I encouraged his parents to consider everything that we have discussed and I would be happy to see them back here in clinic in 2-3 weeks later this month, so we continue to further discuss his exome findings. His parents were interested in continuing this discussion at this next clinic visit.

I spent 80 minutes obtaining the interval history, examination, and discussion with greater than 50% of the time spent in counseling and coordination of care in this extended clinic visit.

SCH refused to release those values.

Addendum (4/22/14): I reviewed the urine organic acid profile collected on April 11, 2013. This profile did show some elevations of some long chain 3-hydroxy-dicarboxylic acids (3OH-DCA), as well as the elevations of adipic and suberic acid. These were fairly high elevations. We also found elevations of other organic acids consistent with him receiving a commercial formula. The elevations of 3OH-DCA would bring some suspicion of either a long-chain fatty acid disorder or generalized mitochondrial dysfunction. If you were then to interpret this with the acylcarnitine profile done about 11 hours later (the next morning), they would not be consistent -- as then the acylcarnitine profile would have to have elevations of 3OH-C16- and 3OH-C18-acylcarntine species, but it did not have these elevations, only the C16 and C16:1 elevations. These 3OH-DCA elevations could be consistent with generalized mitochondrial dysfunction (such as may have been occurring secondary from his liver disease), or possibly commercial formula supplementation at that time. I also noted in his medical record on April 10, 2013 that he was being transitioned to Nutramigen.

SCH used a blood specimen not at critical event to hide the diagnosis, it will be explained in the next slide.

Electronically Authenticated by
J Lawrence Merritt, II, MD 04/22/2014 04:42 P

J Lawrence Merritt, II, MD , Attending Physician, Genetics

JLM/jwm Doc #3407321 d: 04/11/2014 01:50 P t: 04/14/2014 11:52 A (1874451-)
Location: BCG
cc: Simon P Horslen, MB ChB
Evelyn K Hsu, MD
Peggy J Kulpa, MD

[redacted]

Information for Clinicians

http://www.fodsupport.org/diagnostic-approach.htm

Diagnostic Approach to Disorders of Fat Oxidation

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Abstract

The disorders of mitochondrial fat oxidation present clinically with three major clinical phenotypes: Hypoketotic hypoglycemia, Cardiomyopathy, and Myopathy. Although these features can present together in some of the disorders, one will be the dominant presenting problem. This brief review attempts to clarify the clinical phenotypes of these inherited disorders while addressing the diagnostic value of various clinical loading tests and laboratory studies which are often used for making these diagnoses. With knowledge of the clinical presentation, these diagnoses can often be made very rapidly and at relatively low cost by certain methods of analysis, while it can take considerable time and be extremely expensive if multiple specific and nonspecific tests are performed. The relative strengths and weaknesses of the various investigations are discussed.

Key Words:

• Beta Oxidation
• Phenotypes
• Carnitine
• Acylcarnitine

Introduction

The disorders of mitochondrial fat oxidation have received much attention and focus for research over the past decade and represent explanations for some children with Reye-like syndrome, cardiomyopathy, hypotonia and developmental delay, hypoglycemia, as well, in some cases, sudden infant death (1). The diagnosis of these disorders has been difficult in the past and often required long periods of evaluation and highly specific enzyme assays to achieve that diagnosis.

Biochemical investigations of fat oxidation in heart, liver, and muscle have revealed an interesting compartmentalization within the cell for beta-oxidation of long-chain fatty acids. The process is subdivided into three systems: the Carnitine Cycle, the Mitochondrial Inner Membrane System, and the Mitochondrial Matrix System.

The Carnitine Cycle involves mainly long-chain fatty acids and how they are transported into the cell as well as how they pass through both the outer and inner mitochondrial membranes and arrive in the matrix compartment. Specific components of the Carnitine Cycle include: the carnitine uptake system on the plasma membrane, activation of the fatty acid to a co-enzyme A thioester by the acyl-CoA synthetase, conversion to an acylcarnitine by the enzyme carnitine palmitoyl transferase I (CPT I), transport of the acylcarnitine through the inner mitochondrial membrane by Carnitine/Acylcarnitine Translocase, and finally, conversion of the long-chain acylcarnitine to an active Acyl-CoA thioester by carnitine palmitoyl transferase II (CPT II). This reactivated acyl-CoA thioester, now inside the mitochondrion, can be oxidized by the various enzymatic steps of beta oxidation which successively remove 2 carbons as acetyl-CoA thereby shortening the original molecule.

