ADIPOSE ORGAN TRANSPLANT FOR TREATMENT OF MAPLE SYRUP URINE DISEASE (MSUD)

A Thesis in
Laboratory Animal Medicine
by
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ABSTRACT

Maple syrup urine disease (MSUD) is an inborn error of metabolism where affected individuals cannot oxidize the branched-chain amino acids (BCAA) – leucine, isoleucine, and valine. The name of the disease is derived from the distinctive odor of the urine, often described as a “burnt sugar” smell. If left untreated, the disease can lead to seizures, coma, and early death usually before 3 months of age. An estimated 1 in 185,000 infants are affected worldwide, although the incidence is much higher in the Old Order Mennonite population – approximately 1 in 176. Management of any of the five clinical manifestations of MSUD involves a strict diet devoid of BCAA. Several reports in the literature detailing orthotopic liver transplants in children and adults have reported success in controlling plasma BCAA levels to the level of patients with a very mild variant of MSUD, despite non-restrictive diets. However, liver transplantation is a major operative procedure that carries both an elevated surgical risk and a prohibitive financial cost. Recent studies have suggested that adipose tissue has a previously unappreciated high capacity for BCAA metabolism and is already used extensively in reconstructive surgery. Our hypothesis is transplantation of adipose tissue from a wild type mouse into mouse models of MSUD will reduce plasma BCAA levels to that of an unaffected animal. Male and female knockout PP2Cm mice, a model of intermittent MSUD, received either wild type or adipose tissue from affected littermates. Male and female BCATm mice, a mutant model of MSUD, received wild type fat or underwent a sham surgery. Mice were challenged with a variety of diets – BCAA-free, modified BCAA-containing, 18% protein, and 50% protein – and plasma BCAA levels measured. Although circulating plasma BCAA levels were not reduced following abdominal transplant of adipose tissue in PP2Cm mice, they were reduced with subcutaneous transplantation in BCATm mice. Therefore, subcutaneous adipose tissue transplantation may have merit in the treatment of MSUD.
# TABLE OF CONTENTS

List of Figures ........................................................................................................... v

List of Tables ............................................................................................................. vi

List of Abbreviations ................................................................................................. vii

Acknowledgements ..................................................................................................... viii

Chapter 1 Introduction ................................................................................................. 1
  History ..................................................................................................................... 3
  Genetics .................................................................................................................. 7
  Clinical presentation ............................................................................................... 9
  Treatment and management .................................................................................... 10

Chapter 2 Materials and Methods ............................................................................. 14
  Animals ................................................................................................................ 14
    PP2Cm mouse model ......................................................................................... 14
    BCATm mouse model ....................................................................................... 16
  Plasma circulating BCAA levels and body mass measurements ......................... 17
  Surgery ............................................................................................................... 19
  Diet manipulation ................................................................................................. 21
    PP2Cm diet manipulation ................................................................................... 21
    BCATm diet manipulation ............................................................................... 24
  Surgical site examination ...................................................................................... 25
  Statistical analysis ............................................................................................... 26

Chapter 3 Results ....................................................................................................... 27
  PP2Cm ................................................................................................................ 28
  BCATm .............................................................................................................. 36

Chapter 4 Discussion ................................................................................................. 44

Chapter 5 Future Directions ....................................................................................... 48

References ............................................................................................................... 50
LIST OF FIGURES

Figure 2-1. Representative PCR gel results, PP2Cm..........................................................16
Figure 2-2. Representative PCR gel results, BCATm. ..........................................................17
Figure 2-3. H1-NMR analysis..................................................................................18
Figure 2-4. Cohort 1 diet manipulation timeline, PP2Cm ........................................22
Figure 2-5. Cohort 2 diet manipulation timeline, PP2Cm ...........................................23
Figure 2-6. Cohort 3 diet manipulation timeline, PP2Cm ...........................................24
Figure 2-7. BCATm diet manipulation timeline. .........................................................25
Figure 3-1. Pre-surgical plasma BCAA concentration. ...........................................28
Figure 3-2. BCAA plasma concentration levels, PP2Cm. .........................................31
Figure 3-3. Change of body weight in PP2Cm knockout mice on 50% high protein diet. .....32
Figure 3-4. Pre- and post-surgical H1-NMR body composition, PP2Cm. .......33
Figure 3-5. Food consumption, 50% high protein diet, PP2Cm. ................................34
Figure 3-6. Gross examination following whole-body perfusion with toluidine blue dye, PP2Cm Cohorts 1 and 2.................................................................34
Figure 3-7. Gross examination following whole-body perfusion with toluidine blue dye, PP2Cm Cohort 3. ........................................................................................................35
Figure 3-8. Histopathology image of transplanted tissue, PP2Cm. ........................36
Figure 3-9. BCAA plasma concentration levels, BCATm. ..................................39
Figure 3-10. Change of body weight in BCATm knockout mice. ...............................40
Figure 3-11. Pre- and post-surgical H1-NMR body composition, BCATm. .............41
Figure 3-12. BCAA intake, BCATm KO mice..........................................................42
Figure 3-13. Gross examination following whole-body perfusion with toluidine blue dye, BCATm. .................................................................43
LIST OF TABLES

Table 3-1. Cohort 1 characteristics, PP2Cm.................................................................28
Table 3-2. Cohort 2 characteristics, PP2Cm.................................................................29
Table 3-3. Cohort 3 characteristics, PP2Cm.................................................................29
Table 3-4. BCATm study animal population.................................................................37
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSUD</td>
<td>Maple syrup urine disease</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-chain amino acid</td>
</tr>
<tr>
<td>BCKAD</td>
<td>Branched-chain keto acid dehydrogenase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
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<tr>
<td>KIC</td>
<td>α-ketoisocaproate</td>
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<tr>
<td>KMV</td>
<td>α-methyl-β-methylvalerate</td>
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<tr>
<td>KIV</td>
<td>α-ketoisovalerate</td>
</tr>
<tr>
<td>BCKDHA</td>
<td>Branched-chain keto acid dehydrogenase E1, alpha polypeptide</td>
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<tr>
<td>BCKDHB</td>
<td>Branched-chain keto acid dehydrogenase E1, beta polypeptide</td>
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<tr>
<td>DBT</td>
<td>Dihydrolipoamide branched-chain transacylase E2</td>
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<tr>
<td>DLD</td>
<td>Dihydrolipoamide dehydrogenase</td>
</tr>
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<td>MELD</td>
<td>Model end-stage liver disease</td>
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<tr>
<td>PELD</td>
<td>Pediatric end-stage liver disease</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<td>AAALAC</td>
<td>Association for Assessment and Accreditation of Laboratory Animal Care, International</td>
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<td>DPC</td>
<td>Days post-conception</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>H1-NMR</td>
<td>Proton nuclear magnetic resonance</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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Chapter 1

Introduction

Maple syrup urine disease (MSUD) is an inborn error of metabolism where affected individuals cannot process the branched-chain amino acids (BCAA) – leucine, isoleucine, and valine. The name of the disease is derived from the distinctive odor of affected infants’ urine, often described as a “burnt sugar” smell. If left untreated, the disease can lead to seizures, coma, and early death usually before 3 months of age [14, 19, 25].

In normal individuals, the plasma concentration of BCAAs and other essential amino acids are determined by the rate of appearance from protein containing food and tissue protein turnover minus the rates of disappearance (transamination and oxidation) and protein synthesis (ref). The first step in BCAA metabolism occurs in the mitochondria or cytosol (e.g., neuronal tissues) being catalyzed by the product of either the BCAT2 or BCAT1 genes, respectively. In most peripheral tissues BCAT2 (also known as BCATm for mitochondrial location) is the relevant transaminase. BCAT2 has high enzymatic capacity and catalyzes a reversible step in metabolism. It interconverts leucine, isoleucine and valine to or from their respective keto acids (BCKAs), α-ketoisocaproate (KIC), α-methyl-β-methylvalerate (KMV) and α-ketoisovalerate (KIV), respectively.

