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**Protocol Title:** Role of Fatty Acid Oxidation Defects in Insulin Sensitivity

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**OREGON CLINICAL AND TRANSLATIONAL RESEARCH INSTITUTE**
**Oregon Health & Science University**
# RESEARCH PROTOCOL

**Protocol Title:** Role of Fatty Acid Oxidation Defects in Insulin Sensitivity

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## I. BACKGROUND AND SPECIFIC AIMS

The overall goal of this proposal is to investigate the effects of disordered mitochondrial fatty acid oxidation (FAO) on insulin resistance in humans. Mitochondrial dysfunction has been implicated in the development of insulin resistance and type 2 diabetes during excess dietary fat intake and from increased release of endogenous free fatty acids (FFA's), such as occurs in obesity.(49, 70, 75, 88) Controversy exists, however, as to whether this insulin resistance results from intrinsic defects in mitochondrial energy utilization or from abnormalities resulting from excess FFA flux, as well as the role that subsequent accumulation of cellular metabolic intermediates play in impaired insulin signaling.

To address these controversies, we will study a unique population of patients with inherited defects in one of four mitochondrial enzymes in the FAO pathway: 1) carnitine palmitoyltransferase 2 (CPT2); 2) very long-chain acyl-CoA dehydrogenase (VLCAD); 3) trifunctional protein (TFP, which includes long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD)); and 4) medium-chain acyl-CoA dehydrogenase (MCAD). These proteins are required for the oxidation of sequentially shorter fatty acids (long-chain and medium-chain), respectively. We will use these patients to formally test the hypothesis that *intrinsic defects in mitochondrial function involving oxidation of long-chain, <u>but not medium-chain</u>, fatty acids are sufficient to <u>prevent</u> intralipid-induced insulin resistance.*

In a randomized, crossover study design, patients and a healthy control group will receive infusions of either intralipids or a control solution during hyperinsulinemic-euglycemic clamps. Glucose appearance (primarily due to hepatic glucose production) and glucose disposal (primarily by muscle tissue) will be measured, as will intraheptatic and intramyocellular lipid content (ectopic fat) by magnetic resonance spectroscopy, metabolite accumulation in muscle and fat using advanced metabolomics methodology, and insulin signaling pathway component expression and activation in the same biopsy samples.

In the first studies to test the relationships between impaired FAO, accumulation of intracellular lipid and lipid oxidation intermediates, and insulin resistance in humans, we propose the following specific aims:

**A. Specific Aim 1**: **To measure the effect of intralipid infusions on ectopic fat and systemic glucose metabolism in FAO patients and matched controls.** We will measure ectopic lipid deposition in muscle and liver by $^{1}$H magnetic resonance spectroscopy (MRS) as well as glucose appearance (Ra) in the fasting state and insulin-mediated glucose disposal (Rd) during hyerinsulinemic-euglycemic clamps and co-infusion of either intralipids or saline/glycerol in patients with a FAO disorder and matched controls.

**Hypotheses:** During saline/glycerol (control) infusions, patients with CPT2, VLCAD, and LCHAD/TFP deficiency will have similar ectopic lipid stores, and similar (or better) Rd and lower fasting Ra compared to control subjects and patients with MCAD deficiency. In contrast, during the intralipid infusion ectopic lipids will increase in the FAO patients and controls alike, but insulin sensitivity will worsen (decreased Rd) in controls and patients with MCAD deficiency, but not in those with CPT2, VLCAD, or LCHAD/TFP deficiency.

**B. Specific Aim 2**: **To determine the effect of distinct FAO disorders on tissue-specific lipid metabolism and insulin resistance compared to matched controls.** We will measure blood, skeletal muscle, and adipose levels of lipids, intermediates of FAO (including organic acids) and amino acids by metabolomics profiling, and adipose and muscle insulin signaling in FAO patients and matched controls participating in aim 1.

**Hypotheses:** In parallel with our hypothesized outcomes in Aim 1, we expect that CPT2, VLCAD, and LCHAD/TFP-deficient patients, despite intracellular accumulation of lipids and long-chain FAO intermediates in muscle and adipose tissue, will be "protected" from intralipid-induced insulin resistance by exhibiting greater insulin signaling pathway activation and glucose and FFA uptake than tissue from controls. On the other hand, accompanying the accumulation of medium chain FAO intermediates, subjects with MCAD deficiency will have reduced fat and muscle insulin action (similar to controls).

## C. SIGNIFICANCE

Type 2 diabetes (T2DM) affects over 18 million Americans, but the pathogenesis of insulin resistance underlying T2DM remains incompletely understood,(14, 19, 47, 48, 93, 101) and novel therapeutics are still needed. The proposed studies in this application will advance our understanding of the cellular basis for insulin resistance and potentially resolve a major controversy in this field: i.e., whether impaired insulin signaling results from defective mitochondrial function and subsequent accumulation of cytosolic lipid and byproducts of FAO (for review, see ref (62) and below) or from increased flux through the FAO cycle and subsequent generation of metabolic intermediates (for review, see ref (69) and below). In addition to providing a unique opportunity to better understand the interface between FFA metabolism and the expression of insulin resistance in humans, the results of these studies will also help inform the on-going development of potential pharmaceutical targets for the treatment of insulin resistance and T2DM (for review see refs (86, 104)).

## D. INNOVATION

Previous investigators have studied the interactions of lipid and glucose metabolism in patients with existing insulin resistance or a familial predisposition for diabetes. While these studies have yielded important new insights into the importance of mitochondrial dysfunction in insulin resistance, we will recruit and study a novel group of patients with inherited FAO defects. By taking advantage of our current clinical trials program in patients with FAO disorders, autosomal-recessive genetic disorders that severely limit specific enzymatic steps in the FAO cascade, these studies will allow us to pinpoint a specific level of mitochondrial FAO dysfunction that results in either induction of, or protection from, insulin resistance under conditions of high-fat exposure characteristic of developed societies consuming Western diets and exhibiting high rates of obesity. These studies will, therefore, have relevance well beyond the rare patient with a FAO disorder and indeed, by using advanced metabolomics and muscle and adipose tissue analyses to assess tissue-specific metabolite signatures associated with cellular lipid accumulation, impaired insulin signaling, and FAO products, will allow for identification of potentially novel mechanistic candidates of insulin resistance that would not otherwise be possible.

## E. APPROACH

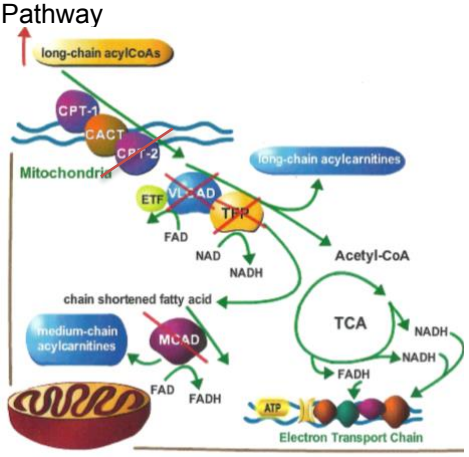
### 1. Specific Aim 1: To measure the effect of intralipid infusions on ectopic fat and systemic glucose metabolism in FAO patients and matched controls.

**Overview and Rationale:**

*Normal Mitochondrial Function and FAO Disorders* (**Figure 1**)

Long-chain fatty acids are transported into mitochondria via the carnitine palmitoyltransferase (CPT) system including CPT-1, carnitine acylcarnitine translocase (CACT) and CPT-2. Long-chain ß-oxidation occurs in a repeating, four-enzyme cycle catalyzed by very-long-chain acyl-CoA dehydrogenase (VLCAD) and trifunctional protein (TFP), resulting in sequential removal of 2-carbon units that become acetyl-CoA and generating both shorter chain-length fatty acids and reducing equivalents (NADH), and releasing electrons directly into the ubiquinone pool via the electron flavin transfer protein (ETF).

**Figure 1:** Fatty Acid Oxidation Pathway




Long and medium chain FAO provides acetyl-coA and reducing equivalents for the TCA and the electron transport chain.

Medium-chain fatty acids are oxidized by a separate set of ß-oxidation enzymes, the first of which is medium-chain acyl-CoA dehydrogenase (MCAD), generating acetyl-CoA, a shorter chain-length fatty acid, and more reducing equivalents ($FADH_2$ and NADH). Acetyl-CoA then enters the tricarboxylic acid (TCA) cycle, where it is further oxidized to $CO_2$ and reducing equivalents (NADH, $FADH_2$), which enter the electron transport chain to generate ATP.

In humans, CPT2, VLCAD, LCHAD/TFP and MCAD deficiency are inherited in an autosomal recessive manner where each parent carries one mutant and one normal allele. All affected patients carry two

mutations in the gene which leads to partial loss of function of these enzymes (see red "X"'s, **Figure 1**) (42, 44, 99) and the accumulation of long-chain acyl-CoA, long-chain hydroxyacyl-CoA, or medium-chain acyl-CoA moieties, respectively, in the mitochondria.(64, 85) These fatty acids exit the mitochondria via the reversal of the CPT system and appear in patient's plasma as long-chain or medium-chain acylcarnitines and their FFAs. As a result of these defects, during periods of fasting and exercise, when demand for FAO increases, circulating and tissue levels of acylcarnitines increase.(31, 32, 80) Infants and children with CPT2, VLCAD, LCHAD/TFP and MCAD deficiency present with fasting or illness induced hypoketotic hypoglycemia, and potentially, sudden infant death, but VLCAD and LCHAD/TFP can also be complicated by cardiomyopathy. Adult patients with CPT2, VLCAD and LCHAD/TFP have recurrent rhabdomoyolysis, especially after a prolonged fast or exercise that is not observed in adults with MCAD deficiency.(6, 15, 41) Early diagnosis and treatment leads to improved survival and normal development through prevention of catastrophic hypoglycemia in infancy and later-onset cardiomyopathy. Under conditions of adequate glucose supply, such as a low-fat, high carbohydrate diet, patients with CPT2, VLCAD, LCHAD/TFP and MCAD deficiencies are medically stable with no symptoms of metabolic decompensation.