The Mitochondrial Inner Membrane System of enzymes proceeds with 2 cycles of beta oxidation removing 2 carbons (acetyl-CoA) with each cycle. Therefore a fatty acid like palmitate is shortened from 16 carbons to 12 carbons while linoleate is shortened from 18 to 14 carbons. Specific components of this inner membrane system include: the Very long-chain acyl-CoA dehydrogenase (VLCAD), the trifunctional protein (TFP) complex which contains the remaining three enzymatic activities required for a single cycle of beta oxidation- namely enoyl-CoA hydratase, L-3-hydroxy acyl-CoA dehydrogenase (LCHAD), and thiolase.

All of these components of both the carnitine cycle and the inner mitochondrial membrane system are involved specifically with the degradation of long-chain length fatty acids in which L-carnitine is required.

The mitochondrial matrix system oxidizes fatty acids of shorter chain-length resulting from the enzymatic steps in the inner membrane system. It is important to realize that L-carnitine is not required for oxidation of these shorter chain-length compounds. In fact, current treatment strategy for the inherited deficiencies involving long-chain fatty acids is based on providing medium chain triglycerides in place of most of the dietary long-chain fats. The advantage is that these fatty acids, containing 10 carbons or less, can be transported directly into the mitochondrion without requiring L-carnitine or its associated acyltransferases such as CPT I or CPT II. In addition, each of the steps required for a cycle of beta-oxidation for these shorter compounds is accomplished by a series of enzymes in the mitochondrial matrix which have shorter carbon chain-length substrate specificity. These are the medium chain acyl-CoA dehydrogenase (MCAD), short-chain acyl-CoA dehydrogenase (SCAD), enoyl-CoA hydratase, short chain L-3-hydroxyacyl-CoA dehydrogenase, and the short chain specific thiolase (acetoacetyl-CoA thiolase).

Clinical Presentations

The approach to the diagnosis of inherited disorders involving mitochondrial fatty acid metabolism can be further focused by knowledge of the phenotypic presentation of each of the known deficiencies. With an understanding of the metabolic pathway and the relative value of various diagnostic tests, the diagnosis can be accomplished efficiently.

Detailed discussions of the individual disorders of mitochondrial fat oxidation are available elsewhere (1). Currently, inherited defects associated with the various components of the carnitine cycle are well-described as are many of those associated with the inner membrane and the mitochondrial matrix. Table 1 summarizes the clinical phenotypes associated with these different disorders.

Table 1 - Phenotypes Observed in Fat Oxidation Disorders

Carnitine Cycle Defects

Defect	Phenotype(s)
Carnitine Transporter	1. Dilated cardiomyopathy 2. Hypoketotic hypoglycemia 3. Skeletal myopathy
CPT 1	1. Hypoketotic hypoglycemia
Translocase	1. Hypertrophic cardiomyopathy with hypoketotic hypoglycemia
CPT 2	1. Neonatal cardiomyopathy with hypoketotic hypoglycemia and death in the first week 2. Hypoketotic hypoglycemia during or after the first year 3. Adult onset rhabomyolysis

Mitochondrial Inner Membrane Defects

Defect	Phenotype(s)
VLCAD	1. Hypoketotic hypoglycemia 2. Hypertrophic cardiomyopathy with Hypoketotic hypoglycemia
LCHAD	1. Hypoketotic hypoglycemia with Skeletal myopathy
Trifunctional Protein	1. Cardiomyopathy, myopathy and Hypoketotic hypoglycemia

Mitochondrial Matrix Defects

Defect	Phenotype(s)

MCAD	1. Hypoketotic hypoglycemia
SCAD	1. Developmental delay,hypotonia, (seizures,microcephaly)
Dienoyl-CoA Reductase	1. Hypotonia

These disorders emphasize three clinical presentations:

1. Hypoketotic hypoglycemia, **2.** Cardiomyopathy, and **3.** Myopathy. As can be seen, these clinical abnormalities can be in one combination or another.

