The Metabolic Investigation of Sudden Unexplained death in Childhood

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Abstract

Inherited metabolic disorders account for a small but significant number of sudden unexplained deaths in neonates, infants and occasionally older children. Post mortem investigations offer the final opportunity to establish a diagnosis. Such diagnoses are of great importance to the families concerned and provide the opportunity for genetic counselling and antenatal diagnosis. Current advances in technology, particularly in the case of electrospray ionisation tandem mass spectrometry MS/MS, have revolutionised the investigation of metabolic disease at post-mortem, facilitating the identification of a wide range of metabolic diseases in tiny samples of blood, plasma and bile. Accurate diagnosis relies on the timely collection of appropriate samples and the subsequent selection of informative testing. In order to maximise the chances of a diagnosis a collaborative approach between the various disciplines is vital. A brief description of the more frequently encountered inherited disorders, collection and processing of appropriate samples and available investigations that may lead to accurate diagnosis are clearly described.

Introduction

Garrod in 1909 used the term *Inborn Errors of Metabolism* in defining a spectrum of genetically inherited disorders characterised by blocks in the metabolic pathway .[1] While Garrod only described 4 such disorders which were known at the time, our current knowledge of the human genome has expanded such that there has been a vast increase in the number of diseases defined and which may now be diagnosed. However inherited metabolic disease that results in premature death is still sadly under diagnosed.[2] While in the United States the concept of "The Metabolic Autopsy" is now well recognised, [3] here in the UK, uncertainties and confusion remain as to which samples should be taken, how these should be processed and the nature of investigations that are currently available. It is primarily the application of mass spectrometry to clinical biochemistry [4,5] coupled with current advances in molecular genetics that has opened up the possibilities for diagnosis in a wide range of

disorders.
Public awareness of the issues surrounding sudden infant death syndrome (SIDS) has served to highlight the importance of collecting and storing appropriate samples that can, if necessary, be reinvestigated at a later date. Metabolic disorders have profound effects not only on patients and their immediate family, but also on the long-term reproductive health of that family, often with implications for the extended family. Only by accurate diagnosis can appropriate genetic counselling and antenatal diagnosis be made available to the families concerned.
The investigation of suspected metabolic disease when there is sudden death of unknown cause, or where investigations in life have not resulted in a diagnosis, requires a co-ordinated plan of action. The importance of good clinical liaison between the different disciplines in facilitating appropriate investigations and in conserving precious post-mortem samples cannot be over-emphasised. Clinical scientists should be in a position to advise on, or at least to know to whom to refer for expert advice, questions relating to the collection of appropriate samples and the types of investigations that are available in cases of suspected metabolic disease.
Many inborn errors of metabolism can present as acute metabolic disease in the newborn period. Most babies with inherited metabolic disorders are born apparently healthy and may show a typical asymptomatic period with clinical manifestation from the second day of life onwards. However, some disorders may present at birth or may be detected by antenatal ultrasonography. Sadly many babies with inherited metabolic disease follow a rapid course of deterioration and may succumb before appropriate investigations can be undertaken. The pattern of deterioration may be a useful indicator of the underlying disorder (*see* table 1). [6]
The intention of this article is to provide a brief description of the metabolic disorders that are more likely to be encountered in the context of unexplained sudden death, or death following a brief illness, in neonates and older infants. The subsequent collection and processing of appropriate samples and types of investigations that are currently available are clearly described.

