	30	0.00652	0.000217		

The difference in the mean values among the different levels of Genotype is greater than would be expected by chance after allowing for effects of differences in Diet. There is a statistically significant difference (P = 0.045). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.295).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.125)

Power of performed test with alpha = 0.0500: for Genotype : 0.419
Power of performed test with alpha = 0.0500: for Diet : 0.0617
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.207

Least square means for Genotype :

Group	Mean	SEM
WT	0.115	0.00340
KO	0.125	0.00359

Least square means for Diet :

Group	Mean	SEM
CD	0.117	0.00368
HFD	0.122	0.00331

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.108	0.00481
WT x HFD	0.121	0.00481
KO x CD	0.126	0.00556
KO x HFD	0.124	0.00454

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
KO vs. WT	0.0104	2	2.977	0.045	Yes

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.00528	2	1.509	0.295	No

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.0131	2	2.724	0.065	No

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	0.00256	2	0.504	0.725	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.0182	2	3.509	0.020	Yes

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.00258	2	0.551	0.700	No

Two Way Analysis of Variance Monday, August 05, 2013, 10:43:19 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: TA

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.359)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.00000179	0.00000179	0.0548	0.817
Diet	1	0.000182	0.000182	5.569	0.026
Genotype x Diet	1	0.0000162	0.0000162	0.495	0.488
Residual	27	0.000883	0.0000327		
Total	30	0.00109	0.0000364		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.817).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.026). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.488)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.534
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.0413	0.00143
KO	0.0418	0.00151

Least square means for Diet :

Group	Mean	SEM
CD	0.0391	0.00154
HFD	0.0440	0.00139

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.0381	0.00202
WT x HFD	0.0445	0.00202
KO x CD	0.0401	0.00233
KO x HFD	0.0435	0.00191

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
KO vs. WT	0.000486	2	0.331	0.817	No

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.00490	2	3.337	0.026	Yes

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.00636	2	3.147	0.035	Yes

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.00344	2	1.614	0.264	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.00195	2	0.892	0.534	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.000975	2	0.496	0.728	No

Two Way Analysis of Variance Monday, August 05, 2013, 10:34:15 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: Heart/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.015)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.00522	0.00522	0.551	0.464
Diet	1	0.0751	0.0751	7.942	0.009
Genotype x Diet	1	0.0115	0.0115	1.219	0.279
Residual	27	0.255	0.00946		
Total	30	0.349	0.0116		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.464).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.009). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.279)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.727
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0699

Least square means for Genotype :

Group	Mean	SEM
WT	0.619	0.0243
KO	0.645	0.0256

Least square means for Diet :

Group	Mean	SEM
CD	0.682	0.0263
HFD	0.582	0.0236

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.688	0.0344
WT x HFD	0.550	0.0344
KO x CD	0.676	0.0397
KO x HFD	0.615	0.0324

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	Diff of Means	p	q	P	P<0.050
KO vs. WT	0.0262	2	1.050	0.464	No

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
CD vs. HFD	0.0996	2	3.985	0.009	Yes

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	0.139	2	4.030	0.008	Yes

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	0.0606	2	1.671	0.248	No

Comparisons for factor: **Genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	0.0128	2	0.344	0.810	No

Comparisons for factor: **Genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.0652	2	1.952	0.179	No

Two Way Analysis of Variance Monday, August 05, 2013, 10:38:26 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: Liver/BW

Normality Test: Failed (P = 0.013)

Equal Variance Test: Passed (P = 0.188)

Source of Variation	DF	SS	MS	F	P
Genotype	1	1.215	1.215	0.905	0.350
Diet	1	2.251	2.251	1.676	0.206
Genotype x Diet	1	0.286	0.286	0.213	0.648
Residual	27	36.259	1.343		
Total	30	40.356	1.345		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.350).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.206).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.648)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.117
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	4.688	0.290
KO	5.088	0.305

Least square means for Diet :

Group	Mean	SEM
CD	4.616	0.313
HFD	5.161	0.282

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	4.318	0.410
WT x HFD	5.057	0.410
KO x CD	4.913	0.473
KO x HFD	5.264	0.386

Two Way Analysis of Variance Monday, August 05, 2013, 10:40:38 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: VF/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.023)

Source of Variation	DF	SS	MS	F	P
Diet	1	5.782	5.782	10.326	0.003
Genotype	1	0.588	0.588	1.050	0.314
Diet x Genotype	1	0.0158	0.0158	0.0282	0.868
Residual	27	15.118	0.560		
Total	30	21.261	0.709		

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.003). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.314).

The effect of different levels of Diet does not depend on what level of Genotype is present. There is not a statistically significant interaction between Diet and Genotype. (P = 0.868)

Power of performed test with alpha = 0.0500: for Diet : 0.853
Power of performed test with alpha = 0.0500: for Genotype : 0.0528
Power of performed test with alpha = 0.0500: for Diet x Genotype : 0.0500

Least square means for Diet :

Group	Mean	SEM
CD	0.943	0.202
HFD	1.817	0.182

Least square means for Genotype :

Group	Mean	SEM
WT	1.519	0.187
KO	1.241	0.197

Least square means for Diet x Genotype :

Group	Mean	SEM
CD x WT	1.060	0.265
CD x KO	0.827	0.305
HFD x WT	1.979	0.265
HFD x KO	1.654	0.249

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.873	2	4.544	0.004	Yes

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	0.279	2	1.449	0.315	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.233	2	0.815	0.569	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	0.324	2	1.261	0.381	No

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.919	2	3.474	0.021	Yes

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.828	2	2.968	0.045	Yes

Two Way Analysis of Variance Monday, August 05, 2013, 10:42:51 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: Gastroc/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.589)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.00379	0.00379	1.095	0.305
Diet	1	0.00592	0.00592	1.710	0.202
Genotype x Diet	1	0.00356	0.00356	1.030	0.319
Residual	27	0.0934	0.00346		
Total	30	0.105	0.00349		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.305).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.202).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.319)

Power of performed test with alpha = 0.0500: for Genotype : 0.0573
Power of performed test with alpha = 0.0500: for Diet : 0.120
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0507

Least square means for Genotype :

Group	Mean	SEM
WT	0.384	0.0147
KO	0.407	0.0155

Least square means for Diet :

Group	Mean	SEM
CD	0.409	0.0159
HFD	0.381	0.0143

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.387	0.0208
WT x HFD	0.381	0.0208
KO x CD	0.431	0.0240
KO x HFD	0.382	0.0196

Two Way Analysis of Variance Monday, August 05, 2013, 10:43:46 AM

Data source: Male tissue weights in KCJ_Tissue wts_7-22-13

General Linear Model

Dependent Variable: TA/BW

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.708)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0000220	0.0000220	0.0590	0.810
Diet	1	0.0000271	0.0000271	0.0726	0.790
Genotype x Diet	1	0.0000829	0.0000829	0.222	0.641
Residual	27	0.0101	0.000373		
Total	30	0.0102	0.000341		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.810).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.790).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.641)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.137	0.00483
KO	0.136	0.00509

Least square means for Diet :

Group	Mean	SEM
CD	0.137	0.00522
HFD	0.136	0.00470

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.137	0.00683
WT x HFD	0.138	0.00683
KO x CD	0.138	0.00789
KO x HFD	0.133	0.00644

Two Way Analysis of Variance Wednesday, July 24, 2013, 7:56:26 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 1

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.653)

Source of Variation	DF	SS	MS	F	P
Genotype	1	1.339	1.339	1.237	0.275
Diet	1	2.861	2.861	2.641	0.115
Genotype x Diet	1	3.363	3.363	3.105	0.089
Residual	29	31.414	1.083		
Total	32	38.881	1.215		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.275).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.115).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.089)

Power of performed test with alpha = 0.0500: for Genotype : 0.0717
Power of performed test with alpha = 0.0500: for Diet : 0.222
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.274

Least square means for Genotype :

Group	Mean	SEM
WT	-0.131	0.260
KO	0.275	0.256

Least square means for Diet :

Group	Mean	SEM
CD	-0.225	0.269
HFD	0.369	0.247

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	-0.750	0.368
WT x HFD	0.488	0.368
KO x CD	0.300	0.393
KO x HFD	0.250	0.329

Two Way Analysis of Variance Wednesday, July 24, 2013, 7:57:33 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 2

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.950)

Source of Variation	DF	SS	MS	F	P
Diet	1	0.955	0.955	0.616	0.439
Genotype	1	0.466	0.466	0.301	0.587
Diet x Genotype	1	0.0941	0.0941	0.0608	0.807
Residual	30	46.481	1.549		
Total	33	48.199	1.461		

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.439).

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.587).

The effect of different levels of Diet does not depend on what level of Genotype is present. There is not a statistically significant interaction between Diet and Genotype. (P = 0.807)

Power of performed test with alpha = 0.0500: for Diet : 0.0500
Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet x Genotype : 0.0500

Least square means for Diet :

Group	Mean	SEM
CD	-0.179	0.314
HFD	0.159	0.295

Least square means for Genotype :

Group	Mean	SEM
WT	-0.128	0.302
KO	0.108	0.307

Least square means for Diet x Genotype :

Group	Mean	SEM
CD x WT	-0.244	0.415
CD x KO	-0.114	0.470
HFD x WT	-0.0125	0.440
HFD x KO	0.330	0.394

Two Way Analysis of Variance Wednesday, July 24, 2013, 7:58:37 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 3

Normality Test: Failed (P = 0.008)

Equal Variance Test: Passed (P = 0.166)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.557	0.557	0.258	0.615
Diet	1	2.422	2.422	1.123	0.298
Genotype x Diet	1	0.0360	0.0360	0.0167	0.898
Residual	30	64.717	2.157		
Total	33	68.041	2.062		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.615).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.298).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.898)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.0601
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.635	0.357
KO	0.894	0.362

Least square means for Diet :

Group	Mean	SEM
CD	0.495	0.370
HFD	1.034	0.348

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.333	0.490
WT x HFD	0.938	0.519
KO x CD	0.657	0.555
KO x HFD	1.130	0.464

Two Way Analysis of Variance Wednesday, July 24, 2013, 7:58:58 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 4

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.690)

Source of Variation	DF	SS	MS	F	P
Genotype	1	3.020	3.020	1.087	0.306
Diet	1	4.737	4.737	1.704	0.202
Genotype x Diet	1	0.217	0.217	0.0781	0.782
Residual	30	83.373	2.779		
Total	33	92.457	2.802		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.306).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.202).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.782)

Power of performed test with alpha = 0.0500: for Genotype : 0.0565
Power of performed test with alpha = 0.0500: for Diet : 0.120
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	0.642	0.405
KO	1.243	0.411

Least square means for Diet :

Group	Mean	SEM
CD	0.566	0.420
HFD	1.319	0.395

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.346	0.556
WT x HFD	0.938	0.589
KO x CD	0.786	0.630
KO x HFD	1.700	0.527

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:01:46 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 5

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.383)

Source of Variation	DF	SS	MS	F	P
Genotype	1	0.0360	0.0360	0.0176	0.895
Diet	1	3.221	3.221	1.572	0.220
Genotype x Diet	1	0.0282	0.0282	0.0138	0.907
Residual	30	61.482	2.049		
Total	33	64.896	1.967		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.895).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.220).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.907)

Power of performed test with alpha = 0.0500: for Genotype : 0.0500
Power of performed test with alpha = 0.0500: for Diet : 0.106
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.0500

Least square means for Genotype :

Group	Mean	SEM
WT	1.073	0.348
KO	1.139	0.353

Least square means for Diet :

Group	Mean	SEM
CD	0.795	0.361
HFD	1.416	0.340

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.733	0.477
WT x HFD	1.413	0.506
KO x CD	0.857	0.541
KO x HFD	1.420	0.453

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:02:21 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 6

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.422)

Source of Variation	DF	SS	MS	F	P
Genotype	1	2.331	2.331	1.848	0.185
Diet	1	6.510	6.510	5.161	0.031
Genotype x Diet	1	5.630	5.630	4.463	0.043
Residual	29	36.583	1.261		
Total	32	51.422	1.607		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.185).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.031). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype depends on what level of Diet is present. There is a statistically significant interaction between Genotype and Diet. (P = 0.043)

Power of performed test with alpha = 0.0500: for Genotype : 0.135
Power of performed test with alpha = 0.0500: for Diet : 0.496
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.424

Least square means for Genotype :

Group	Mean	SEM
WT	1.840	0.273
KO	1.298	0.290

Least square means for Diet :

Group	Mean	SEM
CD	1.117	0.296
HFD	2.021	0.266

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	0.967	0.374
WT x HFD	2.712	0.397
KO x CD	1.267	0.459
KO x HFD	1.330	0.355

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	0.541	2	1.922	0.185	No

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	0.905	2	3.213	0.031	Yes

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	1.746	2	4.524	0.003	Yes

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	0.0633	2	0.154	0.914	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.300	2	0.717	0.616	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	1.382	2	3.670	0.015	Yes

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:29:00 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 7

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.013)

Source of Variation	DF	SS	MS	F	P
Genotype	1	10.082	10.082	2.840	0.102
Diet	1	7.360	7.360	2.073	0.160
Genotype x Diet	1	10.082	10.082	2.840	0.102
Residual	30	106.503	3.550		
Total	33	133.614	4.049		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.102).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.160).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.102)

Power of performed test with alpha = 0.0500: for Genotype : 0.245
Power of performed test with alpha = 0.0500: for Diet : 0.160
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.245

Least square means for Genotype :

Group	Mean	SEM
WT	2.719	0.458
KO	1.620	0.464

Least square means for Diet :

Group	Mean	SEM
CD	1.700	0.475
HFD	2.639	0.447

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	1.700	0.628
WT x HFD	3.737	0.666
KO x CD	1.700	0.712
KO x HFD	1.540	0.596

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:29:27 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 8

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.027)

Source of Variation	DF	SS	MS	F	P
Genotype	1	16.049	16.049	2.779	0.106
Diet	1	18.642	18.642	3.228	0.082
Genotype x Diet	1	30.825	30.825	5.338	0.028
Residual	30	173.232	5.774		
Total	33	238.274	7.220		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.106).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.082).

The effect of different levels of Genotype depends on what level of Diet is present. There is a statistically significant interaction between Genotype and Diet. (P = 0.028)

Power of performed test with alpha = 0.0500: for Genotype : 0.238
Power of performed test with alpha = 0.0500: for Diet : 0.288
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.514

Least square means for Genotype :

Group	Mean	SEM
WT	3.430	0.584
KO	2.044	0.592

Least square means for Diet :

Group	Mean	SEM
CD	1.990	0.605
HFD	3.484	0.570

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	1.722	0.801
WT x HFD	5.137	0.850
KO x CD	2.257	0.908
KO x HFD	1.830	0.760

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	Diff of Means	p	q	P	P<0.050
WT vs. KO	1.386	2	2.358	0.106	No

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
HFD vs. CD	1.494	2	2.541	0.083	No

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	3.415	2	4.136	0.007	Yes

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	0.427	2	0.510	0.721	No

Comparisons for factor: **Genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.535	2	0.625	0.662	No

Comparisons for factor: **Genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	3.307	2	4.104	0.007	Yes

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:30:00 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 9

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.019)

Source of Variation	DF	SS	MS	F	P
Genotype	1	20.021	20.021	2.851	0.103
Diet	1	25.210	25.210	3.590	0.069
Genotype x Diet	1	28.029	28.029	3.991	0.056
Residual	26	182.595	7.023		
Total	29	259.654	8.954		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.103).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.069).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.056)

Power of performed test with alpha = 0.0500: for Genotype : 0.244
Power of performed test with alpha = 0.0500: for Diet : 0.326
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.370

Least square means for Genotype :

Group	Mean	SEM
WT	3.587	0.663
KO	1.950	0.708

Least square means for Diet :

Group	Mean
CD	1.850
HFD	3.687

Std Err of LS Mean = 0.686

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	1.700	0.937
WT x HFD	5.475	0.937
KO x CD	2.000	1.002
KO x HFD	1.900	1.002

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:30:35 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: week 10

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.040)

Source of Variation	DF	SS	MS	F	P
Genotype	1	13.204	13.204	1.943	0.175
Diet	1	14.644	14.644	2.155	0.154
Genotype x Diet	1	32.733	32.733	4.817	0.037
Residual	27	183.471	6.795		
Total	30	246.740	8.225		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.175).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.154).

The effect of different levels of Genotype depends on what level of Diet is present. There is a statistically significant interaction between Genotype and Diet. (P = 0.037)

Power of performed test with alpha = 0.0500: for Genotype : 0.145
Power of performed test with alpha = 0.0500: for Diet : 0.168
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.459

Least square means for Genotype :

Group	Mean	SEM
WT	3.913	0.633
KO	2.601	0.697

Least square means for Diet :

Group	Mean	SEM
CD	2.566	0.657
HFD	3.948	0.675

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	2.189	0.869
WT x HFD	5.637	0.922
KO x CD	2.943	0.985
KO x HFD	2.259	0.985

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	Diff of Means	p	q	P	P<0.050
WT vs. KO	1.312	2	1.971	0.175	No

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
HFD vs. CD	1.382	2	2.076	0.154	No

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	3.449	2	3.850	0.011	Yes

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	0.684	2	0.695	0.627	No

Comparisons for factor: **Genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.754	2	0.812	0.571	No

Comparisons for factor: **Genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	3.379	2	3.542	0.019	Yes

Two Way Analysis of Variance Wednesday, July 24, 2013, 8:32:27 AM

Data source: Data 1 in BW gain by week_KCJ

General Linear Model

Dependent Variable: final wt

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.016)

Source of Variation	DF	SS	MS	F	P
Genotype	1	27.334	27.334	3.364	0.080
Diet	1	29.664	29.664	3.651	0.069
Genotype x Diet	1	37.933	37.933	4.668	0.041
Residual	23	186.898	8.126		
Total	26	289.611	11.139		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.080).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.069).