Generally when a patient has one or more of these problems, there are a variety of ways to proceed for the diagnosis (2). The approach chosen often depends on what methods or technology is available. With the current emphasis on reduced medical costs in the United States and many other countries, the length of hospital stay and the extent of laboratory testing can often be seriously curtailed. These factors can potentially interfere with the ability to make the correct diagnosis in a timely fashion. Therefore it becomes increasingly important to understand which laboratory analyses are most informative and which reference laboratories are most reliable.

In general, the various clinical and laboratory approaches to the diagnosis of inherited disorders of mitochondrial fat oxidation are represented in Table 2.

Table 2 - Diagnostic Approaches to Defects of Fatty Acid Oxidation

Clinical Procedures

Clinical Presentation
Fasting Test with Metabolite Analysis
Loading Tests
a. Long chain triglyceride
b. Medium chain triglyceride
c. Phenylpropionate
d. Carnitine

Fluid Analyses

Blood/Plasma	**Urine**
Plasma Carnitine Levels cis-4-decenoic	Carnitine Levels Organic Acids

Acylcarnitine Profiles	Acylcarnitine Profiles
Molecular Tests	Acylglycine Analysis

Fibroblasts

- Oxidation rates of C1-substrates
- Tritium Release Assays using Palmitate and Myristate
- In Vitro Probe of Fat Oxidation Pathway
- Specific Enzyme or Uptake Assay
- Mutation Analysis

Clinical procedures focus on the clinical presentation (history and physical examination) with special emphasis on: age of onset, dietary preferences, ethnic origin, etc. Many institutions will subject a patient to a diagnostic period of fasting or to additional loading tests. The clinical presentation alone will begin to focus the investigation as to the possibility of a fat oxidation disorder.

Diagnostic fasting, on the other hand, can be dangerous if not properly monitored. If the selected test is appropriate, metabolite analysis can be productive after periods of fasting which correspond to the longest interval between feedings for that particular patient. In most cases, this corresponds to the overnight period.

Oral loading tests are also popular at many institutions during the initial diagnostic evaluation. Long chain triglyceride loading is very common in Europe for elucidating the possibility of a fat oxidation defect. A reduction in ketone production is usually the end-point and suggests the possibility of one of several different long chain enzyme defects (2).

Medium chain triglyceride loading is no longer popular as it can precipitate serious illness or death in children with MCAD deficiency or problems with either Electron Transfer Flavoprotein or its associated dehydrogenase.

After the identification of phenylpropionylglycine (PPG) in the urine of children with MCAD deficiency when ill, an oral load of phenylpropionate was also considered useful. PPG is not seen in the urine in newborns but does appear later when the gut bacteria are well established in patients with MCAD deficiency. However it may also be absent when the child has received oral antibiotics.

Oral carnitine loads were also popular for enhancing the excretion of diagnostic acylcarnitine species analyzed by fast atom bombardment mass spectrometry. This loading test no longer seems necessary since these analyses have become considerably more sensitive and are more appropriate when performed on blood samples as will be discussed below.

Body Fluid Analyses

Carnitine Levels:
L-Carnitine is found in many different dietary sources, especially protein and, surprisingly, in

large quantities in avocado. L-Carnitine is also synthesized in the body through a very complicated pathway involving several cellular compartments. The synthesis of L-carnitine begins with the incorporation of lysine from the diet into proteins like myosin. Lysine is then methylated utilizing s-adenosylmethionine (SAM) to trimethyl-lysine (TML) in the nucleus of the cell. When fragments of myosin with TML are broken down following turnover of the protein, TML is released from the lysosome and becomes available for the remainder of the synthesis. Skeletal muscle (myosin) is the primary source of TML which is exported to other tissues where carnitine synthesis is completed. The final step of synthesis requires butyrobetaine hydroxylase which converts butyrobetaine to L-carnitine. This occurs mainly in liver and kidney but not in muscle.