*FAO and Insulin Sensitivity: Too Little or Too Much?*

Several lines of evidence have led to two current theories linking impaired mitochondrial FAO with the development of insulin resistance.(104) One theory suggests that defective mitochondrial function is associated with accumulation of cytosolic lipid by-products, such as diacylglycerols (DAGs), ceramides, and triglycerides in skeletal muscle and liver, referred to as ectopic fat.(5, 58, 67, 76) In turn, these cytosolic lipid intermediates have been shown to activate serine stress-kinases and signaling pathways that impair normal insulin signaling and are the basis of the "lipotoxicity" hypothesis for insulin resistance.(67, 77) Evidence in support of this theory comes from studies that show that ectopic fat in muscle and liver are closely associated with insulin resistance(33, 45, 54, 58, 74) and that lipid infusions lead to accumulation of intramyocellular lipid (IMCL)(4, 9, 11) and insulin resistance mediated, in part, through activation of protein kinase C Θ (PKC-Θ) and subsequent impairment of glucose transport.(34, 50, 51, 81) In this model, insulin sensitivity would be improved by increasing FAO and reducing ectopic fat, as supported by animal and clinical studies involving drug and genetic manipulations.(1, 12, 13, 43, 77, 102)

An alternative theory, first proposed by Randle,(79) posits that increased flux through the FAO cycle induces insulin resistance.(69) In this model, "metabolic overload" of the mitochondria leads to a mismatch between FAO and TCA oxidation that leads to cellular accumulation of intermediates of FAO and subsequent impaired insulin signaling.(69) (57, 68) This is supported by studies showing that during high-fat feeding, increased production of partially oxidized substrates, such as intermediate metabolites from FAO in the form of fatty acylcarnitines and increased reactive oxygen species (ROS), have been associated with insulin resistance.(2, 40, 57, 59, 65, 66, 84) This model predicts insulin sensitivity will be improved by reducing FAO, thereby alleviating the "metabolic stress" that accompanies increased FFA flux. Indeed, animal studies have shown that blocking FFA entry into mitochondria can alleviate high-fat-diet-induced insulin resistance.(56, 57).

*Medium-chain Acylcarnitines in Expression of Insulin Resistance during Fat Overload*

Plasma elevations in both long- and medium-chain acylcarnitines have been observed in animal and human studies of obesity (2, 52, 57, 66). However, medium-chain acylcarnitine species are reported to be specifically elevated in T2DM and diet-induced insulin resistant animal models,(2, 52, 66) which suggests that long-chain acylcarnitines may be a non-specific marker for obesity and that accumulation of medium-chain acylcarnitines may be more important for expression of insulin resistance. This includes a recent study in which levels of medium-chain acylcarnitines, but not long-chain acylcarnitines or ceramides, were elevated in muscle tissue and inversely correlated (in plasma) with insulin sensitivity in lean men and women fed a high-fat diet.(52) In addition, anecdotal reports of diabetes in patients with MCAD deficiency, but not those with CPT2, VLCAD or LCHAD/TFP deficiencies, have appeared in support groups of FAO patients (personal communication, Deb Gould, Director Parent Support Group for FOD, http://www.fodsupport.org). Although these data and reports do not distinguish whether medium-chain acylcarnitines are causative of, or simply a marker for, insulin resistance and diabetes, in-vitro evidence has linked medium-chain acylcarnitines with insulin resistance in primary skeletal myocytes(55) and with NF-κB activation, thought to be a key mediator of inflammation induced insulin resistance, in primary cell

cultures.(2) In the studies planned here, we will test the role of defective MCAD and accumulation of upstream FOA metabolites in expression of high-fat induced insulin resistance.

**Table 1:** Glucose, insulin and estimates of insulin sensitivity in CPT2 and LCHAD-deficient subjects (n=12) compared to controls (n=12).

| | Subjects | Controls |
|---|---|---|
| Fasting glucose (mg/dl) | 97 ± 14 | 105 ± 19 |
| Fasting insulin (μU/ml) | 17± 8 | 13± 5 |
| HOMA-IR | 4.18 ± 2.39 | 3.50 ± 1.50 |
| Insulin sensitivity index | 2.80 ± 1.80 | 3.13 ± 1.57 |
| Insulinogenic index | 0.68 ± 0.29 | 0.59 ± 0.27 |

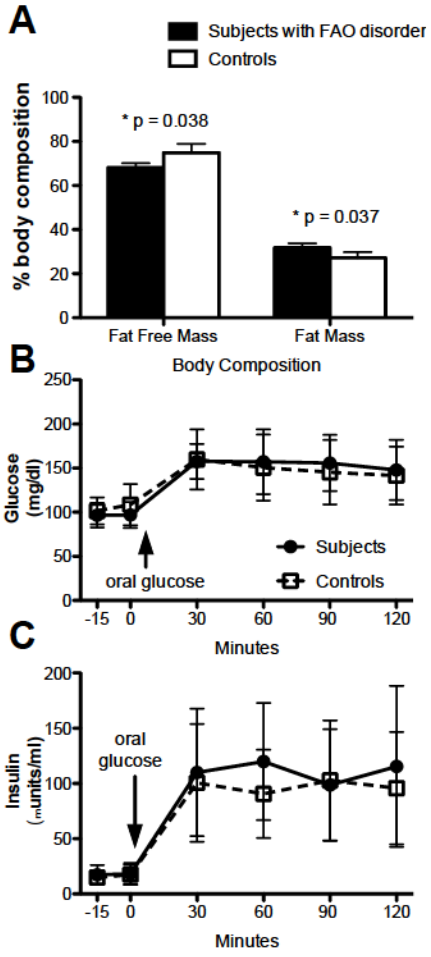
Data are mean ± standard deviation. HOMA-IR = homeostatic model assessment of insulin resistance.

**Preliminary Data:**

In exploratory studies examining the relationships between defective FAO and glucose metabolism, we measured energy expenditure and body composition and performed oral glucose tolerance tests (OGTTs) in 12 subjects with FAO disorders and compared them to 12 age-, sex-, and BMI-matched controls (**Table 1**).(30) Two subjects had CPT-2 and 10 subjects had TFP/LCHAD deficiency. BMIs ranged from 15.3 kg/m$^2$ (underweight) to 29.9 kg/m$^2$ (overweight), and none met criteria for obesity. It should be noted that these FAO subjects and controls were studied during low-fat diets and do not represent true "metabolic stress" conditions that we propose for our studies in this grant application.

*Long-chain FAO (LCFAO)-deficient subjects exhibited higher % fat mass and extramyocellular lipid content (EMCL) in soleus muscle.* Percent fat mass was greater and % fat-free mass lower in LCFAO subjects than controls (**Figure 2A**).**(30)** Intrahepatic lipid (IHL) and IMCL content were not different between FAO patients and controls (**Figure 3A and B**).**(30)** However, subjects with a FAO disorder did have greater EMCL deposition compared to controls (**Figure 3A and B**), which we suspect parallels their higher % body fat (**Figure 2**), which, in turn, likely reflects their reduced capacity to oxidize fat and their more sedentary lifestyle.

**Figure 2**: Body composition and plasma glucose and insulin levels during an OGTT.




*Responses to oral glucose loads were identical in LCFAO-deficient subjects and matched controls.* During a standard OGTT, neither fasting nor post-glucose load concentrations of glucose or insulin were different between FAO-deficient subjects and controls (**Figure 2B and C**). Estimates of insulin sensitivity were also similar between groups (**Table 1**). While our finding that, despite markedly lower FAO and greater body fat compared to controls, patients with CPT-2, VLCAD and TFP deficiency have normal glucose tolerance (and estimated insulin sensitivity and secretion) is supportive of the theory that reducing FAO cycle flux is protective against insulin resistance, these patients did not include subjects with MCAD deficiency, were not truly "metabolically stressed" by virtue of being on a low-fat diet (which could explain similar IMCL levels to controls), and did not undergo a direct measure of insulin sensitivity. Therefore, this aim will address these limitations in our pilot data by performing hyperinsulinemic-euglycemic clamps (HECs) and co-infusions of intralipids (or a control solution) and by including subjects with MCAD defects.

*VLCAD-deficient mice are protected from insulin resistance on a high-fat diet.* VLCAD/- mice with a targeted deletion of exons 7-19 have a phenotype similar to humans with long-chain FAO disorders and have previously been reported to be protected from high-fat-diet-induced insulin resistance.(21, 107) We, too, have shown this when we measured insulin sensitivity by HEC in male VLCAD-/- mice (n=6) compared

to wild-type mice (n=4) fed a high-fat diet for 2 months. The wild-type mice required very little exogenous glucose to maintain euglycemia during the clamp, suggesting insulin resistance, while VLCAD-/- mice required greater glucose infusion rates to maintain euglycemia, indicating better insulin sensitivity (**Figure 4A**). Glucose production (Ra) was lower and glucose utilization (Rd) higher in VLCAD-/- mice compared to wild-type mice (**Figure 4B, and C**). This data and previous reports of VLCAD-/- mice suggest they are protected from high-fat-diet-induced insulin resistance. The studies we propose here in humans will determine if FAO-deficient subjects are similarly protected from intralipid infusion-induced insulin resistance.

**Figure 3**: Lipid deposition in long-chain FAO-deficient subjects and controls.

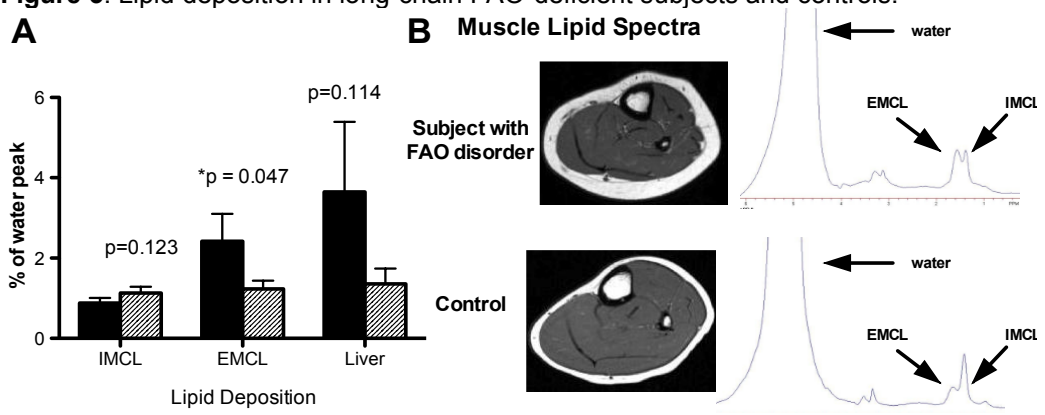
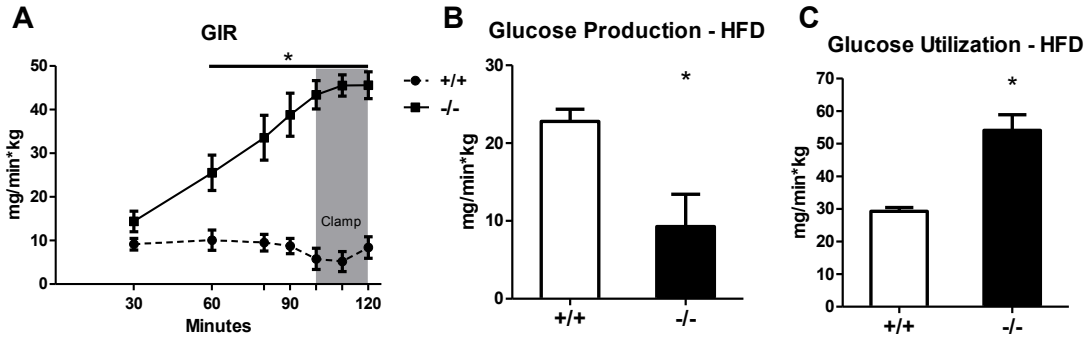