The effect of different levels of Genotype depends on what level of Diet is present. There is a statistically significant interaction between Genotype and Diet. (P = 0.041)

Power of performed test with alpha = 0.0500: for Genotype : 0.299
Power of performed test with alpha = 0.0500: for Diet : 0.330
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.439

Least square means for Genotype :

Group	Mean	SEM
WT	4.886	0.718
KO	2.826	0.863

Least square means for Diet :

Group	Mean	SEM
CD	2.783	0.751
HFD	4.929	0.835

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	2.600	0.950
WT x HFD	7.171	1.077
KO x CD	2.967	1.164
KO x HFD	2.686	1.275

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	2.059	2	2.594	0.080	No

Comparisons for factor: **Diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	2.145	2	2.702	0.069	No

Comparisons for factor: **Diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	4.571	2	4.500	0.004	Yes

Comparisons for factor: **Diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	0.281	2	0.230	0.872	No

Comparisons for factor: **Genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	0.367	2	0.345	0.809	No

Comparisons for factor: **Genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	4.485	2	3.800	0.013	Yes

Two Way Analysis of Variance Monday, August 05, 2013, 11:02:27 AM

Data source: Avg daily intake in daily feed intake data

General Linear Model

Dependent Variable: grams feed intake

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.222)

Source of Variation	DF	SS	MS	F	P
genotype	1	1.879	1.879	3.662	0.063
diet	1	1.158	1.158	2.257	0.141
genotype x diet	1	0.123	0.123	0.239	0.628
Residual	37	18.987	0.513		
Total	40	22.247	0.556		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.063).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.141).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.628)

Power of performed test with alpha = 0.0500: for genotype : 0.340
Power of performed test with alpha = 0.0500: for diet : 0.181
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	3.263	0.177
KO	3.711	0.153

Least square means for diet :

Group	Mean	SEM
CD	3.663	0.143
HFD	3.311	0.185

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	3.382	0.199
WT x HFD	3.145	0.292
KO x CD	3.944	0.207
KO x HFD	3.478	0.227

Two Way Analysis of Variance Friday, August 09, 2013, 1:45:11 PM

Data source: Endurance Ex data in KCJ_endurance ex_7-22-13

General Linear Model

Dependent Variable: Distance (m)

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.960)

Source of Variation	DF	SS	MS	F	P
Genotype	1	89798.542	89798.542	3.086	0.090
Diet	1	80021.301	80021.301	2.750	0.108
Genotype x Diet	1	83679.020	83679.020	2.876	0.101
Residual	28	814796.544	29099.877		
Total	31	1096474.760	35370.154		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.090).

The difference in the mean values among the different levels of Diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Genotype. There is not a statistically significant difference (P = 0.108).

The effect of different levels of Genotype does not depend on what level of Diet is present. There is not a statistically significant interaction between Genotype and Diet. (P = 0.101)

Power of performed test with alpha = 0.0500: for Genotype : 0.271
Power of performed test with alpha = 0.0500: for Diet : 0.234
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.248

Least square means for Genotype :

Group	Mean	SEM
WT	479.211	44.954
KO	371.802	41.445

Least square means for Diet :

Group	Mean	SEM
CD	476.203	41.445
HFD	374.810	44.954

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	581.749	56.862
WT x HFD	376.672	69.642
KO x CD	370.656	60.312
KO x HFD	372.948	56.862

Two Way Analysis of Variance Friday, August 09, 2013, 1:45:47 PM

Data source: Endurance Ex data in KCJ_endurance ex_7-22-13

General Linear Model

Dependent Variable: Maximal Speed (m min)

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.754)

Source of Variation	DF	SS	MS	F	P
Genotype	1	29.429	29.429	3.256	0.082
Diet	1	40.384	40.384	4.467	0.044
Genotype x Diet	1	48.649	48.649	5.382	0.028
Residual	28	253.111	9.040		
Total	31	382.000	12.323		

The difference in the mean values among the different levels of Genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Diet. There is not a statistically significant difference (P = 0.082).

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in Genotype. There is a statistically significant difference (P = 0.044). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Genotype depends on what level of Diet is present. There is a statistically significant interaction between Genotype and Diet. (P = 0.028)

Power of performed test with alpha = 0.0500: for Genotype : 0.290
Power of performed test with alpha = 0.0500: for Diet : 0.423
Power of performed test with alpha = 0.0500: for Genotype x Diet : 0.517

Least square means for Genotype :

Group	Mean	SEM
WT	21.056	0.792
KO	19.111	0.730

Least square means for Diet :

Group	Mean	SEM
CD	21.222	0.730
HFD	18.944	0.792

Least square means for Genotype x Diet :

Group	Mean	SEM
WT x CD	23.444	1.002
WT x HFD	18.667	1.227
KO x CD	19.000	1.063
KO x HFD	19.222	1.002

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **Genotype**

Comparison	Diff of Means	p	q	P	P<0.050
WT vs. KO	1.944	2	2.552	0.082	No

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
CD vs. HFD	2.278	2	2.989	0.044	Yes

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	4.778	2	4.264	0.006	Yes

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	0.222	2	0.215	0.880	No

Comparisons for factor: **Genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	4.444	2	4.302	0.005	Yes

Comparisons for factor: **Genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.556	2	0.496	0.729	No

Two Way Analysis of Variance Thursday, August 01, 2013, 2:38:30 PM

Data source: GTT time points-FINAL in Notebook 3

General Linear Model

Dependent Variable: 0min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.997)

Source of Variation	DF	SS	MS	F	P
Col 1	1	659.060	659.060	1.163	0.290
Col 2	1	1086.919	1086.919	1.919	0.177
Col 1 x Col 2	1	1503.770	1503.770	2.654	0.114
Residual	29	16428.857	566.512		
Total	32	20102.909	628.216		

The difference in the mean values among the different levels of Col 1 is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Col 2. There is not a statistically significant difference (P = 0.290).

The difference in the mean values among the different levels of Col 2 is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Col 1. There is not a statistically significant difference (P = 0.177).

The effect of different levels of Col 1 does not depend on what level of Col 2 is present. There is not a statistically significant interaction between Col 1 and Col 2. (P = 0.114)

Power of performed test with alpha = 0.0500: for Col 1 : 0.0643
Power of performed test with alpha = 0.0500: for Col 2 : 0.143
Power of performed test with alpha = 0.0500: for Col 1 x Col 2 : 0.224

Least square means for Col 1 :

Group	Mean	SEM
WT	132.690	5.997
KO	123.643	5.865

Least square means for Col 2 :

Group	Mean	SEM
CD	133.976	5.997
HFD	122.357	5.865

Least square means for Col 1 x Col 2 :

Group	Mean	SEM
WT x CD	131.667	7.934
WT x HFD	133.714	8.996
KO x CD	136.286	8.996
KO x HFD	111.000	7.527

Two Way Analysis of Variance Thursday, August 01, 2013, 2:39:21 PM

Data source: GTT time points-FINAL in Notebook 3

General Linear Model

Dependent Variable: 30min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.331)

Source of Variation	DF	SS	MS	F	P
genotype	1	4508.916	4508.916	1.677	0.205
diet	1	31571.203	31571.203	11.745	0.002
genotype x diet	1	9825.977	9825.977	3.655	0.066
Residual	29	77954.051	2688.071		
Total	32	120469.333	3764.667		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.205).

The difference in the mean values among the different levels of diet is greater than would be expected by chance after allowing for effects of differences in genotype. There is a statistically significant difference (P = 0.002). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.066)

Power of performed test with alpha = 0.0500: for genotype : 0.117
Power of performed test with alpha = 0.0500: for diet : 0.903
Power of performed test with alpha = 0.0500: for genotype x diet : 0.336

Least square means for genotype :

Group	Mean	SEM
WT	281.222	13.064
KO	257.557	12.775

Least square means for diet :

Group	Mean	SEM
CD	238.079	13.064
HFD	300.700	12.775

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	232.444	17.282
WT x HFD	330.000	19.596
KO x CD	243.714	19.596
KO x HFD	271.400	16.395

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	23.665	2	1.832	0.206	No

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	62.621	2	4.847	0.002	Yes

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	97.556	2	5.280	<0.001	Yes

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	27.686	2	1.532	0.288	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	11.270	2	0.610	0.670	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	58.600	2	3.244	0.029	Yes

Two Way Analysis of Variance Thursday, August 01, 2013, 2:40:06 PM

Data source: GTT time points-FINAL in Notebook 3

General Linear Model

Dependent Variable: 60min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.131)

Source of Variation	DF	SS	MS	F	P
genotype	1	12424.327	12424.327	4.613	0.040
diet	1	9152.621	9152.621	3.399	0.075
genotype x diet	1	3712.568	3712.568	1.379	0.250
Residual	29	78100.614	2693.125		
Total	32	100666.727	3145.835		

The difference in the mean values among the different levels of genotype is greater than would be expected by chance after allowing for effects of differences in diet. There is a statistically significant difference (P = 0.040). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.075).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.250)

Power of performed test with alpha = 0.0500: for genotype : 0.440
Power of performed test with alpha = 0.0500: for diet : 0.307
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0862

Least square means for genotype :

Group	Mean	SEM
WT	233.262	13.076
KO	193.979	12.787

Least square means for diet :

Group	Mean	SEM
CD	196.762	13.076
HFD	230.479	12.787

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	205.667	17.298
WT x HFD	260.857	19.615
KO x CD	187.857	19.615
KO x HFD	200.100	16.411

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	39.283	2	3.038	0.040	Yes

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	33.717	2	2.607	0.076	No

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	55.190	2	2.984	0.044	Yes

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	12.243	2	0.677	0.636	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	17.810	2	0.963	0.501	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	60.757	2	3.360	0.024	Yes

Two Way Analysis of Variance Thursday, August 01, 2013, 2:40:44 PM

Data source: GTT time points-FINAL in Notebook 3

General Linear Model

Dependent Variable: 90min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.677)

Source of Variation	DF	SS	MS	F	P
genotype	1	8757.971	8757.971	5.365	0.028
diet	1	6002.162	6002.162	3.677	0.065
genotype x diet	1	7414.515	7414.515	4.542	0.042
Residual	29	47339.027	1632.380		
Total	32	67708.545	2115.892		

The difference in the mean values among the different levels of genotype is greater than would be expected by chance after allowing for effects of differences in diet. There is a statistically significant difference (P = 0.028). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.065).

The effect of different levels of genotype depends on what level of diet is present. There is a statistically significant interaction between genotype and diet. (P = 0.042)

Power of performed test with alpha = 0.0500: for genotype : 0.516
Power of performed test with alpha = 0.0500: for diet : 0.338
Power of performed test with alpha = 0.0500: for genotype x diet : 0.432

Least square means for genotype :

Group	Mean	SEM
WT	193.603	10.181
KO	160.621	9.955

Least square means for diet :

Group	Mean	SEM
CD	163.460	10.181
HFD	190.764	9.955

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	164.778	13.468
WT x HFD	222.429	15.271
KO x CD	162.143	15.271
KO x HFD	159.100	12.776

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	32.982	2	3.276	0.028	Yes

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	27.304	2	2.712	0.065	No

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	57.651	2	4.004	0.008	Yes

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	3.043	2	0.216	0.880	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	2.635	2	0.183	0.898	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	63.329	2	4.498	0.004	Yes

Two Way Analysis of Variance Thursday, August 01, 2013, 2:41:23 PM

Data source: GTT time points-FINAL in Notebook 3

General Linear Model

Dependent Variable: 120min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.841)

Source of Variation	DF	SS	MS	F	P
genotype	1	2294.731	2294.731	1.694	0.203
diet	1	4738.590	4738.590	3.499	0.072
genotype x diet	1	9122.054	9122.054	6.736	0.015
Residual	29	39274.813	1354.304		
Total	32	54484.970	1702.655		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.203).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.072).

The effect of different levels of genotype depends on what level of diet is present. There is a statistically significant interaction between genotype and diet. (P = 0.015)

Power of performed test with alpha = 0.0500: for genotype : 0.119
Power of performed test with alpha = 0.0500: for diet : 0.318
Power of performed test with alpha = 0.0500: for genotype x diet : 0.640

Least square means for genotype :

Group	Mean	SEM
WT	165.183	9.273
KO	148.300	9.068

Least square means for diet :

Group	Mean	SEM
CD	144.611	9.273
HFD	168.871	9.068

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	136.222	12.267
WT x HFD	194.143	13.909
KO x CD	153.000	13.909
KO x HFD	143.600	11.637

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	16.883	2	1.841	0.203	No

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	24.260	2	2.645	0.072	No

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	57.921	2	4.417	0.004	Yes

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	9.400	2	0.733	0.608	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	16.778	2	1.279	0.373	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	50.543	2	3.941	0.009	Yes

Two Way Analysis of Variance Thursday, August 01, 2013, 2:35:29 PM

Data source: Data 1 in Notebook 2

General Linear Model

Dependent Variable: AUC

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.190)

Source of Variation	DF	SS	MS	F	P
genotype	1	86545487.033	86545487.033	4.355	0.046
Diet	1	122184037.832	122184037.832	6.148	0.019
genotype x Diet	1	88975728.567	88975728.567	4.477	0.043
Residual	29	576298581.429	19872364.877		
Total	32	846460690.909	26451896.591		

The difference in the mean values among the different levels of genotype is greater than would be expected by chance after allowing for effects of differences in Diet. There is a statistically significant difference (P = 0.046). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in genotype. There is a statistically significant difference (P = 0.019). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of genotype depends on what level of Diet is present. There is a statistically significant interaction between genotype and Diet. (P = 0.043)

Power of performed test with alpha = 0.0500: for genotype : 0.412
Power of performed test with alpha = 0.0500: for Diet : 0.589
Power of performed test with alpha = 0.0500: for genotype x Diet : 0.425

Least square means for genotype :

Group	Mean	SEM
WT	25715.000	1123.271
KO	22436.357	1098.425

Least square means for Diet :

Group	Mean	SEM
CD	22127.857	1123.271
HFD	26023.500	1098.425

Least square means for genotype x Diet :

Group	Mean	SEM
WT x CD	22105.000	1485.948
WT x HFD	29325.000	1684.906
KO x CD	22150.714	1684.906
KO x HFD	22722.000	1409.694

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	Diff of Means	p	q	P	P<0.050
WT vs. KO	3278.643	2	2.951	0.046	Yes

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
HFD vs. CD	3895.643	2	3.507	0.019	Yes

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	7220.000	2	4.545	0.003	Yes

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	571.286	2	0.368	0.797	No

Comparisons for factor: **genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	45.714	2	0.0288	0.984	No

Comparisons for factor: **genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	6603.000	2	4.251	0.006	Yes

Two Way Analysis of Variance Sunday, August 04, 2013, 10:35:19 AM

Data source: ITT % baseline-final in ITT time points-final

General Linear Model

Dependent Variable: baseline

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.178)

Source of Variation	DF	SS	MS	F	P
genotype	1	12.718	12.718	0.0170	0.897
diet	1	4093.130	4093.130	5.477	0.026
genotype x diet	1	1171.429	1171.429	1.568	0.220
Residual	31	23167.004	747.323		
Total	34	28468.971	837.323		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.897).

The difference in the mean values among the different levels of diet is greater than would be expected by chance after allowing for effects of differences in genotype. There is a statistically significant difference (P = 0.026). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.220)

Power of performed test with alpha = 0.0500: for genotype : 0.0500
Power of performed test with alpha = 0.0500: for diet : 0.529
Power of performed test with alpha = 0.0500: for genotype x diet : 0.106

Least square means for genotype :

Group	Mean	SEM
WT	196.287	6.484
KO	197.507	6.736

Least square means for diet :

Group	Mean	SEM
CD	185.957	6.736
HFD	207.837	6.484

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	191.200	8.645
WT x HFD	201.375	9.665
KO x CD	180.714	10.332
KO x HFD	214.300	8.645

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
KO vs. WT	1.220	2	0.184	0.897	No

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	21.880	2	3.310	0.026	Yes

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	10.175	2	1.110	0.439	No

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	33.586	2	3.526	0.018	Yes

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	10.486	2	1.101	0.442	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	12.925	2	1.410	0.327	No

Two Way Analysis of Variance Sunday, August 04, 2013, 10:36:40 AM

Data source: ITT % baseline-final in ITT time points-final

General Linear Model

Dependent Variable: change from baseline at 30min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.560)

Source of Variation	DF	SS	MS	F	P
genotype	1	605.403	605.403	2.527	0.122
diet	1	146.367	146.367	0.611	0.440
genotype x diet	1	1027.501	1027.501	4.290	0.047
Residual	31	7425.606	239.536		
Total	34	9215.584	271.047		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.122).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.440).

The effect of different levels of genotype depends on what level of diet is present. There is a statistically significant interaction between genotype and diet. (P = 0.047)

Power of performed test with alpha = 0.0500: for genotype : 0.210
Power of performed test with alpha = 0.0500: for diet : 0.0500
Power of performed test with alpha = 0.0500: for genotype x diet : 0.407

Least square means for genotype :

Group	Mean	SEM
WT	-32.472	3.671
KO	-40.887	3.814

Least square means for diet :

Group	Mean	SEM
CD	-38.748	3.814
HFD	-34.611	3.671

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-40.022	4.894
WT x HFD	-24.922	5.472
KO x CD	-37.475	5.850
KO x HFD	-44.300	4.894

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	Diff of Means	p	q	P	P<0.050
WT vs. KO	8.415	2	2.248	0.122	No

Comparisons for factor: **diet**

Comparison	Diff of Means	p	q	P	P<0.050
HFD vs. CD	4.138	2	1.105	0.440	No

Comparisons for factor: **diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	15.100	2	2.909	0.048	Yes

Comparisons for factor: **diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
CD vs. HFD	6.825	2	1.266	0.378	No

Comparisons for factor: **genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	2.548	2	0.472	0.741	No

Comparisons for factor: **genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	19.378	2	3.733	0.013	Yes

Two Way Analysis of Variance Sunday, August 04, 2013, 10:37:58 AM

Data source: ITT % baseline-final in ITT time points-final

General Linear Model

Dependent Variable: change from baseline 60min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.900)

Source of Variation	DF	SS	MS	F	P
genotype	1	1687.838	1687.838	3.173	0.085
diet	1	262.485	262.485	0.493	0.488
genotype x diet	1	168.400	168.400	0.317	0.578
Residual	31	16491.311	531.978		
Total	34	18508.488	544.367		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.085).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.488).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.578)

Power of performed test with alpha = 0.0500: for genotype : 0.283
Power of performed test with alpha = 0.0500: for diet : 0.0500
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-31.779	5.470
KO	-45.829	5.683

Least square means for diet :

Group	Mean	SEM
CD	-41.574	5.683
HFD	-36.033	5.470

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-36.768	7.294
WT x HFD	-26.789	8.155
KO x CD	-46.381	8.718
KO x HFD	-45.278	7.294

Two Way Analysis of Variance Sunday, August 04, 2013, 10:38:32 AM

Data source: ITT % baseline-final in ITT time points-final

General Linear Model

Dependent Variable: change from baseline 90min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.776)

Source of Variation	DF	SS	MS	F	P
genotype	1	3267.717	3267.717	3.287	0.080
diet	1	1663.856	1663.856	1.674	0.205
genotype x diet	1	40.155	40.155	0.0404	0.842
Residual	31	30816.080	994.067		
Total	34	35255.139	1036.916		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.080).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.205).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.842)

Power of performed test with alpha = 0.0500: for genotype : 0.295
Power of performed test with alpha = 0.0500: for diet : 0.117
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-19.077	7.478
KO	-38.628	7.769

Least square means for diet :

Group	Mean	SEM
CD	-35.828	7.769
HFD	-21.877	7.478

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-27.136	9.970
WT x HFD	-11.019	11.147
KO x CD	-44.519	11.917
KO x HFD	-32.736	9.970

Two Way Analysis of Variance Sunday, August 04, 2013, 10:39:02 AM

Data source: ITT % baseline-final in ITT time points-final

General Linear Model

Dependent Variable: change from baseline 120min

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.948)