The next step in BCAA metabolism is catalyzed by the mitochondrial branched-chain keto acid dehydrogenase (BCKAD) enzyme complex. The BCKAD enzyme complex is associated with the inner mitochondrial membrane and consists of three different catalytic components and two regulatory enzymes. The three catalytic components are known as E1 (branched-chain α-keto acid decarboxylase), E2 (dihydrolipoyl transacylase), and E3 (dihydrolipoyl dehydrogenase); the E1 component consists of two subunits (E1α and E1β) which
form a heterotetramer. The E3 component is not only associated with the BCKAD enzyme complex, but also with the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes [3, 25]. The activities of the enzyme complex itself are regulated by two additional subunits, a kinase and a phosphatase, that reversibly phosphorylate and dephosphorylate, respectively, the BCKAD enzyme complex [3, 19, 25, 29].

This irreversible step in BCAA catabolism is the oxidative decarboxylation of the branched-chain α-keto acid (BCKA) products of the transamination reaction – KIC, KMV, and KIV. The BCAAs/BCKAs are either gluconeogenic (Val/KIV and Ile/KMV) or ketogenic (Leu/KIC and Ile/KMV) precursors. The end products of the reactions catalyzed by the BCKAD enzyme complex are acetoacetate and acetyl-CoA, succinyl-CoA, and acetyl-CoA and succinyl-CoA, from KIC, KIV, and KMV, respectively. Succinyl-CoA enters the citric acid cycle to eventually produce glucose, while acetyl-CoA is eventually converted to acetoacetate. [3, 19, 25, 29].

In individuals with MSUD the BCKAD enzyme complex cannot metabolize BCAA. This causes the branched-chain amino acids to build up in the blood and reach toxic levels. This toxic build-up leads to a variety of serious health problems, including muscular hypo- and hypertonia, cerebral swelling, seizures, coma, and even death [14, 19].

An estimated 1 in 185,000 infants are affected worldwide, although the incidence is much higher in the Old Order Mennonite population – approximately 1 in 176 [3, 14, 19]. As the disease occurs in early infancy, often as soon as the child is fed a meal containing protein, all states within the United States include MSUD testing as part of their newborn screening program. Unfortunately, not many other countries include MSUD as part of their screening programs. As a result, many affected children may die or become severely handicapped before they are diagnosed [14, 25].
History

The first published report of MSUD occurred in 1954 and centered on a family where, over a period of eight years, four of six siblings died within the first three months of life following a rapidly progressive neurologic course. Dr. John Menkes, the lead author on the paper, summarized the clinical signs thusly:

All of these developed cerebral symptoms of progressive severity, commencing within the first week of life, and terminating fatally within three months of their onset. A characteristic feature of their illness was the passage of urine with an odor strikingly similar to that of maple syrup [16].

All of the children enumerated in this paper suffered a similar clinical course that included intermittent tonic seizures, muscle hypertonicity, loss of consciousness, and irregular respiratory patterns. Autopsies were performed on only two of the patients and revealed cerebro-and cardiomegaly and severe aspiration pneumonia, but no other gross or microscopic abnormalities. Although the brains of both infants were larger than normal and grossly edematous, there was no microscopic evidence of what may have caused the clinical signs observed [16].

The most striking feature of these cases was the odor of the urine. Despite the abnormal odor, agreed by all involved to bear a strong resemblance to maple syrup, any routine analysis of the urine was within normal limits. Desperate to find a source for the odor, Menkes and his colleague visited Dr. Louis Fieser, a professor of organic chemistry at Harvard. Menkes describes the interaction:

He was not of much more help, but allowed us to roam through his stock room. Urine bottles in hand, we began to sniff our way through the chemicals. I started at the A’s, Peter Hurst at the Z’s. Not too much later I had what I then thought was the answer: ‘Acid Malic.’ We were dealing with a disorder of the Krebs Cycle, and an enzyme deficiency which … caused malic acid to accumulate, and was responsible for the neurologic symptoms [16].
The urine was subjected to silica gel column chromatography and yielded an organic acid that carried the same maple syrup urine odor, although it had an unknown elution curve. A few months after this, Menkes learned that artificial maple syrup contained a cyclic ketone. Unfortunately it was not until a few years later, in 1957, that Menkes was able to put this knowledge to use by using urine from another affected patient to demonstrate the presence of branched-chain keto acids. Specifically, large amounts of KIC were found in the urine, which is the keto acid of leucine. In addition, smaller amounts of the keto acids of valine (KIV) and isoleucine (KMV) were found. Menkes was on the path to decoding the cause of this disease [15, 16].

It was not until 1960 that Dr. Joseph Dancis, working independently from Menkes, definitively named the cause of maple syrup urine disease as an error in metabolism of BCAAs. Dancis obtained urine and plasma specimens from a patient that had been diagnosed with MSUD by his pediatrician after exhibiting the same neurologic symptoms and unique urine odor as described by Menkes. Analysis of the plasma and urine from this patient showed a considerable increase in BCAA compared with unaffected age-matched children. Dancis concluded that the increased levels of leucine, isoleucine, and valine were due to a block in their metabolic breakdown. Plasma analysis showed that the kidneys were functioning within normal limits, therefore excluding a kidney defect as a cause of the high amino acid levels. It was thought that transaminase, an enzyme that controls the first step in degradation of amino acids, might have been the source of the deficiency. However, tissues collected at autopsy showed normal transaminase activity, leading Dancis and his colleagues to conclude that the block must be at another, latter step in the metabolic pathway [6].

Dancis noted that, while the urine contained higher-than-normal concentrations of BCAA, the relative amounts of the aldehydes and simple amino acids were unchanged. This finding led Dancis to conclude that the metabolic block had to be at a point common to all of the
BCAAs. He theorized that it occurred at an oxidative-decarboxylation step, specifically at the decarboxylation of the branched-chain alpha-keto acids [6].

Dancis continued his work in attempting to discover the specific enzyme or enzymes responsible for the defect in branched-chain amino acid metabolism. Analysis of patient tissues showed that the enzymes necessary for the oxidative decarboxylations of BCAA were present in a wide variety of tissues, including the white blood cell. Working off this knowledge, Dancis developed a method of screening leukocytes for the enzymatic defect. This allowed earlier recognition of affected individuals before they began to show clinical signs. Later on, in 1972, Dancis examined the enzyme activity in skin fibroblasts from recent and past patients and was able to correlate the enzyme activity to the clinical picture. This offered another method by which to study and track the disease progress, as skin fibroblasts were more stable than peripheral blood leukocytes, and could be stored for longer periods of time [4, 5].

The first major breakthrough in treating MSUD was published in 1964 by a team led by Selma Snyderman. By this point it was well known that the underlying problem in MSUD was an inability to metabolize BCAA, resulting in a neurologic disorder stemming from the accumulation of their keto acids in the blood. Realizing this, Snyderman had a simple yet brilliant idea: restrict the intake of BCAA from early infancy [24].

A chance to attempt dietary therapy was offered when a baby girl was born to the family in which MSUD was first characterized. The girl was admitted to the hospital on the 5th day of life, and treatment started on the 17th day of life. Treatment consisted of completely withdrawing the BCAA – leucine, isoleucine, and valine – from the diet, as well as methionine. Within only a few days there was marked clinical improvement in the patient, including resolution of the maple syrup odor. Thereafter, the doctors focused on tailoring her diet based on plasma assays taken at regular intervals [24].
There were many clinical setbacks along the way, including attempts to introduce mother’s milk into the diet that resulted in a complete relapse of MSUD symptoms. In addition, whenever the child became ill such as with a viral or bacterial infection she would suffer a relapse. The diet had to be constantly adjusted, especially as it became apparent that she was losing weight. Eventually the doctors realized that methionine had no bearing on the disease and introduced it back into her diet, resulting in steady weight gain [24].

Despite the success in controlling the disease with dietary therapy, Snyderman and colleagues recognized deficits in growth and mental development. While the child’s weight stabilized and regained a normal developmental curve, her height was seriously affected. In addition, the child’s mental development was characterized as being retarded with difficulties in both gross and fine motor function. Snyderman attributes these deficiencies to starting the therapy too late (on the 17th day of life rather than immediately at admission), or perhaps the total exclusion of methionine for the first few years of life. Examination of other patients placed on dietary trial appears to point to the late start of therapy as a causative factor in the delay of mental development. Patients started much earlier on therapy attained a more normal level of mental development, while others started a few months into life were severely affected [24].

