EVALUATION OF MITOCHONDRIAL DYSFUNCTION IN BIOFLUIDS FROM INDIVIDUALS WITH METHYLMALONIC ACIDEMIA (MMA) AND PROPIONIC ACIDEMIA (PA) AND DEVELOPMENT OF HEK-293 MMA AND PA KNOCKOUT CELL LINES FOR FURTHER INVESTIGATION

by
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Abstract

Methylmalonic acidemia (MMA), caused by mutations in \textit{MUT}, and propionic acidemia (PA), caused by mutations in \textit{PCCA} or \textit{PCCB}, are inborn errors of isoleucine and valine metabolism. The primary biochemical defects of these disorders are well characterized; however, the downstream abnormalities have not been delineated and thus treatment options are limited. There is clinical and experimental evidence supporting a critical role for mitochondrial dysfunction in MMA and PA, and a better understanding of this dysfunction could prove instrumental in development of targeted therapies.

Mitochondrial function was examined in MMA and PA when affected patients are under physiologic stress (in the unwell state) via plasma acylcarnitine analysis, which quantifies intermediates of fatty acid oxidation and amino acid metabolism. To further define mitochondrial dysfunction in individuals with MMA and PA, patient urine samples were examined for markers of oxidative stress via di-tyrosine analysis, and quantified antioxidant reserves.

Acylcarnitine profile analysis revealed significant dysregulation of branch chain amino acid and fatty acid oxidation in patients under physiologic stress compared to control individuals. Sick MMA patients had higher levels of branch chain amino acid metabolites and fatty acid oxidation metabolites as compared to control samples. Sick PA patients had higher levels of branch chain amino acid metabolites as compared to control samples. The urine di-tyrosine assay showed increased levels of protein oxidation in both MMA and PA patient samples compared to controls (p-values<0.05). Urine antioxidant capacity
analysis revealed decreased levels of antioxidant capacity in MMA patients compared to controls (p-value=0.007).

To facilitate further exploration of the cellular mitochondrial phenotype of MMA and PA, Cas9/CRISPR was used to create novel knockout MUT and PCCA HEK-293 cell lines. Based on RT-PCR results, four MUT edited HEK-293 cell lines had reduced (17/22 base pair (bp) deletion, 19/45 bp deletion, and 22 bp homozygous insertion) or no mRNA (10/10 bp deletion) and three PCCA edited HEK-293 cell lines had reduced mRNA (23/13 bp deletion, 45/49 bp deletion, 13 bp homozygous deletion).

These studies provide evidence for mitochondrial dysfunction in MMA and PA, including dysregulation in amino acid metabolism, fatty acid oxidation, and redox balance. Novel cellular models of these disorders were developed, which can be used to validate the markers found in biofluids, and further characterize mitochondrial dysfunction in MMA and PA.

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## Table of Contents

Abstract ii

Table of Contents iv

List of Tables v

List of Figures vi

Chapter 1 Introduction 1

Chapter 2 Methods 10

Chapter 3 Results 16

Chapter 4 Discussion 31

References 35

Appendix A 41

Curriculum Vitae 44
List of Tables

Table 1: gRNA sequences for MUT and PCCA 13
Table 2: Primer Sequences for Surveyor Assay and Sequencing 14
Table 3: RT-PCR Primer Sequences 15
Table 4: Acylcarnitine comparisons between well state MMA and PA patient samples 17
Table 5: Acylcarnitine comparison between sick state MMA and PA patient samples 18
Table 6: Acylcarnitines values for PA, MMA and control samples 20
Table 7: P-values for the comparison between MMA, PA, and control samples 20
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Metabolism of propionyl-CoA to succinyl-CoA</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Schematic of the Cas9 system of <em>S. pyogenes</em></td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Tiglylcarnitine (C5:1) in sick MMA versus sick PA patients</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>Acylcarnitine intermediates in sick MMA versus sick PA patients</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>Urine Oxidation Results</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>The karyotype for a control HEK-293 cell line</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Flow chart of the process from transfection to Surveyor results</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>Subset of Surveyor assay results from <em>MUT</em> edited HEK-293 cell lines</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td><em>MUT</em> sequencing results</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td><em>PCCA</em> sequencing results</td>
<td>27</td>
</tr>
<tr>
<td>11</td>
<td>Example of sequencing results from a <em>MUT</em> and <em>PCCA</em> targeted clonal line</td>
<td>27</td>
</tr>
<tr>
<td>12</td>
<td>RT-PCR for <em>MUT</em> and <em>PCCA</em></td>
<td>29</td>
</tr>
</tbody>
</table>
Figure 13: Band Intensity from the RT-PCR for *MUT* edited

HEK-293 cell lines

Figure 14: Band intensity from the RT-PCR for *PCCA* edited

HEK-293 cell lines
Chapter 1: Introduction

Methylmalonic acidemia (MMA) and propionic acidemia (PA) are autosomal recessive inborn errors of isoleucine and valine metabolism. MMA is caused by mutations in the gene that encodes methylmalonyl-CoA mutase, MUT, located on chromosome 6p12. MMA can also be caused by abnormalities in several genes involved in the metabolism of its cofactor cobalamin. PA is caused by mutations in the genes that encode the two subunits of propionyl-CoA carboxylase located on 13q32 (PCCA) and 3q21 (PCCB) (Deodato et al., 2006).

Methylmalonyl-CoA mutase and propionyl-CoA carboxylase function in the mitochondrial matrix to convert propionyl-CoA to succinyl-CoA, which is an intermediary metabolite of the TCA cycle (Figure 1). In addition to valine and isoleucine metabolism, propionate can also be derived from the metabolism of threonine, methionine, odd-chain fatty acids, and propionate producing gut bacteria (Thompson et al., 1990).
**Figure 1: Metabolism of propionyl-CoA to succinyl-CoA.** Propionyl-CoA is converted into methylmalonyl-CoA by propionyl-CoA carboxylase (PCC). Methylmalonyl-CoA is converted into succinyl-CoA by methylmalonyl-CoA mutase (MUT). Adenosylcobalamin is the cofactor for MUT.

**Clinical Features**

Severe forms of MMA and PA characteristically present in the infantile period with lethargy, vomiting, hyperammonemia, and neutropenia (Matsui et al., 1983 Chapman et al., 2012). Acute and chronic clinical features characterize the subsequent disease course. Acute episodes of metabolic decompensation and metabolic acidosis can occur, typically in the setting of metabolic stress. Other acute clinical events can include pancreatitis and metabolic stroke in the basal ganglia in the brain (Chandler et al., 2009 Manoli et al., 2013). Chronic features include optic nerve atrophy, bone marrow suppression, growth
abnormalities, and learning disabilities/intellectual disability among other features (Deodato et al., 2006 Chandler et al., 2011 Lam et al., 2011).