Source of Variation	DF	SS	MS	F	P
genotype	1	6450.103	6450.103	7.923	0.008
diet	1	4319.649	4319.649	5.306	0.028
genotype x diet	1	111.677	111.677	0.137	0.714
Residual	31	25237.942	814.127		
Total	34	34913.922	1026.880		

The difference in the mean values among the different levels of genotype is greater than would be expected by chance after allowing for effects of differences in diet. There is a statistically significant difference (P = 0.008). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of diet is greater than would be expected by chance after allowing for effects of differences in genotype. There is a statistically significant difference (P = 0.028). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.714)

Power of performed test with alpha = 0.0500: for genotype : 0.730
Power of performed test with alpha = 0.0500: for diet : 0.512
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-3.266	6.767
KO	-30.733	7.031

Least square means for diet :

Group	Mean	SEM
CD	-28.239	7.031
HFD	-5.761	6.767

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-16.312	9.023
WT x HFD	9.780	10.088
KO x CD	-40.165	10.784
KO x HFD	-21.301	9.023

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
WT vs. KO	27.467	2	3.981	0.009	Yes

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
HFD vs. CD	22.478	2	3.258	0.028	Yes

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	26.092	2	2.726	0.063	No

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
HFD vs. CD	18.863	2	1.897	0.190	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	23.853	2	2.399	0.100	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
WT vs. KO	31.081	2	3.248	0.029	Yes

Two Way Analysis of Variance Monday, August 05, 2013, 11:10:12 AM

Data source: Bodipy in Bodipy

General Linear Model

Dependent Variable: IMCL

Normality Test: Passed (P > 0.050)

Equal Variance Test: Failed (P = 0.001)

Source of Variation	DF	SS	MS	F	P
genotype	1	411.043	411.043	4.486	0.034
Diet	1	3017.981	3017.981	32.934	<0.001
genotype x Diet	1	554.360	554.360	6.049	0.014
Residual	825	75601.011	91.638		
Total	828	79620.743	96.160		

The difference in the mean values among the different levels of genotype is greater than would be expected by chance after allowing for effects of differences in Diet. There is a statistically significant difference (P = 0.034). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Diet is greater than would be expected by chance after allowing for effects of differences in genotype. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of genotype depends on what level of Diet is present. There is a statistically significant interaction between genotype and Diet. (P = 0.014)

Power of performed test with alpha = 0.0500: for genotype : 0.449
Power of performed test with alpha = 0.0500: for Diet : 1.000
Power of performed test with alpha = 0.0500: for genotype x Diet : 0.610

Least square means for genotype :

Group	Mean	SEM
WT	40.247	0.468
KO	38.837	0.473

Least square means for Diet :

Group	Mean	SEM
CD	37.632	0.463
HFD	41.451	0.478

Least square means for genotype x Diet :

Group	Mean	SEM
WT x CD	37.519	0.656
WT x HFD	42.974	0.667
KO x CD	37.746	0.653
KO x HFD	39.928	0.686

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	Diff of Means	p	q	P	P<0.050
WT vs. KO	1.409	2	2.995	0.034	Yes

Comparisons for factor: **Diet**

Comparison	Diff of Means	p	q	P	P<0.050
HFD vs. CD	3.819	2	8.116	<0.001	Yes

Comparisons for factor: **Diet within WT**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	5.455	2	8.248	<0.001	Yes

Comparisons for factor: **Diet within KO**

Comparison	Diff of Means	p	q	P	P<0.05
HFD vs. CD	2.182	2	3.260	0.021	Yes

Comparisons for factor: **genotype within CD**

Comparison	Diff of Means	p	q	P	P<0.05
KO vs. WT	0.227	2	0.347	0.806	No

Comparisons for factor: **genotype within HFD**

Comparison	Diff of Means	p	q	P	P<0.05
WT vs. KO	3.046	2	4.504	0.001	Yes

Two Way Analysis of Variance Friday, July 26, 2013, 10:55:25 AM

Data source: Data 1 in KCJ_Respiration measures

General Linear Model

Dependent Variable: Basal

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.168)

Source of Variation	DF	SS	MS	F	P
genotype	1	25.414	25.414	5.330	0.030
diet	1	12.032	12.032	2.524	0.126
genotype x diet	1	0.675	0.675	0.142	0.710
Residual	23	109.663	4.768		
Total	26	148.092	5.696		

The difference in the mean values among the different levels of genotype is greater than would be expected by chance after allowing for effects of differences in diet. There is a statistically significant difference (P = 0.030). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.126).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.710)

Power of performed test with alpha = 0.0500: for genotype : 0.506
Power of performed test with alpha = 0.0500: for diet : 0.207
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-5.483	0.550
KO	-3.498	0.661

Least square means for diet :

Group	Mean	SEM
CD	-3.807	0.575
HFD	-5.174	0.639

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-4.638	0.728
WT x HFD	-6.328	0.825
KO x CD	-2.976	0.891
KO x HFD	-4.019	0.977

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
KO vs. WT	1.986	2	3.265	0.030	Yes

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
CD vs. HFD	1.366	2	2.247	0.126	No

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	1.690	2	2.172	0.138	No

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	1.043	2	1.115	0.439	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	1.662	2	2.043	0.162	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	2.309	2	2.554	0.084	No

Two Way Analysis of Variance Friday, July 26, 2013, 10:56:08 AM

Data source: Data 1 in KCJ_Respiration measures

General Linear Model

Dependent Variable: State 3

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.393)

Source of Variation	DF	SS	MS	F	P
diet	1	3.401	3.401	0.498	0.487
genotype	1	27.791	27.791	4.072	0.055
diet x genotype	1	0.662	0.662	0.0970	0.758
Residual	23	156.975	6.825		
Total	26	188.590	7.253		

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.487).

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.055).

The effect of different levels of diet does not depend on what level of genotype is present. There is not a statistically significant interaction between diet and genotype. (P = 0.758)

Power of performed test with alpha = 0.0500: for diet : 0.0500
Power of performed test with alpha = 0.0500: for genotype : 0.376
Power of performed test with alpha = 0.0500: for diet x genotype : 0.0500

Least square means for diet :

Group	Mean	SEM
CD	-6.297	0.688
HFD	-7.023	0.765

Least square means for genotype :

Group	Mean	SEM
WT	-7.698	0.658
KO	-5.622	0.791

Least square means for diet x genotype :

Group	Mean	SEM
CD x WT	-7.175	0.871
CD x KO	-5.419	1.067
HFD x WT	-8.222	0.987
HFD x KO	-5.825	1.168

Two Way Analysis of Variance Friday, July 26, 2013, 10:56:39 AM

Data source: Data 1 in KCJ_Respiration measures

General Linear Model

Dependent Variable: State 3b

Normality Test: Failed (P = 0.024)

Equal Variance Test: Passed (P = 0.563)

Source of Variation	DF	SS	MS	F	P
genotype	1	30.684	30.684	2.636	0.118
diet	1	0.642	0.642	0.0551	0.816
genotype x diet	1	0.458	0.458	0.0393	0.845
Residual	23	267.718	11.640		
Total	26	299.023	11.501		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.118).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.816).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.845)

Power of performed test with alpha = 0.0500: for genotype : 0.219
Power of performed test with alpha = 0.0500: for diet : 0.0500
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-8.971	0.860
KO	-6.789	1.033

Least square means for diet :

Group	Mean	SEM
CD	-8.038	0.899
HFD	-7.722	0.999

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-8.996	1.137
WT x HFD	-8.947	1.290
KO x CD	-7.080	1.393
KO x HFD	-6.498	1.526

Two Way Analysis of Variance Friday, July 26, 2013, 10:56:59 AM

Data source: Data 1 in KCJ_Respiration measures

General Linear Model

Dependent Variable: State 4

Normality Test: Failed (P = 0.011)

Equal Variance Test: Passed (P = 0.219)

Source of Variation	DF	SS	MS	F	P
genotype	1	28.517	28.517	0.930	0.345
diet	1	40.093	40.093	1.307	0.265
genotype x diet	1	22.649	22.649	0.738	0.399
Residual	23	705.604	30.678		
Total	26	792.561	30.483		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.345).

The difference in the mean values among the different levels of diet is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in genotype. There is not a statistically significant difference (P = 0.265).

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.399)

Power of performed test with alpha = 0.0500: for genotype : 0.0500
Power of performed test with alpha = 0.0500: for diet : 0.0785
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-9.602	1.396
KO	-7.499	1.677

Least square means for diet :

Group	Mean	SEM
CD	-7.303	1.460
HFD	-9.797	1.622

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-9.292	1.846
WT x HFD	-9.912	2.093
KO x CD	-5.314	2.261
KO x HFD	-9.683	2.477

Two Way Analysis of Variance Friday, July 26, 2013, 10:57:26 AM

Data source: Data 1 in KCJ_Respiration measures

General Linear Model

Dependent Variable: State 5

Normality Test: Passed (P > 0.050)

Equal Variance Test: Passed (P = 0.753)

Source of Variation	DF	SS	MS	F	P
genotype	1	64.878	64.878	0.859	0.364
diet	1	392.552	392.552	5.198	0.032
genotype x diet	1	10.856	10.856	0.144	0.708
Residual	23	1736.816	75.514		
Total	26	2233.302	85.896		

The difference in the mean values among the different levels of genotype is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in diet. There is not a statistically significant difference (P = 0.364).

The difference in the mean values among the different levels of diet is greater than would be expected by chance after allowing for effects of differences in genotype. There is a statistically significant difference (P = 0.032). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of genotype does not depend on what level of diet is present. There is not a statistically significant interaction between genotype and diet. (P = 0.708)

Power of performed test with alpha = 0.0500: for genotype : 0.0500
Power of performed test with alpha = 0.0500: for diet : 0.493
Power of performed test with alpha = 0.0500: for genotype x diet : 0.0500

Least square means for genotype :

Group	Mean	SEM
WT	-28.285	2.190
KO	-25.112	2.631

Least square means for diet :

Group	Mean	SEM
CD	-22.796	2.290
HFD	-30.601	2.544

Least square means for genotype x diet :

Group	Mean	SEM
WT x CD	-23.734	2.897
WT x HFD	-32.836	3.284

KO x CD	-21.859	3.548
KO x HFD	-28.366	3.886

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor: **genotype**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
KO vs. WT	3.173	2	1.311	0.364	No

Comparisons for factor: **diet**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
CD vs. HFD	7.804	2	3.224	0.032	Yes

Comparisons for factor: **diet within WT**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	9.102	2	2.939	0.049	Yes

Comparisons for factor: **diet within KO**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
CD vs. HFD	6.507	2	1.749	0.229	No

Comparisons for factor: **genotype within CD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	1.875	2	0.579	0.686	No

Comparisons for factor: **genotype within HFD**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
KO vs. WT	4.471	2	1.243	0.389	No

Appendix C:

Animal Care and Use Documentation & Hazardous Material Approval Form

UNIVERSITY OF
MARYLAND
DIVISION OF RESEARCH
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

0101 Lee Building
College Park, Maryland 20742
301.405.5037 TEL 301.314.1475 FAX

W. Ray Stricklin
IACUC Chair
wrstrick@umd.edu
Phone: (301)405-7044

December 21, 2010

Dr. Espen Spangenburg
Department: Kinesiology
University of Maryland
espen@umd.edu

Dr. Spangenburg,

This letter is to inform you that on **December 16, 2010**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the protocol for:

The role of BRCA1 in lipid utilization by the skeletal muscle

R-10-95

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **December 16, 2013**. Federal laws indicate that protocols must be reviewed yearly. Thus, in order to keep your approved protocol active you **MUST** submit a protocol renewal/update by the first of the month of the anniversary of your approval (December 2011 & December 2012). All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

W. Ray Stricklin
Asst. Dean, College of Ag. & Natural Resources
Chair, IACUC

CC: Doug Powell, Amanda Underwood

*** For IACUC Use only ***
Protocol #: R-10-95 V2
Approval Date _____
Expiration Date _____

IBC # _____

Application for
ANIMAL STUDY PROTOCOL – RESEARCH
University of Maryland, College Park, MD

Attention: Protocols are due to the IACUC Manager by the first of the month. The IACUC generally does not meet in August. Incorporate all protocol related information. For general instructions, see www.umdresearch.umd.edu/IACUC or click on Link.

Section A. Administrative Data: (Link)

1. Title of Project: The role of BRCA1 in lipid utilization by the skeletal muscle.

2. Estimated Start Date: 4/1/2010 Estimated Completion Date: 03/31/2013

3. Type of Protocol. Complete each line.
   a. Initial submission [X] 3 Yr Renewal [] Modification [ ]
   b. Previous protocol number if 3 Yr renewal or Modification: NA [ ]
   c. Field Study: YES [ ] NO [X] If YES, attach required permits or provide documentation that permits are not required.
   d. Location of Research: College Park: YES [X] NO [ ]; Other-than UMD: YES [X] NO []
   e. Location if research is conducted away from UMD: N/A []

4. Principal Investigator.

Name: Espen E Spangenburg, Ph.D. Department: Kinesiology

UMD Address: 2134A SPH Office phone: 5-2483 Lab phone: 5-4579
Fax: 5-5578 E-mail: espen@umd.edu University Title: Assistant Professor

5. Key Personnel. List name, role*, university address, phone and e-mail.
   Kathryn Jackson (GS) 2128 SPH Bldg kacampbe@umd.edu 301-405-4579
   Lindsay Wohlers (GS) 2128 SPH Bldg lwohlers@umd.edu 301-405-4579
   Ana Valencia (GS) 2128 SPH Bldg anapvalencia@gmail.edu 301-405-4579

   * Indicate role of personnel as CO (co-investigator), CS (collaborating scientist), PD (post-doctoral) US (undergraduate student), GS (graduate student), T (technician).

6. Has or will this proposal be submitted through ORAA? YES [X] NO [ ]
   If applicable -
   a. ORAA Proposal ID number(s): unknown
   b. Title of associated proposal(s) or award(s): The role of BRCA1 in regulation of lipid metabolism in skeletal muscle
   c. Name of the PI on the proposal or award application: Espen E. Spangenburg
   d. Other key personnel supported under this proposal or award: [X] NA

7. Funding for animal procurement and care: DRF and submitted grants

(i.e. existing grant, pending grant, submitted grant, departmental, DRF, not yet determined, not applicable)
Type of Grant (i.e. NIH, NSF, Other etc): NIH

Section B. Animal Requirements: (Link)

1. Animals. List species; age or weight at use; sex; strain, stock, common or scientific name; source; holding location (bldg) and total number.

   Species: Mouse
   Age at use: 2 months to 12 months
   Sex: Male and Female
   Strain: C57/BL6---background---transgenic mice (described in detail below)
   Stock:
   Common or Scientific Name: Mus musculus
   Source: BRCA1 (fl/fl) (NCI_Mouse Repository) and HAS-mER-Cre-mER (University of Kentucky_from Dr Karyn Esser)
   Holding Location (bldg): CARF
   Total Number: 188 animals will be used for experiments, however when including the animals necessary for the breeding strategy we will need a total of 268.

2. Description of Animal Usage.

   a) Approximate number of animals used each year: 188 total animals will be used in three experiments. Detailed explanation appears below. Experiment 1 will be done over the course of 1 year. Experiment 2 will be completed in year 2 and experiment 3 in year 3.
   b) Approximate maximum number of animals on hand at any given time: We anticipate less than 100 animals to be housed at CARF.
   c) Approximate maximum number of weeks any single animal may be housed: variable

3. Number of animals by species that will be used in this study that are currently assigned to other protocols: none

4. Animal Accountability.
   a. Approximate number of rodent pups to be euthanized prior to weaning: NA [X]
   b. Approximate number of chicks to be used or euthanized immediately post-hatch: NA [X]

Section C. Transportation: (Link) NA [ ]

We will transport animals to the University of Maryland-Baltimore using standardized procedures established at the University of Maryland-College Park. All of the transported animals will be euthanized upon arrival and the tissue removed (see experiment 3). This is a necessary approach since these experiments must be done on freshly isolated tissue. All euthanasia procedures will be identical to that described in the latter section.

Section D. Lay Summary: (Link)
State your study objectives and goals as they relate to the proposed use of animals. Write for nonspecialists and limit to 300 words.

Loss of ovarian function in females is associated with the development of abdominal obesity, increased risk of type 2 diabetes, and the metabolic syndrome. Currently, we have a poor understanding of how ovarian hormones affect metabolic function of peripheral tissue. This lack of understanding is in part due to a limited knowledge of the effects of estrogens on metabolic function. In previous experiments we have identified that the protein form of the estrogen sensitive Breast Cancer 1 early onset gene (BRCA1) is interacting with and regulating Acetyl CoA-Carboxylase (ACC) activity in skeletal muscle. ACC is a key regulator of lipid metabolism in muscle. Skeletal muscle lipid metabolism is a substantial contributor to an individual's overall metabolic rate, and thus has a huge effect on overall body fat storage. The primary goal of this study is therefore, to determine if endogenous BRCA1 expression is necessary for regulating metabolic mechanisms in female mice. To address this question, we plan to use a breeding strategy to create a mouse model that when induced results in skeletal muscle specific loss of BRCA1 expression. To determine the function of BRCA1 on the regulation of metabolism, we will make a number of metabolic measures in these mice. It is expected that data obtained from these studies will improve our understanding of how estrogens regulate metabolic function of peripheral tissue.

Section E. Rationale for Animal Use: (Link)

1. Justify why animals are required over non-animal alternatives:

To determine if BRCA1 is necessary for optimal skeletal muscle lipid utilization, we must find a way to inhibit or remove BRCA1 from the muscle. One of the most accepted ways to approach this is to genetically ablate BRCA1 from the skeletal muscle, however traditional knockout approaches for BRCA1 cannot be used due to embryonic lethality. Here we are employing a loss of function approach to study the importance of endogenous BRCA1 in skeletal muscle. In preliminary studies we have determined that BRCA1 regulates enzymes that are critical regulators of lipid metabolism during periods of exercise. Thus, we are proposing to use a mouse model that provides us with an inducible Cre-Flox system to, when induced, specifically ablate BRCA1 only from the skeletal muscle. This model will allow us to determine if BRCA1 is playing an important mechanistic role in the regulation of lipid metabolism.

Cell culture is unfortunately not an option since it is not possible to mimic exercise using cell culture models. Further, most skeletal muscle cell lines do not recapitulate the adult phenotype of muscle eliminating them as an option for this study. Finally, it is not possible to use modeling approaches because we know very little about the role of BRCA1 in skeletal muscle.

2. Justify appropriateness of species selected:

   Mice are an appropriate species because we are using a gene targeting strategy to address our specific hypothesis. Other species of animals are not typically suitable for this type of approach.

3. Justify number of animals to be used:

   We have determined in a priori power measure that the minimum number per group needed to maintain statistical power (0.80) for each experiment is 6-12 animals/group depending on the experiment. For a complete breakdown of each group see section F (see below). This proposal has been divided into three independent experiments for ease of reading. The total number of animals to be used is 188.