By this point much work had been done regarding the faulty enzymatic degradation of BCAA. Enzyme activity could be measured and even tracked through multiple samples. This led to earlier diagnosis of the disease, allowing life-saving dietary therapy to begin as soon as possible. However, it was not until 1978 that the enzyme complex responsible for the oxidative-decarboxylation of BCAA was purified. Working with bovine kidney mitochondria, Flora Pettit and colleagues set out to purify the responsible enzyme or enzymes and analyze their homogenous preparations in order to demonstrate that a single dehydrogenase oxidized all three BCAAs [20]. Pettit’s work is credited for showing that the branched-chain alpha-keto acid
dehydrogenase (BCKAD) enzyme complex was responsible for BCAA oxidation and the defect of MSUD [3, 20].

**Genetics**

Maple syrup urine disease is an inborn error in metabolism with an autosomal recessive pattern of inheritance. There are four genes that control the formation of the BCKAD enzyme complex and two genes that function in a regulatory capacity; mutations in any of these six genes result in a defective enzyme complex and manifestations of MSUD. MSUD is a “genetically heterogeneous [3]” disease, where multiple mutations lead to variations of the described clinical presentations. Multiple large-scale genetic studies have correlated the percentages of genes affected to clinical presentations [3, 7, 9, 14, 17, 18, 19, 26].

As mentioned earlier, the BCKAD enzyme complex is composed of three different subunits: E1, E2, and E3. The formation of these three subunits is controlled by four distinct genes: *BCKDHA, BCKDHB, DBT,* and *DLD.* *BCKDHA,* is a designation of the “branched chain keto acid dehydrogenase E1, alpha polypeptide” gene. The human gene has been mapped to a location on chromosome 19. *BCKDHA* encodes the E1-alpha subunit protein, without which the BCKD enzyme complex is inactive. More than 40 different mutations in *BCKDHA* have been identified, while only 15 are associated with the severe, “classic” form of the disease. [3, 7, 9, 14, 17, 18, 19, 26].

“Branched chain keto acid dehydrogenase E1, beta polypeptide,” genename: *BCKDHB,* encodes the beta subunit of BCKD E1-subunit complex. The human gene has been mapped to chromosome 6. Like *BCKDHA* more than 40 mutations of *BCKDHB* have been identified, although only 4 of those mutations are associated with severe, “classic” MSUD. Two alpha
subunits and two beta subunits together form a fully functioning E1 subunit. [3, 7, 9, 14, 17, 18, 19, 26].

“Dihydrolipoamide branched chain transacylase E2” – DBT – provides the proteins critical for function of the E2 subunit of the BCKAD complex. Mutations in DBT, 26 of which are known to cause the thiamine-responsive clinical phenotype, are responsible for milder manifestations of the disease but nonetheless still devastating if left untreated. DBT is found on chromosome 1 [3, 7, 9, 14, 17, 18, 19, 26].

The gene responsible for the E3 subunit of the BCKAD enzyme complex is DLD – “dihydrolipoamide dehydrogenase.” Unlike the other three genes, the portion of the enzyme complex DLD encodes is not unique to BCKAD. The E3 subunit is also part of the pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase enzyme complexes. A genetic abnormality in DLD leads to a variant form of MSUD that includes lactic acidosis. As the E3 subunit is required in multiple enzyme complexes, DLD mutation may include other clinical syndromes in addition to MSUD. Only 4 mutations have been linked to clinical disease. DLD is located on chromosome 7 [3, 7, 9, 14, 17, 18, 19, 26].

Two additional regulatory enzymes alternately associate with the BCKAD enzyme complex: BCKD kinase and BCKD phosphatase. The human gene responsible for encoding the BCKD kinase enzyme has been mapped to chromosome 16. Recent work by this laboratory has demonstrated that the mitochondrial matrix resident type 2C phosphatase gene PP2Cm (also known as PPM1K) is the endogenous BCKD phosphatase critical to the nutrient-induced activation of the BCKAD enzyme complex. The human gene is mapped to chromosome 4 and PP2Cm knockout mice exhibit a similar metabolic phenotype as intermittent MSUD patents [13].

As mentioned previously, a mutation in any one of these genes can lead to MSUD. As MSUD is inherited in an autosomal recessive manner, it takes two copies of a defective gene to produce a disease state. However, because there are six different genes involved in encoding the
branched-chain alpha-keto acid dehydrogenase enzyme complex, it is possible for an affected individual to have multiple genetic mutations that still result in MSUD. It is because of this genetic diversity that multiple clinical forms of MSUD are recognized [3, 7, 9, 14, 17, 18, 19, 26].

**Clinical presentation**

There are five recognized clinical presentations of MSUD: Classic, intermediate, intermittent, thiamine-responsive, and E3-deficient. The classic presentation is the most common, as well as one of the most severe. Affected individuals develop initial clinical signs within the first few days of life; these signs include poor feeding, vomiting, poor weight gain, and increasing lethargy. However, breastfeeding rather than formula feeding may delay the onset of these initial symptoms until the second week of life, due to the relatively lower concentration of BCAA in breast milk compared to formula. Rapidly progressive and debilitating neurologic signs follow these initial clinical signs and include alternating muscular hypotonia and hypertonia, dystonia, seizures, and encephalopathy. Without proper and rapid treatment to lower the amount of circulating keto-acids in the blood, the neurologic signs can and often do lead to coma and death [2, 14, 17, 18, 19].

Intermediate MSUD occurs less frequently than classic MSUD. Patients affected by this clinical syndrome typically have higher residual activity of BCKAD, which means they may present at any age. Intermediate MSUD patients typically present with neurologic impairment, developmental delay, and seizures [2, 14, 17, 18, 19].

Intermittent MSUD is the second-most common manifestation of MSUD. However, patients usually develop with normal growth and intelligence and do not present until times of catabolic stress, such as during illness. During these times of illness, symptoms are similar to those of classic MSUD, including ataxia, lethargy, seizures, and coma. Unfortunately, patients
with this form of MSUD have died during these episodes due to inadequate and inappropriate
treatment, usually because the diagnosis of MSUD has not been made [14, 17, 18, 19].

Thiamine-responsive MSUD is a very rare form of the disease; so far, only the initial
patient described by Scriver in 1971 has been shown to be responsive to the addition of thiamine
to their diet. Since then, the addition of thiamine has become a staple of dietary management of
MSUD, and some patients have regained marginal metabolic control while on it [14, 17, 18, 19].

The final form of MSUD is E3-deficient, another rare form. Patients with E3-deficient
MSUD have combined deficiencies of the BCKAD, pyruvate, and alpha-ketoglutarate
dehydrogenase complexes, resulting in a form of MSUD nearly indistinguishable from
intermediate MSUD. The only differing factor is that E3-deficient patients suffer from lactic
acidosis [14, 17, 18, 19].

**Treatment and management**

Although there are five recognized forms of MSUD, management of these patients is
basically dietary. Once someone is diagnosed with maple syrup urine disease, they are
immediately placed on a low-protein diet that restricts the amount of BCAA leucine, isoleucine,
and valine. It is a delicate balance to restrict the intake of these essential amino acids without
impairing growth and intellectual development of the individual. In addition, as no two
individuals are alike, diets frequently must be tailored to the patient. The amount of leucine one
takes in must be carefully regulated based on plasma measurements of BCAA. Also, thiamine is
often given for a few weeks to determine if the patient is responsive [14, 19].

There are two basic aims of treatment: long-term management and management of acute
crises. As stated before, long-term management is based on dietary restriction of BCAA. In
episodes of acute metabolic crises, an intravenous infusion of glucose is given to control
excessive catabolism of proteins. It is important that the glucose solution is made with NaCl, as glucose rapidly enters the brain and pulls water from the vascular space into the central nervous system unless balanced by sodium. Use of a glucose-only solution causes a rapid influx of fluid and can cause a severe and life-threatening cerebral edema [3, 14, 19].

During the acute crisis, complete cessation of BCAA intake must be practiced, as well as providing additional dietary support with lipids and/or formulas free of BCAA. Intake of BCAA, under strict supervision, can resume once plasma levels normalize. In rare instances, hemodialysis or peritoneal dialysis may be necessary [14].