Sudden Infant Death Syndrome

SIDS is the unexpected death of an apparently well infant over one month of age, for which no cause can be found, in spite of a post-mortem examination.[7] The precise cause of such sudden unexplained death in infants under one year of age remains unknown in approximately 80% of cases.[8] Of the known causes, infections account for the highest number of "natural" causes. [9,10] The diagnosis of SIDS or sudden unexplained death in infancy (SUDI), still remains the largest single cause of death in children in the industrialised world. The frequency is reported at 1:1000 live births and represents 25% of all deaths in the first year of life. Current and future advances in our understanding of human biology along with increasingly sophisticated technology are likely to lead to the recognition of new disorders that may result in sudden death in a previously "well" infant, and provided that stored material is available for future investigation, an opportunity for retrospective diagnosis remains. The recent high profile cases of mothers wrongly imprisoned for infanticide highlights the importance of the opportunity to re-investigate specimens taken at post-mortem. This may be crucial for the family concerned.
Although the majority of SIDS is due to unknown cause(s) it is recognised that a small but significant number of sudden deaths in infancy and occasionally in older children

are a result of inherited metabolic disease. Emery was the first to observe that a wide range of metabolic disorders may present as *sudden infant death syndrome.*[11] It may result from dramatic cardiac failure, shock or cardiac arrest in many metabolic circumstances. Although critical review may suggest that not all these deaths were entirely unexpected, they were initially unexplained. Identification of the cause of death in such cases is consolation to parents and offers the opportunity for future prenatal diagnosis. Although at least 31 metabolic disorders are listed as causes of SIDS, there is some doubt as to the validity of some reports.[12] The most likely metabolic causes of sudden unexplained death are listed below: -

Inherited defects of fatty acid oxidation and ketogenesis.
Urea cycle disorders - most commonly ornithine transcarbamylase (OTC) deficiency.
Organic acidurias e.g. methylmalonic (MMA), propionic (PA) and isovaleric aciduria (IVA).
Congenital lactic acidosis i.e. pyruvate dehydrogenase deficiency (PDH), respiratory chain disorders, biotinidase deficiency.
Carbohydrate disorders e.g. galactosaemia, glycogen storage disease type I (GSD I), hereditary fructose intolerance, fructose 1,6-bisphosphatase deficiency.

In practice with the exception of the fatty acid oxidation defects, the majority of these disorders do not strictly present as SIDS but rather as an acute metabolic crisis with clear clinical symptoms, which precedes death by hours or even a few days.

Non-accidental injury

It is recognised that a number of inherited defects can present in such a way that they may be misinterpreted as being due to non-accidental injury. False accusations of abuse can have catastrophic consequences for the family involved. It is only when all the relevant information has been gathered, which must include all clinical and laboratory information, that a final diagnosis can be made.
A number of metabolic disorders have come to light while infants / children were being investigated for possible abuse, but metabolic disease cannot be diagnosed if it is not suspected and if the appropriate tests are not carried out. When assessing cases of possible abuse one should consider the following: -

Do the injuries accord with the history given by the family?
Has the family suffered a similar “unexpected” death?
Is there a history of consanguinity?
Was there a prodromal illness?
Could this be due to an underlying metabolic disorder?

Glutaric aciduria type I (GA I), Menkes disease, [13] osteogenesis imperfecta OI, [14,15] and haemophilia [16] have all been cited as causing damage to organs and/or tissues that have been wrongly interpreted as signs of non-accidental injury. GA I can lead to subdural haematoma and bilateral retinal haemorrhages very closely mimicking the shaken baby syndrome.[17-19] Similarly defects affecting connective tissue / bone metabolism may lead to multiple fractures and or ruptured blood vessels, which again can be misinterpreted as signs of non-accidental injury. It is vital, therefore, that

adequate material is taken in such cases so as to enable a full investigation and thereby excluding any underlying metabolic disorder.

Metabolic causes of sudden death

The commonest group of metabolic disorders presenting as sudden unexplained death in infancy are the fatty acid oxidation disorders. It is estimated that up to 3-6% of sudden unexpected infant deaths are attributable to this group of disorders (FAO). [20-23] Defects of medium-chain acyl-CoA dehydrogenase, carnitine palmitoyltransferase type II, carnitine-acylcarnitine translocase, the high affinity carnitine transporter (mutations of the *OCTN2* gene causing primary carnitine deficiency), long-chain 3-hydroxyacyl-CoA dehydrogenase, mitochondrial trifunctional protein, very-long chain acyl-CoA dehydrogenase and multiple acyl-CoA dehydrogenase have all been reported as presenting initially as cases of sudden unexplained death. [2, 21] However, of the fatty acid oxidation defects, medium chain acyl-CoA dehydrogenase (MCAD) deficiency is the single most common disorder and warrants further description.