**Figure 4**: Hyperinsulinemic-euglycemic clamps in VLCAD-/- and VLCAD+/+ mice.

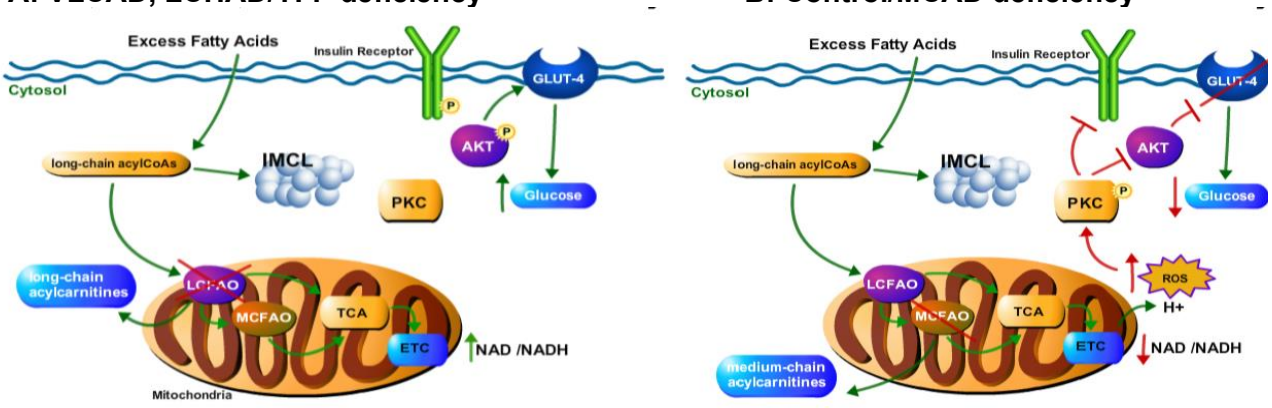



VLCAD-/- mice fed a high-fat diet for 2 months required greater glucose infusion rates (GIR) to maintain euglycemia and had lower glucose production with increased glucose utilization compared to wild-type mice during a hyperinsulinemic-euglycemic clamp. * p≤0.05

**Anticipated Results:**

We anticipate that intralipid

**Figure 5**: Model of protection from FFA-induced insulin resistance by long-chain, but not medium-chain, FAO disorder.




**A**. During fatty acid excess, long-chain acyl-CoAs and IMCL will accumulate, but decreased FAO will prevent an increase of redox equivalents and ROS generation resulting in normal insulin signaling and glucose uptake. **B**. With increased FAO upstream from MCAD, IMCL will still accumulate, but excess redox equivalents will lead to an oxidized mitochondrial environment with ROS generation, activation of serine kinases, inhibition of insulin signaling and decreased glucose uptake. IMCL=intramyocellular lipid. LCFAO = long-chain fatty acid oxidation. MCFAO = medium chain fatty acid oxidation. TCA = tricarboxylic acid cycle. ETC = electron transport chain. ROS= reactive oxygen species. PKC = protein kinase C.

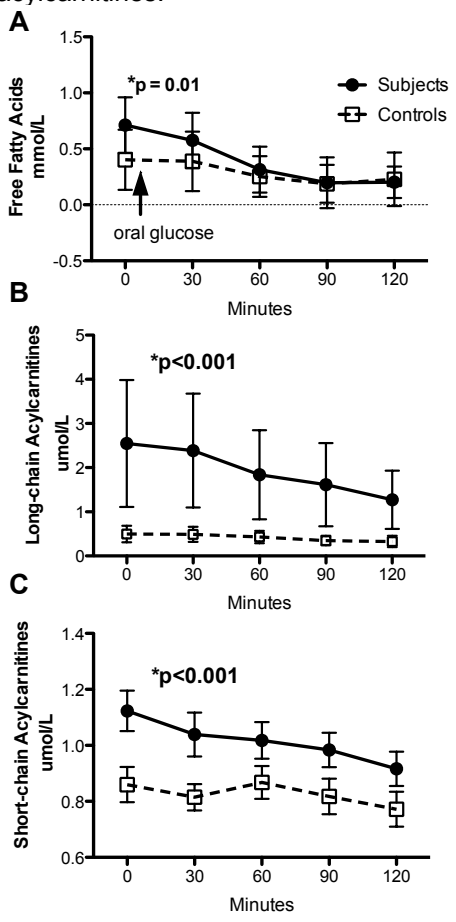
infusion will increase IMCL in FAO patients and controls alike (**Figure 5A and B**). After the intralipid infusion, respiratory quotients will reflect FAO capacity and will be highest (indicating greater carbohydrate oxidation) in subjects with CPT2, VLCAD or LCHAD/TFP deficiency and lower (indicating greater fat oxidation) in MCAD-deficient subjects and controls. Based on our preliminary data of normal OGTTs in patients with inherited defects in the FAO defects, our findings of protection from high-fat-diet-induced insulin resistance in VLCAD -/- mice, as well as studies that have shown that blocking FFA entry into mitochondria can alleviate high-fat-diet-induced insulin resistance in animal models,(56, 57) we anticipate that subjects with defects in CPT2, VLCAD, and LCHAD/TFP enzymes will be protected from high-fat-diet-induced insulin resistance, essentially "blocking" long-chain FA oxidation (flux) at a level that avoids accumulation of metabolic intermediates that adversely affect insulin signaling (**Figure 5A**). On the other hand, we anticipate that increased oxidative flux of fatty acids from long-chain fatty acids to medium chain metabolites will not provide the same protection from excess fatty acid substrate, impairing insulin signaling in patients with MCAD deficiency (**Figure 5B**) who will exhibit insulin resistance (compared to control infusion days) in muscle (reduced glucose disappearance, reduced insulin signaling ex-vivo), and adipose tissue (reduced suppression of FFA during the clamp) similar to controls. We predict that the degree of FFA-induced insulin resistance in these subjects will be similar to control subjects (**Figure 5B**).

## 2. Specific Aim 2: To determine the effect of distinct FAO disorders on tissue-specific lipid metabolism and insulin resistance compared to matched controls.

**Overview and Rationale:**

Skeletal muscle and adipose tissue account for most of whole-body glucose disposal in humans. While skeletal muscle is estimated to be responsible for up to 70% of total glucose uptake,(23) adipose tissue also contributes to whole-body glucose metabolism through Glut-4 glucose transporter translocation and induction of carbohydrate responsive-element-binding protein (ChREBP)-$\alpha$ and $\beta$ isoforms.(26, 37) Adipose tissue also acts as a sink for dietary FFAs and is the source of lipolysis-mediated substrates for the FAO pathway in muscle. In the resting state, skeletal muscle FAO can account for up to 90% of the total energy utilization.(22, 51) Both glucose and FFA uptake in muscle and adipose tissue are under the positive control of insulin through insulin-stimulated translocation of Glut-4(100) and the FATP-1 fatty acid transporter.(61, 91) These actions of insulin are mediated through tyrosine autophosphorylation of the insulin receptor and phosphorylation of insulin receptor substrates (IRS-1/2), followed by activation of phosphoinositol 3-kinase (PI3-K) and the subsequent activation of the downstream protein kinase B/Akt(91, 106) (**Figure 5**). Defects in insulin signaling in skeletal muscle are associated with the development of systemic insulin resistance. Specifically, acute lipid infusion in mice results in intramuscular accumulation of fat metabolites, including fatty acid acyl-CoA and DAG, and increased activity of PKC-Θ.(103) PKC-Θ activation is associated with the reduced tyrosine phosphorylation of IRS-1 and reduced skeletal muscle glucose uptake.(53) The effects of a lipid infusion on adipose insulin sensitivity are largely unknown, although exposure to a high-fat diet or elevated FFA's of obesity are associated with the development of low-grade inflammation and adipose tissue microphage activation.(3, 16, 78) Determination of insulin sensitivity and resistance in adipose and muscle can, therefore, be directly assessed through functional assays of insulin-

**Figure 6**: Plasma FFAs and acylcarnitines.



stimulated glucose uptake in muscle and fat biopsies and FFA uptake in fat biopsies, as well as analysis of insulin receptor, Akt, and (in muscle) PKC-Θ phosphorylation.

**Preliminary Data:**
*Plasma FFAs and long- and short-chain acylcarnitines were higher, medium-chain acylcarnitines were not different, and adiponectin levels were lower among patients with a LCFAO disorder.* FAO-deficient patients had higher fasting FFA levels compared to controls(30) (**Figure 6A**), which decreased after an oral glucose load similarly to controls, suggesting no impairment of post-prandial, insulin-mediated FFA release from adipose tissue. The sum of the long-chain acylcarnitines (LC AC) (C12:1, C12:0, C14:2, C14:1, C14:0, C16:1, C16:0, C18:2, C18:1, C18:0) were significantly increased both before and after oral glucose load in the patients with FAO deficiency, as expected (**Figure 6B**). Levels of short-chain acylcarnitines (SC AC) (C3:0, C4:0, C5:1, C5:0, C4-OH and C5-OH) were also higher before and after the oral glucose load, primarily due to an increase in proprionylcarnitine and C5-OH carnitine in the FAO-deficient patients, that was most pronounced during fasting (**Figure 6C**). These species are derived from branched-chain amino acid (BCAA) oxidation, potentially suggesting increased protein oxidation during fasting. We measured fasting plasma amino acid profiles and found there was no difference in fasting plasma branched-chain or aromatic amino acids between subjects and controls.(30) However, medium-chain acylcarnitine (MC AC) levels were not different between the FAO patients and controls(30) and fasting plasma high-molecular-weight adiponectin levels were approximately 50% lower among FAO-deficient subjects compared to controls.(30) In summary, despite elevated fat mass, fasting FFA, and long-chain acylcarnitines, and lower adiponectin levels, under conditions of chronic low-fat diet feeding, FAO-deficient subjects had normal glucose tolerance compared to healthy controls. That long-chain, but not medium-chain, acylcarnitine

levels, were elevated comparable to controls is compatible with our model that elevations in FAO products upstream from MCAD are not sufficient to induce insulin resistance. Whether this is true during conditions of high-fat metabolic stress, and whether MCAD deficiency and accumulation of metabolic intermediates resulting from this defect are associated with insulin resistance will be addressed in the studies proposed in this application.