While these two disorders are closely related, there are some notable clinical differences. MMA patients have an earlier, more severe renal phenotype (Lam et al., 2011). Kidney pathology in MMA includes tubular atrophy, chronic inflammation, and fibrosis, as well as ultrastructural mitochondrial abnormalities (Zsengeller et al., 2014).

PA patients have a more prevalent cardiomyopathy (Prada et al., 2011). PA patients have been reported to have hypertrophic or dilated cardiomyopathy (Romano et al., 2010). In addition, prolonged QTc intervals are seen in PA patients, which is an indication of rhythm abnormalities (Baumgartner et al., 2007).

**Diagnosis**

MMA and PA can be diagnosed on a biochemical, molecular, or enzymatic basis. The common biochemical findings from urine organic acid analysis are increased levels of tiglylglycine, propionylglycine, 3-hydroxypropionate, and 2-methylcitrate (Chandler et al., 2011). Methylmalonate levels can also be specifically quantified in urine and plasma of MMA patients via a specific stable isotope assay (Deodato et al., 2006). Plasma acylcarnitine profiles of MMA patients have increased levels of propionylcarnitine and methylmalonyl/succinylcarnitine, and PA patients have increased levels of propionylcarnitine (Deodato et al., 2006).

Enzymatic assays using patient-derived fibroblast can determine the function of methylmalonyl-CoA mutase and propionyl-CoA carboxylase using C\(^{14}\)-propionate incorporation into macromolecules (Deodato et al., 2006). MMA can be subdivided into
two groups based on the severity of enzyme deficiency: mut$^i$ (some residual enzyme function) and mut$^0$ (no residual enzyme function) (Worgan et al, 2006 Horster et al., 2007). Some cell lines that exhibit no enzyme function (mut$^0$) show interallelic complementation in somatic cell hybrids. An example is the MUT mutation, p.R93H, which in the homozygous form has no enzyme function (mut$^0$). The p.R93H mutation is in the amino-terminus of the methylmalonyl-CoA mutase protein which interferes with enzyme activity (Crane et al., 1994). Homozygous p.R93H cells can complement with mutations that are located in the C-terminus of the methylmalonyl-CoA mutase protein which interferes with cofactor binding. This complementation can give the cell lines significant function allowing for C$^{14}$-propionate incorporation into macromolecules (Ledley et al., 1997). Interallelic complementation allows for the study of the kinetic properties of combinations of mutations (Janata et al., 1997).

The diagnosis of MMA or PA can be confirmed by sequencing MUT or genes involved in cobalamin metabolism, PCCA, and PCCB. The catalog of mutations that cause the MUT form of MMA are as follows: 54% are missense mutations, 14% are nonsense mutations, 9% are splicing mutations, and 22% are small insertions/deletions (Acquaviva et al., 2005). Mutations in PCCA account for between 35-50% of all PA mutations, and PCCB mutations account for between 50-65% of mutations (Perez et al., 2003 Yang et al., 2004). Approximately 40% of mutations in PCCA and PCCB are missense variants and small insertions/deletions being the second most common variants (Desviat et al., 2006).
Current treatment

Current treatment for MMA and PA focuses primarily on avoidance of metabolic stressors that could precipitate a metabolic crisis and on symptomatic management. Patients are advised to avoid fasting, stress, infection, fever, and increased dietary protein intake. When MMA or PA patients are undergoing metabolic decompensation, the primary treatment is to give fluids, reduce or eliminate protein intake, give carnitine through IV, and to increase glucose intake, which is important to ensure adequate calorie intake and prevent lipolysis (Deodato et al., 2006).

Long-term management includes a low-protein, high-calorie diet (Thompson et al., 1990). Carnitine is given to increase intracellular levels of CoA pools and possibly enhance urine excretion of these metabolites (Touati et al., 2006). Antibiotics (neomycin or metronidazole) are sometimes prescribed with the goal of reducing the amount of propionate producing gut bacteria (Thompson et al., 1990).

Organ transplantation has been shown to protect against metabolic instability in MMA patients; however, this is not curative because the central nervous system pathology continues to progress (Leonard et al., 2001). The reported benefits of liver transplantation in PA are a decreased frequency of metabolic decompensation (Vara et al., 2011) and reversal of cardiomyopathy (Yorifuji et al., 2004). There is evidence that will be presented later that suggests antioxidants may be beneficial in patients with MMA and PA (Manoli et al., 2013). There is a need for targeted therapies in MMA and PA patients to promote better outcomes for the patients and prevent progressive neurologic complications.
Evidence for mitochondrial dysfunction in patients with MMA and PA

While the primary biochemical defects in MMA and PA have been described for decades, many of the downstream biochemical aberrations are still being determined. Based on clinical observations, mitochondrial dysfunction is becoming increasingly apparent as a mechanism of cellular pathology.

Pathological and enzymatic evidence in liver tissue from individuals with MMA have shown decreased respiratory chain complex II+III and IV function. Kidney pathology in a 19-year-old MMA patient showed tubular atrophy, chronic inflammation, and fibrosis. There were changes to the vacuoles in the proximal tubules, in addition to mitochondrial enlargement with loss of cristae in the proximal tubule (Zsengeller et al., 2014). Liver samples from a 5-year-old MMA patient showed that hepatocytes had megamitochondria and abnormal lamellations (Chandler et al., 2009). Deficiency of glutathione, an important mitochondrial redox agent, has also been reported in patients with MMA (Treacy et al., 1996).

While less abundant than the evidence for clinical mitochondrial abnormalities in MMA, there is clinical evidence for mitochondrial dysfunction in PA. Deficiencies of the mitochondrial antioxidant coenzyme Q₁₀ have been reported in propionic acidemia. Multiple organ oxidative phosphorylation deficiency with acute heart failure has also been seen in PA (Fragaki et al., 2011). A kidney biopsy attained from a 29-year-old PA patient showed glomeruli that were unrecognizable and the tubules showed injury including cell blebbing and vacuolization. Mitochondria were enlarged with disorganized cristae. There was moderate interstitial fibrosis and tubular atrophy (Vernon et al., 2013).
Muscle biopsies from two PA patients showed lipid droplets in the muscle fibers, crystalline inclusions, and enlarged mitochondria (Schwab et al., 2006). Abnormal markers of protein and lipid oxidation as well as antioxidant abnormalities have been reported in urine specimens from individuals with PA and forms of MMA (McGuire 2009)

Mitochondrial dysfunction in investigational models of MMA and PA

Mitochondrial dysfunction in mouse models of MMA

A null mouse model of MMA was generated in 2003. These mice had the characteristic biochemical profile of increased methylmalonic acid, 2-methylcitrate, and propionylcarnitine in biofluids; however, they died shortly after birth. (Peters et al., 2003).