Intensive nutritional interventions are generally effective, especially in the long-term. However, maintaining circulating BCAA levels at or below normal plasma concentrations is frequently difficult, especially during times of increased protein catabolism due to stress or infection. This increased efflux of BCAA and branched-chain keto acids accelerates the rate of progressive and irreversible neurological damage [14].

Studies on human tissues have shown that the liver has a high specific activity for branched-chain amino acid oxidation. Although BCKAD is found in a wide variety of tissues, in many of these the enzyme is less active than in liver because of the degree of inactivating phosphorylation. Several reports in the literature detailing orthotopic liver transplants in children and adults have reported success in controlling plasma BCAA levels to the level of patients with a very mild variant of MSUD, despite non-restrictive diets. However, it is important to note that liver transplantation is a major operative procedure that carries a high surgical risk, as well as financial cost that is prohibitive for most. Death is always a possibility as with any major operation, and the post-transplant regimen of immunosuppressive drugs may be just as expensive if not more so as the typical MSUD patient diet. In addition, the model end-stage liver disease and pediatric end-stage liver disease (MELD and PELD, respectively) scores for MSUD patients
may not necessarily allow them to be competitive for liver transplant qualification outside of a domino transplant or clinical trial experiment [3, 20, 25, 27].

Another potential option has been proposed: adipose tissue transplantation. Adipose tissue has a high capacity for catalyzing BCAA metabolism. In addition, adipose tissue has regenerative precursor cells and low oxygen requirements, as well as ability to self-vascularize. It has been widely used in reconstructive surgery, and donors are generally easier to find and usually quite willing to have some of their fat removed. The surgery for both the donor and recipient is far less complicated than that for a liver or any other organ transplant, and can be accomplished with first year costs 10-20 times less than that of a liver transplant. Preliminary studies in a mouse model of MSUD have shown that transplantation of only 0.75 g of adipose tissue from wild type (unaffected) animals into animals affected with MSUD reduced the circulating plasma BCAA levels by half [8, 10, 11, 12].

Our hypothesis is that elective adipose organ transplant can be used for treatment of MSUD. In order to prove this, we used a knockout mouse model of intermittent MSUD and transplanted white adipose tissue from wild type littermates into their abdomens. We also transplanted wild type adipose tissue subcutaneously into a mutant mouse model of classic MSUD. Animals with wild type fat, as well as control groups that received adipose tissue from knockout (affected) littermates or a sham surgery, were challenged with a variety of diets including a diet free of BCAA, a diet with reduced BCAA levels, a normal commercial diet, and a high protein diet. These animals were evaluated using H1-NMR spectroscopy for body composition, as well as plasma concentrations of BCAA. Our results show that the abdominal method of adipose tissue transplantation does not significantly reduce circulating plasma BCAA levels in a mouse model of intermittent MSUD. However, subcutaneous transplantation of wild type adipose tissue into a mutant model of classic MSUD does significantly reduce the circulating
plasma BCAA levels, leading us to believe that this may be a viable treatment option for maple syrup urine disease.
Chapter 2

Materials and Methods

All procedures described herein have been conducted after review and approval by the Penn State Hershey Institutional Animal Care and Use Committee (IACUC). The Animal Resource Program, operated by the Department of Comparative Medicine is accredited by AAALAC International. All animal living conditions are consistent with standards laid forth in the *Guide for the Care and Use of Laboratory Animals*, 8th edition.

Animals

Animals were housed conventionally in open-top, solid-bottom polycarbonate cages (Max75, Alternative Design, Siloam Springs, AR) with wire bar lids and corncob bedding (Teklad 7097 Corn Cob Bedding, Harlan, Frederick, MD). Lighting was controlled with a 12 h:12 h light:dark cycle with no twilight with lights on at 0700 h and off at 1900 h. Humidity was maintained between 30% and 70%, and the temperature set between 70-73°F. Municipal tap water and standard rodent chow (Teklad 2018 Global 18% Protein Rodent Diet, Harlan, Frederick, MD) were available *ad libitum* unless otherwise noted for study purposes.

PP2Cm mouse model

The creation of the PP2Cm knockout mouse model (B6.129Sv-*Ppm1k*<sup>lmiYwa</sup>/Clyn) for intermittent MSUD is detailed elsewhere [13]. In brief, these mice were generated via homologous recombination in ES cells by inserting a LacZ-expressing cassette that replaced the
coding sequence of exon 2. The lack of protein expression was confirmed by western blot analysis on heart extract.

Animals for this project were generated through trio matings with a male and two females, each heterozygous for the PP2Cm mutation. Females were removed from the breeding cage when observed to be pregnant (approximately 10 dpc) and replaced with another heterozygous female. The date of birth and number of pups born to each mating was recorded. Pups were weaned at 21 days and genotyped via polymerase chain reaction (PCR) using an ear biopsy piece. Once a sufficient number of knockout animals had been generated, knockout males were placed in stable monogamous pairs with heterozygous females to take advantage of post-partum estrus. The date of birth and number of pups born to each pair was recorded. Pups remained with their parents until weaning at 21 days of age, but were removed sooner if the dam gave birth to another litter. Pups were genotyped via PCR using an ear biopsy piece.

Ear biopsies were placed in -20°C for at least one hour or overnight to chill them. For DNA extraction, 300 μl of 0.05N NaOH was added to each tube and the tubes heated on a heat block for 14 minutes at 100°C. Once heated, the tubes were vortexed until the ear piece turned white – usually 2 - 3 minutes. Following this 25 μl of 1M Tris HCl (pH 8.0) was added to each tube, the tube vortexed again, then the prepared DNA stored at 4°C until used.

PCR primers used were mPP2Ck forward primer (5′ CCC ATC CTT AGG AGA GGT CG 3′), E2 Rev WT primer (5′ CAG CAG AAT TGG CTC ATC AA 3′), and nLacGL Rev KO primer (5′ ATG GTG GAT CCT GAG ACT GG 3′). The PCR program used was: 1) 94°C for 3 min, 2) 94°C for 30 sec, 3) 60°C for 30 sec, 4) 72°C for 1 min, 5) repeat steps 2 - 4 for 35 cycles, 6) 72°C for 10 min, 7) 4°C hold. A 1.5% agarose gel with 1.5 µl of Ethidium bromide added prior to setting was used to read the PCR results. Each well was loaded with 11 µl of the reaction mixture (23 µl of master mix plus 2 µl of DNA) and the gel run at 70 volts. Products expected were a 900 bp WT (+) band and/or a 700 bp KO (-) band.
The creation of the BCATm knockout mouse model (B6.C-Bcat<sup>2<sup>tm1.2Clyn</sup>Clyn</sup>) is detailed elsewhere [22]. Briefly, conditional and total null alleles were generated using the Cre-<sup>loxP</sup> system of targeted mutation. Although this mouse model does express increased plasma levels of BCAA, there are no recorded human cases of MSUD resulting from a mutation in mitochondrial branched-chain aminotransferase (BCATm) [23].

BCATm knockout animals were generated through a trio mating scheme where two heterozygote females were paired with a heterozygote male, similar to the procedure discussed above for PP2Cm animals. Pups were genotyped by PCR using an ear biopsy piece, identical to the procedure discussed above for PP2Cm animals.

PCR primers used were BCAT primer 1 (5’ GTT CTC AAG GTG GTG GGT GT 3’), BCAT primer 4 (5’ TCC CTG GTG CCT GAG ACT AAA 3’), and BCAT primer 5 (5’ AGA AGC CAC AGG GGA AAT GT 3’). The PCR protocol used was: 1) 95°C for 5 min, 2) 95°C for 45 sec, 3) 65°C for 45 sec (-0.5°C per cycle), 4) 72°C for 1 min, 5) repeat steps 2 – 4 for 30 cycles, 6) 72°C for 5 min, 7) 4°C hold. A 1.2% agarose gel with 1.5 µl of Ethidium bromide added prior to setting was used to read the PCR results. Each well was loaded with 11 µl of the reaction mixture (23 µl of master mix plus 2 µl of DNA) and the gel run at 70 volts. Products
expected were a 534 bp WT (+) band for primers 1 and 5, and/or an approximately 900 bp KO (-) band for primers 4 and 5.

Figure 2-2. Representative PCR gel results, BCATm. Image was pasted together to show DNA ladder in conjunction with DNA bands.