Medium chain acyl-CoA dehydrogenase deficiency

Medium chain acyl-CoA dehydrogenase (MCAD) deficiency was first described in the early 1980s [24, 25] and is the commonest fatty acid oxidation defect occurring in central Europe, affecting Caucasians of north-western European origin with an incidence as high as 1:8000 live births. The first crisis is fatal in up to 25 percent of cases, patients classically presenting with hypoketotic hypoglycaemia.[26] However, a significant percentage of genetically predisposed patients remain asymptomatic throughout life.[26, 27] The disease is primarily of hepatic fatty acid oxidation. Clinically patients may present with lethargy, emesis, encephalopathy, respiratory arrest, hepatomegaly, seizures, apnoea, and cardiac arrest.[26, 28] Although hypoglycaemia with "inappropriate" hypoketosis is usually the major presenting biochemical feature, some patients may present with hypotonia and reduced consciousness while still maintaining blood glucose concentration within the normal range. Patients presenting in crisis may often have detectable ketones in their urine. Indeed there is evidence to suggest that, at least in the well child with MCAD, medium chain fatty acid oxidation and ketone body production and utilisation are within normal limits.[29, 30] Rarely MCAD patients may present in crisis with "paradoxically" gross ketosis (J. Calvin personal communication). Some patients presenting initially as SIDS have subsequently on biochemical testing been shown to have MCAD.[31] Babies can present within the first few days of life as a sudden death.[32, 33] Mean age at presentation is 12 months, but presentation after the age of 5 years is rare, [26] although there are isolated reports of adult presentations following extreme metabolic stress.[34, 35] The common K304E mutation accounts for 85 percent of this disease in most of Western Europe.[36] Neonatal screening programs which now include MCAD are in place in a number of countries including Australia, some states in the USA and a number of European countries. At present there is no national screening programme for MCAD in the UK, but a pilot study involving five UK centres is underway.

Other fatty acid oxidation defects

Following the description of MCAD there has been considerable growth in our knowledge of previously undescribed fatty acid oxidation defects with some 15 or so

defects that directly or indirectly affect fatty acid oxidation having now been reported.[37] This group of disorders is now widely recognised as being an important cause of acute metabolic decompensation and sudden death in the neonatal period, infancy and early childhood.[38] Cumulatively these other disorders probably account for a similar number of sudden deaths as does MCAD. Two main types of presentation are most commonly observed. The first is characterised by hypoketotic hypoglycaemia after a period of prolonged fasting. Patients may present acutely with a life-threatening illness, which may rapidly proceed to coma and death, during intercurrent infections, surgery or other catabolic stress, having apparently been previously well or having only exhibiting mild symptoms such as failure to thrive. Additionally, there may be liver disease with hyperammonaemia and cerebral oedema (Reye-like illness). Cardiac arrhythmias resulting in sudden death may be induced by accumulation of long-chain acylcarnitines, which are particularly arrhythmogenic and there may be acute cardiomyopathy with pericardial effusion.[38]
The second presentation reflects chronic impairment of muscle function with myopathy and / or cardiomyopathy, which can be either hypertrophic or dilated. Sudden death as a result of severe cardiomyopathy has been reported in patients previously thought to be well. [39]

Defects of ketone body production / utilisation

3-Hydroxy-3-methylglutaryl (HMG) CoA lyase is an enzyme required for ketogenesis as well as for the last step in leucine oxidation. Patients with deficiency of this enzyme present in infancy or early childhood with life threatening Reye-like crisis, the picture is one of hypoketotic hypoglycaemia, metabolic acidosis and liver disease. Analysis of urine organic acids gives a specific pattern of abnormal metabolites.

