Sudden and Unexpected Neonatal Death: A Protocol for the Postmortem Diagnosis of Fatty Acid Oxidation Disorders

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Fatty acid oxidation (FAO) disorders are frequently reported as the cause of sudden and unexpected death, but their postmortem identification remains difficult. Over a period of 5 years, the authors have identified 44 cases representing five FAO disorders and 19 additional cases without a diagnosis of a specific defect. Among the two groups, 13 patients died in the neonatal period, 10 in the FAO group, and three from the undetermined defect group. This outcome was consistently associated with exclusive breast feeding and presumably poor caloric intake. The diagnosis of FAO disorder in these cases was based on the analysis of postmortem liver and bile. In postmortem liver, informative findings are microvesicular steatosis, elevated fatty acid concentrations, glucose depletion, and low carnitine concentration. Bile carnitine analysis and acylcarnitine profiling have expanded significantly the effectiveness of the initial protocol and could lead, based on preliminary observations, to better identification of patients who may have been missed or left undetermined by the analysis of liver only. If an autopsy is not performed, informative findings can still be obtained by analysis of blood spots collected for newborn screenings and by biochemical testing of parents and asymptomatic siblings.

Mitochondrial oxidation of fatty acids plays an essential role in energy metabolism during periods of fasting.[1] The production of acetyl-coenzyme A (CoA) fuels the hepatic synthesis of ketone bodies that are utilized by peripheral tissues via the reactions of the Krebs cycle after conversion back to acetyl-CoA.[2] After birth, the sudden interruption of a continuous food supply leads to the mobilization of fatty acids from adipose tissue and the appearance of ketone bodies in blood within the first day of life.[3] Accordingly, for the first 24 to 48 hours after birth, energy metabolism may depend greatly on fatty acid utilization, a need modulated by the timing of feedings and the composition and caloric strength of the nutrients administered.[4]

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At least 22 different disorders of fatty acid oxidation (FAO) have been characterized to date (Table 1).[5] Collectively, they probably represent the most common group of inborn errors of metabolism known to date,[6] with the distinctive features of high morbidity and mortality rates in children with previously normal growth and development. Acute manifestations include hypoglycemia, acute liver disease, and cardiac failure, which are triggered by fasting and common pediatric illnesses.[5-7] Sudden and unexpected death has been reported to occur in the majority of FAO disorders (Table 1), leading to the hypothesis that FAO disorders are the cause of approximately 5% of all cases of sudden death in the first year of life, and a greater proportion of children who die suddenly between 1 and 5 years of life.[8-11] This poor outcome is conspicuously different from the favorable long-term prognosis of patients who survive the first acute episode and receive treatment. When promptly diagnosed, these patients respond very favorably to simple and inexpensive therapeutic measures.[12]

A significant proportion of patients with an FAO disorder experience hypoglycemia within the first 72 hours after birth.[13] Depending on

Table 1. Inborn Errors of Fatty Acid Metabolism and Sudden Death

	Recognized as Cause of Sudden Death
Disorders of membrane-bound enzymes	
Plasma membrane	
Carnitine transport defect	+++
Long-chain fatty acid transport defect	−
Mitochondrial membranes	
Carnitine palmitoyltransferase I deficiency	+
Translocase deficiency	+
Carnitine palmitoyltransferase II deficiency (neonatal onset)	+
Carnitine palmitoyltransferase II deficiency (late onset)	−
Very long-chain acyl-CoA dehydrogenase deficiency	+++
ETF-QO deficiency (glutaric acidemia type 2)	+++
Long-chain 3-hydroxy acyl-CoA dehydrogenase deficiency	+++
α-Trifunctional protein deficiency	+
β-Trifunctional protein deficiency	+
Disorders of mitochondrial matrix enzymes	
Medium-chain acyl-CoA dehydrogenase deficiency	+++
Short-chain acyl-CoA dehydrogenase deficiency	+
α-ETF deficiency (glutaric acidemia type 2)	+++
β-ETF deficiency (glutaric acidemia type 2)	+++
Riboflavin responsive form(s) (glutaric acidemia type 2)	−
Short-chain 3-hydroxy acyl-CoA dehydrogenase deficiency (fibroblasts)	+
Short-chain 3-hydroxy acyl-CoA dehydrogenase deficiency (muscle)	−
Short-chain 3-hydroxy acyl-CoA dehydrogenase deficiency (liver)	+++
Medium-chain 3-ketoacyl-CoA thiolase deficiency	+
2,4-Dienoyl-CoA reductase deficiency	−
3-Hydroxy 3-methylglutaryl-CoA synthase deficiency	−
3-Hydroxy 3-methylglutaryl-CoA lyase deficiency	+