Breeding onto a mixed background C57Bl/6X129 Sv/EV, mitigated early lethality and allowed for further study. Mitochondrial pathology in these mice included megamitochondria and evidence for lipid droplets in liver samples, and mitochondrial lamellations in the proximal tubules. Older mice had the tubulointerstitial nephritis similar to what is seen in patients, in addition, the proximal tubules showed mitochondrial changes including megamitochondria with underdeveloped cristae and lamellations. Liver extracts showed respiratory chain dysfunction including reduction of complex I+II, II+3, and IV, and reduced intracellular glutathione (Chandler et al., 2009).

Several conditional, organ specific knockout mouse lines for MMA were subsequently generated, which allowed for further exploration of organ pathology (Manoli et al., 2013).
**Mouse models for PA**

A null mouse model for PA was generated in 2001. The mutant mice had increased levels of propionylcarnitine, 3-hydroxypropionate, 2-methycitrate, and increased levels of ketones in the urine, indicative of the recapitulation of the primary biochemical human phenotype of PA. All mutant mice died within 24-36 hours after birth with evidence of acidosis. Pathology in these mice showed increased levels of fat deposition in the liver and decreased levels of glycogen. Mitochondrial abnormalities were not explored in these mice, likely due to the early lethality (Miyazaki et al., 2001).

A subsequent hypomorphic PA mouse knockout model was developed. These animals were able to escape early lethality. Biochemical analysis of biofluids in these mice showed markers for mitochondrial dysfunction including elevated glycine, lysine and ammonia. Further studies in this mouse model are ongoing (Guenzel et al., 2013).

**Mitochondrial dysfunction in cellular models of MMA and PA**

Patient-derived primary fibroblast cultures have been used as a model for both MMA and PA. In the fibroblast model systems, methylmalonic acid significantly inhibits complex I-III, I, II-III in the electron transport chain (Brusque et al., 2001).

Patient-derived fibroblast primary cultures have been used to look at the mitochondrial dysfunction in MMA. In one study, there was a significant increase in the reactive oxygen species (ROS) produced in patient-derived fibroblast, in addition to a significant increase in the levels of mitochondrial superoxide dismutase (Richard et al., 2007).
**Project Aims**

In order to better define the nature of the mitochondrial dysfunction in individuals with MMA and PA, specific metabolites in both urine and plasma of affected individuals were measured. These metabolites included markers of fatty acid oxidation and branch chain amino acid metabolism, protein and lipid oxidation, and antioxidant content.

Once these markers were defined, cell models were developed for further exploration of mitochondrial phenotypes. In order to overcome the limitations of fibroblast cell models for MMA and PA, which include senescence, transfection inefficiency, and differing genetic backgrounds; isogenic HEK-293 knockout cell models of MMA and PA were developed using Cas9/CRISPR genome editing technology.

These cell lines will be used for future exploration of mitochondrial and cellular phenotypes of these two disorders. The importance of creating knockout HEK-293 cell lines for both disorders is to understand the similarities and differences between the mitochondrial phenotypes in these disorders in isogenic cell lines. In addition, screening potential treatment options like antioxidants is much simpler in HEK-293 cell lines than in fibroblast. Knockout HEK-293 cell lines for MMA and PA were made to further understand the etiology of mitochondrial dysfunction in MMA and PA, in a model system that small molecule screens can be done with in a high-throughput manner.
Chapter 2: Methods

Acylcarnitine Profiles

LC-MS analysis of acylcarnitines was performed in plasma of individuals with MMA and PA to evaluate for acyl chain lengths C2-C18, representing intermediates in amino acid metabolism and fatty acid oxidation. Acylcarnitines were measured in plasma in sick and well MMA and PA patients.

Samples were analyzed under IRB: NA_00069372, Metabolic analysis of archived biofluids. The criteria for inclusion in the sick cohort was blood pH less than 7.30, serum bicarbonate less than 16 meq/L, serum amylase greater than 100 U/L, serum lipase greater than 60 U/L, plasma ammonia >100 μmol/L, acute neurologic findings, and/or acute abdominal pain or recurrent vomiting. Well samples were obtained during a routine outpatient clinic visit. For plasma samples, 40 μl of plasma was placed in a 1.5 ml Eppendorf tube. 100 μl of an internal standard was added to each sample. The internal standard was composed of heavy free carnitine, C2, C3, C4, C5, C8, C14, and palmitoylcarnitine (Cambridge Isotope Laboratories Inc., Tewksbury, MA). Samples were spun in a microcentrifuge at 13,000 rpm for 3 minutes. The supernatant was then dried under a slow stream of nitrogen. Once the samples were dry, 60 μl of 3N HCl-butanol was added to each sample and heated at 65°C for 15 minutes. After heating, samples were dried again under a slow stream of nitrogen; then 100 μl of acetonitrile-H2O-formic acid was added to the sample. The samples were transferred to a new filter Eppendorf tube and spun at 13,000 rpm for 30 seconds. The samples were placed on the LC-MS for analysis.
URINE OXIDATION STUDIES

Urine di-tyrosine autofluorescence analysis

Urine di-tyrosine analysis, as a proxy measurement for protein oxidation, was performed on urine samples from 7 well MMA patients, 4 well PA patients, and 5 controls. Di-tyrosine levels were measured by autofluorescence. 50 µl of urine was added to 950 µl 6 mol/l urea in 20 mmol/l sodium phosphate buffer pH 7.4. After a 30-minute incubation, the samples were read using excitation of 315 nm and emission of 410 nm using the auto-gain function of the plate reader.

15-F2t-isoprostane analysis

Urine 15-F2t-isoprostanes was measured in urine samples from 7 well MMA patients, 4 well PA patients, and 5 controls, as a proxy for lipid oxidation. The level of 15-F2t-isoprostane was measured in the urine using a competitive ELISA assay (Oxford Biomed, Michigan). In each sample, the amount of 15-F2t-isopratane competed with 15-F2t-isoprostane conjugated to horseradish peroxidase, which bound to a polyclonal antibody coated on the microplate. The amount of 15-F2t-isoprostane in the sample was inversely proportional to the amount of color development measured at 630 nm.

Antioxidant measurements

Antioxidant capacity was measured in urine samples from 7 well MMA patients, 4 well PA patients, and 5 controls as determined by the Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI). The assay measured the inhibition of 2,2’-azino-di-3-ethylbenzthiazoline sulfonate oxidation by metmyoglobin by the antioxidants in a patient’s urine sample. The ability of the antioxidants in a urine sample to prevent the
oxidation was measured directly to a standard curve generated by Trolox, which is a tocopherol analogue.

**CAS9/CRISPR**

**Karyotype and Fluorescent in situ hybridization Analysis**

A G banded karyotype analysis was performed on the control HEK-293 cell lines and 10 cells were analyzed (Seabright, 1971). The assay was performed by the Cytogenetics laboratory at Kennedy Krieger Institute (now affiliated with Johns Hopkins hospital).