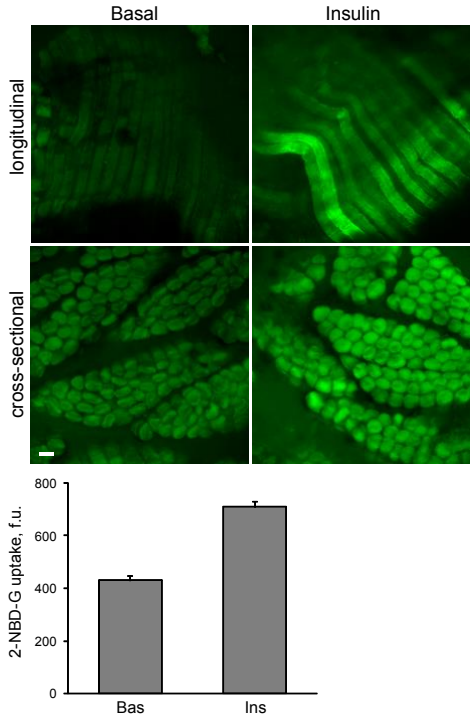
*Insulin signaling cascade and FFA and glucose uptake in muscle and white adipose tissue (WAT).* Glucose and FFA are the two major substrates used for the synthesis of triglycerides in WAT. We have developed a fluorescent-based approach for simultaneous measurement of glucose and FFA uptake using green fluorescent 2-NBD-glucose and red fluorescent BODIPY-C12(97) (**Figure 7**). This approach will allow determination of insulin sensitivity and lipid and glucose accumulation of primary WAT explants. In addition, small soleus muscle biopsies will be analyzed for insulin sensitivity using 2-NBD-glucose (**Figure 8**).

**Anticipated results:**

Mirroring increases in ectopic fat we expect to see in Aim 1, we anticipate that intralipid infusion, but not infusion of the control solution, will increase plasma FFA's associated with increased muscle and adipose triglycerides and DAG's in all FAO patients and controls (**Figure 5**). Plasma and tissue long-chain acylcarnitines will be greatly elevated in CPT2, VLCAD or LCHAD/TFP-deficient subjects; medium-chain acylcarnitines will be greatly elevated among subjects with MCAD deficiency; and both long-chain and medium-chain acylcarnitines will be only modestly elevated in normal controls. Based on our preliminary data, we expect that short-chain acylcarnitines may be elevated in subjects with CPT2, VLCAD, LCHAD/TFP, and MCAD deficiency compared to controls.

We predict that these cellular changes will be associated with a decrease in phospho-(p)IR and pAKT and an increase in pPKC-Θ in subjects with MCAD deficiency and control subjects, but not in CPT2, VLCAD, and LCHAD/TFP-deficient subjects, paralleling the expected outcomes of system insulin sensitivity measurements obtained by the HEC's in Aim 1. These expected decreases in insulin signaling pathway component expression and activation will also be reflected by decreased glucose uptake into muscle and adipose tissue and decreased FFA uptake into adipose in controls and subjects with MCAD deficiency. Decreased glucose uptake will be correlated with medium-chain acylcarnitines, as has been reported in human studies(52) and in cultured myocytes.(52) We anticipate no change in glucose and FFA uptake in muscle or adipose tissue from the glycerol/saline to the intralipid co-infusion samples in CPT2, VLCAD and LCHAD/TFP-deficient subjects.

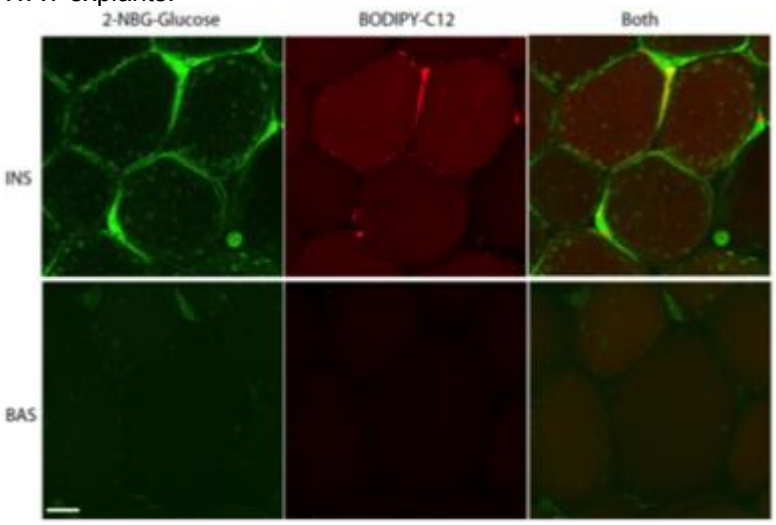
**Figure 8**: Fluorescence analysis of glucose uptake in monkey skeletal muscle.




Skeletal muscle (vastus lateralis) explants obtained at necropsy from a female rhesus macaque were treated with 10 nM insulin at 37$^0$C for 30 min., followed by 0.05 mM 2-NBD-glucose for an additional 30 min., and analyzed by confocal microscopy. A) Upper panels are images of longitudinal muscle fibers and lower panels are cross-sectional images of muscle fiber bundles. Bars are means ± SEM of 36 individual muscle fibers. Scale bar=50 μm; f.u.=fluorescent units. 2-NBD-glucose incorporation into individual fibers was quantified using image J. Mean fluorescence per fiber was determined using a randomly positioned circular region of interest of cross-sectional images.

Metabolites will be measured by metabolomics, including the acylcarnitines, amino acids, organic acids and ketone bodies (**Figure 5**. Based on previous observations, we anticipate controls will have a decrease in plasma BCAAs,(72) but we anticipate normal amino acid profiles among subjects with a FAO disorder.(30) Tissue long-chain ayclcarnitines will be much higher and ketones such as acetoacetate will be lower in CPT2, VLCAD and LCHAD/TFP-deficient subjects compared to controls due to the decreased long-chain FAO. Subjects with MCAD deficiency will have higher medium-chain acylcarnitines compared to subjects with long-chain FAO disorders and ketone concentrations similar to controls.

**Figure 7:** Concurrent measurement of FFA and glucose uptake in WAT explants.




Subcutaneous biopsy-derived WAT explants from a female rhesus macaque were pretreated for 1 hour with basal or 10 nM insulin-containing media. Explants were co-labeled with 2-NBD-glucose and BODIPY-C12, imaged using confocal microscopy, and processed as described. Green patches enriched in 2-NBD-glucose represent adipocyte cytoplasm. BODIPY-C12 accumulates in large and small lipid droplets (red). Scale =10 μm.

## II. Study Population

**Subjects with a fatty acid oxidation disorder:**

Inclusion Criteria: 32 subjects with a confirmed diagnosis of VLCAD (n=8), CPT2 (n=8) LCHAD/TFP (n=8), or MCAD (n=8) deficiency will be recruited for this study. Subjects must be ≥ 18 years of age, and be willing to complete 2 CRC admissions for hyperinsulinemic euglycemic clamp studies and muscle and adipose tissue biopsies. Subjects with disorders in FAO will be recruited to participate in this study through announcements on the FAO family support network website, through OHSU's clinical population, and through letters sent to metabolic specialists across the US. Inclusion and exclusion criteria are in **Table 2**.

Confirming the Diagnosis: Each subject must have a confirmed diagnosis of carnitine palmitoyltransferase 2 (CPT2), very long-chain acylCoA dehydrogenase (VLCAD), long-chain 3-hydroxyacyl CoA dehydrogenase (LCHAD), trifunctional protein (TFP), or medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Diagnosis of a disorder in fatty acid oxidation will be confirmed by obtaining medical record results of acylcarnitine profiles, fatty acid oxidation probe studies in cultured fibroblasts and/or mutation analysis (44, 71). Establishing the diagnosis of these disorders is a complex process that varies across metabolic centers in the US. Not all potential subjects will have had a skin biopsy and FAO probe studies in cultured fibroblasts. In many cases mutation analysis can identify only one recognized mutation in the sequenced exons of the gene. Thus, a combination of methods is most often used to establish the diagnosis. If two of the three diagnostic tests suggest a diagnosis of CPT2, VLCAD, LCHAD/TFP, or MCAD, the subject will be eligible to participate in the trial (Table 3).

| **Table 2:** Participant Selection Criteria | | | |
|---|---|---|---|
| **Subjects with an FAO disorder** | | **Matched controls** | |
| **Inclusion criteria** | **Exclusion criteria** | **Inclusion criteria** | **Exclusion criteria** |
| Confirmed CPT2, VLCAD, LCHAD, TFP or MCAD | Hgb <10g/dl<br>INR>1.20<br>PTT>36 seconds<br>Platelets<150 K/mm$^3$ | Same gender and similar age/BMI as subject with FAO disorder | Hgb <10g/dl<br>INR>1.20<br>PTT>36 seconds<br>Platelets<150 K/mm$^3$ |
| ≥18 years | Pregnant /lactating females | ≥18 years | Endocrine disorder such as diabetes, or untreated thyroid disease |
| Ability to travel to OHSU | Endocrine disorder such as diabetes, or untreated thyroid disease | Good health, exercise < 30 min, 3 x per week for 2 weeks prior to study visits. | Cardiovascular disease or elevated plasma lipids |
| Ability and willingness to complete protocol | Cardiovascular disease or elevated plasma lipids | Ability and willingness to complete protocol | Taking lipid lowering meds |
| | Regularly taking meds that strongly affect bleeding, bruising or platelets. | | Regularly taking meds that strongly affect bleeding, bruising or platelets |

Exclusion Criteria: Subjects with CPT2, VLCAD, LCHAD/TFP, or MCAD deficiency may not be actively participating in another research project that prohibits their participation in this study such as a project that requires a specific diet or supplement that must be consumed daily. All subjects will be screened for anemia prior to study participation. Female subjects of childbearing potential will be screened for pregnancy. Anemic (Hgb < 10 g/dl) or pregnant subjects will be excluded from the study. Subjects regularly taking medications that strongly affect bleeding, bruising, or platelets such as Coumadin, Plavix, Aggrenox, Ticlid, Agrylin, Xagrid, Aricept, Namenda, Exelon, Razadyne, or aspirin will be excluded from the study. Subjects may not have diabetes or be on medications to treat diabetes. We do not anticipate any subjects with CPT2, VLCAD, or LCHAD/TFP will have diabetes, as it has never been reported. Several cases of diabetes have been mentioned on disease specific list serves among subjects with MCAD deficiency.