+++, frequently reported; +, reported in a few cases; −, not yet reported.

several factors (eg, type of feeding, schedule), the severity of these episodes may vary from relatively mild hypoglycemia, which responds rapidly to intravenous glucose or resolves spontaneously with feeding, to sudden and unexpected death.[14-18] Those who recover may not suffer another episode for several years, and diagnosis is made only when another sibling becomes symptomatic or dies suddenly.[19]

Until recently, the effort to investigate the collective contribution of inborn errors of organic acid and fatty acid metabolism to sudden death cases was directed to the biochemical analysis of body fluids.[20-22] The limitations of these methods are of a dual nature: (1) Several disorders are not associated with informative metabolite profiles in blood and urine[23] and (2) The collection of postmortem samples for metabolic investigations is frequently overlooked, or these specimens are completely consumed for drug and toxicology screenings. The diagnostic contribution of these routine procedures in pediatric autopsies is likely to be inferior to the potential for detection of metabolic disorders in sudden death cases. As more data become available, serious consideration should be given to shifting existing laboratory resources from toxicological to biochemical studies.

Figure 1 summarizes a diagnostic protocol that allows detection of multiple disorders based on the evaluation of independent diagnostic criteria. If parental permission to perform an autopsy is not granted, an immediate effort should be made to retrieve specimens that may still be available: if death occurred in a nursery or hospital setting, the laboratory should be contacted immediately and asked to hold any unused portions of blood and urine specimens previously sent for routine tests. If available, these specimens should undergo a complete metabolic workup including levels of plasma carnitine, acylcarnitines, free fatty acids, urine organic acids, and acylglycines.[23-26] If death occurs at home after discharge, retrieval of any unused portion of the blood spots collected for newborn screenings could be easily arranged via a request sub-