**Construct design**

A Cas9 nickase cutting strategy was used to make the knockout HEK-293 cell lines because of the increased specificity at the target site (Figure 2). Targeted exons were chosen based on the human gene mutation database based on the location of reported human disease causing mutations; exon 5 of *MUT* was targeted and exon 12 of *PCCA* was targeted. The gRNAs (Table 1) were designed using http://tools.genome-engineering.org (Ran et al., 2013). Synthesized gRNAs were inserted into a CR-BluntII-TOPO plasmid (Invitrogen, Grand Island, NY) and transformed into *E.coli*. DNA was collected for each of the gRNAs and used to transfect the HEK-293 cells. Transfection was done using lipofectamine (Invitrogen, Grand Island, NY) following a previously established protocol (Yang et al, 2014). The plasmid that the Cas9 protein was encoded by has a GFP tag, which allowed for FACS to be done (Addgene 44720, Cambridge, MA). Single cells were isolated from a mixture of both GFP + and GFP- cells by FACS into 96-well plates.
Figure 2: Schematic of the Cas9 system of *S. pyogenes*. The Cas9 protein is shown in yellow. The blue signifies the gRNA for the genomic locus and the red signifies the scaffold for the gRNA. The Cas9 protein cuts three bp upstream of the PAM site (NGG) (Ran et al., 2013).

<table>
<thead>
<tr>
<th>Exon</th>
<th>gRNA 1</th>
<th>gRNA 2</th>
<th>Off-target sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-MUT</td>
<td>CACACTGTCAGACATCTGGATGG</td>
<td>CCTAAAAACTCAAATCTTTCCTTCT</td>
<td>gRNA 1: 31 genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gRNA 2: 29 genes</td>
</tr>
<tr>
<td>12-PCCA</td>
<td>TGGGACCCTGGAGTCTTGTGG</td>
<td>CCAGAAGCTAAATATCTTCTTCT</td>
<td>gRNA 1: 31 genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gRNA 2: 24 genes</td>
</tr>
</tbody>
</table>

Table 1: gRNA sequences for *MUT* and *PCCA*. PAM sequences are highlighted in red.

**Targeted clone selection**

DNA was extracted from the clonal populations isolated from single cells. The surveyor assay, a screening system used to identify potentially targeted exons, was performed using the primers in Table 2 for each targeted exon (IDTDNA, Coralville, IA). A PCR
was done with the DNA extracted from the isolated clonal populations of edited HEK-293 cells and the control HEK-293 cell using the GoTaq Green Master Mix (Promega, Madison, WI). 10 µl of the PCR product from the edited HEK-293 cells and 10 µl of the PCR product from the control HEK-293 cells were combined to create heteroduplexes using a thermocycler. 10 µl of the hybridized DNA product was added to 1 µl MgCl₂ solution, 1 µl Surveyor Enhancer S, and 1 µl Surveyor Nuclease S. The mixture was vortexed and incubated at 42°C for 1 hour. The samples were run on a 2% agarose gel.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Exon 5-MUT</td>
<td>tgtacgtgcaetgtetaatc</td>
<td>ttaaccaggagggagacaa</td>
</tr>
<tr>
<td>Exon 12- PCCA</td>
<td>aaagtgtatattgagcatgtgtag</td>
<td>gatgtcttagacaagcataaaaaac</td>
</tr>
</tbody>
</table>

Table 2: Primer Sequences for Surveyor Assay and Sequencing

Surveyor positive samples were sequenced at the sequencing core at Johns Hopkins University School of Medicine. A positive sequencing result was determined to be either a homozygous or compound heterozygous insertion or deletion.

**MUT and PCCA transcript analysis**

The positive sequencing samples were grown and RNA was extracted from the cells (Qiagen, Netherlands). cDNA was synthesized from the RNA. The Omniscript Reverse Transcription Kit (Qiagen, Netherlands) was used to make the cDNA. Master mix was prepared according to the protocol in the kit. Briefly, 2 µl of 10xBuffer RT, 2 µl dNTP mix, 1µl Oligo dT primer (Promega, Madison, WI), 1 µl Omniscript RT, 1 µg of RNA, and a variable amount of water up to the total volume of 20 µl per cDNA reaction. The mixture was vortexed for <5seconds and centrifuged briefly to collect liquid from the
tube. The samples were incubated at 37°C for 60 minutes. The cDNA was used for RT-PCR. The primers used for RT-PCR are in Table 3. RT-PCR was done using 2 µl of cDNA, 13 µl of GoTaq Green Master Mix (Promega, Madison, WI), 2.5 µl of forward primer, 2.5 µl of reverse primer, and 5 µl of water. The samples were transferred to a thermocycler for 30 cycles at 57°C. The samples were run on a 1% agarose gel and the bands were stained with ethidium bromide. The intensity of the bands for each of the PCCA and MUT edited HEK-293 cell lines were compared to the intensity of the GADP band for the same cDNA sample using ImageJ to determine the amount of mRNA from the PCCA and MUT locus in comparison to the amount of mRNA from the GADP locus.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Exons</th>
</tr>
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<tbody>
<tr>
<td>MUT</td>
<td>gctggccctgacaattgatga</td>
<td>tttctagcttgtcttcgggca</td>
<td>F:4 R:7</td>
</tr>
<tr>
<td>PCCA</td>
<td>ttgcatgttggtattagaa</td>
<td>aggggtcctcagcataaacc</td>
<td>F:3-4 R:13-14</td>
</tr>
<tr>
<td>GADP</td>
<td>tgcacccaacctgtttagc</td>
<td>ggcatggacttggtcatag</td>
<td>F:7 R:7-8</td>
</tr>
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</table>