4. Justify duplicative research: NA [X]

Section F. Experimental Design and Animal Procedures:

1. Brief description of experimental design and animal procedures including summary table(s) of experimental groups and the number in each group: (Link)

Breeding/utilization Strategy: We plan to breed male HSA-mER-Cre-mER (n=5) (hemizygous_obtained from Dr. Karyn Esser_University of Kentucky) with female BRCA1 fl/fl (n=5) (obtained from NCI Mouse repository). We are using male HSA-mER-Cre-mER for breeding to reduce any chance of developing Cre toxicity. Approximately 50% of the offspring will be HSA-BRCA $^{fl/+}$. We will then breed male HSA-BRCA $^{fl/+}$ to female BRCA1 fl/fl to generate HSA-BRCA1$^{fl/fl}$ mice. All animals will be genotyped before use. Genotyping will be done with a tail snip prior to 21 days of age. For this breeding strategy we are estimating it will take ~80 animals to generate the necessary breeding colony of HSA-BRCA1$^{fl/fl}$ mice. We have accounted for these animals in our total animal numbers (Section B, number 1). To ease the reading, we completed two section M from the new protocol. The first section M describes the animals we will need to breed to generate HSA-BRCA1$^{fl/fl}$ breeder mice. Once we generate the necessary animals, we will euthanize all the remaining animals. The second attached section M, describes the animals we will need to breed to generate enough animals to complete the specific experiments described in this proposal. Please note prior to the time of weaning, any males generated from the breeding of the HSA-BRCA1$^{fl/fl}$ mice will be euthanized since this particular study is focused on women's health issues and thus the male mice are irrelevant.

To induce ablation of BRCA1 in skeletal muscle from the HSA-BRCA1$^{fl/fl}$ animals, we will treat animals with tamoxifen (T) for 5 consecutive days at (0.5mg/day_500µL IP). Control HSA-BRCA1$^{fl/fl}$ animals will receive vehicle injections for 5 consecutive days (85% sunflower oil/15% ethanol mixture_500µL IP). After 5 days, animals will remain in their cages for 14 days to allow washout of the (T). We do not expect any non-specific effects of (T) since complete elimination of serum (T) occurs within 7-9 days and these animals will undergo a 14 day washout. We will also use (T) treated HSA-mER-Cre-mER in some experiments to account for any potential Cre-toxicity. Please note Cre-toxicity is nothing harmful to the animal, but a biological effect that could effect our results therefore we must control for this effect in our design. We expect to find no indications of Cre-toxicity since none has been detected by Dr. Esser's Lab (personal communication). We will randomly test treated animals to ensure BRCA1 ablation occurs only in skeletal muscle. In the rest the application animals treated with (T) are referred to as HSA-BRCA1$^{fl/fl}$ (T+) and those not receiving (T) termed HSA-BRCA1$^{fl/fl}$ (T-).

Experimental Design

Three different experiments will be conducted to determine the metabolic effect of ablated BRCA1 expression on metabolic function of skeletal muscle tissue. We have divided the total number of animals into three distinct experiments. This experimental design is needed because each experiment requires us to process the samples using completely different methods.

In experiment 1, we will divide both the HSA-BRCA1$^{fl/fl}$ (T+) and HSA-BRCA1$^{fl/fl}$ (T-) animals into 4 different groups with 12 animals per group. The distinction of the groups of animals will be their age when the experiments are conducted. Specifically, we will age the animals for 12 weeks, 16 weeks, 20 weeks, and 24 weeks after we treat the animals with T or vehicle. At each specific time point, we will perform GTTs and ITTs (see below for specific methods). Animals will be

euthanized and tissues collected will take place at this point. We will treat one small set (n=6) of HSA-mER-Cre-mER in some experiments to account for any potential Cre-toxicity and they will be tested at 24 weeks. We will use 102 animals for these experiments.

In experiment 2, we will divide both the HSA-BRCA1$^{fl/fl}$ (T+) and HSA-BRCA1$^{fl/fl}$ (T-) animals into 2 different groups with 21 animals per group. At 16 weeks of age, we will perform exercise treadmill testing on the animals (see below for specifics). In 7 animals from each group, we will euthanize the animals immediately after the exercise bout and tissue will be collected. In the remaining 28 animals, we will euthanize half of them at 30 minutes after the exercise bout, with the rest being euthanized 1 hr after the exercise bout. We will collect the necessary tissues and snap freeze the tissue for cell signaling and gene expression measures. We will treat one small set (n=12) of HSA-mER-Cre-mER in some experiments to account for any potential Cre-toxicity We will use 56 animals for these experiments.

In experiment 3, we will divide both the HSA-BRCA1$^{fl/fl}$ (T+) and HSA-BRCA1$^{fl/fl}$ (T-) animals into 2 different groups with 12 animals per group. At 16 weeks of age, we will euthanize the animals and remove their skeletal muscle for specific experiments. We will treat one small set (n=6) of HSA-mER-Cre-mER in some experiments to account for any potential Cre-toxicity. We will use 30 animals for these experiments.

Experimental procedures:

Acute Exercise: The acute exercise protocol will be performed using a mouse specific treadmill. The animals will be placed on a treadmill and run at 25 m/min for 30 minutes. This is an easy protocol for the mice therefore we do not anticipate any major issues. There is a mild electrical stimulus to encourage the animals to run. If we encounter a mouse that refuses to run (sits at the back of the treadmill for longer than 15-20 seconds), it will be eliminated from the study. In our experience, we very rarely encounter mice that are not capable of running at this speed or duration.

GTT/ITT: See below for specific methods (Section F; part 2)

2. Administered substances other than aesthetics and analgesics. NA [ ] (Link)
   a. List substance, dose or concentration, route, volume, frequency, site, and needle size.

Genetic removal of BRCA1 from skeletal muscle. To induce ablation of BRCA1 in skeletal muscle from the HSA-BRCA1$^{fl/fl}$ animals, we will treat animals with tamoxifen (T) for 5 consecutive days at (0.5mg/day_500μL IP). Control HSA-BRCA1$^{fl/fl}$ animals will receive vehicle injections for 5 consecutive days (85% sunflower oil/15% ethanol mixture_500μL IP). After 5 days of injection treatments, animals will remain in their cages for 14 days to allow washout of the (T). We do not expect any non-specific effects of (T) since complete elimination of serum (T) occurs in 7-9 days and these animals will undergo a 14 day washout.

Glucose Tolerance Test (GTT) Procedures: Circulating levels of blood glucose will be measured using a standard glucometer in a subset of animals from each group (n=6/group). We will remove food but maintain access to water 8-12 hours before the experiment. Prior to the start of the GTT the next morning, we will weigh the mice and nick the tail with a sterile scalpel blade at the very end to remove roughly 0.25 cm of the tail. A new sterile scalpel blade will be used for each mouse tested. Baseline blood glucose will be measured using a glucose meter (AlphaTRAK, Abbott Labs). A ~3 μL droplet is required for each measurement. Sterilized D-glucose (200 mg/ml, i.p.) warmed to 37°C will be injected at 2 mg/g body weight in normal saline. Blood glucose will be

measured again at 30, 60, and 120 minutes by gentle massage of the tail and spotting the blood onto the glucometer strip. The first drop of blood is discarded to ensure accurate analysis of blood glucose. Mice are monitored throughout the procedure for excessive bleeding or other adverse conditions. If for any reason an adverse reaction is detected the test will be discontinued and the campus veterinarian will be contacted. Following the final test, food will be returned and mice will be monitored for 2 hours to assure complete recovery.

Insulin Tolerance Test (ITT) Procedures: Circulating levels of blood glucose will be measured using a standard glucometer in a subset of animals from each group (n=6/group). We will remove food for 6 hrs prior to the experiment but maintain access to water. Before the start of the ITT the following morning, we will weigh the mice and nick the tail with a sterile scalpel blade at the very end to remove roughly 0.25 cm of the tail. A new sterile scalpel blade will be used for each mouse tested. Baseline blood glucose will be measured using a glucose meter (AlphaTRAK, Abbott Labs). A ~3 μL droplet is required for each measurement. Animals will then receive 0.75U/kg body weight (approximately 11 units) of bovine insulin (Sigma) via intraperitoneal injection. Intraperitoneal injections will be administered once using a sterile 1/2 inch 29 gauge needle. Blood glucose will be measured at 30, 60, and 120 minutes by gentle massage of the tail and spotting the blood onto the glucometer strip. The first drop of blood is discarded to ensure accurate analysis of blood glucose. Mice are monitored throughout the procedure for excessive bleeding or other adverse conditions. If for any reason an adverse reaction is detected the test will be discontinued and the campus veterinarian will be contacted. Following the final test, food is returned and mice are monitored for 2 hours to assure complete recovery.

b. List and provide justification for any non-pharmaceutical grade substances including anesthetics, analgesic and injectable euthanasia solutions that will be used. NA [X]

All anesthetics (i.e. isoflurane) are pharmaceutical grade.

3. Blood collected from live animals. NA [ ] (Link)

   a. List method, site, volume and frequency.

      Blood will be collected for glucose tolerance tests and insulin tolerance tests via tail snip. These tests require sampling of a single drop of blood over time following an intraperitoneal injection of glucose or insulin. See above response in #2 for specifics of testing procedures.

   b. Identify if terminal bleed. N/A

   c. Will animals be anesthetized (local or general) for blood collection? YES [ ] NO [X]
      If YES, add to table under Section H.

4. Describe methods of restraint: NA [X] (Link)

5. Survival surgery: NA [X] (Link)

   a. Describe the surgical procedure:

   .

   b. Describe aseptic methods:

c. List who will perform the surgery and their qualifications:

d. Describe post-operative care and who will provide it: We will monitor the animals and if we observe any situations where the animal appears to sick, not eating/drinking, or stressed we will immediately contact the campus veterinarian.

e. Has major survival surgery been performed on any animal prior to being placed on this study? YES [ ] NO [X] If yes, justify:

f. Will more than one major survival surgery be performed on any animal while on this study? YES [ ] NO [X] If yes, justify:

6. Describe anticipated resultant effects: (Link)

The anticipated resultant effects of this study are that genetic ablation of BRCA1 will result in peripheral metabolic alterations that ultimately will increase visceral adipose tissue mass in the mice. We expect to find that skeletal muscle from mice that have had BRCA1 genetically removed will demonstrate an inability to utilize lipid due to a lipid transport defect. We predict that this effect will be exacerbated by a single bout of treadmill exercise. Finally, we anticipate that these mice will exhibit other indicators of metabolic dysfunction such as poor GTT and ITT results.

7. Describe experimental and humane endpoints: (Link) Include description of pain scoring, by whom, how often, intervention criteria and method to intervene.

All animals will be euthanized and specific tissues will be collected from the animal. None of the experiments will result in any pain or distress for the animal. If any animal appears to be in pain, we will remove it from the study and contact the campus veterinarian.

8. Locations (Link)

Animal Procedure	Building(s)	Room(s)
Non-survival Surgery		
Survival Surgery		
Euthanasia	SPH	2128
Tissue Harvesting	SPH	2128
Behavior Testing		
Imaging		
Other Experimental Procedures: GTT and ITT	SPH	2128
Treadmill running	SPH	2128

Section G. Pain and Distress:

1. Categorize based on the most severe procedure to which they will be subjected. (Link)

Procedure	Species	Number of animals	Category I (Minimal, transient or no pain or distress)	Category II (Pain or distress relieved by appropriate means)	Category III (Pain or distress not relieved)
Isoflurane inhalation	Mouse	268		X	
Treadmill running	Mouse	21	X		

2. Literature Search for Alternatives to Painful or Distressful Procedures: (Link) Required for procedures under Category II and III to determine if other methods are available that could reduce or eliminate pain or distress.

   a. Sources used (at least 2): PubMed; UMD Research Port

   b. Date search completed: 10/10/10

   c. Years covered: 1900-2010

   d. Key words used: anesthesia mice isoflurane safety treadmill exercise rodent

   e. Summary of the outcome of the search including a statement that no acceptable alternatives were found or why alternatives cannot be used:

We anesthetize our animals with isoflurane (induction 4-5%, maintenance 2-3%) and remove all of the necessary skeletal muscles (including the diaphragm) while the animal is anesthetized. Other alternatives found include using injectable cocktails. These methods are less ideal for us due to the accumulation of needles and a number of these drugs are regulated by the DEA, while isoflurane is not. By using isoflurane we can control the depth and reduce the handling of the mice to ensure lower stress levels whereas with injectable cocktails this capability is not possible. In addition, we do not use CO_2 inhalation because a number of our experimental measures require blood flow to remain intact for as long as possible before the tissue is removed. Thus, no acceptable alternatives were found for our chosen form of anesthesia.

In order to understand the acute effects of exercise on physiological systems investigators either employ swimming or treadmill running. Our lab uses treadmill running because it results in a substantially lower stress response in the mice and we can determine the intensity of the exercise bout. In order to ensure that the mice run and do not sit at the end of the treadmill there is an electrified grid at the end of the treadmill. The shock pad can deliver a maximum of 160 volts but only at 1.0 milli-amps. Thus, this is insufficient to induce anymore than a moderate discomfort to the animal's feet, in other words no tissue damage will occur. Note: these are the maximum settings for the machine, we do not increase the settings above the 50% threshold. Further, we discontinue the test if the mouse becomes stationary on the shock pad for longer than a few seconds. I have been studying the responses to acute exercise in rodents since 1995 and I know of no other alternative model to perform these studies. Further, I was unable to locate any alternative approaches with my literature search.

Section H. Anesthesia and Analgesia: (Link)

1. List agent, procedure requiring agent, dose, route, and frequency.

Anesthesia will be initially delivered by inhalation of 4-5% isoflurane. After the induction, the isoflurane is lowered 2-3%.

2. Provide details on fasting prior to anesthesia, methods of monitoring anesthesia, and care during anesthesia recovery if not mentioned in Section F: NA []

Food will be removed 4-5 hours prior to anesthesia. The animals will be initially induced in an induction box and then isoflurane exposure will be maintained by using a mouse specific nose cone. The level of induction will be monitored by reflex responses to toe pinches throughout the entire process of the tissue removal. No response to a toe pinch would indicate a sufficient level of induction, if the animal exhibits a toe pinch response we will not perform any procedures until the animal reaches a suitable anesthetic plane.

Section I. Biological Materials for Use in Animals: (Link)

1. Will animals be exposed to any of the following materials? This information will help to prevent introductions of infectious agents to University animals.

Animal tissue, fluids or cells YES [ ] NO [X]

2. If YES, explain:

3. Has the material been tested for murine pathogens? YES [ ] NO [ ]

Section J. Animal Disposition and Euthanasia: (Link)

1. Will animals be euthanized? YES [X] NO [ ]

2. Method of euthanasia: NA [ ] List agent, dose, and route.

Animals will be anesthetized via inhalation of 4-5% isoflurane gas and maintained under 2-3% isoflurane. When animals have reached an appropriate plane of anesthesia (as determined by lack of toe pinch reflex), we will remove numerous tissues (heart, liver, skeletal muscle, brain, and adipose tissue) from the mice and snap freeze the tissues. It is important that we use anesthesia since we need the tissues to be blood flow intact. Thus, the animal will be euthanized by exsanguinations, which is a necessity since we remove the heart and diaphragm for subsequent biochemical measures.

3. Method to ensure death: NA [ ]

The tissues to be removed following anesthetization include the heart and diaphragm, such that the animals will be euthanized through exsanguination. The animals will remain anesthetized throughout the entire procedure.

4. Justification for conditionally acceptable or unacceptable methods: NA [X]

5. Method to dispose of euthanized animals: NA [ ]

Final disposal of euthanized animals will be performed via incineration through CARF.

6. Final disposition of animals if not euthanasia: NA [X]

Section K. Hazardous Agents: (Link)

1. Identify all hazardous agents that will be administered to animals.

	Check all that apply	List agents & Registration Document # (if applicable)
Radionuclides		
Biological Agents		
Hazardous Chemicals		
Recombinant DNA including recombinant microorganisms and transgenic animals	XXXX	In progress

2. Study conducted at Animal Biosafety Level: NA [X]

3. Describe precautions and procedures: NA [X]

4. Has approval been obtained from the Division of Environmental Safety: Yes [ ] No [X]

5. Identify any agents administered and expected concentration/activity that will be expelled in animal waste: NA [X]

6. Identify any agents administered and expected concentrations/activity that will be in animal tissues when the animal is euthanized/disposed: NA [X]

Section L. Special Concerns or Requirements: (Link) None [ ]

1. Describe deviations from standard housing and animal care: None [X]

2. Describe special equipment requirements: None [X]

Half of the animals will be treated with Tamoxifen (T) for 5 days with IP injections (0.5mg/day_500µL IP). This is necessary for induction of the mechanism that will result in genetic ablation of BRCA1 only in the skeletal muscle of the mice.

3. Describe deviations from standard diet including amount and frequency: None [ ]

Prior to glucose tolerance testing and insulin tolerance testing food and bedding will be removed for 6-12 hours before the test. Upon completion of the test the food will be immediately returned to them.

4. Describe water restrictions: None [X]

5. Describe phenotypes and care of any animals which may be associated with morbidity or shortened lifespan: None [X]

6. Justify any deviations from the *Guide for the Care and Use of Laboratory Animals*. None [X]

7. Other: None [X]

Section M. Training: (Link) To be completed for each person named in this protocol including the PI.

Name: Espen E. Spangenburg, Ph. D.
Animal activities performed on this protocol: Will assist with tissue removal and oversee the experiments
Credentials: BS, MS, Ph. D.
Experience working with each species: since 1995
Experience with animal procedures listed above: greater than 10 years
Training the individual will receive: none
Year completed UMD PI/Animal User training: 2006

Name: Lindsay M. Wohlers
Animal activities performed on this protocol: Will monitor animals throughout the study, perform all injections and tissue removal
Credentials: BS, MA
Experience working with each species: since 2007
Experience with animal procedures listed above: 2007
Training the individual will receive: No new training is necessary
Year completed UMD PI/Animal User training: 2007

Name: Kathryn C. Jackson
Animal activities performed on this protocol: Will monitor animals throughout the study, perform all injections and tissue removal
Credentials: BS, MS
Experience working with each species: 2008
Experience with animal procedures listed above: 2008
Training the individual will receive: No new training is necessary
Year completed UMD PI/Animal User training: 2008

Name: Ana Valencia
Animal activities performed on this protocol: Will monitor animals throughout the study, perform all injections and tissue removal
Credentials: BS
Experience working with each species: 2010
Experience with animal procedures listed above: 2010
Training the individual will receive: Will be trained by Dr. Spangenburg on proper animal handling and the specific experiments to be performed.
Year completed UMD PI/Animal User training: 2010

Section N. Principal Investigator Certifications and Acknowledgments:

1. I acknowledge responsibility for the conduct of these procedures and the care of these animals.

2. I will conduct this work with animals in accordance with the protocol as approved by the

IACUC and the campus animal care and use guidelines. I will obtain approval from the IACUC before initiating any changes in the protocol.

3. I certify that I have determined that the research proposed herein is not unnecessarily duplicative of previously reported research.