The extent to which the body gets L-carnitine from synthesis is not known. However, the fact that children and adults become deficient while receiving total parenteral nutrition (TPN) suggests that endogenous turnover of protein is a major contributor to body carnitine stores. Despite the fact that TPN amino acid solutions contain very high levels of lysine, endogenous synthesis is suppressed by continuous provision of substrate via TPN. The kidney attempts to conserve L-carnitine by a transport system involving its reabsorption.

Carnitine deficiency can be due to a primary deficiency in which endogenous synthesis is decreased (TPN, renal carnitine loss due to transport), or a secondary deficiency may occur due to excessive renal loss as in Fanconi syndrome or for secondary biochemical effects due to inherited metabolic disorders. A good example is any disorder affecting the availability of SAM for methylation. The first inherited disorder described with carnitine deficiency was the methylene tetrahydrofolate reductase deficiency. Another example is the carnitine deficiency associated with ascorbic acid deficiency (Scurvy). Ascorbate is a required co-factor for the synthesis of L-carnitine. L-Carnitine deficiency is associated with many inherited biochemical defects involving branched chain amino acid degradation and fatty acid oxidation but the mechanism causing these deficiency states is not yet clear.

Carnitine deficiency is also commonly associated with anticonvulsant therapy - especially with valproate and multi drug therapy for seizures. It has become routine to monitor carnitine levels with these treatments to prevent secondary metabolic complications associated with its deficiency.

Urine carnitine levels are usually expressed as uMol/L or uMol/gm creatinine and as free carnitine, esterified carnitine (acylcarnitines), and total carnitine. The ratio of esterified to free carnitine is normally less than 4. When higher than this, it reflects increased production of acylcarnitines at the expense of free carnitine which represents a metabolic process. The simplest example is during ketosis where the total carnitine may be normal while the esterified fraction (acetylcarnitine) is greatly increased and the free fraction is depressed thus elevating the ratio of acylcarnitines to free carnitine. Urine carnitine levels are not of great value, in general, unless one is specifically looking for conditions associated with excessive loss via the kidneys. Further, the acylcarnitines excreted by the kidney rarely exceed a 10 carbon chain length which makes the assessment of long chain fatty acid defects impossible from the analysis of carnitine levels in urine. (See "Acylcarnitine Profiles" below.)

Plasma levels of carnitine are of greater utility and are expressed as uM concentrations for free carnitine, short - medium chain acylcarnitines, and by some techniques, long-chain acylcarnitines. The sum is the plasma total carnitine level. The carnitine level in the plasma may give a clue to several of the fat oxidation defects. First, in the hepatic presentation of CPT I deficiency, the plasma total carnitine is usually significantly increased largely due to the free carnitine fraction. In contrast, the carnitine transporter defect frequently presents with levels under 10 um (normal = ~46 um).

Most of the other disorders have an associated milder secondary deficiency. Although carnitine levels may give a clue to the presence of a metabolic disorder, the levels are not specific to any disorder.

Organic Acids and other Metabolites:

Urine organic acid analysis is generally more specific for the diagnosis of disorders of branched chain amino acids than it is for the mitochondrial fat oxidation disorders. For these, MCAD deficiency is the most consistently recognized due to the excretion of hexanoyl-, suberyl-, and occasionally phenylpropionyl-glycines. These same metabolites can also be seen in the multiple acyl-CoA dehydrogenase (MADD) deficiency. Plasma levels of cis-4-decenoic can also be observed in both MCAD and MADD. The latter can be confused with SCAD deficiency since ethylmalonate can be elevated in both. In the case of CPT I deficiency, it is the only one in which organic acid analysis is consistently normal.