Table 3: RT-PCR Primer Sequences
Chapter 3: Results

Acylcarnitine Profiles

There is prior evidence that biochemical abnormalities can be exacerbated under stressful conditions including sickness; therefore, acylcarnitines were extracted in both sick and well MMA and PA patients. Comparisons between acylcarnitines in PA in the well versus sick state showed a significant elevation in propionylcarnitine in the sick state (32.55 µM +/- 8.04 vs. 54.53 µM +/- 7.88; p-value=0.003). Comparisons between acylcarnitines in MMA in the well versus sick patients showed a significant elevation in tiglylcarnitine in the sick state (0.23 µM +/- 0.08 vs. 0.56 µM +/- 0.25; p-value=0.021). Comparisons between acylcarnitines in the well state between MMA and PA showed many differences in acylcarnitines corresponding to metabolites of amino acid metabolism (Table 4). Comparisons between acylcarnitines in the sick state between MMA and PA showed many differences in acylcarnitines corresponding to metabolites of amino acid metabolism and fatty acid oxidation (Table 5, Figures 3 & 4). Comparisons between acylcarnitines in the well and sick MMA and PA patient samples and control samples showed differences in acylcarnitines corresponding to metabolites of amino acid metabolism and fatty acid oxidation (Tables 6 & 7).
<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>MMA</th>
<th>PA</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionylcarnitine (C3)</td>
<td>44.86 µM +/- 7.88</td>
<td>32.55 µM +/- 5.22</td>
<td>0.036</td>
</tr>
<tr>
<td>Isobutyryl/butyrylcarnitine (C4)</td>
<td>0.89 µM +/- 0.72</td>
<td>0.16 µM +/- 0.09</td>
<td>0.044</td>
</tr>
<tr>
<td>Tigylycarnitine (C5:1)</td>
<td>0.23 µM +/- 0.08</td>
<td>0.07 µM +/- 0.03</td>
<td>0.003</td>
</tr>
<tr>
<td>Isovaleryl/2-methylbutyrylcarnitine(C5)</td>
<td>0.59 µM +/- 0.22</td>
<td>0.07 µM +/- 0.03</td>
<td>0.002</td>
</tr>
<tr>
<td>3-hydroxyisovaleryl/2methyl-3-hydroxybutyryl carnitine (C5OH)</td>
<td>0.22 µM +/- 0.06</td>
<td>0.11 µM +/- 0.05</td>
<td>0.017</td>
</tr>
<tr>
<td>Methylmalonyl/succinylcarnitine (C4DC)</td>
<td>3.09 µM +/- 1.1</td>
<td>0.03 µM +/- 0.02</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 4: Comparison between well state MMA and PA patient samples. MMA has significantly increased levels of the branch chain amino acid intermediates. P-values are from the comparison of well state MMA to well state PA.
<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>MMA</th>
<th>PA</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoyl carnitine (C8)</td>
<td>0.43 µM+/−0.36</td>
<td>0.06 µM+/−0.03</td>
<td>0.045</td>
</tr>
<tr>
<td>Decanoyl carnitine (C10)</td>
<td>0.32 µM+/−0.2</td>
<td>0.07 µM+/−0.03</td>
<td>0.026</td>
</tr>
<tr>
<td>Glutaryl/3-hydroxydecanoyl carnitine (C5DC)</td>
<td>0.22 µM+/−0.15</td>
<td>0.03 µM+/−0.02</td>
<td>0.023</td>
</tr>
<tr>
<td>Tiglylcarnitine (C5:1)</td>
<td>0.56 µM+/−0.25</td>
<td>0.09 µM+/−0.05</td>
<td>0.005</td>
</tr>
<tr>
<td>3-hydroxydodecenoyl (C12:1-OH)</td>
<td>0.17 µM+/−0.12</td>
<td>0.03 µM+/−0.02</td>
<td>0.027</td>
</tr>
<tr>
<td>3-hydroxydodecanoyl (C12)</td>
<td>0.16 µM+/−0.14</td>
<td>0.02 µM+/−0.01</td>
<td>0.044</td>
</tr>
<tr>
<td>3-hydroxytetradecenoyl (C14:1-OH)</td>
<td>0.14 µM+/−0.11</td>
<td>0.03 µM+/−0.01</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Table 5: Comparison between sick state MMA and PA patient samples. MMA has significantly elevated levels of branch chain amino acid intermediates and fatty acid oxidation intermediates. P-values are from the comparison of sick state MMA to sick state PA patients.
Figure 3: *Tiglylcarnitine (C5:1) in sick MMA versus sick PA patients:* Plasma from sick MMA patients had significantly higher levels of C5:1 compared to plasma from sick PA patients. Error bars denote standard deviation.

Figure 4: *Acylcarnitine intermediates in sick MMA versus sick PA patients:* Plasma from sick MMA patients had significantly higher levels of octanoylcarnitine (C8), decanoylcarnitine (C10), glutaryl/3-hydroxydecanoylcarnitine (C5DC), 3-hydroxydodecenoyl (C12:1-OH), 3-hydroxydecanoyl (C12), and 3-hydroxytetradecenoyl (C14:1-OH) compared to plasma from sick PA patients. Error bars denote standard deviation.
<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Well MMA</th>
<th>Sick MMA</th>
<th>Well PA</th>
<th>Sick PA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionylcarnitine (C3)</td>
<td>44.86 µM +/- 7.88</td>
<td>54.13 µM +/- 17.40</td>
<td>32.55 µM +/- 5.22</td>
<td>54.53 µM +/- 7.88</td>
<td>0.40 µM +/- 0.13</td>
</tr>
<tr>
<td>Isobutyryl/butyrylcarnitine (C4)</td>
<td>0.89 µM +/- 0.72</td>
<td>4.10 µM +/- 6.36</td>
<td>0.16 µM +/- 0.09</td>
<td>0.22 µM +/- 0.05</td>
<td>0.18 µM +/- 0.05</td>
</tr>
<tr>
<td>Tiglylcarnitine (C5:1)</td>
<td>0.23 µM +/- 0.08</td>
<td>0.56 µM +/- 0.25</td>
<td>0.07 µM +/- 0.03</td>
<td>0.09 µM +/- 0.05</td>
<td>0.02 µM +/- 0.01</td>
</tr>
<tr>
<td>Isovaleryl/2-methylbutyrylcarnitine (C5)</td>
<td>0.59 µM +/- 0.22</td>
<td>3.78 µM +/- 4.99</td>
<td>0.07 µM +/- 0.03</td>
<td>0.13 µM +/- 0.08</td>
<td>0.12 µM +/- 0.03</td>
</tr>
<tr>
<td>3-hydroxyisovaleryl/2methyl-3- hydroxybutyrylcarnitine (C5OH)</td>
<td>0.22 µM +/- 0.06</td>
<td>0.82 µM +/- 0.71</td>
<td>0.11 µM +/- 0.05</td>
<td>0.15 µM +/- 0.10</td>
<td>0.04 µM +/- 0.01</td>
</tr>
<tr>
<td>Methylmalonyl/succinylcarnitine (C4DC)</td>
<td>3.09 µM +/- 1.1</td>
<td>4.50 µM +/- 3.17</td>
<td>0.03 µM +/- 0.02</td>
<td>0.04 µM +/- 0.02</td>
<td>0.04 µM +/- 0.01</td>
</tr>
<tr>
<td>Octanoylcarnitine (C8)</td>
<td>0.18 µM +/- 0.05</td>
<td>0.43 µM +/- 0.36</td>
<td>0.05 µM +/- 0.02</td>
<td>0.06 µM +/- 0.03</td>
<td>0.09 µM +/- 0.02</td>
</tr>
<tr>
<td>Decanoylcarnitine (C10)</td>
<td>0.21 µM +/- 0.06</td>
<td>0.32 µM +/- 0.2</td>
<td>0.06 µM +/- 0.02</td>
<td>0.07 µM +/- 0.03</td>
<td>0.16 µM +/- 0.04</td>
</tr>
<tr>
<td>Glutaryl/3-hydroxydecanoylcarnitine (C5DC)</td>
<td>0.12 µM +/- 0.05</td>
<td>0.22 µM +/- 0.15</td>
<td>0.03 µM +/- 0.01</td>
<td>0.03 µM +/- 0.02</td>
<td>0.06 µM +/- 0.01</td>
</tr>
<tr>
<td>3-hydroxydodecenoyl (C12:1-OH)</td>
<td>0.07 µM +/- 0.01</td>
<td>0.17 µM +/- 0.12</td>
<td>0.03 µM +/- 0.02</td>
<td>0.03 µM +/- 0.02</td>
<td>0.03 µM +/- 0.00</td>
</tr>
<tr>
<td>3-hydroxydodecanoyl (C12)</td>
<td>0.06 µM +/- 0.02</td>
<td>0.16 µM +/- 0.14</td>
<td>0.01 µM +/- 0.01</td>
<td>0.02 µM +/- 0.01</td>
<td>0.02 µM +/- 0.01</td>
</tr>
<tr>
<td>3-hydroxytetradecenoyl (C14:1-OH)</td>
<td>0.06 µM +/- 0.02</td>
<td>0.14 µM +/- 0.11</td>
<td>0.02 µM +/- 0.01</td>
<td>0.03 µM +/- 0.01</td>
<td>0.02 µM +/- 0.01</td>
</tr>
</tbody>
</table>