Table 3: Medical Records to Confirm the Diagnosis

| Disorder | Acylcarnitine Profiles | Fatty acid oxidation Probe studies | Mutation analysis |
|---|---|---|---|
| VLCAD/CPT2 | ↑ C14:0 or C14:1 | ↓ FAO flux with ↑ disease specific acylcarnitines | 1 or 2 known mutations in specific gene |
| LCHAD/TFP | ↑ OH-acylcarnitines | | |
| MCAD | ↑ C8:0 or C10:0 | | |

**Matched Control Subjects:** Inclusion criteria: In addition, 32 age-, sex-, and BMI-matched controls will be recruited to participate in the study through local newspaper and OHSU web site announcements. Control subjects must be a similar age, sex and BMI (± 2 kg/m$^2$) as a study subject with a FAO disorder, be able to complete and comply with the protocol, and be in good health. Subjects must be weight stable (± 5 lbs) for the past 3 months and exercise < 30 minutes, three times a week (low activity) for two weeks prior to each study visit.

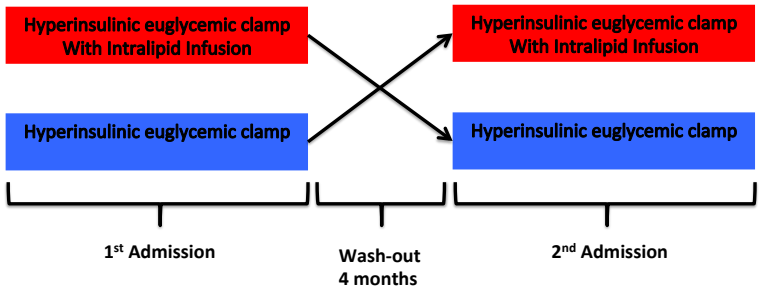
Prior to the starting the study in subjects with FAO disorders, individual study procedures such as the muscle and fat biopsies, the HEC and/or the MRS may be performed to practice the procedures and perfect them in up to 3 healthy volunteers. These control subjects may not specifically match data from FAO disorders. A separate consent form has been developed to consent these subjects.

Exclusion Criteria: Control subjects may not be participating in another study that alters their macronutrient intake such as low carbohydrate diet. All subjects will be screened for anemia prior to study participation. Female subjects of childbearing potential will be screened for pregnancy. Anemic (Hgb < 10 g/dl) or

pregnant subjects will be excluded from the study. Subjects regularly taking medications that strongly affect bleeding, bruising, or platelets such as Coumadin, Plavix, Aggrenox, Ticlid, Agrylin, Xagrid, Aricept, Namenda, Exelon, Razadyne or aspirin will be excluded from the study. Subjects may not have diabetes or be on medications to treat diabetes. Control subjects may not have a history of an endocrine disorder such as thyroid disease, renal disease or cardiovascular disease.

**Inclusion of Children, Women and Minorities***:* All participants will be adults able to provide informed consent. We believe the complex full-day testing schedule planned for Day 2 of each inpatient admission, including two biopsies, is best conducted in the adult population. Both genders will be included in this research project. FAO disorders appear to occur equally in males and females so we expect to include similar numbers of males and females. Women of child-bearing potential are included in this research. Pregnant woman will be excluded because the DEXA scan would increase risk to the fetus and pregnancy may affect insulin sensitivity. Recruitment should result in study demographics similar to that of the national disease demographics. Caucasians will comprise the majority of subjects in this study. To date, the majority of patients with fatty acid oxidation disorders have been of Caucasian ancestry. We will welcome subjects from other racial backgrounds but anticipate that more, if not all, subjects will be white.

**Figure 9:** Randomized cross-over study design

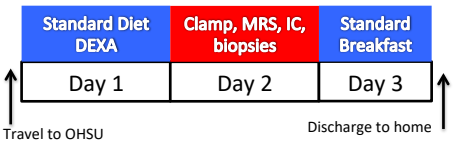



Participants will complete 2 inpatient admissions with a 4 month wash-out period in between. The order of the clamp procedures, with or without intralipid, will be randomly assigned.

## III. Study Design

This is a randomized cross-over study in which all participants will complete 2 inpatient admissions with a 4 month washout period in between visits (**Figure 9**). Participants will be consented prior to the first admission and medical records will be reviewed to document the participants meet the inclusion criteria. Most if not all of the subjects with an FAO disorder will be traveling from other parts of the US to participate. Travel arrangements will be made and the 1st inpatient visit scheduled once the consent is complete, and the participant meets all the inclusion criteria. The outline for each inpatient visit is provided in **figure 10**. Subjects living locally will be consented in person at an outpatient screening visit while the others will be consented by mail.

**Figure 10:** Inpatient visit schedule

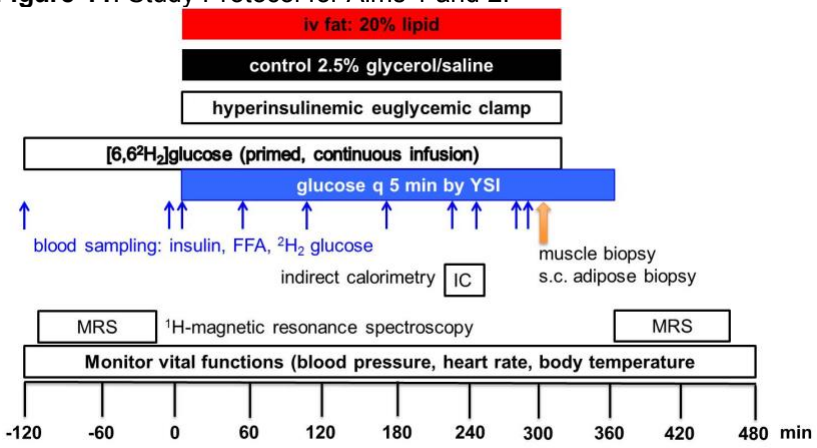



Subjects will travel to OHSU if necessary prior to day 1. Day 1 they will be fed a standardized diet and have a DEXA scan. On day 2, a hyperinsulinemic, euglycemic clamp with MRS, indirect calorimetry and tissue biopsies will be completed. The following day, subjects will consume a standard breakfast and then be discharged to home.

All subjects (those with an FAO disorder and controls) will be counseled to consume a standard diet consisting of 20% of total calories from fat, 20% from protein, and 60% from carbohydrates for the three days before the study by a registered dietitian. The dietitian will provide written and oral instructions to the participants on the phone. Subjects will keep a record of foods consumed during these 3 days (3-day diet record) prior to the inpatient admission.

Participation will include two admissions to the OHSU CTRC inpatient unit. Subjects will be admitted the day before the clamp for measurement of body composition by DEXA scans and to be fed the standardized diet (20% of total calories from fat, 20% from protein, and 60% from carbohydrates) prior to the clamp procedure (Day 1). The two intravenous lines needed for the clamp will be placed the evening before the procedure or in the morning as determined by nursing assessment.

**Figure 11**: Study Protocol for Aims 1 and 2.




Participants will start a dideuterated glucose infusion 2 hours prior to the clamp. Lipid deposition will be measured by $^{1}H$ spectroscopy. The insulin infusion with either co-infusion of glycerol/saline or intralipid will begin at time 0 and last for 5 hours. Glucose will be infused at a variable rate to maintain euglycemia. At the end of the clamp, a muscle and adipose biopsy will be collected and lipid deposition re-measured.

DEXA: Whole body and regional fat, lean, and bone mass will be measured by DEXA (Lunar iDXA, GE Healthcare Lunar, Madison, WI) through the OHSU Clinical and Translational Research Center (CTRC) Bionutrition Unit.

The following day, the clamp study will begin at approximately 6:00 AM and end at about 4:00 PM (Day 2; **Figure 11**). Indirect calorimetry to measure energy expenditure and respiratory quotient for estimation of fat oxidation will be performed in the last hour of the clamp. The order of the intralipid vs. glycerol/saline infusion during HEC will be randomly assigned. Subjects will rest overnight and consume a standardized breakfast the following day (Day 3) and be discharged to home. Subjects will return 4 months later and repeat the admission with the alternative clamp procedure. If accurate clamp results are not achieved at Visit 1 or 2, the subject may be asked to return for another study visit to repeat the clamp procedure, DEXA, indirect calorimetry and/or biopsies. During periods of hospital recommendations for decreased in-person contact, study visits will be postponed until study staff are notified of return to normal research visit operations.

$^{1}H$ and $^{13}C$ MRS Procedure: of the liver and muscle: will be performed as previously described(18, 30) prior to (-100 min) and following both clamp procedures. Each subject will complete 4 MRS measurements of liver and muscle lipid content (**Figure 11**). Image-guided, $^{1}H$ localized, MRS following high-resolution T-weighted spin-echo imaging will be performed on a Siemens 3T whole-body system. Radiofrequency surface coils will be used for region-of-interest (ROI) voxel localization and spectroscopy data acquisition. Depending on the schedule for the MRS instruments and to allow for more time for a meal before the testing, the MRS measurements may not be completed until approximately 5:30 PM.

MRI of the abdomen to quantify visceral and subcutaneous fat: Although not a primary outcome, these measures will be acquired using a Siemens 3T system as previously described (18, 30).

HEC with co-infusion of [6,6-$^{2}H_2$] glucose and intralipids will be used to determine whole-body [$S_I$], liver [$S_I$-H], and skeletal muscle [$S_I$-M] insulin sensitivity. Following a 10-hour overnight fast, at approximately 0600 hr, the intravenous catheters placed the evening before in one arm for infusions and in the contralateral hand for blood withdrawal will be warmed to 70°C using a warming mitt for sampling of arterialized venous blood. A primed (200 mg/m$^2$) IV bolus followed by 2.0 mg/m$^2$ body surface area per minute constant infusion of [6,6-$^{2}H_2$] glucose (Cambridge Isotope Laboratories, Andover, MA) will be infused to achieve ~1.0 mol percent excess for all subjects. Plasma samples will be obtained at -125, -120, -20, -15, -10, and -5 minutes to estimate basal endogenous glucose production and/or fasting insulin concentration. At the completion of the 2-hr infusion with glucose isotope, a primed, constant infusion of regular insulin at 40 mU/m$^2$/min will be started and continued for 5 hours. Concurrent with the insulin infusion, either a

saline/2.5% glycerol or 20% intralipid infusion of 1.5 ml/min will be started and continued until the 5-hour clamp has been completed and the muscle and fat biopsy have been performed. Blood glucose will be monitored every 5-10 min during the insulin infusion using a Hemocue Glucose 201(HemoCue America, Brea, CA), or an i-STAT analyzer (Abbott Point of Care Inc, Abbott Park, IL) and euglycemia will be maintained throughout the clamp by infusing 20% dextrose at a variable rate. To maintain a constant enrichment of [6,6-$^2H_2$] glucose in blood during the clamp, [6,6-$^2H_2$] glucose infusion will be continued throughout the clamp and at 2% enrichment in the 20% dextrose solution. Additional blood samples will be collected every every 60 minutes for insulin, ketones,and FFA, and at the beginning and end of the clamp for $^2H_2$-glucose and metabolomics. Using the blood samples collected during the 30-minute period just preceding and at the end of the glucose clamp, glucose disposal rates (Rd) and basal endogenous glucose production ($R_a$) will be calculated according to the steady-state equations of Steele.(92) At the end of the 5 hour infusion and completion of the muscle and fat biopsies, the insulin and intralipid or glycerol/saline infusions will be stopped. After 10 minutes, the subject will be fed a low-fat meal. Blood glucose will continue to be monitored for an additional hour after the insulin infusion is stopped with adjustment to the glucose infusion as indicated to ensure subjects reestablish endogenous glucose regulation.