**Plasma circulating BCAA levels and body mass measurements**

Plasma circulating BCAA levels were assessed prior to the start of study, one week post-surgery, and when the diet was switched. Animals were fasted for approximately two hours before collection via the facial vein. A 5mm Goldenrod animal lancet was used (MEDIpoint, Inc., Mineola, NY) and approximately 100 to 200 μl of blood collected in a lithium-heparinized capillary collection tube (CB 300 LH, Microvette®, SARSTEDT INC., Newton, NC). The animal was given 0.5 cc of warmed normal saline subcutaneously before being returned to its cage. Animals were closely observed for at least 15 minutes following blood collection to assess any adverse effects.

Plasma levels of BCAA were analyzed using a spectrophotometric assay previously described [1]. Standards and samples were plated in duplicate on a 96-well plate and read twice on a spectrophotometer at a wavelength of 340 nm, the second reading thirty minutes after the addition of 10 μl of leucine dehydrogenase to each well. Values were entered into an Excel spreadsheet for analysis and a standard curve prepared from the absorbance values of the standards. To determine the BCAA plasma levels, unknown sample values were fitted to the
standard curve. Animals whose BCAA levels were consistently lower than 1000 μM (i.e., equal to wild type controls) were excluded from study.

Prior to the start of study, one week post-surgery, and when the diet was switched the body composition of each animal was assessed using proton nuclear magnetic resonance (H1-NMR) [21]. Animals were weighed and placed within a sample holder (animal restrainer) with adequate ventilation holes. The animal’s movement was limited via a restraining disk, adjusted so the animal could breathe freely but not turn around. The sample holder was placed in the machine (LF90, Bruker Optics, Billerica, MA; Figure 2-2), the animal’s weight entered, and the program started. Each measurement took no more than 2 minutes, after which the animal was returned to its home cage with no adverse effects. Values for fat, lean body mass, and free body fluid were displayed in grams and percentages of body weight and recorded for each animal.

Figure 2-3. H1-NMR analysis. Pictorial representation of the Bruker LF90 minispec (A). The mouse is held securely within the animal restrainer (B). A disassembled animal restrainer (C).
Surgery

All surgical procedures were performed in a dedicated surgical room. Animals were placed upon a warmed heating pad (SnuggleSafe®) during the surgical procedure. Donor animals (wild type littermates of knockouts, both males and females) were placed in an induction chamber with 4 - 5% isoflurane in 1L O₂. Once the mice were deeply anesthetized (i.e., did not react to a toe-pincho), the mice were removed and euthanized via cervical dislocation. The abdomen was shaved and cleaned with two alternating scrubs of povidone-iodine (Betadine®) and 70% isopropyl alcohol. Following the last alcohol rinse, iodine was painted onto the abdomen and remained on for the procedure. The abdomen was opened from xyphoid to pubis using autoclaved iris scissors and perigonadal and perirenal fat harvested. The fat was originally placed in an autoclaved metal cup containing approximately 3.0 ml of sterile saline and weighed; the surgical procedure was later amended to where the adipose tissue was placed within a covered Petri dish with sterile saline before transplant to reduce the occurrence of surgical site abscesses. For PP2Cm surgeries approximately 1.0 g of fat was harvested per transfer, while BCATm animals received 2.0 g of fat per transfer.

Recipient knockout animals were placed in an induction chamber with 2% isoflurane in 1L O₂. Once the mouse lost its righting reflex (i.e., did not right itself when placed on its side), the mouse was removed from the chamber and attached to a nose cone with 2% isoflurane in 1L O₂. The eyes were lubricated with a sterile ophthalmic ointment. The abdomen was shaved from xyphoid process to pubis and cleaned with two alternating scrubs of povidone-iodine (Betadine®) and 70% isopropyl alcohol. Following the last alcohol rinse, iodine was painted onto the abdomen and remained on for the procedure. BCATm recipient animals had a small (2.5 x 1.0 cm) area on the dorsum prepared in an identical manner, in addition to the abdominal site.
A separate sterile pack was used for the recipient transfer surgeries. The initial incision was made on midline then the skin bluntly dissected from the underlying musculature with a pair of iris scissors. A stab incision through the musculature was made on midline, then the incision widened by opening the scissors. Prior to removal of the fat, a few drops (less than 0.05 ml) of a 50:50 bupivicaine:lidocaine mix was introduced onto the incision. The gonads were exteriorized and the surrounding adipose tissue removed using both blunt and sharp dissection. Excess bleeding was controlled via pressure hemostasis or tying off bleeding vessels with absorbable suture. The removed adipose tissue was placed on a moistened sterile surgical drape to be used in the knockout-to-knockout transfer surgeries (PP2Cm animals only).

For both PP2Cm and BCATm animals, 1.0 g of the harvested fat was cut into smaller pieces and introduced into the abdominal cavity. The abdominal wall was closed with two to three interrupted sutures of 4-0 polydioxanone absorbable suture (PDS II, Ethicon). The skin was closed in a continuous pattern with 4-0 PDS.

BCATm mice were laid in sternal recumbency and an incision made into the skin of the dorsum with a pair of iris scissors; the skin was bluntly dissected away from the underlying musculature, creating a large subcutaneous pocket. Approximately 1.0 g of harvested wild type adipose tissue was cut into smaller pieces and introduced into the subcutaneous pocket. Prior to closure, a few drops (less than 0.05 ml) of a 50:50 bupivicaine:lidocaine mix was instilled into the incision. The skin was closed in a continuous pattern with 4-0 PDS.

Isoflurane was discontinued and the mouse was allowed to breathe room air. Buprenorphine (0.05 mg/kg, Buprenex) in 1.0 ml of warmed saline was given subcutaneously.

The mouse was returned to its home cage, then the cage placed upon a temperature-controlled heating pad (HotDog® patient warming system) set to 40°C. Mice were returned to their room in the animal facility once all animals moved around the cage reliably and did not exhibit lasting effects from the anesthesia.
As a general rule, mice receiving wild type fat were operated on first, and then the
removed perigonadal fat used in the remaining knockout-to-knockout control surgeries in the
PP2Cm model. BCATm control animals underwent a sham surgery, where the abdomen and
dorsal skin were opened and closed in the same fashion as the animals that received wild type
adipose tissue.

**Diet manipulation**

All animals on study were weighed and their food intake measured for the first three to
five days following surgery and any time the diet was changed, then weekly thereafter. Animals
were maintained starting at weaning on a mixed diet of Teklad 18% protein rodent chow and a
custom diet of BCAA-free pellets isocaloric to the 18% chow (DYET 510081, Dyets, Inc.,
Bethlehem, PA). Following PCR results, only knockout animals were maintained on the mixed
diet (hereafter referred to as “Diet 1”); all others (heterozygous and wild type) were maintained
on the Teklad 18% rodent diet.

**PP2Cm diet manipulation**

The first surgical group was placed on the BCAA-free diet alone for the week prior to
and the week following surgery. While on this diet, animals lost an unacceptable amount of
weight and so the practice was discontinued. This group appeared to suffer no adverse effects
and rapidly gained weight on Diet 1. However, while on Diet 1, they preferentially ate only the
18% chow and did not eat the BCAA-free pellets. Therefore, they remained on this diet for only
one week before being moved to a total 18% chow diet. This group remained on the 18% chow
diet for two weeks before being placed on a specialty diet with 50% protein (TD.94266, Harlan Teklad Research Diet, Harlan) until the end of the study.

Cohort 1 Diet Manipulation Timeline, PP2Cm

- BCAA-free diet
- Diet 1 (Choice of BCAA-free and 18% protein rodent chow)
- 18% protein diet
- 50% protein diet

Figure 2-4. Cohort 1 diet manipulation timeline, PP2Cm.

The second surgical group was placed on the BCAA-free diet for the week prior to surgery, then immediately moved to the 18% rodent chow the day of surgery. This group was maintained on the 18% diet for approximately 2 weeks, and then placed on the 50% protein diet for the remainder of the study.
The diet manipulation for the final group reflects refinements made as a result of the outcomes of the initial groups. Therefore, Group 3 was moved from the Mix diet immediately to the 50% protein diet on the day of surgery. They were maintained on the 50% protein diet until the end of the study.
A group of gender-matched control wild type animals underwent the same diet manipulations as a part of Group 3.