Table 6: Acylcarnitines values for PA, MMA and control samples.

<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Well MMA</th>
<th>Sick MMA</th>
<th>Well PA</th>
<th>Sick PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionylcarnitine (C3)</td>
<td>0.000</td>
<td>0.002</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>Isobutyryl/butyrylcarnitine (C4)</td>
<td>0.079</td>
<td>0.173</td>
<td>0.330</td>
<td>0.216</td>
</tr>
<tr>
<td>Tiglylcarnitine (C5:1)</td>
<td>0.002</td>
<td>0.007</td>
<td>0.012</td>
<td>0.031</td>
</tr>
<tr>
<td>Isovaleryl/2-methylbutyrylcarnitine (C5)</td>
<td>0.009</td>
<td>0.135</td>
<td>0.028*</td>
<td>0.432</td>
</tr>
<tr>
<td>3-hydroxyisovaleryl/2methyl-3- hydroxybutyrylcarnitine (C5OH)</td>
<td>0.002</td>
<td>0.061</td>
<td>0.028</td>
<td>0.056</td>
</tr>
<tr>
<td>Methylmalonyl/succinylcarnitine (C4DC)</td>
<td>0.003</td>
<td>0.030</td>
<td>0.072</td>
<td>0.269</td>
</tr>
<tr>
<td>Octanoylcarnitine (C8)</td>
<td>0.017</td>
<td>0.087</td>
<td>0.020*</td>
<td>0.115</td>
</tr>
<tr>
<td>Decanoylcarnitine (C10)</td>
<td>0.125</td>
<td>0.124</td>
<td>0.002*</td>
<td>0.006*</td>
</tr>
<tr>
<td>Glutaryl/3-hydroxydecanoylcarnitine (C5DC)</td>
<td>0.036</td>
<td>0.064</td>
<td>0.002*</td>
<td>0.023*</td>
</tr>
<tr>
<td>3-hydroxydodecenoyl (C12:1-OH)</td>
<td>0.002</td>
<td>0.054</td>
<td>0.395</td>
<td>0.271</td>
</tr>
<tr>
<td>3-hydroxydodecanoyl (C12)</td>
<td>0.027</td>
<td>0.077</td>
<td>0.022*</td>
<td>0.288</td>
</tr>
<tr>
<td>3-hydroxytetradecenoyl (C14:1-OH)</td>
<td>0.014</td>
<td>0.065</td>
<td>0.218</td>
<td>0.218</td>
</tr>
</tbody>
</table>

Table 7: P-values for the comparison between MMA, PA, and control samples.

*denotes when the average for the control samples is significantly higher than the MMA or PA sample.
**Urine Oxidation Studies**

The average urine di-tyrosine measurement in MMA was $4.87 \times 10^5$ FU/ mg Cr+/-1.28$\times10^5$, in PA was $9.39 \times 10^5$ FU/ mg Cr+/-2.72$\times10^5$, and in control was $1.43 \times 10^5$ FU/mg Cr+/-5.74$\times10^4$. Both the MMA and PA urine samples had significantly higher levels of di-tyrosine as compared to controls (p-values<0.05), and the PA urine samples had significantly higher di-tyrosine than the MMA urine samples (p-value=0.038) (Figure 5a).

15-F2t- isoprostane levels were not significantly different between the MMA, PA, and control urine samples. MMA samples had a value of 1.92 ng/mg Cr+/-0.99, PA samples had a value of 2.68 ng/mg Cr+/-1.69, and control samples had a value of 2.07 ng/mg Cr+/-0.89 (p-values> 0.05) (Figure 5b).

The urine antioxidant capacity in MMA was 0.83 mM Trolox+/-0.53, in PA was 1.34 mM Trolox+/-1.37, and in controls was 2.71 mM Trolox+/-1.12. The controls had significantly higher urine antioxidant capacity than the MMA cohort (p-value of 0.007) but not the PA cohort (Figure 5c).
Figure 5: Urine Oxidation Results. A. Urine from MMA patients (n=7) and from PA patients (n=4) had significantly higher di-tyrosine compared to controls (n=5) (p-values<0.05). Urine from PA patients had significantly higher di-tyrosine than MMA patients (P-value 0.038). B. Urine from MMA patients and from PA patients had 15-F2t-isoprostane levels that were not significantly different from controls. C. Urine from MMA patients had significantly lower antioxidant capacity compared to controls (p-value=0.007). Urine from PA patients had antioxidant capacity that was lower than control but not significantly different (p-value=0.1575).
Cas9/CRISPR

Aneuploidy was determined via standard karyotyping

The karyotype for the HEK-293 cell line was aneuploid: 69-73,XX,+X add 1(q21)+der(1)del(1)p32 add (1)(q21),+7,-8,-9, add (9)(p22), -13, -18, +19, -20, +21, +22, +22, +3mar, inc[cp10] (Figure 6). Neither the 6p (where MUT is located) nor 13q region (where PCCA is located) was aneuploid.

Figure 6: The karyotype for a control HEK-293 cell line. Karyotype was 69-73,XX,+X add 1(q21)+der(1)del(1)p32 add (1)(q21),+7,-8,-9, add (9)(p22), -13, -18, +19, -20, +21, +22, +22, +3mar, inc[cp10].

Cas9/CRISPR development

Figure 7 shows the workflow from transfection with gRNAs for MUT and PCCA to the results from the surveyor assay. The surveyor assay was done on the 158 clonal
populations of cells. A subset of the surveyor assay results from the 158 clonal populations is shown in Figure 8.