3-Ketothiolase (mitochondrial 2-methylacetoacetyl-CoA thiolase) is a ketolytic enzyme but is also an enzyme of isoleucine metabolism. Patients with a deficiency of this enzyme usually present in infancy between 6-24 months of age, but occasionally as older children, with episodes of vomiting often precipitated by intercurrent infection or occasionally by high protein intake. There is acute life threatening ketoacidotic crisis with severe metabolic acidosis, occasionally low, but usually normal glucose and normal or moderately raised ammonia. Patients may rapidly succumb to overwhelming metabolic acidosis. Urine organic acid and blood acylcarnitine analysis will identify this disorder.

Urea Cycle Defects

Defects of the urea cycle may present in the neonatal period, infancy, and childhood or in adolescents and adults, cumulatively they have an incidence of 1:20000. Hyperammonaemia is a potent cause of cerebral oedema in this patient group. The presentation at different age groups shows some variability but it is particularly in neonates and infants where the presentation may be of a sudden acute decompensation rapidly proceeding to coma and death. Neonates typically present on day 2 of life, following a brief asymptomatic period. There is usually disinterest in feeding, lethargy, hyperventilation, seizures and progressive encephalopathy and coma with loss of reflexes and frequently intracranial haemorrhages resulting from coagulation defects. Respiratory alkalosis develops with marked hyperammonaemia. A misdiagnosis of sepsis is frequently made.[40, 41]

Diagnosis, depending on the defect, is by amino acid analysis in plasma and urine, demonstration of elevated argininosuccinic acid in urine in the case of argininosuccinate lyase deficiency, increased orotic acid in urine in ornithine transcarbamylase (OTC) deficiency, followed by enzyme studies in leucocytes, fibroblasts or liver and mutation studies.

Organic acidurias

This large group of disorders result from defects of intermediary metabolism with the characteristic accumulation of carboxylic acids in urine. The majority of the important organic acidurias are caused by disorders involving the metabolism of branch chain amino acids. Patients classically present in the neonatal period with metabolic encephalopathy resulting from 'metabolic intoxication' (see table 1). There is lethargy, feeding problems, dehydration, limb hypertonia with truncal hypotonia. Patients show multi-system failure, neurovegetative dysregulation and coma. These disorders include isovaleric aciduria (IVA), propionic aciduria (PA), methylmalonic aciduria (MMA) and multiple carboxylase deficiency. The multiple carboxylases are biotin dependent enzymes important in the metabolism of branched chain amino acids and disorders of these enzymes encompass biotinidase deficiency, holocarboxylase synthetase deficiency and 3-methylcrotonylglycinuria.

GA I (glutaryl-CoA dehydrogenase deficiency) is a disorder of lysine and tryptophan metabolism. Patients are normal at birth but usually present during infancy or early childhood with acute encephalopathic crisis and metabolic decompensation during intercurrent infection or other metabolic stress. There is often macrocephaly with fronto-temporal atrophy. The first episode may be fatal but those patients that survive are left with severe dystonic-dyskinetic movement disorder, intellect however is frequently intact. Patients presenting with this condition may be mistaken as cases of possible non-accidental injury (refer to section on NAI).

Mitochondrial Respiratory Chain Disorders

Mitochondriopathies are disorders of enzymes or enzyme complexes that are directly involved in the production of chemical energy by the process of oxidative phosphorylation. These include pyruvate dehydrogenase (PDH) complex and the respiratory chain complexes including ATP synthase. These disorders lead to a wide spectrum of clinical disease involving virtually all organ systems. Almost any organ or system can be affected, often a combination of seemingly unrelated symptoms with clinical disease in several tissues, suggests the possibility of respiratory chain disease. [42] Cardiac disease in infants and older children is often a result of genetic defects of oxidative phosphorylation and may present as an isolated cardiomyopathy or with other multiorgan involvement. Some infants with respiratory chain disease die early in the neonatal period and many of these are not fully investigated in life.