**BCATm diet manipulation**

BCATm knockout animals were maintained on an *ad libitum* diet consisting of a mixture of BCAA-free pellets and standard 18% protein rodent chow (Diet 1) prior to surgery. On the day of surgery, all animals were switched to a measured mixture of the BCAA-free pellets and a modified MSUD pelleted diet (DYET 510090, Dyets, Inc., Bethlehem, PA) containing decreased and defined amounts of the branched-chain amino acids. Mice remained on this diet (Diet 2) for two weeks from the date of surgery, and then were returned to a measured Diet 1 for one week. The mice were then switched to the 18% protein standard rodent diet for three days, before being
placed back on the *ad libitum* Diet 1 until time of euthanasia and surgical site examination. A group of WT females were placed on the same diet regimen in order to ascertain circulating plasma BCAA levels in a wild type animal on each of the different diets.

**BCATm Diet Manipulation Timeline**

<table>
<thead>
<tr>
<th>Diet 1 (Choice of BCAA-free and 18% protein rodent diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet 2 (Choice of BCAA-free and BCAA containing amino acid purified diet)</td>
</tr>
<tr>
<td>18% protein rodent chow</td>
</tr>
</tbody>
</table>

Figure 2-7. BCATm diet manipulation timeline.

**Surgical site examination**

Animals were euthanized by CO\(_2\) asphyxiation followed by cervical dislocation and the abdominal surgical site examined grossly for signs of integration of the transplant (i.e., vascularization, visceral attachment). Four animals (one each of male and female WT and KO fat recipients) were subjected to whole body perfusion of saline followed by toluidine blue to examine vascular patterns of the transplantation. One animal (female, KO fat) underwent histological examination of the transplant site.
Statistical analysis

Data was analyzed using GraphPad Prism statistical software. Results were calculated using a paired t-test, with transplanted fat type (wild type vs. knockout) and plasma BCAA levels across the different diets analyzed. Pre- and post-surgical body composition (percent fat and lean mass) and pre- and post-diet manipulation weights were also analyzed. Significance was set at $p \leq 0.05$. 
Chapter 3

Results

Two colony animals were used as sentinels to determine health status of the colony as a whole. Animals were free of external and internal parasites, and were seronegative for the following: Minute virus of mice, generic parvovirus, mouse parvovirus, Theiler’s murine encephalitis virus, epizootic diarrhea of infant mice, Sendai virus, Mycoplasma pulmonis, pneumonia virus of mice, reovirus, lymphocytic choriomeningitis virus, ectromelia virus, mouse adenovirus 1 and 2, polyoma virus, Encephalitozoon cuniculi, cilia-associated respiratory bacillus, Clostridium piliforme, and mouse cytomegalovirus. Sentinel animals were seropositive for mouse hepatitis virus and mouse norovirus. Animals were also positive for Helicobacter ganmani via PCR profile of fecal pellets.

All animals were test-naïve at the start of the study. Prior to surgery, knockout animals had blood drawn following a two-hour fast to determine circulating BCAA levels. These results were compared to wild type colony animals also following a two-hour fast. Animals whose BCAA levels were consistently lower than 1000 μM (i.e., equal to wild type controls) were excluded from study. Animals were randomly assigned to surgical treatment with the goal of maintaining roughly equal numbers of males and females in each treatment group.
Figure 3-1. Pre-surgical plasma BCAA concentration. Values for both PP2Cm (A) and BCATm animals (B) are represented here.

**PP2Cm**

Tables 3-1 through 3-3 show a brief overview of the animals on study arranged by diet group and include weights at the start and end of study, as well as make note of any animal that was removed from study early. All animals spent roughly six weeks on study from start to finish.

Table 3-1. Cohort 1 characteristics, PP2Cm.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Treatment (fat type)</th>
<th>Start weight (g)</th>
<th>End weight (g)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td>F</td>
<td>WT</td>
<td>14.6</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>162</td>
<td>M</td>
<td>WT</td>
<td>20.0</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>172</td>
<td>F</td>
<td>WT</td>
<td>14.1</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>177</td>
<td>M</td>
<td>KO</td>
<td>18.3</td>
<td>25.3</td>
<td>Sentinel animal</td>
</tr>
<tr>
<td>167</td>
<td>F</td>
<td>KO</td>
<td>16.6</td>
<td>21.8</td>
<td>Sentinel animal</td>
</tr>
<tr>
<td>155</td>
<td>M</td>
<td>WT</td>
<td>14.5</td>
<td>23.2</td>
<td></td>
</tr>
<tr>
<td>158</td>
<td>F</td>
<td>WT</td>
<td>17.6</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>Gender</td>
<td>Treatment (fat type)</td>
<td>Start weight (g)</td>
<td>End weight (g)</td>
<td>Comments</td>
</tr>
<tr>
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<td>------------------</td>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>119</td>
<td>M</td>
<td>WT</td>
<td>25.4</td>
<td>--</td>
<td>Euthanized due to declining health; large abscess in abdomen</td>
</tr>
<tr>
<td>159</td>
<td>F</td>
<td>KO</td>
<td>17.0</td>
<td>21.8</td>
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<tr>
<td>120</td>
<td>M</td>
<td>KO</td>
<td>22.3</td>
<td>26.5</td>
<td></td>
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</tbody>
</table>

Table 3-2. Cohort 2 characteristics, PP2Cm.

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<th>ID</th>
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<th>End weight (g)</th>
<th>Comments</th>
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<tr>
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<td>--</td>
<td>24.6</td>
<td>--</td>
<td>Died under anesthesia</td>
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<tr>
<td>149</td>
<td>F</td>
<td>WT</td>
<td>20.2</td>
<td>23.5</td>
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<tr>
<td>152</td>
<td>F</td>
<td>WT</td>
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<td></td>
</tr>
<tr>
<td>139</td>
<td>M</td>
<td>KO</td>
<td>23.9</td>
<td>20.5</td>
<td></td>
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<td>18.5</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>M</td>
<td>WT</td>
<td>28.4</td>
<td>28.3</td>
<td></td>
</tr>
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<tr>
<td>136</td>
<td>F</td>
<td>--</td>
<td>20.1</td>
<td>--</td>
<td>Died under anesthesia</td>
</tr>
</tbody>
</table>

Table 3-3. Cohort 3 characteristics, PP2Cm.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Treatment (fat type)</th>
<th>Start weight (g)</th>
<th>End weight (g)</th>
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<td>WT</td>
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<td>147</td>
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<td>WT</td>
<td>29.7</td>
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</tr>
<tr>
<td>132</td>
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<td>19.7</td>
<td></td>
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<td>WT</td>
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<tr>
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<td>F</td>
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<td>22.4</td>
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<td>94</td>
<td>M</td>
<td>KO</td>
<td>29.8</td>
<td>29.3</td>
<td></td>
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<tr>
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<td>M</td>
<td>KO</td>
<td>31.3</td>
<td>29.8</td>
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<tr>
<td>640</td>
<td>M</td>
<td>KO</td>
<td>36.2</td>
<td>32.8</td>
<td></td>
</tr>
<tr>
<td>CM1</td>
<td>M</td>
<td>--</td>
<td>36.9</td>
<td>36.3</td>
<td>Non-surgical control animal; diet manipulation only</td>
</tr>
<tr>
<td>CM2</td>
<td>M</td>
<td>--</td>
<td>32.5</td>
<td>32.8</td>
<td>Non-surgical control animal; diet manipulation only</td>
</tr>
</tbody>
</table>
Prior to undergoing surgery male and female PP2Cm knockout animals were placed on an 18% protein rodent diet (Harlan) for several days. The knockout animals, along with male and female wild type littermates, were fasted for two hours before having blood drawn for plasma BCAA levels. Animals whose plasma BCAA levels were less than 1000 µM were excluded from the study and did not undergo adipose tissue transplantation. Final study populations analyzed were: animals receiving WT fat (n=18), animals receiving KO fat (n=13), and control animals undergoing diet manipulation only (n=6).