*Rationale for HEC approach:* Patients will undergo insulin sensitivity measurement using HECs along with determination of ectopic fat accumulation by $^1H$ magnetic resonance spectroscopy (MRS). If the defective mitochondrial function and "lipotoxicity" hypothesis is true, then insulin resistance should be common among patients with long-chain FAO disorders in whom FAO capacity is greatly reduced. However, insulin resistance and diabetes have not been previously reported in case reports of patients with CPT2, VLCAD, or LCHAD/TFP deficiency(35) and, in our preliminary data(30) (see also above), these patients have comparable levels of IMCL, IHL, and glucose tolerance to controls while consuming a low-fat diet.

Because a low-fat diet is standard therapy for these patients, previous clinical reports and our preliminary data could be argued to lack the proper conditions to permit accumulation of insulin resistance-inducing cytosolic lipid byproducts. However, prolonged high-fat feeding is contraindicated in FAO patients due to increased risk of hypoglycemia and rhabdomyolysis (see also Human Subjects). Instead, we propose creating a "fat challenge" by infusing intralipids (20% IL, 1.5 mL/min+heparin) during a HEC, which has been shown to increase IMCL and induce insulin resistance within 4 to 6 hours.(4, 9, 11, 94) From studies using an identical intralipid infusion protocol, levels of non-esterified free fatty acids in the blood can range between 0.3 to 4.5 mmol/L (mean 1.49 mmol/L).(4, 8, 9, 11, 17, 27, 28, 36, 39, 60, 73, 81, 82, 89, 90) In comparison, non-esterified fatty acid (NEFA) levels have been reported to rise up to 0.8 - 2 mmol/L in adults and children after an overnight fast(7, 10, 83, 95) and up to 0.8 - 1.0 mmol/L after a high-fat meal.(87)

*Safety:* By performing a HEC during the intralipid infusion, hypoglycemia and rhabdomyolysis are avoided through the variable glucose infusion to maintain glucose levels in a safe range and NEFA levels remain closer to physiological levels. Therefore, we feel the intralipid + HEC protocol proposed can be conducted safely among FAOD subjects while achieving high insulin and high NEFA levels. By using a co-infusion of dideuterated glucose, we will be able to separately measure glucose appearance in the fasting state (Ra; mostly hepatic glucose output) as well as insulin-induced glucose disposal (Rd; mostly muscle), as there is reason to predict that FAO defects may affect these two organs separately.(104, 105) Using a randomized, crossover design, the change in insulin sensitivity with co-infusion of intralipid will be compared between FAO patients and matched controls to a control infusion (glycerol / saline).

*Tissue Biopsies*: Blood, muscle, and adipose tissue samples will be collected near the end of each clamp to allow determination of both changes in serum and intracellular energy metabolites and tissue-specific insulin sensitivity *ex vivo*.

*Muscle Biopsy Technique:* Biopsies of the vastus lateralis muscle from study subjects will be obtained by percutaneous puncture using a Bergstrom side-cut needle(142) (144). Briefly, after local infiltration with lidocaine (2%), a small incision is made with a scalpel on the overlying skin. The needle is then advanced and the fascia traversed. Once in the muscle, the tip of the needle is advanced ≈3 cm and aspiration

applied through the back of the needle to enhance the amount of tissue protruding into the side-port cutting window. A sharp and quick cut is made and the specimen extracted. Usually more than 100 mg of wet-weight tissue is obtained. This tissue is dabbed dry, weighed, and cut into pieces that are immediately frozen in liquid nitrogen. The incision is closed with a simple steristrip; a pad dressing is applied at the end of the visit. Complications can include pain/soreness and superficial hematoma.

*Adipose Biopsy Techniques:* At the time of biopsy, ~ 1.0 gram of fat tissue will be extracted from just lateral to the umbilicus on the abdomen by aspiration under local anesthesia. We have successfully and safely used this technique to harvest samples in more than 100 patients to date participating in ongoing studies at our institution. Immediately after harvest, the sample is divided two ways by the lab technician. The first sample is placed in buffer and taken for size and insulin sensitivity measures. The second sample is then stored at –80° C for later analysis.

*Biopsy Anticipated adverse events:* Prior to the aspiration fat and muscle biopsies, a local anesthetic is injected into the skin. An allergic reaction to the anesthetic is rare (less than 1 in 10,000). Significant bleeding from the fat or muscle biopsy is rare. Baseline lab work will include measurement of platelets, INR and PTT to assure these levels are normal prior to the biopsies. Potential subjects taking regular medications that may have an anticoagulant affect will also be excluded from participation in the study. Infection of a fat biopsy may occur in up to 1 in 10 cases. A small scar will result at the biopsy site. The scar is usually smaller than the biopsy incision and is frequently almost invisible.

Blood and tissue metabolites collected from subjects with a FAO disorder and controls during clamps with and without co-infusion of intralipid will be measured by the West Coast Metabolomics Center (UC Davis, see specific methods below). Activation and expression of the insulin-signaling cascade components IR, AKT, PKC-Θ, will be measured in muscle and WAT samples as described in detail below. Basal and insulin-stimulated glucose and FFA uptake into muscle and WAT explants from biopsies will be measured by the novel florescence technique described above. Changes in insulin signaling and tissue fatty acid and glucose uptake between the intra-lipid and glycerol infusions will be compared between CPT2, VLCAD, and LCHAD/TFP-deficient subjects, subjects with MCAD deficiency, and normal controls.

## IV. Study Procedures

| Table 4. Schedule of Study Procedures | | | | | |
|---|---|---|---|---|---|
| | Screening Visit (Local Subjects) | Prior to Admit | At Admit or on Day 1 | Day 2 | Day 3 |
| Informed Consent/HIPAA Authorization | X | X | | | |
| Inclusion/Exclusion Assessment | X | X | X | | |
| Demographics and Medical History | X | X | | | |
| CBC with platelets, INR, PTT | | | X | | |
| Pregnancy Test (if applicable) | | | X | | |
| Vital Signs and Weight | X | | X | X | X |
| Height | X | | X | | |
| Physical Exam | | | X | | |
| Standard FAO Low Fat Diet | | X | X | X | X |
| Low Fat Diet Instruction | X | X | | | |
| 3-Day Diet Diary | | X | | | |
| DEXA Scan | | | X | | |
| Intravenous (IV) Catheter Placement | | | X | | |
| Indirect Calorimetry | | | | X | |
| Magnetic Resonance Spectroscopy (MRS) | | | | X | |
| Hyperinsulinemic-Euglycemic Clamp (Figure 11) | | | | X | |
| Muscle Biopsy | | | | X | |
| Fat Biopsy | | | | X | |
| Discharge from CTRC | | | | | X |

**Laboratory Methods:**

Lab Assays: Insulin by RIA, leptin and total and high-molecular-weight adiponectin by ELISA, and FFA levels by enzymatic colorimetric kit will be determined by the OHSU CTRC Laboratory Core.

Metabolomics: Plasma, muscle, and subcutaneous adipose tissue aliquots will be extracted, derivatized, centrifuged, decanted and dried as previously described.[42] A set of 13 C8-C30 fatty acid methyl ester internal standards will be added, samples derivatized, and analytes separated using an Agilent 6890 gas chromatograph (Santa Clara, CA). Chromatography will be performed followed by mass spectrometry by a Leco Pegasus IV time-of-flight mass spectrometer (St. Joseph, MI) with 280°C transfer line temperature, electron ionization at 270eV and an ion source temperature of 250°C. Mass spectra will be acquired from m/z 85–500 at 17 spectra s21 and 1850 V detector voltage. Result files will be exported to our servers and further processed by the metabolomics BinBase database. All database entries in BinBase will be matched against the Fiehn mass spectral library of 1,200 authentic metabolite spectra. Identified metabolites will be reported if present within at least 50% of the samples per study design group. Peak heights of quantifier ions defined for each metabolite in BinBase will be normalized to the sum intensities of all known metabolites and used for statistical investigation. Compiled data sets will be analyzed using an appropriate array of multivariate data analyses techniques including principal components analysis, hierarchical cluster analyses, and projection to latent structures discriminate analyses using the an R-based Microsoft Excel Add-in developed in the laboratory of Dr. Newman and SIMPCA-P v.12.0.

Laboratory analysis of Tissue Biopsies: Subcutaneous WAT and muscle biopsies will be processed for explant assays of glucose and FFA uptake as well as for the preparation of lysates for Western immunoblot

analysis of insulin signaling. *Insulin-stimulated FFA and glucose uptake in WAT explants* from umbilical biopsies will be performed as previously established for non-human primate tissue and as shown in our previous publications and in **Figure 8**.(96-98) *Glucose uptake in skeletal muscle biopsies* will be evaluated in samples treated alone or with 10 nM insulin for 30 min and then exposed to 50 μM 2-NBD-glucose for an additional 30 min. Samples are then washed, fixed in 4% paraformaldehyde, washed in HBSS with 50 mM $CoCl_2$, and stored in HBSS with 25 mM $CoCl_2$ until analyzed by confocal microscopy. 2-NBD-glucose incorporation into individual fibers is quantified using image J. Mean fluorescence per fiber is determined using a randomly positioned circular region of interest of cross-sectional images. *Insulin signaling pathway expression and activation* will be analyzed by preparing total protein lysates in RIPA buffer after homogenization in a motor-driven glass-glass homogenizer for muscle and in X buffer for WAT. Lysates are then subjected to standard Western immunoblotting for phosphorylated (activated) IR, Akt, and (in muscle) PKC-Θ, followed by stripping and probing with pan-IR, Akt, and PKC-Θ antibodies to determine general expression levels and for normalization of phospho-antibody signals as previously described.(24, 25, 63) We acknowledge that insulin receptor substrates (IRS)-1 and 2 are important components of the insulin signaling pathway in fat and muscle and are involved in insulin resistance, but it is our experience that analysis of IRS-1/2 activation requires significant amounts of lysate and immunoprecipitation prior to Western immunoblotting, which we are unlikely to acquire from the biopsies that will be obtained. The proposed analysis of IR, Akt, and PKC-Θ should, however, provide sufficient data to ascertain the status of the insulin-signaling pathway in samples from the different treatment groups.