4. I certify that all individuals working on this proposal who have significant animal contact are participating in the Laboratory Animal Handler's Medical Surveillance Program.

5. I will maintain appropriate animal records (e.g. census, health, veterinary care, surgery, diagnostic, treatment, etc.)

6. For Category II and III proposals, I certify that I have reviewed the pertinent scientific literature, the sources and/or databases (2 or more) as noted in Section G, and have found no alternatives to any procedures described herein which may cause more than a momentary pain or distress whether it is relieved or not.

7. I certify that the individuals listed in Section A are authorized to conduct procedures involving animals under the proposal and have attended training. (Link) Training may include but not be limited to the biology, handling and care of the species, aseptic surgical techniques, research methods that limit the use of animals or minimize distress, proper use of anesthetics and analgesics, and procedures for reporting animal welfare concerns.

Principal Investigator: Signature_____ Date _____

Section O. Concurrences: Protocol number _____ (leave blank) (Link)

O.1. Department Chair certification of approval of resources.

Name: _____ Signature: _____ Date: _____

O.2. Division of Environmental Safety Representative:
Required for studies utilizing hazardous agents. Required prior to approval but not at submission.

Name: _____ Signature: _____ Date: _____

Name: _____ Signature: _____ Date: _____

O.3. Facility Manager or Veterinarian certification of resource capability:

Name: _____ Signature: _____ Date: _____

Facility: _____

Section P. Approval:

Certification of review and approval by the IACUC chairperson.

Name: _____ Signature: _____ Date: _____

ARAC Approved – October 8, 2008
Revised –

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)
SECTION M: BREEDING COLONY (INCLUDING TRANSGENIC/KO BREEDING AND USE)

M1. JUSTIFICATION

Could the animals that will be bred be purchased from commercial sources in the required number?

- (•) No. The animals are not available commercially in sufficient number.
- ( ) Yes. The animals are available commercially.

Please describe the rationale for breeding these animals at the University of Maryland:

Breeding colony 2: The breeding of the HSA-BRCA1 fl/fl mice will be used to establish the necessary animals to complete the experiments described in the attached animal protocol.

Please identify the source of breeders:

- [X] Colony managed by a UMCP Investigator. Specify source protocol #: pending this application
- [ ] Obtained from another institution. Identify source: _____
- [ ] Purchased from a vendor (e.g., Harlan, Charles River, etc.) Identify source: _____

M2. BREEDING COLONY SPECIES AND NUMBERS

List species to be bred, indicate the number of breeders required, and provide **estimates** for the numbers of offspring expected and their ultimate disposition. Protocols have a life span of 3 years, list numbers anticipated over a 3 year period.

	Species	Strain	# of Male Breeders	# of Female Breeders	Expected # of Offspring	Estimated # of Offspring used for this Protocol (over 3 year)	Estimated # Of Offspring transferred to another Protocol	Estimated # Of Offspring euthanized without use
Add Species	Mouse	C57/Bl6	5	5	4-6	188		~150-200

M3. DISPOSITION OF BREEDERS AND UNNEEDED OFFSPRING

Indicate the final disposition of retired breeders:

[X] Euthanasia according to protocol [ ] Used in experiments [ ] Other

Please explain any planned experimental use and/or other disposition:

If euthanasia without use is indicate in section Q3 above, please explain why the surplus offspring cannot be used for this protocol or by another investigator.

This study is specific to female health issues, thus we will not use any males except for breeding purposes.

M4. BREEDING PLAN

The breeding method will be:

- ( ) Monogamous (single male and female per cage)
- (•) Harem (single male and multiple females). Please indicate which of the following will apply:
  - [X] Males will be removed once females are confirmed pregnant.
  - [ ] Females will not bred again until the offspring are weaned.
  - [X] Individual pregnant females will be moved to new cages prior to delivery of offspring.
  - [ ] Females and their litters will be moved to larger cages to provide required floor space.

Special care (feed, water, temp, humidity, air flow) required for this breeding colony:

- (•) No special care is required.
- ( ) Special care is necessary to keep these animals healthy. The required special care is as follows *(describe)*:

Weaning of rodents will occur at:

- [X] 21 days of age or earlier
- [ ] 22 days of age or later (specify strains affected and justification of extending weaning beyond 21 days)

(•) Special care beyond routine animal care IS NOT necessary.

( ) Special care IS necessary to keep these animals healthy. The required special care is as follows *(describe)*:

Genotyping:

( ) Genotyping is not necessary for this protocol.

(•) Genotyping will be performed on tissue obtained by the method(s) defined below.

☒ Tail snipping (mice).

( ) The tail snip will be taken after 21 days of age.

(•) The tail snip will be taken prior to 21 days of age.

Justify the delayed genotyping and describe the anesthetic regimen to be used for the procedure:

☐ Oral swabs.

☐ Blood collection.

☐ Other *(describe)*:

M5. SPECIES AND GENOTYPE

(Complete this section only if breeding genetically engineered animals. Otherwise, go to section M6.)

List species to be used, identify genetic lines, and indicate any induction method that is necessary.

	Species	Strain / Genotype
Add Species	Mouse	C57/Bl6/HSA-BRCA fl/fl

M6. RECORD KEEPING *(Applies to all breeding colonies of conventional and genetically engineered animals.)*

Indicate the record-keeping system that will be used to document health surveillance and the maintenance of well-being for the conventional and/or genetically-engineered animals.

☒ Special care is not required and documentation will be provided by standard observation records.

☐ Special care outlined in section M4 will be documented by the record-keeping sheet attached to this application.

☐ Special care outlined in section M4 will be documented as follows:

PART II: PROTOCOL SPECIFIC INFORMATION (Append to Part I, if applicable)
SECTION M: BREEDING COLONY (INCLUDING TRANSGENIC/KO BREEDING AND USE)

M1. JUSTIFICATION

Could the animals that will be bred be purchased from commercial sources in the required number?

- (•) No. The animals are not available commercially in sufficient number.
- ( ) Yes. The animals are available commercially.

Please describe the rationale for breeding these animals at the University of Maryland:

Breeding colony 1: These mice will be used to establish the breeding colony of HSA-BRCA fl/fl. This is a necessary step to establish the mouse line we need for the described experiments in this protocol. This mouse line that we are proposing to develop does not exist anywhere in the world, thus it is necessary for us to perform this breeding strategy to create these mice. The breeding strategy is explained in the animal protocol. None of these mice will be used for specific experiments, they will be bred to create breeder pairs for the specific transgenic mouse line we need for the experiments described in the protocol. Thus, the breeding of these mice will establish a colony of HSA-BRCA fl/fl mice at UMD, which would be a new transgenic mouse line.

Please identify the source of breeders:

- ☐ Colony managed by a UMCP Investigator. Specify source protocol #: _____
- ☒ Obtained from another institution. Identify source: University of Kentucky
- ☒ Purchased from a vendor (e.g., Harlan, Charles River, etc.) Identify source: NCI-Mouse Repository (Frederick, MD)

M2. BREEDING COLONY SPECIES AND NUMBERS

List species to be bred, indicate the number of breeders required, and provide **estimates** for the numbers of offspring expected and their ultimate disposition. Protocols have a life span of 3 years, list numbers anticipated over a 3 year period.

	Species	Strain	# of Male Breeders	# of Female Breeders	Expected # of Offspring	Estimated # of Offspring used for this Protocol (over 3 year)	Estimated # Of Offspring transferred to another Protocol	Estimated # Of Offspring euthanized without use
Add Species	Mouse	C57/Bl6	5	5	4-6	80		50-60

M3. DISPOSITION OF BREEDERS AND UNNEEDED OFFSPRING

Indicate the final disposition of retired breeders:

☒ Euthanasia according to protocol ☐ Used in experiments ☐ Other

Please explain any planned experimental use and/or other disposition:

If euthanasia without use is indicate in section Q3 above, please explain why the surplus offspring cannot be used for this protocol or by another investigator.

M4. BREEDING PLAN

The breeding method will be:

- ( ) Monogamous (single male and female per cage)
- (•) Harem (single male and multiple females). Please indicate which of the following will apply:
  - ☒ Males will be removed once females are confirmed pregnant.
  - ☐ Females will not bred again until the offspring are weaned.
  - ☒ Individual pregnant females will be moved to new cages prior to delivery of offspring.
  - ☐ Females and their litters will be moved to larger cages to provide required floor space.

Special care (feed, water, temp, humidity, air flow) required for this breeding colony:

- (•) No special care is required.
- ( ) Special care is necessary to keep these animals healthy. The required special care is as follows (describe):

Weaning of rodents will occur at:

- ☒ 21 days of age or earlier
- ☐ 22 days of age or later (specify strains affected and justification of extending weaning beyond 21 days)

- ◉ Special care beyond routine animal care IS NOT necessary.
- ○ Special care IS necessary to keep these animals healthy. The required special care is as follows *(describe)*:

Genotyping:

- ○ Genotyping is not necessary for this protocol.
- ◉ Genotyping will be performed on tissue obtained by the method(s) defined below.
  - ☒ Tail snipping (mice).
    - ○ The tail snip will be taken after 21 days of age.
    - ◉ The tail snip will be taken prior to 21 days of age.

      Justify the delayed genotyping and describe the anesthetic regimen to be used for the procedure:
  - ☐ Oral swabs.
  - ☐ Blood collection.
  - ☐ Other *(describe)*:

M5. SPECIES AND GENOTYPE

(Complete this section only if breeding genetically engineered animals. Otherwise, go to section M6.)

List species to be used, identify genetic lines, and indicate any induction method that is necessary.

	Species	Strain / Genotype
Add Species	Mouse	C57/Bl6/BRCA1 fl/fl
Add Species	Mouse	C57/Bl6/HSA-mER-Cre-mER

M6. RECORD KEEPING *(Applies to all breeding colonies of conventional and genetically engineered animals.)*

Indicate the record-keeping system that will be used to document health surveillance and the maintenance of well-being for the conventional and/or genetically-engineered animals.

- ☒ Special care is not required and documentation will be provided by standard observation records.
- ☐ Special care outlined in section M4 will be documented by the record-keeping sheet attached to this application.
- ☐ Special care outlined in section M4 will be documented as follows:

Research Registration Form

The current status of this research registration is:
Approved

Approved on: 01/24/2011

Tracking Number: 10-63 (E-849)

The BSO's comments on this research are: IBC approval on 13 January 2011 at ABSL1 for construction of transgenic mice by breeding.

(Required information is designated in black)

1. **Principal Investigator (PI):**
   First Name: Espen
   Last Name: Spangenburg
2. **College:** Health and Human Performance
3. **Department:** Kinesiology
4. **Phone Number:** 301-405-2483
5. **Email:** espen@umd.edu
   Send email to: espen@umd.edu
6. **Project Title:** The role of BRCA1 in lipid utilization by the skeletal muscle
7. **Other Personnel working on Project:** Katie Jackson, Lindsay Wohlers, Ana Valencia
8. **Lab Building:** 87 - Central Animal Resources Facility
9. **Lab Room Number:** 2128
10. **Department Chair Name:** Brad Hatfield
11. **Department Chair Email Address:** bhatfiel@umd.edu
    Send email to: bhatfiel@umd.edu

12. **Generation of transgenic animals by breeding**
    a. **Species of animal:** mice
    b. **Are two transgenic (or knockout) mouse strains being bred to create a new strain?:** Yes
    c. **Is a transgenic (or knockout) mouse strain being bred to a new background strain?:** No
    d. **Is the transgene under the control of a gamma-retroviral long terminal repeat (LTR)?:** No

e. **Is more than 50% of the genome of an exogenous eukaryotic virus from a single family present?:** No
f. **Describe the expected unique characteristics of viable offspring:** Obesity
g. **Describe the precautions that will be taken to minimize the possibility that animals could escape confinement:** Animals will be maintained only in approved cages
h. **Describe the effect on the wild population if an animal were to escape and mate with wild-type?:** none transgene can only induced by tamoxifen

13. Containment equipment available:
Biological safety cabinet: No
Is biological safety cabinet certified annually: No
Date of last certification:
Containment centrifuge: No
Other:
14. **Overview.** Please briefly describe the experimental design, highlighting the recombinant DNA methodology used and/or the use of infectious microorganisms if either are part of the study. Write for non-specialists and limit your response to 300 words. Do not cut and paste from a grant application.
Our goal is develop a transgenic mouse where we can genetically ablate BRCA1 specifically from skeletal muscle in adult mice. Thus, our model we be controlled through a drug-inducible gene strategy. In order to develop the model, we need to complete a breeding strategy. We plan to breed male HSA-mER-Cre-mER (n=5) (hemizygous_obtained from Dr. Karyn Esser_University of Kentucky) with female BRCA1 fl/fl (n=5) (obtained from NCI Mouse repository). Approximately 50 of the offspring will be HSA-BRCA fl/+. We will then breed male HSA-BRCA fl/+ to female BRCA1 fl/fl to generate HSA-BRCA1fl/fl mice. All animals will be genotyped before use. Genotyping will be done with a tail snip prior to 21 days of age. To induce ablation of BRCA1 in skeletal muscle from the HSA-BRCA1fl/fl animals, we will treat animals with tamoxifen (T) for 5 consecutive days at (0.5mg/day_500μL IP). Control HSA-BRCA1fl/fl animals will receive vehicle injections for 5 consecutive days (85 sunflower oil/15 ethanol mixture_500μL IP). After 5 days, animals will remain in their cages for 14 days to allow washout of the (T).
15. **Risk Assessment and Control.** Please attach a brief protocol-specific risk assessment. Include consideration of parent and recombinant agent pathogenicity, virulence, infectious dose, route of transmission, host range, and stability, as well as the likelihood of exposure and consequences of exposure. How will identified risks be controlled (e.g. PPE, work practices, etc.)?
All animals will be handled with appropriate PPE, which includes lab coats, gloves, and eye protection. There is no pathogencity with these animals and since the animals are transgenic there is not a likelihood of exposure.
16. **Responisble Conduct of Life Sciences Research**
a. **Will the intermediate or final product of your experiments:**

Enhance the harmful consequences of the agent or toxin?
Disrupt immunity or the effectiveness of an immunization against the agent or toxin without

clinical or agricultural justification?
Confer to the agent or toxin resistance to clinically or agriculturally useful prophylactic or therapeutic interventions against that agent or toxin or facilitate their ability to evade detection methodologies?
Increase the stability, transmissibility, or the ability to disseminate the agent or toxin?
Alter the host range or tropism of the agent or toxin?
Enhance the susceptibility of a host population to the agent or toxin?
Generate or reconstitute an eradicated or extinct agent or toxin?

b. **After considering the above answers, do you believe there is the potential for your research data / product to be readily utilized to cause public harm?**
c. **For any question that was answered "yes" above, please provide sufficient information to allow for review of the indicated concern:**
d. Researcher checked the box, "I confirm that I have carefully reviewed my proposed research for its potential to generate data / product that could be readily utilized to cause public harm."

17. **Post-exposure procedures. Please describe post-exposure procedures that will be followed in the event of an accidental exposure.**
Describe what first aid will be implemented: Exposure to the transgene is very unlikely, due to this being a transgenic mouse. However, if an animal handler is bit or is somehow exposed to tissue from the animals. The area will be thoroughly cleaned through washing with soap and water. In addition, the area with be cleaned with an aspectic agent. If this is due to animal bite, we will contact the UMD Health Center for further directions.
Will medical follow-up be obtained? No
If yes, where?
Describe the reporting procedures after the exposure.
Note: Spills resulting in release or accidents involving rDNA must be reported to the BSO in compliance with the requirements of the NIH Guidelines.
18. **Section of NIH Guidelines:** III-E-3
19. **Containment:** BL1

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Date: October 29, 2010

To: Espen Spangenburg, PhD
Assistant Professor
Department of Kinesiology
University of Maryland College Park

Cc: Kathryn C. Jackson
Student
Department of Kinesiology
University of Maryland College Park

From: Joseph M. Smith, MA, CIM
IRB Manager
University of Maryland, College Park

Re: Request for Human Subject Research Determination

Title: "Is BRCA1 expressed in human skeletal muscle and does it play a role in regulating lipid metabolism?"

The request for determination of Non Human Subject Research for the above-cited project has been reviewed by the University of Maryland College Park Institutional Review Board Office. According to the information provided, it has been determined that this project does not meet one or both of the following definitions and therefore does not require further evaluation by the University of Maryland College Park Institutional Review Board.

§46.102 - (d) *Research* means a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge.

§46.102 - (f) *Human subject* means a living individual about whom an investigator (whether professional or student) conducting research obtains:

(1) Data through intervention or interaction with the individual, or
(2) Identifiable private information.

If the scope of your project changes and meets one of the above definitions, an IRB protocol must be created and submitted to the UMCP IRB for approval. For further clarification, questions or concerns please contact the IRB Office at 301-405-0678.

Literature Cited:

1. **Abildgaard J**, **Pedersen AT**, **Green CJ**, **Harder-Lauridsen NM**, **Solomon TP**, **Thomsen C**, **Juul A**, **Pedersen M**, **Pedersen JT**, **Mortensen OH**, **Pilegaard H**, **Pedersen BK**, **Lindegaard B**. Menopause is associated with decreased whole body fat oxidation during exercise. *AJP: Endocrinology and Metabolism* 304: E1227–E1236, 2013.

2. **Abu-Elheiga L**, **Brinkley WR**, **Zhong L**, **Chirala SS**, **Woldegiorgis G**, **Wakil SJ**. The subcellular localization of acetyl-CoA carboxylase 2. *Proc. Natl. Acad. Sci. U.S.A.* 97: 1444–1449, 2000.

3. **Abu-Elheiga L**, **Oh W**, **Kordari P**, **Wakil SJ**. Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets. *Proc. Natl. Acad. Sci. U.S.A.* 100: 10207–10212, 2003.

4. **Abumrad NA**, **el-Maghrabi MR**, **Amri EZ**, **Lopez E**, **Grimaldi PA**. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. *J. Biol. Chem.* 268: 17665–17668, 1993.

5. **Adams JM**, **Pratipanawatr T**, **Berria R**, **Wang E**, **DeFronzo RA**, **Sullards MC**, **Mandarino LJ**. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes* 53: 25–31, 2004.

6. **Aloia JF**, **McGowan DM**, **Vaswani AN**, **Ross P**, **Cohn SH**. Relationship of menopause to skeletal and muscle mass. *Am. J. Clin. Nutr.* 53: 1378–1383, 1991.

7. **Amati F**, **Dubé JJ**, **Alvarez-Carnero E**, **Edreira MM**, **Chomentowski P**, **Coen PM**, **Switzer GE**, **Bickel PE**, **Stefanovic-Racic M**, **Toledo FGS**, **Goodpaster BH**. Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes* 60: 2588–2597, 2011.

8. **Arabi A**, **Garnero P**, **Porcher R**, **Pelissier C**, **Benhamou CL**, **Roux C**. Changes in body composition during post-menopausal hormone therapy: a 2 year prospective study. *Hum. Reprod.* 18: 1747–1752, 2003.