Disorders of galactose and fructose metabolism

Patients with classical galactosaemia resulting from galactose-1-phosphate uridyltransferase deficiency or hereditary fructose intolerance only develop clinical symptoms of disease after ingestion of lactose (milk and dairy products) or fructose / sucrose respectively. Galactose-1-phosphate and fructose-1-phosphate which accumulate in classical galactosaemia and hereditary fructose intolerance are toxic metabolites that lead to organ damage, particularly in liver, kidneys and brain.

Galactosaemia usually presents in the neonatal period on commencement of milk feeds and is frequently fatal unless detected at an early stage. Hypoglycaemia with jaundice, deranged liver function and renal failure are the common features often with accompanying sepsis. Patients may succumb with the incorrect primary diagnosis of sepsis. Diagnosis for galactosaemia is by enzyme assay in whole blood or on a fresh Guthrie card blood spot, quantitation of galactose-1-phosphate in erythrocytes, or by mutation detection. Blood transfusion will of course invalidate the enzyme assay in blood.
Hereditary fructose intolerance may present with similar features to galactosaemia at the time of weaning with the first introduction of fructose containing feeds. Patients present with hypoglycaemia, vomiting, progressive liver dysfunction with hepatomegaly, renal tubular damage and coma. Diagnosis is by demonstration of reducing substance in the urine (fructose) and often by demonstrating the presence of the common A149P mutation in the *aldolase B* gene. Galactosaemia and hereditary fructose intolerance show similar pathologic changes e.g. fatty change in the liver, giant cell transformation, pseudoacinar arrangement of hepatocytes and cirrhosis.
Fructose-1,6-bisphosphatase deficiency results in severely impaired gluconeogenesis. Neonatal presentation of this disorder follows a precipitous and often lethal course. There is hypoglycaemia, hyperventilation, ketosis, lactic acidosis, apnoea, seizures and cardiac arrest. However infants that are rapidly treated with glucose and bicarbonate may then remain symptom free for weeks or months before having a further attack. Episodes in older infants are often triggered by fasting and febrile episodes. There is frequently mild hepatomegaly and urine organic acids contain glycerol, glycerol-3-phosphate and 2-ketoglutaric acid. Confirmation is by enzyme assay in liver or by mutation analysis of the *aldolase A gene*.

Glycogen Storage Disease

Glycogen storage disease (GSD) presents invariably either with pathological accumulation of glycogen (e.g. isolated hepatomegaly) and corresponding organ dysfunction (e.g. liver disease / myopathy), or with hypoglycaemia. The enzyme defect is frequently organ specific and therefore there may be primary hepatopathic, myopathic or mixed symptoms. The cumulative incidence of GSD is 1:20000.
Patients may present in early infancy with hypotonia and severe cardiomyopathy (type II - Pompe) or with hypoglycaemic seizures, recurrent hypoglycaemia with acidosis, truncal obesity, hepatomegaly, nephromegaly, muscle atrophy and bleeding tendency (type I). Infants with type I disease may present in acute hypoglycaemic crisis with lactic acidosis which may rapidly proceed to death. Diagnosis is confirmed by biopsy / enzyme studies or mutation analysis.

Post-mortem findings

A co-ordinated plan of investigation should include accurate details of clinical and family history. Information on ethnicity, consanguinity and previous obstetric history followed by physical examination and preliminary post-mortem findings should be assessed. This information, together with results from any pre-mortem biochemical and haematological testing may provide important clues that may help to direct the selective use of appropriate laboratory analysis. Clearly unexplained death in an infant or child can be due to a number of non-metabolic causes and these of course need also