V. **Statistical Analysis:**

The primary analyses to compare disease groups with healthy controls will use mixed models with a random effect for matched pair to allow for correlation between the observations on the matched observations. In addition to the random pair effect, a disease effect (disease versus healthy), and a diagnostic group effect (CPT2, VLCAD, LCHAD/TFP, MCAD), we will also incorporate a fixed effect for the order of testing (interlipid infusion tested first or second). The hypotheses focus on the comparisons between diseased individuals and their matched controls with regard to the difference in outcomes under the two conditions (glycerol vs interlipid infusion). For these analyses, we will use the difference in a given outcome (e.g., Rd uptake) between infusion conditions as the dependent variable and compare these differences between diseased and matched controls, separately for the four diagnostic groups (CPT2, VLCAD, LCHAD/TFP, MCAD). Although we will conduct a formal test of interaction of disease (disease vs healthy) and diagnostic group, we are not powered for such a test and, hence, will focus on separate inferences for the four disease groups. Note that, as indicated in the hypotheses, we expect to find differences between the controls and the CPT2, VLCAD, and LCHAD/TFP subjects, but not between the controls and the MCAD subjects. For the latter comparison, our emphasis will be on estimating the magnitude of the mean differences via 95% confidence intervals rather than formal hypothesis testing. We will use a significance level of 0.05 for each outcome comparison, with no adjustment for multiple comparisons due to the limited sample sizes. We are unable to increase sample sizes due to the small study populations and budgetary constraints, so cannot power the study to take into account the multiple comparisons. However, we will present for each outcome, both unadjusted p-values as well as p-values adjusted for the two multiple comparisons (one for each disease group) using the Holm procedure.(38)

**Power Considerations:**

For each outcome, we have two measurements on every subject, one for each infusion condition. Our analyses will focus on comparisons between patients and controls of the *differences between outcomes* under the two conditions. We expect to see meaningful differences between controls CPT2, LCHAD/TFP, and VLCAD-deficient participants. These are the groups of interest for power calculations, whereas for MCAD, our aim is to provide useful descriptive information. There are two sets of correlations

between measures that need to be considered in power calculations (and statistical analyses). The first is the correlation between outcome measures on a given subject under the two infusion conditions, the second is the correlation of the differences in outcomes under the two infusion conditions, between each subject and his/her matched pair. For power calculations, we considered various values for each of these two correlations and used standard deviation estimates for each infusion condition as reported for glucose infusion rate (GIR) and for PDK4 mRNA levels (81, 94). GIR is a primary result during the HEC, closely related to insulin resistance (specific aim 1). PDK4 message is associated with our hypothesized mechanisms for insulin resistance in muscle and adipose tissue (Specific Aim 2). Mean and SD values for these outcomes in healthy controls are given in **Table 5**. We assume that normal controls and MCAD subjects will have mean outcomes as given in the table but that subjects with CPT2, VLCAD, and LCHAD/TFP disorder will have mean decreases when given the interlipid infusion that are less than 10% from that under the glycerol infusion condition for GIR and a mean increase of less than 100% for PDK4 mRNA. That is, we expect the mean change from glycerol infusion condition to be less than 5.7, and 0.18 for GIR, and PDK4 mRNA, respectively, for the CPT2, VLCAD, and LCHAD/TFP subjects.

**Table 5**: Glucose Infusion Rate (GIR), and PDK4 mRNA/actin in healthy controls and hypothesized mean differences in subjects.

| Outcome | Glycerol Infusion | Interlipid Infusion | $H_a$: mean Δ In Controls and MCAD | $H_a$: mean Δ In VLCAD and LCHAD/TFP |
|---|---|---|---|---|
| GIR (μmol/kg/min) (n=9) | 57.2 ± 9.3 | 25.7 ± 7.8 | 31.5 | 5.7 |
| PDK4 (mRNA/actin) (n=10) | 0.18 ± 0.06 | 0.8 ± 0.38 | -0.62 | -0.18 |

Data are mean ± standard deviation from Roden et al and Tsintzas et al. GIR = glucose infusion rate; PDK4 = pyruvate dehydrogenase kinase 4; $H_a$: mean Δ= hypothesized mean difference.

In **Table 6**, we show the differences between groups in mean change that can be detected with 80% power at level 0.05 when the sample size per long-chain disorders is 20. For n=10 subjects, we have adequate power for detecting expected differences for GIR and PDK4 mRNA with modest correlation between repeated observations on the same subject and between matched pairs. For GIR, even with zero correlation, we would have about 97% power at level 0.05 to detect the expected difference in change between groups (31.5 vs 5.7) with n=10. For PDK4 mRNA, we could detect the expected difference in change (-0.62 vs -0.18) with 63% power if zero correlation and with 78% power if the correlation is 0.25 between and within n=10 matched patients.

**Table 6**: Change between glycerol infusion and interlipid infusion that can be detected between control and the VLCAD, LCHAD/TFP and CTP2 groups with 80% power at level 0.05 with n=20 subjects long-chain FAO disorders.

| Outcome | Mean Δ (Glycerol – Intralipid) Controls | Correlation between observations on the same subjects | Correlation of Δ between matched controls and patients | Mean Detectable Δ (Glycerol – Intralipid)* LCHAD/TFP subjects |
|---|---|---|---|---|
| GIR | 31.5 | .25 | .25 | 17.6 |
| | 31.5 | .50 | .25 | 20.0 |
| | 31.5 | .50 | .50 | 22.1 |
| PDK4 mRNA | 0.62 | .25 | .25 | 0.17 |
| | 0.62 | .50 | .25 | 0.19 |
| | 0.62 | .50 | .50 | 0.27 |

Δ= change; * Note that changes smaller than these can be detected with greater power as smaller changes are further from the changes expected in the control groups.

**Timeline:** Enrollment and clamp procedures will be completed in years 1, 2, and 3. Biopsy assays and insulin signaling measures will be completed real time with collection of the muscle and adipose samples during years 1-3. In year 4, we will batch analyze stored plasma, muscle, and adipose samples for metabolomics to control for run variation of the metabolomics methods, prepare manuscripts and present the results of the project.

## VI. Protection of Human Subjects

### A. Potential Risks

Risks from the procedures in this study may include: pain, bleeding, bruising and infection from the catheter placement, the muscle biopsy and the subcutaneous fat biopsy; and exposure to a small amount of radiation (1.5 – 3.0 mrem) during the DEXA scan. Nonradioactive stable isotopes of $^{2}H$ will be given to subjects. The safety of stable isotopes is well documented and does not pose an added risk to the subjects (46). Alterations in dietary intake associated with poor intake or prolonged fasting in subjects with CPT2, VLCAD, LCHAD/TFP or MCAD deficiency carry the risk of associated hypoglycemia, or rhabdomyolysis. The research meals during the study will be designed to provide adequate total energy and pose little risk to subjects. Dextrose will be infused at a variable rate during the hyperinsulinemic clamp and will prevent hypoglycemia.

Infusion of intralipid in subjects with a fatty acid oxidation disorder could increase partial fatty acid oxidation metabolites in plasma and tissues such as long-chain, long-chain hydroxyl and medium-chain acylcarnitines. *When normal amounts of nonfat energy are provided and the subject is not dependent upon FAO at the time, we do not believe the acylcarnitines in themselves induce metabolic decompensation.* The chronic provision of high fat diets or intravenous lipid is not recommended clinically for this population. One case report of intravenous lipid infusion raised plasma acylcarnitines but did not result in any adverse event or metabolic decompensation (20). The effects of high fat diet feeding have been documented in only one case report where a child was inadvertently fed a high fat, low carbohydrate formula and developed lethargy, rhabdomyolysis and acidosis after several days (29). It is important to note that the clinical symptoms of metabolic decompensation observed in this case report and in subjects with FAO disorders occur in the absence of adequate glucose or energy to provide an alternative fuel. Episodes of rhabdomyolysis or hypoglycemia occur with prolonged fasting, exercise, or intercurrent illness. We will avoid metabolic decompensation in this study by providing a dextrose infusion to maintain euglycemia and provide substrate for energy production.

### B. Subject Recruitment

Subjects with CPT2, VLCAD, LCHAD/TFP or MCAD deficiency will be recruited to participate in this study. Recruitment techniques will include announcements on Fatty Acid Oxidation Support group web pages and chat boards and through our center's clinic population. Each subject must have a confirmed diagnosis of CPT2, VLCAD, LCHAD/TFP or MCAD deficiency, be 18 years of age or older, and be able to complete and comply with the protocol. Subjects may not be participating in another research project that alters macronutrient content of the diet.

We will also recruit normal matched control subjects. These subjects will be recruited from the local Portland area via the OHSU webpage, and informational flyers on campus. Control subjects will be chosen to be similar in age, BMI, gender, and activity level as a subject with a fatty acid oxidation disorder, and must be in good health and able to complete the protocol. As many as 100 subjects (both with FAO disorders and normal controls) may be screened for this study.

### C. Informed Consent

A principal investigator or the study coordinator will explain and discuss all procedures and tests to be completed during the study and will be available to answer questions for the subjects, and their families. Informed consent will be obtained from each subject prior to beginning the study. Because these disorders are rare diseases, many subjects with CPT2, VLCAD, LCHAD/TFP or MCAD deficiency will live out of state. Local subjects will be brought in for an outpatient screening visit to consent them in person. If informed consent cannot be obtained in person, it will be obtained using the following process:

1. A potential subject will contact the PI or study staff.

2. A packet of information including a cover letter, consent form, HIPAA authorization form, medical release form and a stamped return envelope will be mailed to their address.
3. The consent form and HIPAA authorization will be on colored paper and copies of the forms will be provided on white paper for the subject to retain for their records.
4. The PI or authorized study staff will review the consent on the phone, confirm subject understanding and answer any questions.
5. Once the investigator is convinced that the subject verbally demonstrates understanding and agrees to the process, the consent, HIPAA and medical release documents will be signed and mailed to the investigator.
6. When the forms arrive at OHSU and are signed by the researcher who obtained verbal consent, the HIPAA authorization will be copied and mailed back to the subject.
7. When the investigator receives the consent and medical release forms, study staff will obtain the necessary records to verify the diagnosis of a fatty acid oxidation disorder.
8. If the subject is eligible to participate, a visit will be scheduled and travel arrangements to OHSU will be made.
9. Individuals authorized to obtain written consent are the principal investigator, co-investigators and assigned research staff specifically designated by the principal investigator to work on this project.