9. **Arabi A**. Changes in body composition during post-menopausal hormone therapy: a 2 year prospective study. *Human Reproduction* 18: 1747–1752, 2003.

10. **Bachelier R**, **Dalla Venezia N**, **Mazoyer S**, **Frappart L**, **Lenoir GM**, **Vincent A**. Differential expression and subcellular localization of murine

BRCA1 and BRCA1-delta 11 isoforms in murine and human cell lines. *Int. J. Cancer* 88: 519–524, 2000.

11. **Bandyopadhyay GK**, **Yu JG**, **Ofrecio J**, **Olefsky JM**. Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects. *Diabetes* 55: 2277–2285, 2006.

12. **Beckett T**, **Tchernof A**, **Toth MJ**. Effect of ovariectomy and estradiol replacement on skeletal muscle enzyme activity in female rats. *Metab. Clin. Exp.* 51: 1397–1401, 2002.

13. **Blaak E**. Gender differences in fat metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care* 4: 499–502, 2001.

14. **Bogardus C**, **Ravussin E**, **Robbins DC**, **Wolfe RR**, **Horton ES**, **Sims EA**. Effects of physical training and diet therapy on carbohydrate metabolism in patients with glucose intolerance and non-insulin-dependent diabetes mellitus. *Diabetes* 33: 311–318, 1984.

15. **Bonen A**, **Holloway GP**, **Tandon NN**, **Han XX**, **McFarlan J**, **Glatz JFC**, **Luiken JJFP**. Cardiac and skeletal muscle fatty acid transport and transporters and triacylglycerol and fatty acid oxidation in lean and Zucker diabetic fatty rats. *AJP: Regulatory, Integrative and Comparative Physiology* 297: R1202–R1212, 2009.

16. **Bonen A**, **Luiken JJ**, **Liu S**, **Dyck DJ**, **Kiens B**, **Kristiansen S**, **Turcotte LP**, **Van Der Vusse GJ**, **Glatz JF**. Palmitate transport and fatty acid transporters in red and white muscles. *Am. J. Physiol.* 275: E471–8, 1998.

17. **Bonen A**, **Miskovic D**, **Kiens B**. Fatty acid transporters (FABPpm, FAT, FATP) in human muscle. *Can J Appl Physiol* 24: 515–523, 1999.

18. **Bonen A**, **Parolin ML**, **Steinberg GR**, **Calles-Escandon J**, **Tandon NN**, **Glatz JFC**, **Luiken JJFP**, **Heigenhauser GJF**, **Dyck DJ**. Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. *The FASEB Journal* 18: 1144–1146, 2004.

19. **Boyle KE**, **Canham JP**, **Consitt LA**, **Zheng D**, **Koves TR**, **Gavin TP**, **Holbert D**, **Neufer PD**, **Ilkayeva O**, **Muoio DM**, **Houmard JA**. A High-Fat Diet Elicits Differential Responses in Genes Coordinating Oxidative Metabolism in Skeletal Muscle of Lean and Obese Individuals. *Journal of Clinical Endocrinology & Metabolism* 96: 775–781, 2011.

20. **Bradbury MW**, **Berk PD**. Mitochondrial aspartate aminotransferase: direction of a single protein with two distinct functions to two subcellular sites does not require alternative splicing of the mRNA. *Biochem. J.* 345 Pt 3: 423–427, 2000.

21. **Braun B**, **Horton T**. Endocrine regulation of exercise substrate utilization in women compared to men. *Exerc Sport Sci Rev* 29: 149–154, 2001.

22. **Bruce CR**, **Hoy AJ**, **Turner N**, **Watt MJ**, **Allen TL**, **Carpenter K**, **Cooney GJ**, **Febbraio MA**, **Kraegen EW**. Overexpression of Carnitine Palmitoyltransferase-1 in Skeletal Muscle Is Sufficient to Enhance Fatty Acid Oxidation and Improve High-Fat Diet-Induced Insulin Resistance. *Diabetes* 58: 550–558, 2008.

23. **Brunet J**, **Vazquez-Martin A**, **Colomer R**, **Graña-Suarez B**, **Martin-Castillo B**, **Menendez JA**. BRCA1 and acetyl-CoA carboxylase: The metabolic syndrome of breast cancer. *Mol. Carcinog.* 47: 157–163, 2008.

24. **Burkholder TJ**, **Fingado B**, **Baron S**, **Lieber RL**. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. *J. Morphol.* 221: 177–190, 1994.

25. **Calles-Escandon J**, **Sweet L**, **Ljungqvist O**, **Hirshman MF**. The membrane-associated 40 KD fatty acid binding protein (Berk's protein), a putative fatty acid transporter is present in human skeletal muscle. *Life Sci.* 58: 19–28, 1996.

26. **Cameron-Smith D**, **Burke LM**, **Angus DJ**, **Tunstall RJ**, **Cox GR**, **Bonen A**, **Hawley JA**, **Hargreaves M**. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. *Am. J. Clin. Nutr.* 77: 313–318, 2003.

27. **Campbell SE**, **Febbraio MA**. Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 281: E803–8, 2001.

28. **Campbell SE**, **Febbraio MA**. Effects of ovarian hormones on exercise metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care* 4: 515–520, 2001.

29. **Campbell SE**, **Mehan KA**, **Tunstall RJ**, **Febbraio MA**, **Cameron-Smith D**. 17beta-estradiol upregulates the expression of peroxisome proliferator-activated receptor alpha and lipid oxidative genes in skeletal muscle. *J. Mol. Endocrinol.* 31: 37–45, 2003.

30. **Cao J**, **Li J-L**, **Li D**, **Tobin JF**, **Gimeno RE**. Molecular identification of

microsomal acyl-CoA:glycerol-3-phosphate acyltransferase, a key enzyme in de novo triacylglycerol synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 103: 19695–19700, 2006.

31. **Carling D**, **Clarke PR**, **Zammit VA**, **Hardie DG**. Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur. J. Biochem.* 186: 129–136, 1989.

32. **Carr MC**. The Emergence of the Metabolic Syndrome with Menopause. *Journal of Clinical Endocrinology & Metabolism* 88: 2404–2411, 2003.

33. **Cases S**, **Smith SJ**, **Zheng YW**, **Myers HM**, **Lear SR**, **Sande E**, **Novak S**, **Collins C**, **Welch CB**, **Lusis AJ**, **Erickson SK**, **Farese RV**. Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 95: 13018–13023, 1998.

34. **Chomczynski P**, **Sacchi N**. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156–159, 1987.

35. **Clemons M**, **Goss P**. Estrogen and the risk of breast cancer. *N. Engl. J. Med.* 344: 276–285, 2001.

36. **Cnop M**, **Havel PJ**, **Utzschneider KM**, **Carr DB**, **Sinha MK**, **Boyko EJ**, **Retzlaff BM**, **Knopp RH**, **Brunzell JD**, **Kahn SE**. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia* 46: 459–469, 2003.

37. **Coene ED**, **Hollinshead MS**, **Waeytens AAT**, **Schelfhout VRJ**, **Eechaute WP**, **Shaw MK**, **Van Oostveldt PMV**, **Vaux DJ**. Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria. *Mol. Biol. Cell* 16: 997–1010, 2005.

38. **Colberg SR**, **Simoneau JA**, **Thaete FL**, **Kelley DE**. Skeletal muscle utilization of free fatty acids in women with visceral obesity. *J. Clin. Invest.* 95: 1846–1853, 1995.

39. **Colditz GA**. Economic costs of obesity and inactivity. *Medicine & Science in Sports & Exercise* 31: S663, 1999.

40. **Corpeleijn E**, **Saris WHM**, **Blaak EE**. Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle. *Obesity Reviews* 10: 178–193, 2009.

41. **Cressman VL**, **Backlund DC**, **Hicks EM**, **Gowen LC**, **Godfrey V**, **Koller BH**. Mammary tumor formation in p53- and BRCA1-deficient mice. *Cell Growth Differ.* 10: 1–10, 1999.

42. **D'Eon T**, **Braun B**. The roles of estrogen and progesterone in regulating carbohydrate and fat utilization at rest and during exercise. *J Womens Health Gend Based Med* 11: 225–237, 2002.

43. **D'Eon TM**, **Souza SC**, **Aronovitz M**, **Obin MS**, **Fried SK**, **Greenberg AS**. Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J. Biol. Chem.* 280: 35983–35991, 2005.

44. **Dean D**, **Daugaard JR**, **Young ME**, **Saha A**, **Vavvas D**, **Asp S**, **Kiens B**, **Kim KH**, **Witters L**, **Richter EA**, **Ruderman N**. Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. *Diabetes* 49: 1295–1300, 2000.

45. **DeFronzo RA**, **Sherwin RS**, **Kraemer N**. Effect of physical training on insulin action in obesity. *Diabetes* 36: 1379–1385, 1987.

46. **Dela F**, **Mikines KJ**, **Larsen JJ**, **Galbo H**. Training-induced enhancement of insulin action in human skeletal muscle: the influence of aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 51: B247–52, 1996.

47. **Deng C-X**, **Xu X**. Generation and analysis of Brca1 conditional knockout mice. *Methods Mol. Biol.* 280: 185–200, 2004.

48. **Deng CX**. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Research* 34: 1416–1426, 2006.

49. **Devries MC**. 17 -Estradiol Supplementation Decreases Glucose Rate of Appearance and Disappearance with No Effect on Glycogen Utilization during Moderate Intensity Exercise in Men. *Journal of Clinical Endocrinology & Metabolism* 90: 6218–6225, 2005.

50. **Duncan JA**, **Reeves JR**, **Cooke TG**. BRCA1 and BRCA2 proteins: roles in health and disease. *MP, Mol. Pathol.* 51: 237–247, 1998.

51. **Dzamko N**, **Schertzer JD**, **Ryall JG**, **Steel R**, **Macaulay SL**, **Wee S**, **Chen ZP**, **Michell BJ**, **Oakhill JS**, **Watt MJ**, **Jorgensen SB**, **Lynch GS**, **Kemp BE**, **Steinberg GR**. AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. *The Journal of Physiology* 586: 5819–5831, 2008.

52. **Enns DL**, **Tiidus PM**. The Influence of Estrogen on Skeletal Muscle. *Sports Medicine* 40: 41–58, 2010.

53. **Erion DM**, **Shulman GI**. Diacylglycerol-mediated insulin resistance. *Nat Med* 16: 400–402, 2010.

54. **Evers B**, **Jonkers J**. Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. *Oncogene* 25: 5885–5897, 2006.

55. **Febbraio M**. A Null Mutation in Murine CD36 Reveals an Important Role in Fatty Acid and Lipoprotein Metabolism. *Journal of Biological Chemistry* 274: 19055–19062, 1999.

56. **Feil S**, **Valtcheva N**, **Feil R**. Inducible Cre Mice. In: *Methods in Molecular Biology*. Totowa, NJ: Humana Press, 2009, p. 343–363.

57. **Finkelstein EA**, **Trogdon JG**, **Cohen JW**, **Dietz W**. Annual Medical Spending Attributable To Obesity: Payer-And Service-Specific Estimates. *Health Affairs* 28: w822–w831, 2009.

58. **Flegal KM**, **Williamson DF**, **Pamuk ER**, **Rosenberg HM**. Estimating deaths attributable to obesity in the United States. *Am J Public Health* 94: 1486–1489, 2004.

59. **Frezza C**, **Cipolat S**, **Scorrano L**. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts. *Nat Protoc* 2: 287–295, 2007.

60. **Friedenson B**. BRCA1 and BRCA2 pathways and the risk of cancers other than breast or ovarian. *MedGenMed* 7: 60, 2005.

61. **Fu MHH**, **Maher AC**, **Hamadeh MJ**, **Ye C**, **Tarnopolsky MA**. Exercise, sex, menstrual cycle phase, and 17 -estradiol influence metabolism-related genes in human skeletal muscle. *Physiological Genomics* 40: 34–47, 2009.

62. **Galgani JE**, **Johannsen NM**, **Bajpeyi S**, **Costford SR**, **Zhang Z**, **Gupta AK**, **Ravussin E**. Role of Skeletal Muscle Mitochondrial Density on Exercise-Stimulated Lipid Oxidation. *Obesity* (June 16, 2011). doi: 10.1038/oby.2011.166.

63. **Galgani JE**, **Moro C**, **Ravussin E**. Metabolic flexibility and insulin resistance. *AJP: Endocrinology and Metabolism* 295: E1009–E1017, 2008.

64. **Gambacciani M**, **Ciaponi M**, **Cappagli B**, **Benussi C**, **De Simone L**,

Genazzani AR. Climacteric modifications in body weight and fat tissue distribution. *Climacteric* 2: 37–44, 1999.

65. **Garcia-Cazarin ML**, **Snider NN**, **Andrade FH**. Mitochondrial Isolation from Skeletal Muscle. *JoVE*, 2011.

66. **Glover JNM**, **Williams RS**, **Lee MS**. Interactions between BRCT repeats and phosphoproteins: tangled up in two. *Trends Biochem. Sci.* 29: 579–585, 2004.

67. **Goodpaster BH**, **He J**, **Watkins S**, **Kelley DE**. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *Journal of Clinical Endocrinology & Metabolism* 86: 5755–5761, 2012.

68. **Gorres BK**, **Bomhoff GL**, **Morris JK**, **Geiger PC**. In vivo stimulation of oestrogen receptor α increases insulin-stimulated skeletal muscle glucose uptake. *The Journal of Physiology* 589: 2041–2054, 2011.

69. **Gorzek JF**, **Hendrickson KC**, **Forstner JP**, **Rixen JL**, **Moran AL**, **Lowe DA**. Estradiol and Tamoxifen Reverse Ovariectomy-Induced Physical Inactivity in Mice. *Medicine & Science in Sports & Exercise* 39: 248–256, 2007.

70. **Goudriaan JR**. CD36 deficiency increases insulin sensitivity in muscle, but induces insulin resistance in the liver in mice. *The Journal of Lipid Research* 44: 2270–2277, 2003.

71. **Gower BA**, **Muñoz J**, **Desmond R**, **Hilario-Hailey T**, **Jiao X**. Changes in intra-abdominal fat in early postmenopausal women: effects of hormone use. *Obesity (Silver Spring)* 14: 1046–1055, 2006.

72. **Greising SM**, **Baltgalvis KA**, **Lowe DA**, **Warren GL**. Hormone Therapy and Skeletal Muscle Strength: A Meta-Analysis. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 64A: 1071–1081, 2009.

73. **Griffin ME**, **Marcucci MJ**, **Cline GW**, **Bell K**, **Barucci N**, **Lee D**, **Goodyear LJ**, **Kraegen EW**, **White MF**, **Shulman GI**. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48: 1270–1274, 1999.

74. **Guo SS**, **Zeller C**, **Chumlea WC**, **Siervogel RM**. Aging, body composition, and lifestyle: the Fels Longitudinal Study. *Am. J. Clin. Nutr.* 70: 405–411, 1999.

75. **Gupta P**, **Ho P-C**, **Huq MDM**, **Khan AA**, **Tsai N-P**, **Wei L-N**. PKCε Stimulated Arginine Methylation of RIP140 for Its Nuclear-Cytoplasmic Export in Adipocyte Differentiation. *PLoS ONE* 3: e2658, 2008.

76. **Hamilton MT**, **Etienne J**, **McClure WC**, **Pavey BS**, **Holloway AK**. Role of local contractile activity and muscle fiber type on LPL regulation during exercise. *Am. J. Physiol.* 275: E1016–22, 1998.

77. **Hancock CR**. Contraction-mediated phosphorylation of AMPK is lower in skeletal muscle of adenylate kinase-deficient mice. *Journal of Applied Physiology* 100: 406–413, 2006.

78. **Handschin C**, **Chin S**, **Li P**, **Liu F**, **Maratos-Flier E**, **LeBrasseur NK**, **Yan Z**, **Spiegelman BM**. Skeletal Muscle Fiber-type Switching, Exercise Intolerance, and Myopathy in PGC-1 Muscle-specific Knock-out Animals. *Journal of Biological Chemistry* 282: 30014–30021, 2007.

79. **He J**, **Watkins S**, **Kelley DE**. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* 50: 817–823, 2001.

80. **Herrero MC**, **Remesar X**, **Bladé C**, **Arola L**. Muscle amino acid pattern in obese rats. *Int J Obes Relat Metab Disord* 21: 698–703, 1997.

81. **Hiatt WR**, **Regensteiner JG**, **Wolfel EE**, **Ruff L**, **Brass EP**. Carnitine and acylcarnitine metabolism during exercise in humans. Dependence on skeletal muscle metabolic state. *J. Clin. Invest.* 84: 1167–1173, 1989.

82. **Ho GH**, **Phang BH**, **Ng IS**, **Law HY**, **Soo KC**, **Ng EH**. Novel germline BRCA1 mutations detected in women in singapore who developed breast carcinoma before the age of 36 years. *Cancer* 89: 811–816, 2000.

83. **Hoeg L**, **Roepstorff C**, **Thiele M**, **Richter EA**, **Wojtaszewski JFP**, **Kiens B**. Higher intramuscular triacylglycerol in women does not impair insulin sensitivity and proximal insulin signaling. *Journal of Applied Physiology* 107: 824–831, 2009.

84. **Holloszy JO**. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J. Biol. Chem.* 242: 2278–2282, 1967.

85. **Holloszy JO**. Muscle metabolism during exercise. *Arch Phys Med Rehabil* 63: 231–234, 1982.

86. **Holloway GP**, **Benton CR**, **Mullen KL**, **Yoshida Y**, **Snook LA**, **Han XX**, **Glatz JFC**, **Luiken JJFP**, **Lally J**, **Dyck DJ**, **Bonen A**. In obese rat muscle transport of palmitate is increased and is channeled to

triacylglycerol storage despite an increase in mitochondrial palmitate oxidation. *AJP: Endocrinology and Metabolism* 296: E738–E747, 2009.

87. **Horowitz JF**, **Mora-Rodriguez R**, **Byerley LO**, **Coyle EF**. Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. *Am. J. Physiol.* 273: E768–75, 1997.

88. **Hulver MW**, **Berggren JR**, **Cortright RN**, **Dudek RW**, **Thompson RP**, **Pories WJ**, **MacDonald KG**, **Cline GW**, **Shulman GI**, **Dohm GL**, **Houmard JA**. Skeletal muscle lipid metabolism with obesity. *Am. J. Physiol. Endocrinol. Metab.* 284: E741–7, 2003.

89. **Hurley BF**, **Nemeth PM**, **Martin WH**, **Hagberg JM**, **Dalsky GP**, **Holloszy JO**. Muscle triglyceride utilization during exercise: effect of training. *J. Appl. Physiol.* 60: 562–567, 1986.

90. **Høeg LD**, **Sjøberg KA**, **Jeppesen J**, **Jensen TE**, **Frøsig C**, **Birk JB**, **Bisiani B**, **Hiscock N**, **Pilegaard H**, **Wojtaszewski JFP**, **Richter EA**, **Kiens B**. Lipid-induced insulin resistance affects women less than men and is not accompanied by inflammation or impaired proximal insulin signaling. *Diabetes* 60: 64–73, 2011.