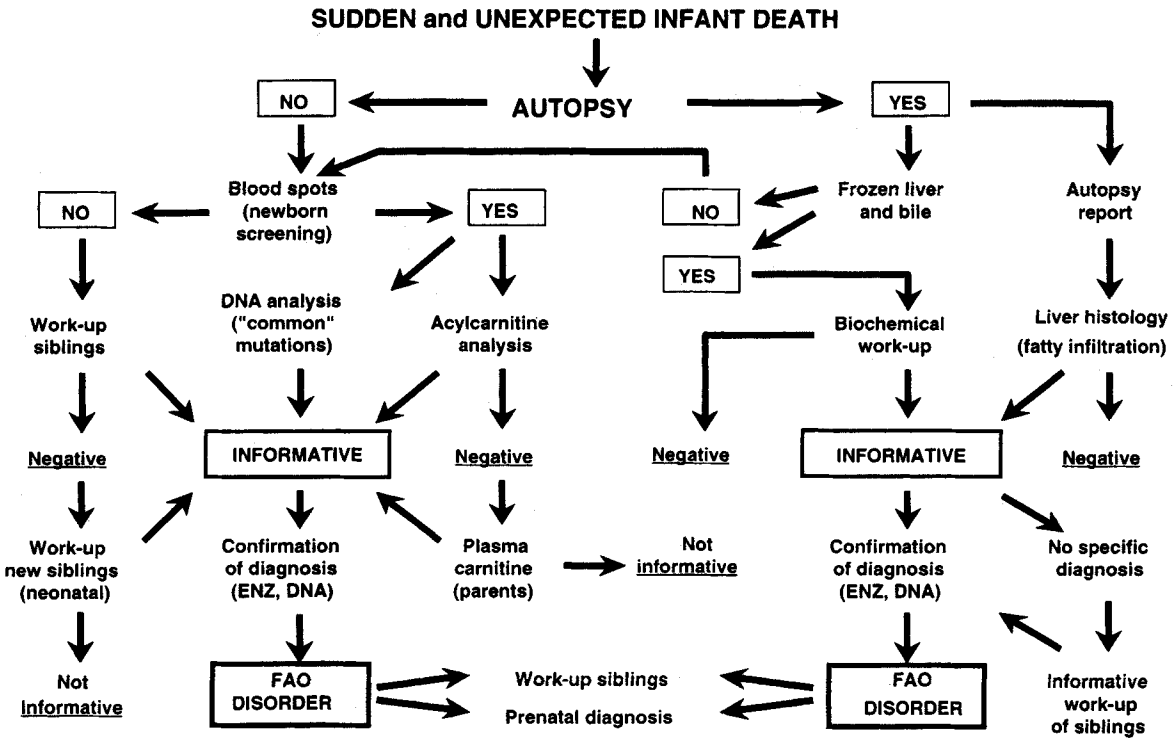

Figure 1. Protocol for the postmortem screening of fatty acid oxidation disorders.

mitted by a physician to the state laboratory. Blood spots should be sent for acylcarnitine analysis by electrospray tandem mass spectrometry.[24,26] This approach is preferable to the direct molecular analysis of relatively "common" mutations, which is too limited in scope and has the disadvantage of precluding an effective screening of multiple conditions, not to mention the tangible risk of missing patients who carry less common mutations.[27,28] If the acylcarnitine profile is not informative, testing of parental plasma carnitine levels is indicated to rule out the possibility that they are heterozygous for carnitine uptake defect.[15,29]

When the procedure results listed above are negative or not available, a biochemical workup of all siblings should be considered. Although this approach needs to take into consideration the potentially concealed biochemical phenotype of several FAO disorders, it has been very effective in the specific case of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency.

MCAD deficiency is the most common FAO disorder and possibly one of the most frequent metabolic diseases overall. According to the most recent estimates, it occurs in approximately 1 in 10,000 newborns.[27] Although growth and development are not affected, acute episodes of decompensation often result in sudden and unexpected death. This outcome is estimated to occur in 30% to 50% of cases, especially at the first episode.[30] These are fatalities that could be prevented with simple dietary treatment and basic prophylactic measures. Currently, analysis of blood acylcarnitines and urine acylglycines,[24,31] a combination capable of identifying asymptomatic patients under any circumstances, is the best method for biochemical diagnosis of MCAD deficiency. Table 2 summarizes the results of a 5-year prospective study of urine acylglycine analysis among 469 patients in three groups at risk: (1) Asymptomatic siblings of patients with newly diagnosed deficiency, (2) Siblings of patients who died of sudden infant death syndrome, and (3) Sudden death. In these groups, we diagnosed twenty-six cases of MCAD deficiency, including 13 asymptomatic siblings of known MCAD patients, 11 cases of which were diagnosed in the first day of life, and six older

Table 2. Diagnosis of MCAD Deficiency by Urine Acylglycine Analysis, Including Postmortem Urine Specimens (1988-1993)

At-Risk Group	*No. of Cases*	*No. of Affected Patients*
Asymptomatic MCAD siblings	70	13
SIDS siblings	303	6
Sudden death (0-4 yr) (postmortem urine)	96	7

siblings of infants whose deaths were diagnosed as sudden infant death syndrome.[32]

If the biochemical workup of asymptomatic siblings is not informative, consideration should be given to performing a skin biopsy and establishing a line of cultured fibroblasts. The evaluation of cultured cells in vitro should include measuring the oxidation rate of multiple substrates. Ideally, this would include octanoate (C_8) and oleate ($C_{18:1}$), in addition to myristate (C_{14}) and palmitate (C_{16}).[33] Impaired oxidation of octanoate with normal activity using longer chain substrates[34] and a compromise of the oxidation rate, which becomes more severe with longer chain substrates,[35] are potential outcomes that could be missed or overlooked. The natural history of many FAO disorders is still poorly defined, so it is plausible to pursue the recognition of patients who remained clinically asymptomatic for the first few years of life. In spite of an extensive workup, there are defects that are not expressed in skin fibroblasts. For these disorders, specifically 3-hydroxy 3-methylglutaryl-CoA synthase deficiency[36] and liver-specific short-chain acyl-CoA dehydrogenase deficiency,[37] a liver biopsy may offer the only available diagnostic tool in vitro, pending the future development of molecular methods.[38]