to be excluded. Any unexplained sudden death will include an infective cause as a key part of the differential diagnosis and this needs to be excluded where possible. Features that may suggest a metabolic cause of death include a range of dysmorphic features, some of which may suggest a specific diagnosis e.g. microcephaly, micrognathia, anteverted nostrils, syndactyly of second and third toes strongly indicating the possibility of Smith-Lemli-Opitz syndrome S-L-O (*see* table 1). However, dysmorphology, particularly if it is subtle, is best assessed by a clinical geneticist. It is therefore important to carefully undertake an external examination, which must include careful evaluation of any dysmorphic features. Newer techniques such as MRI may allow targeted biopsies in cases with limited consent, and in the future, some of the subtle internal changes e.g. cerebral dysgenesis, agenesis of the corpus callosum and congenital heart anomalies may be amenable to detection by post-mortem MRI. In cases of early neonatal death, assessment of the placenta may also yield vital information e.g. vacuolation of the syncytiotrophoblast may arise in a number of storage disorders. Examination of tissue samples for fat deposition at an early stage of the post-mortem may give an indication for further investigations e.g. coarse vacuolation of renal tubular epithelium with fat deposition in proximal renal tubules, is highly suggestive of a fat oxidation defect. Storage material in various organs and / or tissues may also offer other vital clues to a diagnosis.

Samples

The collection of appropriate samples at post-mortem is of paramount importance in the investigation of metabolic disease. All too frequently further investigations are requested in cases of unexplained death but appropriate samples are not available. It is essential to collect representative post-mortem samples and to discuss their analysis with a metabolic specialist. Appropriate investigations on often very small samples of body fluids and tissues must be well co-ordinated if precious material is not to be used up unnecessarily. In cases of expected death, whenever possible, collect samples of blood, urine and tissues prior to death. This applies especially to skeletal muscle biopsies and CSF for the investigation of suspected mitochondrial respiratory chain disease, as samples collected after death are highly susceptible to post-mortem deterioration. Liver and other internal tissues undergo proteolysis quickly after death. Changes are apparent in mitochondria within two hours, and respiratory chain enzyme activity deteriorates rapidly. Consequently for the purpose of many biochemical analyses liver, heart and skeletal muscle biopsies must be taken promptly if accurate results are to be obtained. Unless an autopsy is going to be conducted immediately, biopsies of internal organs for enzyme analysis, especially those for respiratory chain assay, should be done soon after death. Small open muscle and open liver biopsies are easily obtained. However, some enzymes of intermediary metabolism are more stable, so biopsies of liver, muscle, heart, kidney and even brain may still provide essential diagnostic or confirmatory information, even when obtained a day or two after death during a routine autopsy.

It is of paramount importance to obtain a biopsy for fibroblast culture, as many of the enzymes known to be deficient in inborn errors of metabolism are expressed in fibroblasts, and this will frequently be the only tissue where an enzyme confirmation of a suspected diagnosis can be made. Cultured fibroblasts also provide important

reference material for future prenatal diagnosis for the family concerned as these can be cryopreserved for an indefinite period in liquid nitrogen.

Urine

This should be collected by catheterisation or suprapubic puncture into a container with no preservative, as little as 100µL can be sufficient for organic acid analysis by gas chromatography mass spectrometry (GC-MS). If the sample is contaminated with blood, centrifuge the sample to remove the cells prior to freezing the urine at -20^{0}C. Washing out the bladder with a small volume of sterile saline may also yield enough sample for organic acid analysis.

Blood

Liquid blood is frequently obtainable by cardiac puncture up to several days after death. If only small quantities of blood are available however, whole blood acylcarnitine analysis is likely to be single most informative test that can be performed in spite of the potential problems of interpretation [2] (*see* Investigations). Spot a few drops of whole blood onto a filter paper (i.e. a Guthrie card), as this is the most convenient method of collection and importantly most suitable for uniformity of analytical technique. If more blood is available, (in addition to the Guthrie card) collect up to 10 mL of heparinised blood as this can be separated and the plasma stored at -20^{0}C, while the cells should be stored at +4^{0}C (*do not freeze*). If DNA analysis is likely to be required collect a further 5 mL of whole blood (EDTA) and freeze immediately (at least -20^{0}C) until DNA is required. Collect also 5 mL of blood into a fluoride oxalate tube for the measurement of intermediary metabolites. This last sample however, is only applicable to samples taken before or very soon after death.