Blood was drawn for plasma BCAA concentration every time the diet was changed. No significant differences were seen between animals receiving wild type and those reciving knockout adipose tissue on any of the diets, although only data from the 50% protein diet is shown. No significant differences were seen between manipulated animals and wild type control littermates, although plasma BCAA concentration was lower in the wild type littermates than those of the PP2Cm knockouts. This lack of a significant difference was maintained once the genders were separated out.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
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<tbody>
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<td>CM3</td>
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<td>24.7</td>
</tr>
<tr>
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<td>F</td>
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<td>24.6</td>
</tr>
<tr>
<td>CF3</td>
<td>F</td>
<td>--</td>
<td>25.5</td>
</tr>
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</table>
Figure 3-2. BCAA plasma concentration levels, PP2Cm. Bars indicate the mean ± SEM for each of the groups: animals receiving WT fat (n=18, Males n=8, Females n=10), animals receiving KO fat (n=13, Males n=7, Females n=6), and control animals undergoing diet manipulation only (n=6, Males n=3, Females n=3).

Body weights of PP2Cm and wild type control animals did not differ significantly pre-surgery, and body weight gain was steady and did not differ significantly between animals receiving WT or KO fat, or between the manipulated animals and their wild type controls. Males weighed more than females, but not significantly so. Weight was only lost in Groups 1 and 2 while on the BCAA-free diet (data not shown); Group 3 did not receive the BCAA-free diet. Only data from the 50% protein diet is shown.
Figure 3-3. Change of body weight in PP2Cm KO mice on 50% high protein diet. Data shown represent the mean ± SEM for males receiving WT fat (n=8), females receiving WT fat (n=10), males receiving KO fat (n=7), and females receiving KO fat (n=6).

H1-NMR body mass spectrophotometry was performed on all animals prior to surgery and any time the diet was changed. Percent fat and percent lean body mass did not differ significantly between male and female animals prior to surgery. Following adipose tissue transplant body mass remained roughly similar across all genders and surgical manipulations for all diets; only data from the 50% protein diet is shown.
Figure 3-4. Pre- and post-surgical H1-NMR body composition. Bars indicate the mean ± SEM for each of the groups: pre-surgical male KO animals (n=15), pre-surgical female KO animals (n=16). Post-surgical groups on the 50% protein diet are animals receiving WT fat (n=18, Males n=8, Females n=10), and animals receiving KO fat (n=13, Males n=7, Females n=6).
Food consumption was tracked for the first three to five days following the diet change. Although only data from the 50% high protein diet is shown, there were no significant differences in food consumed across any of the diet groups.

![Food Intake on 50% High Protein Diet, PP2Cm](image)

Figure 3-5. Food consumption, 50% high protein diet, PP2Cm.

![Gross examination following whole-body perfusion with toluidine blue dye, PP2Cm](image)

Figure 3-6. Gross examination following whole-body perfusion with toluidine blue dye, PP2Cm Cohorts 1 and 2. Arrows indicate transplanted fat (block arrows) and re-growth of native fat (line arrows). Animals represent males that received KO (A) and WT (B) fat.
All animals in Groups 1 and 2 exhibited abscessation of the transplanted fat with regrowth of native perigonadal adipose tissue. The transplanted fat was a distinct buttery yellow color and contained a creamy yellow purulent material that grew *Klebsiella pneumonia* and *Pasteurella multocida* on aerobic culture. Cytology of the abscess contained a brisk, suppurative response with numerous bacteria of at least three different morphologies. Animals in Group 3 did not exhibit abscessation of the surgical site, but the transplanted fat was non-vascular, firm, and often found freely floating in the abdomen.

Figure 3-7. Gross examination following whole-body perfusion with toluidine blue dye, PP2Cm Cohort 3 animal. The transplanted fat (white arrow) was found freely floating in the abdomen. Images are of transplanted tissue *in situ* (A), and with the transplanted tissue removed (B).

On histology adipocytes of WT animals are morphologically indistinguishable from adipocytes of KO animals; therefore, it was difficult to definitively distinguish between transplanted and native adipose tissue. However, it was easy to see the difference between dead (assumed) transplanted tissue and living, native adipose tissue. The body was mounting a bland
granulomatous immune response against the presumed transplanted fat, which was necrotic and non-viable. No viable angiogensis was present within the histologic samples.

Figure 3-8. Histopathology image of transplanted tissue. Abdominal fat transplant showing coagulative necrosis at top and an encapsulated bland granulomatous response at the bottom of the figure.

**BCATm**

Table 3-4 shows a brief overview of the animals on study and includes weights at the start and end of study, as well as makes note of any animal that was removed from study early. All animals spent roughly six weeks on study from start to finish.
Table 3-4. BCATm study animal population.

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Prior to undergoing surgery male and female BCATm knockout animals were placed on an 18% protein rodent diet (Harlan) overnight. The knockout animals, along with male and female wild type littermates, were fasted for two hours before having blood drawn for plasma BCAA levels. Animals whose plasma BCAA levels were less than 1000 µM were excluded from the study and did not undergo adipose tissue transplantation. Final study populations analyzed were: animals receiving WT fat (n=10), animals undergoing sham surgery (n=9), and control animals undergoing diet manipulation only (n=5).

Blood was drawn for plasma BCAA concentration every time the diet was changed. Both animals receiving WT fat and those undergoing Sham surgeries had circulating plasma BCAA levels significantly different ($p = 0.0020$ and $p = 0.0005$, respectively) than those of the
Control animals. Male animals that received WT fat had circulating plasma BCAA levels significantly different ($p = 0.0032$) from Sham males while on the mixture of the BCAA-free and the modified MSUD diet pellets, although there were no significant differences between females receiving WT fat and females undergoing Sham surgeries ($p = 0.1306$). Plasma BCAA levels differed significantly between both males and females with WT fat compared to their Sham counterparts while on the mixed BCAA-free and 18% protein pelleted diet ($p = 0.0100$ and $p = 0.0107$, respectively), as well as while on the 18% protein diet alone ($p = 0.0169$ male WT vs male Sham; $p = 0.0029$ female WT vs female Sham).
Figure 3-9. BCAA plasma concentration levels, BCATm. Bars represent mean ± SEM, while asterisks (*) represent significantly different values ($p < 0.05$) from their counterparts. Males receiving WT fat (n=5) were compared to males undergoing Sham surgeries (n=4), while the females were compared similarly (n=5 and n=5, respectively).
Body weights of BCATm and wild type control animals did not differ significantly pre-surgery, and body weight gain was steady and did not differ significantly between animals receiving WT fat or undergoing Sham surgery, or between the manipulated animals and their wild type controls. Males weighed more than females, but not significantly so. Data from all three diets is shown.

Figure 3-10. Change of body weight in BCATm knockout mice. Data shown represent the mean ± SEM for males receiving WT fat (n=5), females receiving WT fat (n=5), males undergoing Sham surgery (n=4), and females undergoing Sham surgery (n=5).

H1-NMR body mass spectrophotometry was performed on all animals prior to surgery and any time the diet was changed. Percent fat and percent lean body mass did not differ
significantly between male and female BCATm knockout animals prior to surgery. Following WT adipose tissue transplant or Sham surgery body mass remained roughly similar across all genders and surgical manipulations for all diets; pre-surgical and post-surgical data for all three diets is shown.

Figure 3-11. Pre- and post-surgical H1-NMR body composition, BCATm. Bars indicate the mean ± SEM for each of the groups: pre-surgical male KO animals (n=10), pre-surgical female KO animals (n=10). Post-surgical groups on all three diets include animals receiving WT fat (n=10, Males n=5, Females n=5), and animals undergoing Sham surgery only (n=9, Males n=4, Females n=5). Figures included are pre-surgical body composition (A), body composition on the mixed BCAA-free and modified MSUD diet (B), body composition on the mixed BCAA-free and 18% protein diet (C), and body composition on the 18% protein diet (D).
The amount of food consumed was recorded for three to five days following surgery and whenever the diet was changed. The BCAA-free diet contained no branched-chain amino acids (leucine, isoleucine, and valine), while the modified MSUD diet with defined and decreased BCAA levels contained 2.5% BCAA and the 18% protein rodent chow contained 3.5% BCAA. The amount of BCAA for each animal was calculated and graphed; no significant differences were found in BCAA consumption between the four treatment groups.