Autopsy Specimen Collection

During postmortem examination, a small (1 to 5 g) fragment of liver is collected and stored frozen. For biochemical analyses, timing is less of a critical factor, and specimen collection could be delayed to a maximum of 72 hours after the time of death.[39] Bile is collected by direct puncture of the gallbladder and stored frozen. In most cases, 2 to 3 mL of bile are recovered; smaller volumes could be spotted on filter paper and stored at room temperature. Macroscopic examination of the bladder should include a special effort to collect even the smallest volume of urine available. Traces of urine embedded in the bladder posterior wall could be recovered effectively with a cotton swab, later to be washed with distilled water. This specimen is suitable for quantitative analyses in ratio to creatinine to be measured separately. As reported by Lundemose et al,[40] cultured skin fibroblasts can be grown successfully from biopsy specimens of the Achille's tendon obtained up to 4 days after death.

Analysis to be Performed and Methods

Liver homogenate is separated by ultracentrifugation into supernatant and pellet.[39] The pellet is washed with methanol, and an aliquot is analyzed by gas chromatography–mass spectrometry to provide quantitative determination of C_8-C_{18} fatty acids and glucose.[39] Total and free carnitine concentration are measured in the liver supernatant by an enzymatic radioisotopic methods.[41] In bile, carnitine fractions are measured by the same method with some modifications.[42] Qualitative acylcarnitine profiles of bile and blood spots are performed by electrospray tandem mass spectrometry.[24,26,43] If available, urine samples are tested for organic acids and acylglycines according to established methods.[23,44]

Informative Findings

Informative biochemical criteria are represented by microvesicular steatosis (Oil Red O stain is recommended), elevated fatty acid concentrations, glucose depletion, and low total carnitine concentration in liver homogenate,[39,45] and by carnitine and acylcarnitine profiles in bile.[43]

No single test is likely to be sufficiently specific to be used alone for screening purpose. In particular, it is important to underscore the unreliability of steatosis as the sole criteria to suspect or exclude a possible underlying FAO disorder during the postmortem evaluation of a case of sudden death.[45] The outcome of the postmortem protocol is considered abnormal when two or more abnormal findings are detected among the four diagnostic criteria for liver specimens. Table 3 summarizes the abnor-

Table 3. Sudden and Unexpected Death Cases of a Confirmed or Suspected Defect of Fatty Acid Metabolism: Postmortem Diagnosis by Analysis of Liver and Bile (1993-1998)

			Liver findings				
FAO Disorder	*No. of Cases*	*Neonatal Death*	*Fatty Infiltration**	*Abnormal Fatty Acids†*	*Glucose Depletion‡*	*Carnitine Depletion§*	*Elevated Bile AC/FC Ratio‖*
Carnitine uptake defect	7	2	4/7	0/7	6/7	7/7	0/0
VLCAD deficiency	7	2	4/7	6/7	6/7	3/7	3/3
LCHAD deficiency	7	0	6/7	4/7	5/7	1/7	4/4
MCAD deficiency	14	3	13/14	11/14	10/14	7/14	3/3
ETF/ETF-QO deficiency	9	3	8/9	9/9	8/9	1/9	0/0
No. of cases with known diagnosis (%)	44	10 (23%)	35/44 (80%)	30/44 (68%)	35/44 (80%)	19/44 (43%)	10/10 (100%)
Undetermined¶ diagnosis (%)	19	3 (16%)	16/19 (84%)	11/19 (58%)	10/19 (53%)	6/19 (32%)	
Total of all cases (%)	63	13 (21%)	51/63 (81%)	41/63 (65%)	45/63 (71%)	25/63 (40%)	