CSF

CSF may be useful in certain circumstances (e.g. organic acid analysis, acylcarnitine analysis), but is often only reliably informative (e.g. analysis of certain amino acids), if collected prior to death. Collect two 1mL samples, one into a plain tube and one with fluoride oxalate and store these immediately at -80^{0}C.

Vitreous humour

This can be collected into a fluoride bottle by needle aspiration and stored at -20^{0}C.

Bile

Bile may be the only available analysable fluid in cases where the interval between death and post-mortem has been protracted. It is recommended that in all cases where there is the possibility of underlying metabolic disease that a sample of bile should be obtained. Bile is most conveniently spotted onto a Guthrie card for acylcarnitine profiling. If a Guthrie card is not available bile can be collected at post-mortem into a plain tube for storage at -20^{0}C.

Skin or other samples for culturing fibroblasts

A skin biopsy should be a routine part of all post-mortem investigations where an infant or child has died from unknown cause. Sterility is of paramount importance when taking a skin biopsy. Ideally two punch biopsies taken from different sites and

placed in separate sterile vials containing appropriate culture medium (Ham's F10, Eagle's MEM, Dulbecco's medium) is the preferred option. It is recommended that all pathologists who undertake paediatric post-mortems should invest in a biopsy punch. In the absence of a punch, it should be borne in mind that small biopsies carry a lower risk of infection, two 3mm x 3mm full thickness biopsies are all that is required. Large pieces of skin and tissue frequently fail because of infection. It is recommended that the skin biopsy be taken at the beginning of the post-mortem to reduce the risk of contamination. Skin fibroblasts remain viable for 2 to 3 days after death, and in some cases up to a week. However, the biopsy should be obtained as soon as possible, as this increases the likelihood of a successful culture. The addition of Fungizone to the culture medium can help to reduce the risk of fungal infections, but it is no substitute for good aseptic technique. Alternatively where infection is likely to be a particularly problematic or where there has been a post-mortem delay of some days and viability is likely to be low, it is worth taking small biopsies from a number of separate sites into separate containers to increase the chances of successful culture e.g. pericardium, fascia and / or cartilage. Once the biopsies have been taken they should be sent immediately to the cell culture laboratory, but can if necessary, be stored overnight at $+4^0$C *(do not freeze as this will destroy any viable cells)* prior to dispatch. In an emergency sterile normal saline can be used instead of culture medium, but do not use agar.

Tissue samples

The selection of organ biopsies depends on the clinical picture and is best discussed with a metabolic specialist. Liver, heart muscle, skeletal muscle and kidney are usually only suitable for biochemical analysis if taken *within 2-4 hours of death*. Open biopsies are preferable, but if not possible, two or three needle biopsies should be taken. All such biopsies for biochemical analysis should be wrapped in aluminium foil and snap frozen in liquid nitrogen or solid carbon dioxide. The samples should then be stored in a -80^0C freezer.

Tissue samples for histological examination

Liver

A small piece of liver is collected into formalin for staining and light microscopy and a piece into glutaraldehyde for electron microscopy.

Kidney

A piece of kidney for fat stain.

Muscle biopsy

Small piece for formalin fixation and paraffin preparation (do not freeze). This is for light microscopy. Thin muscle fibre, tied to a stick (to avoid contraction and preserve orientation) in 2% glutaraldehyde on ice. This is for electron microscopy.

Investigations and Analysis

The types of samples and analyses along with examples of the disorders that may be diagnosed are outlined in table 2.

Blood acylcarnitine profiles

The introduction of acylcarnitine profiling on Guthrie card blood spots by electrospray ionisation-tandem mass spectrometry has revolutionised the investigation of metabolic disease and is *the* major advance in the post-mortem investigation of these disorders, facilitating the identification of a wide range of metabolic diseases in tiny samples of blood, plasma and bile.[3] Acylcarnitine profiling has the potential to detect many inherited metabolic defects including most fatty acid oxidation disorders and many organic acidaemias.[2] Acylcarnitine profiles are likely to be more informative when expressed as ratios e.g. C8/C10 or C8/C12 ratios in the detection of MCAD.[2] This is because post-mortem changes often lead to a non-specific rise in a number of medium and short chain acylcarnitines and the expression of results as a ratio gives a more reliable indication of abnormalities resulting from specific defects in fatty acid oxidation. However, few of the profiles from post-mortem blood are in themselves reliably diagnostic and will need further tests, often on fibroblasts, to confirm a diagnosis. As interpretation of acylcarnitine profiles can be especially problematic in post-mortem samples it is always worth seeking out the stored newborn screening Guthrie card (if this is available) for acylcarnitine analysis, as this is potentially more reliably informative.