Sequences of surveyor positive clones were analyzed in Codoncode (Centerville, MA), which revealed 25 clones with identifiable mutations in at least one copy of either \textit{MUT} or \textit{PCCA}. 19 of the clones were in \textit{MUT} edited HEK-293 cell lines (numbers 1-81) and 6 of the clones were in \textit{PCCA} edited HEK-293 cell lines (numbers 114-154) (Figures 9, 10, & 11).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{flowchart.png}
\caption{Flow chart of the process from transfection to Surveyor results}
\end{figure}
Figure 8: Subset of Surveyor assay results from MUT edited HEK-293 cell lines. The expected band size for MUT was 400 bp. A positive surveyor results has multiple bands present, which is indicative of a mismatch between the control HEK-293 DNA and the edited HEK-293 DNA.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutation 1</th>
<th>Mutation 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21 bp del</td>
<td>Homozygous</td>
</tr>
<tr>
<td>8</td>
<td>21 bp del</td>
<td>22 bp del</td>
</tr>
<tr>
<td>13</td>
<td>34 bp del</td>
<td>34 bp del</td>
</tr>
<tr>
<td>20</td>
<td>17 bp del</td>
<td>22 bp del</td>
</tr>
<tr>
<td>27</td>
<td>14 bp del</td>
<td>16 bp del</td>
</tr>
<tr>
<td>29</td>
<td>54 bp del</td>
<td>53 bp del</td>
</tr>
<tr>
<td>31</td>
<td>47 bp del</td>
<td>53 bp del</td>
</tr>
<tr>
<td>38</td>
<td>19 bp del</td>
<td>16 bp del</td>
</tr>
<tr>
<td>52</td>
<td>19 bp del</td>
<td>45 bp del</td>
</tr>
<tr>
<td>53</td>
<td>13 bp ins</td>
<td>14 bp ins</td>
</tr>
<tr>
<td>59</td>
<td>6 bp del</td>
<td>11 bp ins</td>
</tr>
<tr>
<td>60</td>
<td>39 bp del</td>
<td>38 bp del</td>
</tr>
<tr>
<td>62</td>
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</tr>
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<td>63</td>
<td>7 bp del</td>
<td>10 bp del</td>
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<td>68</td>
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<tr>
<td>70</td>
<td>24 bp del</td>
<td>24 bp del</td>
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<tr>
<td>79</td>
<td>38 bp del</td>
<td>38 bp del</td>
</tr>
<tr>
<td>80</td>
<td>22 bp ins</td>
<td>Homozygous</td>
</tr>
<tr>
<td>81</td>
<td>10 bp del</td>
<td>10 bp del</td>
</tr>
</tbody>
</table>

**Figure 9: MUT sequencing results.** Insertions are abbreviated as ins and deletions are abbreviated as del.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutation 1</th>
<th>Mutation 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>114</td>
<td>16 bp del</td>
<td>33 bp del</td>
</tr>
<tr>
<td>117</td>
<td>30 bp del</td>
<td>42 bp del</td>
</tr>
<tr>
<td>121</td>
<td>23 bp del</td>
<td>13 bp del</td>
</tr>
<tr>
<td>128</td>
<td>85 bp del</td>
<td>85 bp del</td>
</tr>
<tr>
<td>152</td>
<td>45 bp del</td>
<td>49 bp del</td>
</tr>
<tr>
<td>154</td>
<td>13 bp del</td>
<td>Homozygous</td>
</tr>
</tbody>
</table>

**Figure 10: PCCA sequencing results.** Insertions are abbreviated as ins and deletions are abbreviated as del.

**Figure 11: Example of sequencing results from a MUT and PCCA targeted clonal line.** Homozygous sequencing results for a MUT edited HEK-293 cell line and for a PCCA edited HEK-293 cell line. There is a 22 bp insertion in MUT edited HEK-293 clone ID# 80. There is a 13 bp deletion in PCCA edited HEK-293 clone ID# 154.
RT-PCR

The ratios of the MUT or PCCA band intensity to the GADP band intensity are shown in figure 13 and 14. No MUT cDNA is detected in HEK-293 clonal cell line 81, and clonal lines 20, 52, and 80 have a decrease in the intensity of the MUT band. (Figure 12a, Figure 13).

In addition to reduced transcript amount, there is evidence for other MUT clonal cell lines with altered transcript size, corresponding to sequencing findings of deletions. MUT edited HEK-293 line 1 was expected to have a homozygous 21 bp deletion, MUT edited HEK-293 line 8 was expected to have a compound heterozygous 21/22 bp deletion, and MUT edited HEK-293 line 20 had a compound heterozygous 17/22 bp deletion. These three samples appear to run below the expected band size of 556 bp and at a similar size to each other, however further characterization is required to verify this (Figure 12a).

PCCA edited HEK-293 lines 121, 152, and 154 have a decrease in the intensity of the PCCA band as seen through RT-PCR, representing reduced PCCA transcript level (Figure 12b, Figure 14). PCCA edited HEK-293 cell line 117 has a smaller sized band than the control HEK-293 cell lines. This size reduction in transcript corresponds to the sequencing results showing a compound heterozygous deletion of 30/42 bp (Figure 12b).

These data show that the Cas9/CRISPR genome editing technique altered the expected targeted sites in the MUT and PCCA edited HEK-293 cell lines.
Figure 12: RT-PCR for MUT and PCCA. Original unaltered gels appear in the appendix A.

Figure 13: Band Intensity from the RT-PCR for MUT edited HEK-293 cell lines
Figure 14: Band intensity from the RT-PCR for *PCCA* edited HEK-293 cell lines
Chapter 4: Discussion

MMA and PA are two genetic disorders of propionate metabolism with many secondary downstream biochemical effects, including mitochondrial abnormalities. Understanding the nature of these mitochondrial abnormalities could prove crucial for developing novel therapies. The above-described studies provided further evidence for mitochondrial dysfunction in individuals with MMA and PA.

Acylcarnitines profiles were studied in MMA and PA patients, and evidence was found for mitochondrial fatty acid oxidation abnormalities in individuals with MMA when they were sick, which is during catabolism and when biochemical abnormalities should be most apparent. Widespread changes were observed in multiple acylcarnitines corresponding to amino acid metabolism in well and sick MMA and PA patients. For example, tiglylcarnitine was significantly elevated in the sick MMA patients as compared to the sick PA patients. Tiglylcarnitine is an intermediate of the isoleucine metabolic pathway and is converted to 2-methyl-3hydroxybutyrylcarnitine and is converted from 2-methylbutyrylcarnitine. These results suggest that in sick MMA patients there could be increased disruption of the isoleucine metabolic pathway. The most striking differences between the comparisons between MMA and PA patients were in the 3-hydroxy fatty acid oxidation intermediates, which were exclusively elevated in the sick MMA patients. Increases in 3-hydroxy fatty acid oxidation intermediates have been shown to cause mitochondrial damage and dysfunction; in particular, 3-hydoxylated fatty acids act as potent uncouplers of oxidative phosphorylation (Tonin et al., 2013). The finding of increased levels of medium and 3-hydroxy fatty acid intermediates in sick
MMA patient plasma is a novel finding and one that points to another potential target for treatment during a metabolic crisis or sickness.