Abbreviations: ETF/ETF-QO, electron transfer flavoprotein/electron transfer flavoprotein ubiquinone oxidoreductase (glutaric acidemia type 2); LCHAD, long chain 3-hydroxy acyl-CoA dehydrogenase.
* Moderate to diffuse microvesicular changes.[45]
† Elevated concentrations in liver homogenate of unsaturated fatty acids; reference values (μmol/100 mg protein): $C_{10:1}$, <0.01; $C_{14:1}$, <0.08; $C_{16:1}$, <1.36.[39]
‡ Low concentration of glucose in liver homogenate; reference values: <0.2 μmol/100 mg protein.[39]
§ Low concentration of total carnitine in liver homogenate; reference values: <0.34 μmol/g wet weight.[39]
‖ Bile specimens were available in 10 of 44 cases with established diagnoses. Acylcarnitine profiles were typical of the underlying disorder (data not shown). Reference values for acylcarnitine-free carnitine ratio: 0.2 ± 0.1 (0.1-0.5)[43]
¶ Cases with multiple abnormal results but without a confirmed diagnosis.
Data from references 15, 39, 45, and 46 and unpublished observations (Piero Rinaldo, 1993-1998).

mal results obtained over a 5-year period by application of this protocol to more than 500 cases of sudden death.[15,39,45,46] Twenty-three percent (10 of 44) of the cases diagnosed postmortem with either MCAD deficiency, glutaric acidemia type II, carnitine uptake deficiency, or very long–chain acyl-CoA dehydrogenase (VLCAD) deficiency died within the first week of life. In retrospect, a common denominator among these cases of neonatal death was that the patients were exclusively breast-fed, indicating excessive energy consumption or limited caloric intake.[12]

Nineteen cases showed two or more abnormal findings suggesting an underlying FAO disorder, but it was not possible to reach a conclusive diagnosis. In three of the confirmed cases, the diagnosis was actually based on a characteristic bile acylcarnitine profile (Yoon et al, unpublished results). If bile had not been available, these cases would have been classified as undetermined too.[45] The significance of bile acylcarnitine analysis has been confirmed recently in the mouse knockout model of long-chain acyl-CoA dehydrogenase deficiency, a disorder not yet identified in humans.[47]

The lack of a specific postmortem diagnosis in these cases mirrors what happens frequently in clinical practice. Although our understanding of the biochemical and molecular basis of FAO disorders has improved dramatically in recent years, clinically suspected patients often remain without a specific diagnosis.[48] Such patients are considered to be affected with an "unspecified" fatty acid oxidation disorder, a less than ideal but nevertheless appropriate clinical concept, because new discoveries often stem from the study of these atypical and valuable cases.[34-36,49] Currently, the main factor limiting the interpretation of results obtained according to this protocol is the unavailability of positive controls with FAO disorders other than the five defects listed in Table 3.

Depending on the tentative diagnosis under consideration, the availability of specimens, and the degree of access to family members, confirmatory and conclusive testing is available by a variety of methods, including enzyme assays, im-

munoblotting, and molecular analyses. Although a reliable measurement of selected enzyme activities is possible in postmortem liver only under carefully monitored conditions,[50] targeted molecular studies are feasible in virtually any specimen, including paraffin-fixed tissues.[51]

The postmortem diagnosis of FAO disorders in sudden death victims is important for accurate genetic counseling and the evaluation of siblings who may be at risk for significant, yet often preventable, morbidity and mortality. Although a systematic biochemical workup of all cases of pediatric sudden death is not a feasible option, it is crucial that pediatric pathologists perform a comprehensive autopsy on all newborns and infants who die suddenly; at minimum, freeze liver and bile specimens; and study further the cases of fatty infiltration of the liver and other risk factors for metabolic disorders.[52] These risk factors include the finding of fatty infiltration of the liver and other organs, a possible family history (sudden death, Reye's syndrome, myopathy), a history of lethargy, and vomiting or fasting before death.[45] In addition, a history of exclusive breast feeding is an important and potentially overlooked element in the evaluation of cases of sudden and unexpected death in the first week of life.

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