91. **Ibrahimi A**. Muscle-specific Overexpression of FAT/CD36 Enhances Fatty Acid Oxidation by Contracting Muscle, Reduces Plasma Triglycerides and Fatty Acids, and Increases Plasma Glucose and Insulin. *Journal of Biological Chemistry* 274: 26761–26766, 1999.

92. **Irrcher I**, **Ljubicic V**, **Hood DA**. Interactions between ROS and AMP kinase activity in the regulation of PGC-1 transcription in skeletal muscle cells. *AJP: Cell Physiology* 296: C116–C123, 2008.

93. **Isken F**, **Pfeiffer AFH**, **Nogueiras R**, **Osterhoff MA**, **Ristow M**, **Thorens B**, **Tschop MH**, **Weickert MO**. Deficiency of glucose-dependent insulinotropic polypeptide receptor prevents ovariectomy-induced obesity in mice. *AJP: Endocrinology and Metabolism* 295: E350–E355, 2008.

94. **Issekutz B**, **Miller HI**, **Rodahl K**. Lipid and carbohydrate metabolism during exercise. *Journal of Applied Physiology* 16: 1415–1420, 1961.

95. **Issekutz B**, **Miller HI**, **Rodahl K**. Lipid and carbohydrate metabolism during exercise. *Fed. Proc.* 25: 1415–1420, 1966.

96. **Jackson KC**, **Sallstedt EK**, **Norrbom J**, **Thomson DM**, **Schuh RA**, **Neufer PD**, **Spangenburg EE**. BRCA1 is a Novel Regulator of Lipid Metabolism in Skeletal Muscle. *Unpublished Manuscript*.

97. **Jackson KC**, **Wohlers LM**, **Lovering RM**, **Schuh RA**, **Maher AC**, **Bonen A**, **Koves TR**, **Ilkayeva O**, **Thomson DM**, **Muoio DM**, **Spangenburg EE**. Ectopic lipid deposition and the metabolic profile of skeletal muscle in ovariectomized mice. *AJP: Regulatory, Integrative and Comparative Physiology* 304: R206–17, 2013.

98. **Jackson KC**, **Wohlers LM**, **Valencia AP**, **Cilenti M**, **Borengasser SJ**, **Thyfault JP**, **Spangenburg EE**. Wheel running prevents the accumulation of monounsaturated fatty acids in the liver of ovariectomized mice by attenuating changes in SCD-1 content. *Appl. Physiol. Nutr. Metab.* 36: 798–810, 2011.

99. **Jeong S**, **Yoon M**. Swimming's prevention of ovariectomy-induced obesity through activation of skeletal-muscle PPARα. *Int J Sport Nutr Exerc Metab* 22: 1–10, 2012.

100. **Kelley DE**, **Goodpaster B**, **Wing RR**, **Simoneau JA**. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am. J. Physiol.* 277: E1130–41, 1999.

101. **Kiens B**. Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *Journal of Applied Physiology* 97: 1209–1218, 2004.

102. **Kiens B**. Skeletal Muscle Lipid Metabolism in Exercise and Insulin Resistance. *Physiological Reviews* 86: 205–243, 2006.

103. **Kim JK**, **Fillmore JJ**, **Sunshine MJ**, **Albrecht B**, **Higashimori T**, **Kim D-W**, **Liu Z-X**, **Soos TJ**, **Cline GW**, **O'Brien WR**, **Littman DR**, **Shulman GI**. PKC-θ knockout mice are protected from fat-induced insulin resistance. *J. Clin. Invest.* 114: 823–827, 2004.

104. **Kim JY**, **Hickner RC**, **Cortright RL**, **Dohm GL**, **Houmard JA**. Lipid oxidation is reduced in obese human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 279: E1039–44, 2000.

105. **Kolleck I**, **Guthmann F**, **Ladhoff A-M**, **Tandon NN**, **Schlame M**, **Rüstow B**. Cellular cholesterol stimulates acute uptake of palmitate by redistribution of fatty acid translocase in type II pneumocytes. *Biochemistry* 41: 6369–6375, 2002.

106. **Koves TR**, **Sparks LM**, **Kovalik JP**, **Mosedale M**, **Arumugam R**, **Debalsi KL**, **Everingham K**, **Thorne L**, **Phielix E**, **Meex RC**, **Kien CL**, **Hesselink MKC**, **Schrauwen P**, **Muoio DM**. PPARγ coactivator-1α contributes to exercise-induced regulation of intramuscular lipid droplet programming in mice and humans. *The Journal of Lipid Research* 54: 522–534, 2013.

107. **Koves TR**, **Ussher JR**, **Noland RC**, **Slentz D**, **Mosedale M**, **Ilkayeva O**, **Bain J**, **Stevens R**, **Dyck JRB**, **Newgard CB**, **Lopaschuk GD**, **Muoio DM**. Mitochondrial Overload and Incomplete Fatty Acid Oxidation Contribute to Skeletal Muscle Insulin Resistance. *Cell Metabolism* 7: 45–56, 2008.

108. **Krebs M**, **Krssak M**, **Bernroider E**, **Anderwald C**, **Brehm A**, **Meyerspeer M**, **Nowotny P**, **Roth E**, **Waldhäusl W**, **Roden M**. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes* 51: 599–605, 2002.

109. **Kubista M**, **Rosner M**, **Kubista E**, **Bernaschek G**, **Hengstschläger M**. Brca1 regulates in vitro differentiation of mammary epithelial cells. *Oncogene* 21: 4747–4756, 2002.

110. **Kusminski CM**, **Shetty S**, **Orci L**, **Unger RH**, **Scherer PE**. Diabetes and apoptosis: lipotoxicity. *Apoptosis* 14: 1484–1495, 2009.

111. **Langie SAS**, **Lara J**, **Mathers JC**. Early determinants of the ageing trajectory. *Best Practice & Research Clinical Endocrinology & Metabolism* 26: 613–626, 2012.

112. **Lanza IR**, **Sreekumaran Nair K**. Regulation of skeletal muscle mitochondrial function: genes to proteins. *Acta Physiologica* 199: 529–547, 2010.

113. **Layne AS**, **Nasrallah S**, **South MA**, **Howell MEA**, **McCurry MP**, **Ramsey MW**, **Stone MH**, **Stuart CA**. Impaired Muscle AMPK Activation in the Metabolic Syndrome May Attenuate Improved Insulin Action after Exercise Training. *Journal of Clinical Endocrinology & Metabolism* 96: 1815–1826, 2011.

114. **Lee JS**. Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites. *Journal of Applied Physiology* 100: 1467–1474, 2006.

115. **Lee MS**, **Green R**, **Marsillac SM**, **Coquelle N**, **Williams RS**, **Yeung T**, **Foo D**, **Hau DD**, **Hui B**, **Monteiro ANA**, **Glover JNM**. Comprehensive Analysis of Missense Variations in the BRCT Domain of BRCA1 by Structural and Functional Assays. *Cancer Research* 70: 4880–4890, 2010.

116. **Lefort N**, **Glancy B**, **Bowen B**, **Willis WT**, **Bailowitz Z**, **De Filippis EA**, **Brophy C**, **Meyer C**, **Højlund K**, **Yi Z**, **Mandarino LJ**. Increased Reactive Oxygen Species Production and Lower Abundance of Complex I Subunits and Carnitine Palmitoyltransferase 1B Protein Despite Normal Mitochondrial Respiration in Insulin-Resistant Human Skeletal Muscle. *Diabetes* 59: 2444–2452, 2010.

117. **Leite RD**, **Prestes J**, **Bernardes CF**, **Shiguemoto GE**, **Pereira GB**, **Duarte JO**, **Domingos MM**, **Baldissera V**, **de Andrade Perez SE**. Effects of ovariectomy and resistance training on lipid content in skeletal muscle, liver, and heart; fat depots; and lipid profile. *Appl. Physiol. Nutr. Metab.* 34: 1079–1086, 2009.

118. **Leung DW**. The structure and functions of human lysophosphatidic acid acyltransferases. *Front. Biosci.* 6: D944–53, 2001.

119. **Levine EG**, **Raczynski JM**, **Carpenter JT**. Weight gain with breast cancer adjuvant treatment. *Cancer* 67: 1954–1959, 1991.

120. **Little JP**, **Safdar A**, **Wilkin GP**, **Tarnopolsky MA**, **Gibala MJ**. A practical model of low-volume high-intensity interval training induces mitochondrial biogenesis in human skeletal muscle: potential mechanisms. *The Journal of Physiology* 588: 1011–1022, 2010.

121. **Liu L**, **Zhang Y**, **Chen N**, **Shi X**, **Tsang B**, **Yu Y-H**. Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *Journal of Clinical Investigation* 117: 1679–1689, 2007.

122. **Ljubicic V**, **Joseph A-M**, **Saleem A**, **Uguccioni G**, **Collu-Marchese M**, **Lai RYJ**, **Nguyen LMD**, **Hood DA**. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: Effects of exercise and aging. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1800: 223–234, 2010.

123. **Ludlow AT**, **Lima LCJ**, **Wang J**, **Hanson ED**, **Guth LM**, **Spangenburg EE**, **Roth SM**. Exercise alters mRNA expression of telomere-repeat binding factor 1 in skeletal muscle via p38 MAPK. *Journal of Applied Physiology* 113: 1737–1746, 2012.

124. **Luiken JJ**, **Turcotte LP**, **Bonen A**. Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. *The Journal of Lipid Research* 40: 1007–1016, 1999.

125. **Lynch NA**, **Ryan AS**, **Berman DM**, **Sorkin JD**, **Nicklas BJ**. Comparison of VO2max and disease risk factors between perimenopausal and postmenopausal women. *Menopause* 9: 456–462, 2002.

126. **Magnard C**, **Bachelier R**, **Vincent A**, **Jaquinod M**, **Kieffer S**, **Lenoir GM**, **Venezia ND**. BRCA1 interacts with acetyl-CoA carboxylase through its tandem of BRCT domains. *Oncogene* 21: 6729–6739, 2002.

127. **Maher AC**, **Akhtar M**, **Tarnopolsky MA**. Men supplemented with 17 -estradiol have increased -oxidation capacity in skeletal muscle.

Physiological Genomics 42: 342–347, 2010.

128. **Maher AC**, **Akhtar M**, **Vockley J**, **Tarnopolsky MA**. Women have higher protein content of beta-oxidation enzymes in skeletal muscle than men. *PLoS ONE* 5: e12025, 2010.

129. **Maher AC**, **Mohsen A-W**, **Vockley J**, **Tarnopolsky MA**. Low expression of long-chain acyl-CoA dehydrogenase in human skeletal muscle. *Molecular Genetics and Metabolism* 100: 163–167, 2010.

130. **Makowski L**, **Noland RC**, **Koves TR**, **Xing W**, **Ilkayeva OR**, **Muehlbauer MJ**, **Stevens RD**, **Muoio DM**. Metabolic profiling of PPAR -/- mice reveals defects in carnitine and amino acid homeostasis that are partially reversed by oral carnitine supplementation. *The FASEB Journal* 23: 586–604, 2008.

131. **Maniccia AW**, **Lewis C**, **Begum N**, **Xu J**, **Cui J**, **Chipitsyna G**, **Aysola K**, **Reddy V**, **Bhat G**, **Fujimura Y**, **Henderson B**, **Reddy ESP**, **Rao VN**. Mitochondrial localization, ELK-1 transcriptional regulation and growth inhibitory functions of BRCA1, BRCA1a, and BRCA1b proteins. *J. Cell. Physiol.* 219: 634–641, 2009.

132. **Mattingly KA**, **Ivanova MM**, **Riggs KA**, **Wickramasinghe NS**, **Barch MJ**, **Klinge CM**. Estradiol Stimulates Transcription of Nuclear Respiratory Factor-1 and Increases Mitochondrial Biogenesis. *Molecular Endocrinology* 22: 609–622, 2007.

133. **Mauvais-Jarvis F**. Estrogen and androgen receptors: regulators of fuel homeostasis and emerging targets for diabetes and obesity. *Trends in Endocrinology & Metabolism* 22: 24–33, 2011.

134. **Maxwell KN**, **Domchek SM**. Cancer treatment according to BRCA1 and BRCA2 mutations. *Nat Rev Clin Oncol* 9: 520–528, 2012.

135. **McCarthy JJ**, **Srikuea R**, **Kirby TJ**, **Peterson CA**, **Esser KA**. Inducible Cre transgenic mouse strain for skeletal muscle-specific gene targeting. *Skelet Muscle* 2: 8, 2012.

136. **McClung JM**, **Davis JM**, **Carson JA**. Muscle: Ovarian hormone status and skeletal muscle inflammation during recovery from disuse in rats. *Experimental Physiology* 92: 219–232, 2006.

137. **McGarry JD**, **Mills SE**, **Long CS**, **Foster DW**. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem. J.* 214: 21–28, 1983.

138. **McGarry JD**, **Stark MJ**, **Foster DW**. Hepatic malonyl-CoA levels of fed, fasted and diabetic rats as measured using a simple radioisotopic assay. *J. Biol. Chem.* 253: 8291–8293, 1978.

139. **Menshikova EV**. Effects of weight loss and physical activity on skeletal muscle mitochondrial function in obesity. *AJP: Endocrinology and Metabolism* 288: E818–E825, 2004.

140. **Merrill GF**, **Kurth EJ**, **Rasmussen BB**, **Winder WW**. Influence of malonyl-CoA and palmitate concentration on rate of palmitate oxidation in rat muscle. *J. Appl. Physiol.* 85: 1909–1914, 1998.

141. **Merrill RM**, **Layman AB**, **Oderda G**, **Asche C**. Risk Estimates of Hysterectomy and Selected Conditions Commonly Treated with Hysterectomy. *Annals of Epidemiology* 18: 253–260, 2008.

142. **Miki Y**, **Swensen J**, **Shattuck-Eidens D**, **Futreal PA**, **Harshman K**, **Tavtigian S**, **Liu Q**, **Cochran C**, **Bennett LM**, **Ding W**. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266: 66–71, 1994.

143. **Millot GA**, **Carvalho MA**, **Caputo SM**, **Vreeswijk MPG**, **Brown MA**, **Webb M**, **Rouleau E**, **Neuhausen SL**, **Hansen TVO**, **Galli A**, **Brandão RD**, **Blok MJ**, **Velkova A**, **Couch FJ**, **Monteiro ANA**, **on behalf of the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) Consortium Functional Assay Working Group**. A guide for functional analysis of BRCA1variants of uncertain significance. *Hum. Mutat.* 33: 1526–1537, 2012.

144. **Miranda DA**, **Koves TR**, **Gross DA**, **Chadt A**, **Al-Hasani H**, **Cline GW**, **Schwartz GJ**, **Muoio DM**, **Silver DL**. Re-patterning of skeletal muscle energy metabolism by fat storage-inducing transmembrane protein 2. *J. Biol. Chem.* 286: 42188–42199, 2011.

145. **Mixon M**, **Kittrell F**, **Medina D**. Expression of Brca1 and splice variant Brca1delta11 RNA levels in mouse mammary gland during normal development and tumorigenesis. *Oncogene* 19: 5237–5243, 2000.

146. **Mok MTS**, **Henderson BR**. A comparison of BRCA1 nuclear localization with 14 DNA damage response proteins and domains: Identification of specific differences between BRCA1 and 53BP1 at DNA damage-induced foci. *Cellular Signalling* 22: 47–56, 2010.

147. **Moran AL**. Removal of ovarian hormones from mature mice detrimentally affects muscle contractile function and myosin structural distribution. *Journal of Applied Physiology* 100: 548–559, 2006.

148. **Moreau K**, **Dizin E**, **Ray H**, **Luquain C**, **Lefai E**, **Foufelle F**, **Billaud M**, **Lenoir GM**, **Venezia ND**. BRCA1 affects lipid synthesis through its interaction with acetyl-CoA carboxylase. *J. Biol. Chem.* 281: 3172–3181, 2006.

149. **Mulla NA**, **Simonsen L**, **Bülow J**. Post-exercise adipose tissue and skeletal muscle lipid metabolism in humans: the effects of exercise intensity. *The Journal of Physiology* 524 Pt 3: 919–928, 2000.

150. **Newgard CB**, **An J**, **Bain JR**, **Muehlbauer MJ**, **Stevens RD**, **Lien LF**, **Haqq AM**, **Shah SH**, **Arlotto M**, **Slentz CA**. A Branched-Chain Amino Acid-Related Metabolic Signature that Differentiates Obese and Lean Humans and Contributes to Insulin Resistance. *Cell Metabolism* 9: 311–326, 2009.

151. **Nicklas BJ**, **Rogus EM**, **Colman EG**, **Goldberg AP**. Visceral adiposity, increased adipocyte lipolysis, and metabolic dysfunction in obese postmenopausal women. *Am. J. Physiol.* 270: E72–8, 1996.

152. **Noland RC**, **Koves TR**, **Seiler SE**, **Lum H**, **Lust RM**, **Ilkayeva O**, **Stevens RD**, **Hegardt FG**, **Muoio DM**. Carnitine Insufficiency Caused by Aging and Overnutrition Compromises Mitochondrial Performance and Metabolic Control. *Journal of Biological Chemistry* 284: 22840–22852, 2009.

153. **Norrbom J**, **Wallman SE**, **Gustafsson T**, **Rundqvist H**, **Jansson E**, **Sundberg CJ**. Training response of mitochondrial transcription factors in human skeletal muscle. *Acta Physiol (Oxf)* 198: 71–79, 2010.

154. **Norris J**, **Fan D**, **Aleman C**, **Marks JR**, **Futreal PA**, **Wiseman RW**, **Iglehart JD**, **Deininger PL**, **McDonnell DP**. Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. *J. Biol. Chem.* 270: 22777–22782, 1995.

155. **O'Neill HM**, **Maarbjerg SJ**, **Crane JD**, **Jeppesen J**, **Jorgensen SB**, **Schertzer JD**, **Shyroka O**, **Kiens B**, **van Denderen BJ**, **Tarnopolsky MA**, **Kemp BE**, **Richter EA**, **Steinberg GR**. AMP-activated protein kinase (AMPK) 1 2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proceedings of the National Academy of Sciences* 108: 16092–16097, 2011.

156. **Paquette A**, **Chapados N**, **Bergeron R**, **Lavoie JM**. Fatty Acid Oxidation is Decreased in the Liver of Ovariectomized Rats. *Horm Metab Res* 41: 511–515, 2009.

157. **Peeters A**. Obesity in Adulthood and Its Consequences for Life Expectancy: A Life-Table Analysis. *Ann Intern Med* 138: 24, 2003.

158. **Pickersgill L**, **Litherland GJ**, **Greenberg AS**, **Walker M**, **Yeaman SJ**. Key Role for Ceramides in Mediating Insulin Resistance in Human Muscle Cells. *Journal of Biological Chemistry* 282: 12583–12589, 2007.