**Compensation of Study Participants:**

Both subjects with a fatty acid oxidation disorder and controls will be compensated for their participation in this study and reimbursed for travel expenses. The compensation plan for each group is outlined below.

1. Subjects with a fatty acid oxidation disorder:
    a. The majority of subjects with CPT2, VLCAD, LCHAD/TFP or MCAD will be traveling from a distant US city to Portland to participate. Subjects will not be compensated directly for airline tickets but their travel costs will be covered. Study staff will book their airfare and purchase the airline ticket. In rare situations where subjects live quite a distance from the airport and need to take an early flight, a night in a hotel will be booked for them. They will be compensated for miles traveled in their own car and airport parking. Miles are reimbursed based on the current IRS rate per mile traveled for business. Baggage fees, if needed, will also be covered. Study staff will pick-up most subjects at PDX and return them to the airport, but if a taxi is needed the fare will be reimbursed.
    b. A few subjects may be local. Local subjects may be compensated for miles traveled in their own car. They will travel to OHSU for 3 separate visits.
    c. If subjects decide to extend their stay in Portland before or after the study visit and stay in a hotel or eat food not provided by OHSU, they will do it at their own expense.
    d. If the local FAOD subjects come in for a screening visit (requires 2 hours of their time), they will be compensated $25.
    e. At the completion of the 1$^{st}$ visit (including 3 nights, 2 days at the CTRC, and muscle/fat biopsies), subjects will be compensated $300.
    f. At the completion of the 2$^{nd}$ visit 4 months later (including 3 nights, 2 days at the CTRC, and muscle/fat biopsies), subjects will be compensated $700. **Maximum compensation for 1 screening visit and 2 inpatient visits will be $1025 plus travel reimbursement.**
    g. If subjects are asked to return for an extra visit to repeat the clamp procedure, DEXA, indirect calorimetry and/or muscle/fat biopies, they will have their airfare covered, the same travel reimbursements noted in (a) above and will receive additional compensation of $300. Subjects may return for up to two additional visits.
    h. If subjects are traveling from a country other than the United States, they will be asked to purchase their own airline tickets. Travel reimbursement for airfare will be a maximum of $1000 for each of the two inpatient visits. They will also receive additional travel reimbursement as noted in (a) above and $300 compensation for Visit 1 and $700 compensation for Visit 2.
2. Control subjects who do not have a fatty acid oxidation disorder:
    a. All of the control subjects will be recruited from the Portland metro area. Participation will require 3 trips to OHSU: 1 screening visit and 2 separate inpatient admissions. They will be compensated for miles traveled in their own car using the current IRS rate per mile..

b. At the completion of the screening visit (requires about 2 hours of their time), subjects will be compensated $25.
c. At the completion of the 1st visit (including 3 nights, 2 days at the CTRC, and muscle/fat biopsies), subjects will be compensated $300.
d. At the completion of the 2nd visit 4 months later (including 3 nights, 2 days at the CTRC, and muscle/fat biopsies), subjects will be compensated $700. **Maximum compensation for a screening visit and 2 inpatient visits will be $1025 plus travel reimbursement.**

3. Control subjects participating for study procedure practice:
   a. All subjects will be recruited from the Portland metro area. Compensation will depend on which practice procedures they elect to participate in. If an HEC will be performed then there will be an overnight admission with discharge the next afternoon. Compensation will be $200. If a muscle and fat biopsy are performed, compensation will be $100. If an MRS study is performed, compensation will be $25. **Maximum compensation for all three procedures will be $325 plus travel reimbursement**.

**D. Protection Against Risk**

Studies will be reviewed and approved by the OHSU Institutional Review Board (IRB). All subjects will be admitted to the inpatient unit of the Oregon Clinical and Translational Research Institute (OCTRI). Nursing staff and the investigative team will monitor subjects and care for wound or bleeding complications as a result of catheter placement and biopsy. To prevent radiation exposure to a fetus, a urine pregnancy test will be completed for females of child bearing potential prior to each DEXA Scan. Subjects who are pregnant will be excluded. A stable isotope $^2H_2$ glucose will be given to subjects. As noted above, the safety of stable isotopes is well documented and does not pose an added risk to the subjects (46). Special care will be taken to explain the use of stable isotopes to the subjects and their families. Plasma glucose will be monitored during the clamp procedure. The MD will discharge subjects at the end of each admission after a physical exam, vital signs are normal and they have no complaints of muscle pain. If they do have muscle pain, we will check a plasma creatine kinase (CK) level prior to discharge.

**E. Confidentiality**

Upon enrollment, subjects will be assigned a unique identifier that will be used instead of their name, medical record number or other personally identifying information. To ensure confidentiality, all data and specimens collected will be kept in locked laboratory facilities. Urine, blood, muscle and adipose samples will be stored with a unique identifier in locked laboratory facilities at OHSU. Long-term storage of blood muscle and adipose will be stored at -80°C and urine samples will be stored at -20 °C in the laboratory of Dr. Gillingham. The laboratory is a locked facility in the Richard T. Jones building, room 4549 at OHSU, Portland, Oregon. Only the PI and study staff will have access to paper records and the key code which will be stored in locked filing cabinets in restricted access offices at OHSU. Computer files of collected data will be password protected and stored on a restricted drive behind the OHSU firewall. The study coordinator will code urine, blood, muscle and adipose samples with the study ID code to remove any identifiable information on the sample prior to sending the samples to other laboratories for analysis. Subject's names or other identifiable information will not be published or shared with outside research facilities.

**F. Potential Benefits of the Proposed Research to the Subjects and Others**

The proposed studies may provide new insights into the relationship between fatty acid oxidation and the development of insulin resistance in humans. This knowledge will be useful in designing future pharmaceutical targets for patients with insulin resistance and type 2 diabetes. The participants in this study will not benefit from the proposed research.

**G. Data and Safety Monitoring Plan**

1. Data quality and management. The principal investigators, Jonathan Purnell, and Melanie Gillingham, will review the data collection forms on an annual basis for completeness and accuracy of the data as well as

protocol compliance. A statement reflecting the results of the review will be sent to the NIH in the annual report (non-competing continuation). The data that will be reported to and monitored by the PI will include:

- Subject anthropometric data including height, weight and body composition
- Subject laboratory results
- Indirect calorimetry analysis of energy needs
- Data from the hyperinsulinemic-euglycemic clamp study
- Data from the magnetic resonance spectroscopy procedure
- Subject reports of adverse events or unanticipated problems

2. Adverse Event Definitions and Grading: An adverse event (AE) is defined as any unfavorable or unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the use of a medical treatment or procedure that may or may not be considered related to the medical treatment or procedure. Adverse events in this study are evaluated for relationship to study intervention by a physician co-investigator using the Common Terminology Criteria for Adverse Events version 4.0. This grading criterion is listed as follows:

   Grade 1: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
   Grade 2: Moderate; minimal, local, or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living.
   Grade 3: Severe or medically significant but not immediately life-threatening; hospitalization indicated; disabling; limiting self-care activities of daily living.
   Grade 4: Life-threatening consequences; urgent intervention needed.
   Grade 5: Death related to AE.

   A Serious Adverse Event (SAE) is defined as an event that results in death, is life threatening, requires in-patient hospitalization, results in persistent or significant disability or is an important medical event that does not meet the above criteria but may require medical or surgical intervention. SAE's that are unexpected and possibly related will be reported to the Independent Safety Monitor for the study, and the IRB as indicated in the OHSU Research Integrity Office policy on Reporting Unanticipated Problems and Adverse Events.

   Some risks are considered expected in this study. They are considered mild to moderate risks and are addressed in the consent form. The expected risks include:

   - Inserting a venous catheter needle for blood sampling and infusion can be associated with some discomfort, bruising, and, very rarely, with inflammation and infection of the arm veins.
   - Muscle and adipose biopsies can be associated with some discomfort, bleeding, bruising, and, very rarely, with inflammation and infection of the biopsy site.
   - Undergoing DEXA scans is associated with a small degree of radiation exposure.
   - Insulin infusion has a risk of hypoglycemia.
   - Heparin infusion may cause bruising or heavy bleeding, and rarely low platelet levels.

3. Plan for Reporting Both Anticipated and Unanticipated Adverse Events.
   a) Each subject will be evaluated for any adverse events at study visits.
   b) Any event that is reported to either the principal investigator or his or her designated research associates by the subject or medical staff caring for the subject and which meets adverse event criteria will be documented as such. An MD Co-Investigator will review AEs real-time.
   c) Any AE or protocol deviation that represents an unanticipated problem /will be reviewed by the PI and submitted to the OHSU IRB. The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the reporting of the event.

d) AEs will be reported as outlined by the OHSU Research Integrity Office policy on Reporting Unanticipated Problems and Adverse Events.

e) AEs and unanticipated problems will be summarized annually and submitted to the IRB at continuing review.

f) Any action resulting in a temporary or permanent suspension of the study (e.g. IRB actions, or actions by the investigators or co-investigators) will be reported to the appropriate NIH program official.

4. Safety Review and Monitoring

a) Independent Safety Monitor. The study will be monitored by the principal investigator, MD co-investigator and an Independent Safety Monitor. The Safety Monitor will be Penelope Hogarth MD, Associate Professor of Neurology and Molecular & Medical Genetics at OHSU. Dr. Hogarth will be notified immediately if a significant adverse event occurs, and then she, as Safety Monitor, together with the PI, will notify the OHSU IRB, the NIH, and the CRC. The role of the Safety Monitor will be:

i) The Safety Monitor will review the annual report and all adverse events prior to submission of the IRB review

ii) She will prepare a memo of her findings and recommendations to be included in the submission to the IRB with the annual report.

iii) If any serious unexpected adverse event occurs, the safety monitor will be notified with 24 hours and she will review the medical records.

iv) The Safety Monitor, together with the PI, will notify the OHSU IRB, the NIH, and the CRC of serious unexpected adverse events.

b) Annual Review. The principal investigator and MD co-investigator will review this protocol on a continuing basis for subject safety and include results of the review in the annual progress reports submitted to the IRB and NIH.

c) Annual Report. The annual report will include a list of adverse events and the content listed below as well as a memo of findings and recommendations provided by the Safety Monitor.

d) Content of the Annual Report. The annual report will address: a) Whether adverse event rates are consistent with pre-study assumptions; b) Reason for dropouts from the study; c) Whether all participants met entry criteria; d) Whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims on the study; and e) Conditions whereby the study might be terminated prematurely.

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