The most consistent clue to a fat oxidation defect by organic acid analysis is the presence of dicarboxylic acids such as adipic, suberic, dehydrosuberic, sebacic, dehydrosebacic, and modest amounts of 3-OH dicarboxylics - especially 3-OH-decanedioic. These compounds are the result of defective mitochondrial fat oxidation and are the products of omega oxidation and peroxisomal chain shortening. Their presence clearly indicates defective mitochondrial oxidation but does not specifically identify the enzyme defect. There is no dicarboxylic aciduria in CPT I deficiency. Artifactual dicarboxylic aciduria can occur due to dietary medium chain triglycerides (MCT). This can be recognized by the absence of unsaturated dicarboxylic acids since MCT contains only saturated species of 6, 8, and 10 carbons (adipic, suberic, and sebacic). Large amounts of 3-hydroxy-dicarboxylic acids of 10, 12, and 14 carbon chain length often point to LCHAD deficiency but this profile can not be distinguished from deficiency of the trifunctional protein (TFP) of which LCHAD is one component of the alpha gene of the TFP. In all of these situations, ketosis, if present, is rather unimpressive due to the compromise in mitochondrial beta-oxidation. By contrast, when there is real ketoacidosis as in a ketone utilization defect like beta ketothiolase or succinyl-CoA transferase deficiencies, or acute idiopathic ketoacidosis, dicarboxylic acids are detectable in greater than normal quantities but insignificant compared to the quantities of ketones being produced. This is due to flooding the beta-oxidation pathway in the mitochondrion and does not signify an isolated defect of the pathway.

In Vitro Oxidation Studies:

On many occasions, it is desirable to screen fibroblasts in culture for their ability to oxidize various precursors to the pathway of beta oxidation. Typically, the rates of oxidation of 14C1 - butyrate, - octanoate, and - palmitate are determined compared to normal cell lines as an index as to where in the pathway the defect could be found. Similar information can also be determined

by the in vitro analysis of the amount of tritiated HOH released when suspect cell lines are incubated with compounds such as [9,10] - 3H - palmitate or [9,10]- 3H -myristate. The specificity of these assays depends on which enzyme is deficient, how close that step is to the chain length of the compound being tested, conditions of assay, and the experience of the investigator.

Acylcarnitine Profiles:
When available, this test is the most specific and direct approach for the specific diagnosis of most of the disorders of mitochondrial fat oxidation as well as many of those involving branched chain amino acids. Table 3 summarizes the main features of the acylcarnitine profile analyzed by tandem or electrospray mass spectrometry.

Table 3 - Analysis of Acylcarnitine Profiles from Guthrie Cards

Blood Spot (PKU Card) Analysis

Defect	Phenotype(s)
1. Diagnostic for:	a. Translocase - CPT 2 b. VLCAD Deficiency c. LCHAD - Trifunctional Protein Deficiency d. MCAD Deficiency e. SCAD Deficiency f. Dienoyl-CoA Reductase Deficiency
2. Neonatal screening demonstrated for most disorders 3. Postmortem diagnosis possible from actual newborn screening card or postmortem toxicology blood sample	

In each disorder where a missing enzyme results in the accumulation of an acyl-CoA intermediate which was the substrate, this compound is converted into an acylcarnitine (by cellular carnitine acyltransferases) which can then be detected by tandem or electrospray mass spectrometry. For example, in CPT I deficiency there is no enzyme present to convert long-chain acyl-CoA compounds to the corresponding acylcarnitines, therefore there are no disease specific acylcarnitines produced in this deficiency. However, from Translocase deficiency down the pathway to SCAD deficiency, acyl-CoA intermediates (representing substrates for those enzymes) accumulate and are converted to acylcarnitines by the carnitine acyltransferases. In each case, a disease specific profile of acylcarnitine species is produced which can be readily detected in blood samples, fibroblasts, or amniocytes.

Whole blood on a Guthrie (PKU) card is the preferred sample since, unlike urine, blood contains the full chain-length spectrum of acylcarnitines reflecting the fat oxidation defects. The profiles are specific and diagnostic for all defects except for the distinction between the identical profile observed in both LCHAD and TFP deficiency or the different but indistinguishable profile which characterizes Translocase and CPT II deficiency. It now appears that the clinical phenotypes are

sufficiently different that one can distinguish these disorders despite the similarity in the acylcarnitine profile (See Table 1). In two of the disorders of mitochondrial fat oxidation, LCHAD and SCAD deficiency, the acylcarnitine profile from blood can be intermittently negative for the disorder when they are clinically well. This fact emphasizes the need to analyze urine organic acids as well as the acylcarnitine profiles from blood spots to ensure recognizing the disorder. Ethylmalonate is very consistently present in SCAD deficiency although it can be observed in other defects. For LCHAD deficiency, sample analysis for organic acids or acylcarnitines is best carried out on samples from when the patient is ill.