Figure 3-12. BCAA intake, BCATm KO mice.
The appearance of the transplanted abdominal fat was similar to that of the PP2Cm animals – the fat was firm, non-vascular, a distinct buttery-yellow color, and was often found freely floating in the abdomen. The subcutaneous fat, however, differed in appearance from a soft, semi-liquid mass with areas of dark necrosis, to having been nearly completely resorbed by the body.

Figure 3-13. Gross examination following whole-body perfusion with toluidine blue dye, BCATm. Images represent the various states in which the subcutaneous transplanted adipose tissue was found: necrotic and semi-liquid (A); solid and a healthy, lighter color with evidence of vascular ingrowth (B); and nearly complete resorption by the body (C). White block arrows indicate the transplanted abdominal tissue.

Due to time constraints, histologic examination of the transplanted WT tissue into the subcutaneous and intra-abdominal spaces of male and female BCATm knockout animals was not performed.
Chapter 4

Discussion

No significant differences were seen in circulating plasma BCAA levels between PP2Cm animals receiving wild type and those receiving knockout fat in the intra-abdominal space when challenged with any of the diets. This was likely due to the fact that the transplanted adipose tissue did not survive. Although adipose tissue has a great capacity for angiogenesis, it needs vessel-to-vessel contact to encourage growth [12]. Straight transplantation into the abdomen does not allow for this. The differences seen in circulating plasma BCAA levels in BCATm animals receiving subcutaneous transplantation of wild type adipose tissue is likely due to the fact that the tissue was able to survive and vascularize, suggesting this approach may be a more viable option for future surgeries. Histologic examination of the transplant site will be needed to determine whether the tissue is viable in this location, as gross examination suggests.

As stated previously, although adipose tissue does have a great capacity for angiogenesis it needs contact with vasculature to promote vascular growth. To encourage this contact, we have begun transplanting harvested adipose tissue into the subcutaneous space on the dorsum of experimental animals. Gross examination of the transplanted fat shows a healthier and more viable tissue when compared to the abdominally transplanted fat. Vascular ingrowth from the subcutis to the adipose tissue transplant is evident grossly. Histologic examination of these transplant sites has not been completed at the time of this writing, but based on preliminary data discussed below we feel this is a more viable alternative than the abdominal transplantation.

The polymicrobial nature of the abscessation noted in the PP2Cm surgeries points to a break in aseptic technique. Although the transplanted tissue was stored within an autoclaved container with sterile saline, the container itself remains uncovered until the time of
transplantation. The air in the room where surgeries are performed is unfiltered, and therefore the open container is susceptible to microbial contamination. In addition, on histologic section of the PP2Cm surgical site there were pigmented hairs embedded within the presumed transplanted fat, showing gross contamination of the surgical site. To remedy this, donor animals for the BCATm surgeries were carefully shaved on the ventrum and dorsum and all loose fur removed before being transferred to a secondary clean area. The animals were scrubbed and fully opened for maximal exposure of perirenal and perigonadal fat stores. Excised fat was placed in warmed sterile saline, but covered Petri dishes temporarily stored this fat until time of transplant. The covered Petri dishes added an extra layer of protection from the unfiltered air of the surgical room. Since using this modified surgical procedure, the rate of abscess formation dropped to zero.

The bland, granulomatous immune response to the transplanted tissue suggests that the transplanted fat was dead and the body was simply cleaning it up. The tissue inside the granulomatous capsule was non-viable; however, it was impossible to tell whether the fat had been dead at the time of transplantation or died during the course of the study. Serial necropsies with histologic examination of the transplant site would be needed to determine whether the tissue had been viable at one point or not. Histologic examination of the surgical site in the BCATm animal was not performed, but gross examination of the site as well as the significant difference in circulating plasma BCAA levels suggests the tissue was viable.

Sentinel animals were seropositive for mouse hepatitis virus and mouse norovirus. This does not necessarily indicate an active infection, but rather that animals were exposed to these viruses at some point in their lives. It is important to be aware that MHV can alter biological responses in mice; however, both the PP2Cm and the BCATm mouse strains are immunocompetent. Therefore, any infection with MHV or MNV would likely be subclinical.
Finally, the PP2Cm knockout animal is a model of intermittent MSUD, a significantly less severe form of the disease when compared to the classic and intermediate MSUD forms. These animals can be maintained on a normal (18% protein) rodent diet with no adverse effects, whereas animal models of classic MSUD must be supported with BCAA-free diets in order to survive to and past weaning age. Although some PP2Cm knockout animals had higher circulating plasma BCAA levels when compared to age-matched wild type animals, often the difference was not significant. This is due to the fact that, like their human counterparts with intermittent MSUD, the PP2Cm knockout mouse retains anywhere from 5-20% residual BCKAD enzyme activity [23]. Indeed, individual variation may disclose a knockout animal with BCKAD enzyme activity closer to that of its wild type littermates. Due to this, we took fasting circulating plasma BCAA levels of both PP2Cm knockout animals as well as their wild type littermates. This permitted us to come up with a range of circulating plasma BCAA levels indicative of normal BCKAD enzyme activity, allowing us to choose animals with impaired BCKAD enzyme activity. We felt that using animals with higher than normal circulating plasma BCAA levels would help us judge whether the transplant was successful or not.

Models of classic MSUD have circulating plasma BCAA levels several times greater than those of wild type. The branched-chain aminotransferase mutant (BCATm) mouse is a model of classic MSUD with severely increased plasma BCAA levels, failure to thrive without early dietary intervention, and when challenged with an injectable anesthetic cocktail (ketamine + xylazine + acepromazine) enters a state of metabolic crisis that responds to oral gavage of a glucose solution – all features of the human MSUD condition. However, this mouse is affected due to a random mutagenesis; the BCKAD enzyme complex is normal. There are no reported cases of humans suffering from MSUD due to random mutagenesis in BCATm. Therefore, although we are seeing a significant reduction in circulating plasma BCAA concentration in BCATm knockout animals given subcutaneous transplantation of wild type adipose tissue, this
model is not an accurate representation of the human condition, and is not ideal for testing this possible disease correction strategy.

Our initial results are promising, and have allowed us to further refine our model and experimental procedures in the hopes of moving forward with future studies.
Chapter 5

Future Directions

Significant changes to the surgical protocol have been made to hopefully correct the difficulties seen with this project, namely the abscessation of the surgical site and the non-survival of the transplanted adipose tissue.

As stated previously, although adipose tissue does have a great capacity for angiogenesis it needs contact with vasculature to promote vascular growth. To encourage this contact, we have begun transplanting harvested adipose tissue into the subcutaneous space on the dorsum of experimental animals. Gross examination of the transplanted fat shows a healthier and more viable tissue when compared to the abdominally transplanted fat. Another alternative to the subcutaneous transplantation would be transplantation under the renal capsule. Unfortunately this method of transplantation severely limits the amount of adipose tissue that can be transplanted, and the procedure is significantly more difficult and delicate than the one currently in use.

A knockout mouse model of classic MSUD was created by targeted deletion of portions of the E2 subunit. Plasma BCAA levels of heterozygous animals are equal to wild type controls, while homozygous knockout have plasma BCAA concentrations greatly elevated above wild type and heterozygous littermates. These animals also show a failure to thrive, and will not survive a few days past birth without dietary intervention. To help correct this issue a human liver rescue gene was inserted into this animal, enabling them to survive until weaning where they could be placed on a pelleted BCAA-free diet. Heterozygous knockout animals show phenotypic characteristics nearly identical to the human condition, including the distinctive maple syrup odor to their urine, decreased BCKAD enzyme complex activity, and neurological damage that can be improved with a BCAA-restricted diet.
The next step for our laboratory is to attempt the refined transplantation procedure on homozygous E2 knockout animals. We would like to attempt a “dose-response curve,” utilizing different amounts of subcutaneously transplanted abdominal fat to find an ideal balance between reduction in circulating plasma BCAA concentration and the amount of fat needed to be transplanted as a percent of body weight.

It is our hope that, with refinement and utilization of different models of classic maple syrup urine disease, we will eventually be able to offer an alternative to life-long dietary restriction or liver transplantation as a treatment option for this debilitating disease.
References


27. The University of Utah. [Internet]. Maple Syrup Urine Disease. [Cited Feb 2011] Available at: http://learn.genetics.utah.edu/content/disorders/whataregd/msud.