Further evidence for mitochondrial dysfunction was found in the urine of affected individuals. Di-tyrosine autofluorescence assay showed evidence for increased protein oxidation in the patient samples as compared to the control samples, which could be due to increased levels of reactive oxygen species arising from dysfunctional oxidative phosphorylation. The MMA patients had significantly decreased levels of antioxidants in their urine samples. A significant decrease in antioxidant capacity would inhibit the patient’s ability to reduce reactive oxygen species to oxygen or hydrogen peroxide. Reactive oxygen species can damage DNA, RNA, protein, and lipids. These data suggest that supplementation with antioxidants could be a beneficial treatment for MMA and PA patients.

In order to further characterize the mitochondrial phenotype in MMA and PA, isogenic HEK-293 knockout cell lines for MUT and PCCA were successfully created. These cell lines will be an invaluable tool for detailed functional investigation and eventual investigation for therapeutic targets. Four MUT edited HEK-293 cell lines had reduced or no mRNA expression based on RT-PCR results. MUT edited HEK-293 cell line 81 has no RNA for the MUT locus. This data suggests that non-sense mediated decay occurred in MUT edited HEK-293 cell line 81. Three of the PCCA edited HEK-293 cell lines appeared to have reduced mRNA expression based on RT-PCR, likely indicating reduced transcript levels.
Further work is needed to characterize these cell lines. Cell lines could be analyzed via Western blot analysis for protein expression of both methylmalonyl-CoA mutase and propionyl-CoA carboxylase. Enzymatic analysis of targeted cell lines could be performed via C\textsuperscript{14}-incorporation into macromolecules (an established mechanism for assaying propionate metabolism) (Deodato et al., 2006). Further characterization could include determination if any of the \textit{MUT} edited HEK-293 cell lines are cobalamin responsive.

Once fully characterized for the primary phenotype, detailed characterization of mitochondrial function of \textit{MUT} and \textit{PCCA} edited HEK-293 cell lines could be pursued. To look at the oxidative phosphorylation in the \textit{MUT} and \textit{PCCA} edited HEK-293 cell lines, the XF Mito Stress Test from Seahorse Biosciences (North Billerica, MA) could be used. This test looks at 5 measurements of mitochondrial function including basal respiration, ATP synthesis, proton leak, spare respiratory capacity, and maximum respiratory capacity. Changes in the respiratory chain capacity could cause disruption in electron flow to the electron transport chain or the inability of substrates to be utilized appropriately by the electron transport chain. To analyze the fatty acid oxidation in the \textit{MUT} and \textit{PCCA} edited HEK-293 cell lines, the XF Palmitate-BSA FAO kit can be used in conjunction with the XF Mito Stress Test from Seahorse Biosciences. The test would look at the same five parameters as the XF Mito Stress Test in addition to oxygen consumption rate (OCR) due to utilization of endogenous fatty acids, OCR due to utilization of exogenous fatty acids, and OCR due to uncoupling by free fatty acids. This would allow for identification of fatty acid oxidation deficits in the \textit{MUT} and \textit{PCCA} edited HEK-293 cell lines. To further elucidate the mitochondrial phenotypes, the \textit{MUT} and \textit{PCCA} edited HEK-293 cell lines could be stressed to further exacerbate
mitochondrial dysfunction. In the future, edited MUT and PCCA HEK-293 cell lines could be used for small molecule screens designed to provide targeted treatment options for MMA and PA patients.
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RT-PCR for *MUT* edited HEK-293 cell lines. Expected band size is 556 bp.

RT-PCR for *MUT* edited HEK-293 cell line 81 and *PCCA* edited HEK-293 cell lines. Expected band for *MUT* is 556 bp and expected band size for *PCCA* is 790 bp.
RT-PCR for *GADP* and *HPRT1* for the *MUT* and *PCCA* edited HEK-293 cell lines.

Black bar represents the *GADP* RT-PCR results. *GADP* has a band size of 87 bp and *HPRT1* has a band size of 94 bp.
RT-PCR for GADP and HPRT1 for the MUT and PCCA edited HEK-293 cell lines.

Black bar represents the GADP RT-PCR results. GADP has a band size of 87 bp and HPRT1 has a band size of 94 bp.
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Evaluation of mitochondrial dysfunction in biofluids from individuals with
Methylmalonic acidemia (MMA) and propionic acidemia (PA) and development of
HEK-293 MMA and PA knockout cell lines for further investigation

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University of California, Riverside, Riverside, CA

Graduated Cum Laude

Skills:

Extensive experience in molecular biology techniques including gene knockout using
Cas9/CRISPR, DNA isolation, RNA isolation, PCR, RT-PCR, qPCR, and sequencing
siRNA technology  Extensive mass spectrometry experience in both assay
development and assay utilization  Experience in protein isolation and purification

Western analysis  Sterile technique  Mammalian tissue cell culture  Mitochondrial
Function Assay  Subcellular Fractionation  Flow Cytometry  ELISA Assay
development  Confocal Microscopy
Computer Proficiencies:

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Prism  CodonCode Aligner  Sequencer Aligner  ImageJ

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August, 2012 – February, 2016

Graduate student in McKusick-Nathans Institute of Genetic Medicine
Johns Hopkins University School of Medicine

Main project focused on the secondary mitochondrial dysfunction in Methylmalonic Acidemia and Propionic Acidemia  Modeling diseases in primary cell culture and immortalized cell lines  Utilization of archived patient samples for fluorescent and absorbance assays  Mitochondrial function analysis in primary patient-derived fibroblast cultures  ELISA analysis of reactive oxygen species and immunologic response  Created knockout HEK293 cell lines using Cas9/CRISPR to model mitochondrial dysfunction in Methylmalonic Acidemia and Propionic Acidemia  Protocol development and troubleshooting  Statistical analysis

December, 2010- June, 2012

Undergraduate Research Assistant

University of California, Riverside

Main project focused on NSD1 and its role in inflammation  Experience in
protein isolation  Western blots  qPCR  Confocal microscopy  ELISA

Confocal and ELISA with *Anaplasma phagocytophilum*

**Presentations:**

Poster Presentation: Oxidative stress in three disorders of mitochondrial dysfunction: Methylmalonic Aciduria, Propionic Aciduria and Barth Syndrome. **Kaitlin Victor** and Hilary Vernon, ASHG Conference, Baltimore, MD, October, 2015


**Publications:**

Sakhon OS, **Victor KA**, Choy A, Tsuchiya T, Eulgem T, Pedra JH., 2013, NSD1 mitigates caspase-1 activation by listeriolysin O in macrophages, PLoS One, 8(9)