These reservations do not apply to diagnosis of these disorders in the neonatal period. When blood spots are obtained on the second day from a neonate, the infant has been essentially fasted and the acylcarnitine profiles are accentuated and consistent. This is the reason for searching for the original newborn screening card in those cases in which an infant has died without explanation but with post-mortem findings such as steatosis or cerebral edema. The Guthrie card analysis of acylcarnitines represents an excellent opportunity for neonatal and, at times, post-mortem diagnosis of fat oxidation defects.

In vitro analysis probing the fat oxidation pathway with fatty acid precursors:
This extremely powerful diagnostic method is also based on the measurement by tandem or electrospray mass spectrometry of disease-specific acylcarnitines produced when stable isotope labeled fatty acid precursors are incubated with cells in the presence of excess L-Carnitine. The deuterium label is placed on the omega end of the molecule (16-2H3-palmitate or 17,17,18,18, -2H4-linoleate) such that every intermediate produced through beta-oxidation will be labeled as an acylcarnitine. This represents a single test for all enzymes from Translocase down through SCAD accounting for every enzymatic step involved in the degradation of saturated and unsaturated fatty acids. This technique is highly specific and can be applied to fibroblasts (3,4) or for prenatal diagnosis from amniocytes (5). Since it is performed on cells under selected conditions, the diagnosis will always be apparent unlike the intermittent results seen with some defects from blood analysis.

Molecular Diagnosis:
Currently, molecular diagnosis of mitochondrial fat oxidation defects is limited to MCAD and LCHAD deficiencies. In the former, a very common mutation A985G (K329E) was identified which accounts for 90% of affected individuals. However, one out of five families with MCAD deficiency is compound heterozygous for this mutation making it difficult to diagnose affected and carriers by analysis only for the A985G mutation (1). This necessitates testing individuals who appear heterozygous with biochemical markers to identify which are actually affected.

A similar situation seems to exist for isolated LCHAD deficiency in whom a common mutation , G1528C, has been identified (6). In a recent study of eleven new cases, this mutation was found on at least one allele in all patients. The frequency of compound heterozygotes appeared much higher than in MCAD deficiency - seven of the 11 cases. The presence of the G1528C seems to be a good indicator for LCHAD deficiency, but unless both parents are known to carry this mutation, biochemical markers must be examined to identify compound heterozygous individuals. The DNA analysis for the G1528C mutation is complicated by the presence of a "pseudogene" with 93 % homology. Standard analysis by PCR may produce erroneous

impressions such as true homozygotes appearing heterozygous or problems with RT-PCR resulting in compound heterozygous individuals appearing to be homozygous. These problems are overcome by a newly developed method which eliminates the contribution of the "pseudogene" using nested primers. Despite this, one is still faced with the issue of whether an apparent carrier of the G1528C mutation is compound heterozygous (7).

For both MCAD and LCHAD, it is recommended that PKU cards with whole blood be analyzed for both the appropriate mutation and the acylcarnitine profile simultaneously. This way, affected individuals will not be overlooked.

Summary

The clinical presentations of the various inherited disorders of mitochondrial fatty acid oxidation are frequently characteristic of one or more deficiencies and can direct specific laboratory testing to establish the diagnosis rapidly and economically. If the technology is available, the combination of whole blood acylcarnitine analysis and urine organic acid analysis is frequently the most direct approach to the diagnosis. Virtually all of the disorders can be diagnosed from specific individual assays involving fibroblasts. With the advent of the metabolic probe of the beta-oxidation pathway in fibroblasts, disorders ranging from translocase deficiency down to and including SCAD deficiency can be accomplished efficiently and simultaneously using tandem mass spectrometry.

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