159. **Powell DJ**, **Turban S**, **Gray A**, **Hajduch E**, **Hundal HS**. Intracellular ceramide synthesis and protein kinase Czeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochem. J.* 382: 619–629, 2004.

160. **Rasmussen BB**, **Winder WW**. Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. *J. Appl. Physiol.* 83: 1104–1109, 1997.

161. **Rasmussen BB**. Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. *Journal of Clinical Investigation* 110: 1687–1693, 2002.

162. **Rector RS**, **Uptergrove GM**, **Morris EM**, **Borengasser SJ**, **Laughlin MH**, **Booth FW**, **Thyfault JP**, **Ibdah JA**. Daily exercise vs. caloric restriction for prevention of nonalcoholic fatty liver disease in the OLETF rat model. *AJP: Gastrointestinal and Liver Physiology* 300: G874–G883, 2011.

163. **Ribas V**, **Nguyen MTA**, **Henstridge DC**, **Nguyen AK**, **Beaven SW**, **Watt MJ**, **Hevener AL**. Impaired oxidative metabolism and inflammation are associated with insulin resistance in ER -deficient mice. *AJP: Endocrinology and Metabolism* 298: E304–E319, 2010.

164. **Ritov VB**, **Menshikova EV**, **Azuma K**, **Wood R**, **Toledo FGS**, **Goodpaster BH**, **Ruderman NB**, **Kelley DE**. Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *AJP: Endocrinology and Metabolism* 298: E49–E58, 2009.

165. **Rockl KSC**, **Hirshman MF**, **Brandauer J**, **Fujii N**, **Witters LA**, **Goodyear LJ**. Skeletal Muscle Adaptation to Exercise Training: AMP-Activated Protein Kinase Mediates Muscle Fiber Type Shift. *Diabetes* 56: 2062–2069, 2007.

166. **Roepstorff C**, **Helge JW**, **Vistisen B**, **Kiens B**. Studies of plasma membrane fatty acid-binding protein and other lipid-binding proteins in human skeletal muscle. *Proc Nutr Soc* 63: 239–244, 2004.

167. **Rogers NH**, **Perfield JW**, **Strissel KJ**, **Obin MS**, **Greenberg AS**.

Reduced Energy Expenditure and Increased Inflammation Are Early Events in the Development of Ovariectomy-Induced Obesity. *Endocrinology* 150: 2161–2168, 2008.

168. **Rooyackers OE**, **Adey DB**, **Ades PA**, **Nair KS**. Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* 93: 15364–15369, 1996.

169. **Ruderman NB**, **Saha AK**, **Vavvas D**, **Witters LA**. Malonyl-CoA, fuel sensing, and insulin resistance. *Am. J. Physiol.* 276: E1–E18, 1999.

170. **Saengsirisuwan V**, **Pongseeda S**, **Prasannarong M**, **Vichaiwong K**, **Toskulkao C**. Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement. *Metabolism* 58: 38–47, 2009.

171. **Safdar A**, **Little JP**, **Stokl AJ**, **Hettinga BP**, **Akhtar M**, **Tarnopolsky MA**. Exercise Increases Mitochondrial PGC-1 Content and Promotes Nuclear-Mitochondrial Cross-talk to Coordinate Mitochondrial Biogenesis. *Journal of Biological Chemistry* 286: 10605–10617, 2011.

172. **Saha T**, **Rih JK**, **Rosen EM**. BRCA1 down-regulates cellular levels of reactive oxygen species. *FEBS Letters* 583: 1535–1543, 2009.

173. **Saltin B**, **Grimby G**. Physiological analysis of middle-aged and old former athletes. Comparison with still active athletes of the same ages. *Circulation* 38: 1104–1115, 1968.

174. **Schaffer JE**, **Lodish HF**. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell* 79: 427–436, 1994.

175. **Schuh RA**, **Jackson KC**, **Khairallah RJ**, **Ward CW**, **Spangenburg EE**. Measuring mitochondrial respiration in intact single muscle fibers. *AJP: Regulatory, Integrative and Comparative Physiology* 302: R712–9, 2012.

176. **Schwieterman W**, **Sorrentino D**, **Potter BJ**, **Rand J**, **Kiang CL**, **Stump D**, **Berk PD**. Uptake of oleate by isolated rat adipocytes is mediated by a 40-kDa plasma membrane fatty acid binding protein closely related to that in liver and gut. *Proc. Natl. Acad. Sci. U.S.A.* 85: 359–363, 1988.

177. **She P**, **Zhou Y**, **Zhang Z**, **Griffin K**, **Gowda K**, **Lynch CJ**. Disruption of BCAA metabolism in mice impairs exercise metabolism and endurance. *Journal of Applied Physiology* 108: 941–949, 2010.

178. **Shukla PC**, **Singh KK**, **Quan A**, **Al-Omran M**, **Teoh H**, **Lovren F**,

Cao L, **Rovira II**, **Pan Y**, **Brezden-Masley C**, **Yanagawa B**, **Gupta A**, **Deng C-X**, **Coles JG**, **Leong-Poi H**, **Stanford WL**, **Parker TG**, **Schneider MD**, **Finkel T**, **Verma S**. BRCA1 is an essential regulator of heart function and survival following myocardial infarction. *Nat Comms* 2: 593, 2011.

179. **Simoneau JA**, **Colberg SR**, **Thaete FL**, **Kelley DE**. Skeletal muscle glycolytic and oxidative enzyme capacities are determinants of insulin sensitivity and muscle composition in obese women. *FASEB J.* 9: 273–278, 1995.

180. **Simoneau JA**, **Veerkamp JH**, **Turcotte LP**, **Kelley DE**. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J.* 13: 2051–2060, 1999.

181. **Singh KK**, **Shukla PC**, **Yanagawa B**, **Quan A**, **Lovren F**, **Pan Y**, **Wagg CS**, **Teoh H**, **Lopaschuk GD**, **Verma S**. Regulating cardiac energy metabolism and bioenergetics by targeting the DNA damage repair protein BRCA1. *J. Thorac. Cardiovasc. Surg.* (January 12, 2013). doi: 10.1016/j.jtcvs.2012.12.046.

182. **Sitnick M**. Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass. *Journal of Applied Physiology* 100: 286–293, 2006.

183. **Smith BK**, **Perry CGR**, **Koves TR**, **Wright DC**, **Smith JC**, **Neufer PD**, **Muoio DM**, **Holloway GP**. Identification of a novel malonyl-CoA IC(50) for CPT-I: implications for predicting in vivo fatty acid oxidation rates. *Biochem. J.* 448: 13–20, 2012.

184. **Somasundaram K**, **Zhang H**, **Zeng YX**, **Houvras Y**, **Peng Y**, **Zhang H**, **Wu GS**, **Licht JD**, **Weber BL**, **El-Deiry WS**. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Nature* 389: 187–190, 1997.

185. **Sorensen LP**, **Sondergaard E**, **Nellemann B**, **Christiansen JS**, **Gormsen LC**, **Nielsen S**. Increased VLDL-Triglyceride Secretion Precedes Impaired Control of Endogenous Glucose Production in Obese, Normoglycemic Men. *Diabetes* 60: 2257–2264, 2011.

186. **Sorrentino D**, **Stump D**, **Potter BJ**, **Robinson RB**, **White R**, **Kiang CL**, **Berk PD**. Oleate uptake by cardiac myocytes is carrier mediated and involves a 40-kD plasma membrane fatty acid binding protein similar to that in liver, adipose tissue, and gut. *J. Clin. Invest.* 82: 928–935, 1988.

187. **Southgate RJ**, **Bruce CR**, **Carey AL**, **Steinberg GR**, **Walder K**,

Monks R, **Watt MJ**, **Hawley JA**, **Birnbaum MJ**, **Febbraio MA**. PGC-1alpha gene expression is down-regulated by Akt- mediated phosphorylation and nuclear exclusion of FoxO1 in insulin-stimulated skeletal muscle. *The FASEB Journal* 19: 2072–2074, 2005.

188. **Spangenburg EE**, **Brown DA**, **Johnson MS**, **Moore RL**. Exercise increases SOCS-3 expression in rat skeletal muscle: potential relationship to IL-6 expression. *The Journal of Physiology* 572: 839–848, 2006.

189. **Spangenburg EE**, **Pratt SJP**, **Wohlers LM**, **Lovering RM**. Use of BODIPY (493/503) to Visualize Intramuscular Lipid Droplets in Skeletal Muscle. *Journal of Biomedicine and Biotechnology* 2011: 1–8, 2011.

190. **Spangenburg EE**. SOCS-3 Induces Myoblast Differentiation. *Journal of Biological Chemistry* 280: 10749–10758, 2005.

191. **Stanley WC**. Malonyl-CoA decarboxylase inhibition suppresses fatty acid oxidation and reduces lactate production during demand-induced ischemia. *AJP: Heart and Circulatory Physiology* 289: H2304–H2309, 2005.

192. **Steffensen CH**, **Roepstorff C**, **Madsen M**, **Kiens B**. Myocellular triacylglycerol breakdown in females but not in males during exercise. *Am. J. Physiol. Endocrinol. Metab.* 282: E634–42, 2002.

193. **Steinberg GR**, **Rush JWE**, **Dyck DJ**. AMPK expression and phosphorylation are increased in rodent muscle after chronic leptin treatment. *Am. J. Physiol. Endocrinol. Metab.* 284: E648–54, 2003.

194. **Stewart LK**, **Wang Z**, **Ribnicky D**, **Soileau JL**, **Cefalu WT**, **Gettys TW**. Failure of dietary quercetin to alter the temporal progression of insulin resistance among tissues of C57BL/6J mice during the development of diet-induced obesity. *Diabetologia* 52: 514–523, 2009.

195. **Stirone C**. Estrogen Increases Mitochondrial Efficiency and Reduces Oxidative Stress in Cerebral Blood Vessels. *Molecular Pharmacology* 68: 959–965, 2005.

196. **Stremmel W**. Fatty acid uptake by isolated rat heart myocytes represents a carrier-mediated transport process. *J. Clin. Invest.* 81: 844–852, 1988.

197. **Stump DD**, **Zhou SL**, **Berk PD**. Comparison of plasma membrane FABP and mitochondrial isoform of aspartate aminotransferase from rat liver. *Am. J. Physiol.* 265: G894–902, 1993.

198. **Tarnopolsky LJ**, **MacDougall JD**, **Atkinson SA**, **Tarnopolsky MA**, **Sutton JR**. Gender differences in substrate for endurance exercise. *J.*

Appl. Physiol. 68: 302–308, 1990.

199. **Tarnopolsky MA**, **Rennie CD**, **Robertshaw HA**, **Fedak-Tarnopolsky SN**, **Devries MC**, **Hamadeh MJ**. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *AJP: Regulatory, Integrative and Comparative Physiology* 292: R1271–R1278, 2006.

200. **Tarnopolsky MA**. Gender differences in substrate metabolism during endurance exercise. *Can J Appl Physiol* 25: 312–327, 2000.

201. **Tarnopolsky MA**. Sex Differences in Exercise Metabolism and the Role of 17-Beta Estradiol. *Medicine & Science in Sports & Exercise* 40: 648–654, 2008.

202. **Thakur S**, **Zhang HB**, **Peng Y**, **Le H**, **Carroll B**, **Ward T**, **Yao J**, **Farid LM**, **Couch FJ**, **Wilson RB**, **Weber BL**. Localization of BRCA1 and a splice variant identifies the nuclear localization signal. *Molecular and Cellular Biology* 17: 444–452, 2012.

203. **Thauer RK**. Citric-acid cycle, 50 years on. Modifications and an alternative pathway in anaerobic bacteria. *Eur. J. Biochem.* 176: 497–508, 1988.

204. **Thomson DM**, **Brown JD**, **Fillmore N**, **Condon BM**, **Kim HJ**, **Barrow JR**, **Winder WW**. LKB1 and the regulation of malonyl-CoA and fatty acid oxidation in muscle. *AJP: Endocrinology and Metabolism* 293: E1572–E1579, 2007.

205. **Thyfault JP**, **Cree MG**, **Tapscott EB**, **Bell JA**, **Koves TR**, **Ilkayeva O**, **Wolfe RR**, **Dohm GL**, **Muoio DM**. Metabolic profiling of muscle contraction in lean compared with obese rodents. *AJP: Regulatory, Integrative and Comparative Physiology* 299: R926–R934, 2010.

206. **Thyfault JP**, **Rector RS**, **Uptergrove GM**, **Borengasser SJ**, **Morris EM**, **Wei Y**, **Laye MJ**, **Burant CF**, **Qi NR**, **Ridenhour SE**, **Koch LG**, **Britton SL**, **Ibdah JA**. Rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury. *The Journal of Physiology* 587: 1805–1816, 2009.

207. **Timmers S**, **Nabben M**, **Bosma M**, **van Bree B**, **Lenaers E**, **van Beurden D**, **Schaart G**, **Westerterp-Plantenga MS**, **Langhans W**, **Hesselink MKC**, **Schrauwen-Hinderling VB**, **Schrauwen P**. Augmenting muscle diacylglycerol and triacylglycerol content by blocking fatty acid oxidation does not impede insulin sensitivity.

Proceedings of the National Academy of Sciences 109: 11711–11716, 2012.

208. **TK M**, **W E-D**. Functional Interactions Between BRCA1 and the Cell Cycle. : 1–7, 2013.

209. **Turcotte LP**, **Swenberger JR**, **Tucker MZ**, **Yee AJ**, **Trump G**, **Luiken JJ**, **Bonen A**. Muscle palmitate uptake and binding are saturable and inhibited by antibodies to FABP(PM). *Mol Cell Biochem* 210: 53–63, 2000.

210. **van Loon LJ**, **Greenhaff PL**, **Constantin-Teodosiu D**, **Saris WH**, **Wagenmakers AJ**. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *The Journal of Physiology* 536: 295–304, 2001.

211. **Wang Y**, **Cortez D**, **Yazdi P**, **Neff N**, **Elledge SJ**, **Qin J**. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes & Development* 14: 927–939, 2000.

212. **Wilson CA**, **Payton MN**, **Elliott GS**, **Buaas FW**, **Cajulis EE**, **Grosshans D**, **Ramos L**, **Reese DM**, **Slamon DJ**, **Calzone FJ**. Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b. *Oncogene* 14: 1–16, 1997.

213. **Winder WW**, **Hardie DG**. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am. J. Physiol.* 270: E299–304, 1996.

214. **Winer J**, **Jung CK**, **Shackel I**, **Williams PM**. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal. Biochem.* 270: 41–49, 1999.

215. **Witte MM**, **Resuehr D**, **Chandler AR**, **Mehle AK**, **Overton JM**. Female mice and rats exhibit species-specific metabolic and behavioral responses to ovariectomy. *General and Comparative Endocrinology* 166: 520–528, 2010.

216. **Wohlers LM**, **Jackson KC**, **Spangenburg EE**. Lipolytic signaling in response to acute exercise is altered in female mice following ovariectomy. *J. Cell. Biochem.* 112: 3675–3684, 2011.

217. **Wohlers LM**, **Powers BL**, **Chin ER**, **Spangenburg EE**. Using a novel coculture model to dissect the role of intramuscular lipid load on skeletal muscle insulin responsiveness under reduced estrogen conditions. *AJP:*

Endocrinology and Metabolism 304: E1199–E1212, 2013.

218. **Wohlers LM**, **Spangenburg EE**. 17beta-estradiol supplementation attenuates ovariectomy-induced increases in ATGL signaling and reduced perilipin expression in visceral adipose tissue. *J. Cell. Biochem.* 110: 420–427, 2010.

219. **Wohlers LM**, **Sweeney SM**, **Ward CW**, **Lovering RM**, **Spangenburg EE**. Changes in contraction-induced phosphorylation of AMP-activated protein kinase and mitogen-activated protein kinases in skeletal muscle after ovariectomy. *J. Cell. Biochem.* 107: 171–178, 2009.

220. **Woldegiorgis G**, **Dai J**, **Arvidson D**. Structure-Function Studies with the Mitochondrial Carnitine Palmitoyltransferases I and II. *Monatsh. Chem.* 136: 1325–1340, 2005.

221. **Wolf AM**, **Colditz GA**. Current estimates of the economic cost of obesity in the United States. *Obes. Res.* 6: 97–106, 1998.

222. **Wolf AM**. What is the economic case for treating obesity? *Obes. Res.* 6 Suppl 1: 2S–7S, 1998.

223. **Wolfe RR**, **Jahoor F**. Recovery of labeled CO2 during the infusion of C-1- vs C-2-labeled acetate: implications for tracer studies of substrate oxidation. *Am. J. Clin. Nutr.* 51: 248–252, 1990.

224. **Writing Group for the Women's Health Initiative Investigators**. Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women: Principal Results From the Women's Health Initiative Randomized Controlled Trial. *JAMA: The Journal of the American Medical Association* 288: 321–333, 2002.

225. **Wu JM**, **Wechter ME**, **Geller EJ**, **Nguyen TV**, **Visco AG**. Hysterectomy rates in the United States, 2003. *Obstet Gynecol* 110: 1091–1095, 2007.

226. **Xiang T**, **Ohashi A**, **Huang Y**, **Pandita TK**, **Ludwig T**, **Powell SN**, **Yang Q**. Negative Regulation of AKT Activation by BRCA1. *Cancer Research* 68: 10040–10044, 2008.

227. **Xu S**, **Pratt SJP**, **Spangenburg EE**, **Lovering RM**. Early metabolic changes measured by 1H MRS in healthy and dystrophic muscle after injury. *Journal of Applied Physiology* 113: 808–816, 2012.

228. **Yuzefovych LV**, **Musiyenko SI**, **Wilson GL**, **Rachek LI**. Mitochondrial DNA damage and dysfunction, and oxidative stress are associated with endoplasmic reticulum stress, protein degradation and apoptosis in high

fat diet-induced insulin resistance mice. *PLoS ONE* 8: e54059, 2013.

229. **Zderic TW**. High-fat diet elevates resting intramuscular triglyceride concentration and whole body lipolysis during exercise. *AJP: Endocrinology and Metabolism* 286: 217E–225, 2003.

230. **Zhai P**, **Eurell TE**, **Cooke PS**, **Lubahn DB**, **Gross DR**. Myocardial ischemia-reperfusion injury in estrogen receptor-alpha knockout and wild-type mice. *Am. J. Physiol. Heart Circ. Physiol.* 278: H1640–7, 2000.

231. **Zhai P**, **Eurell TE**, **Cotthaus R**, **Jeffery EH**, **Bahr JM**, **Gross DR**. Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats. *Am. J. Physiol. Heart Circ. Physiol.* 279: H2766–75, 2000.

232. **Zhai P**, **Eurell TE**, **Cotthaus RP**, **Jeffery EH**, **Bahr JM**, **Gross DR**. Effects of dietary phytoestrogen on global myocardial ischemia-reperfusion injury in isolated female rat hearts. *Am. J. Physiol. Heart Circ. Physiol.* 281: H1223–32, 2001.i thi