Blood spot amino acid profiles

Electrospray ionisation-tandem mass spectrometry also allows the identification of amino acid profiles in Guthrie card blood spots in a similar manner as is done with acylcarnitine analysis. This may be particularly useful in cases when larger volumes of liquid blood are not available at post mortem. Such analysis has the potential to identify certain disorders affecting amino acid metabolism / urea cycle defects [43] even on post mortem samples e.g. argininosuccinic acid due to argininosuccinate lyase deficiency or citrulline in argininosuccinate synthase deficiency. Some other amino acid disorders such as maple syrup urine disease are also potentially detectable by blood spot amino acid profiling.[2]

Plasma intermediary metabolites

Blood samples collected prior to or just after death into a fluoride oxalate tube, may be useful for the measurement of intermediary metabolites, namely glucose, lactate, non-esterified fatty acids (NEFA) and 3-hydroxybutyrate (HB). Hypoglycaemic patients with fatty acid oxidation defects show high (>2.0) NEFA / HB ratios, while hypoglycaemic infants with hyperinsulinism show an inadequate or absent lipolytic and ketogenic response.

Amino acids in plasma, CSF and urine

Aminoacidopathies and urea cycle defects will give abnormal amino acid profiles as measured by more conventional amino acid analysis in samples taken prior to death, but as with Guthrie blood spots, interpretation of post mortem material is often problematic. Abnormal elevations of certain amino acids may indicate the possibility of specific disorders but need to be interpreted in relation to the interval between death and sampling. Suspected defects of amino acid metabolism will require enzyme / biochemical confirmation in the appropriate tissue or molecular analysis in order to confirm a diagnosis. It is worth noting that some of the urea cycle defects can only be enzymatically confirmed on fresh liver biopsy i.e. OTC deficiency or in the case of

carbamylphosphate synthase 1 (CPS 1) deficiency in fresh liver or colon. Mutation analysis circumvents the need for a fresh liver sample in these disorders when a specific diagnosis is strongly suspected.

Organic acids in urine

Organic acidurias, including many of the fatty acid oxidation defects and a significant number of other defects of intermediary metabolism will give abnormal organic acid results, some profiles may in themselves be pathognomonic, as in the case of MMA, classical IVA**,** GA I or MCAD, but other findings may be less specific and highlight the need for further investigations. The presence of significant ketones in urine at post mortem does *NOT* exclude a fatty acid oxidation defect.

Bile

Acylcarnitine and bile acid profiles can be measured in small quantities of post mortem bile [2, 32] but need careful interpretation and require supporting biochemical evidence for accurate diagnosis. Bile acylcarnitine analysis is likely to prove more informative when interpreted in conjunction with a blood spot acylcarnitine profile. Interpretation of post mortem acylcarnitine profiles is best achieved by expression of acylcarnitine ratios. An example of this is in the diagnosis of MCAD (as is the case with blood) where C8/C10 or C8/C12 ratios are far more diagnostically reliable than measurement of octanoylcarnitine alone. [2]

Cis-4-decenoic acid in plasma

This compound is significantly raised in cases of MCAD and although not entirely specific for this disorder, also raised in multiple acyl-CoA dehydrogenase deficiency, it serves as a very useful indicator of these disorders.

Plasma very long chain fatty acids and sterols

Very long-chain fatty acids (VLCFAs), phytanic acid, pristanic acid and bile acids can be measured in plasma or serum by GC-MS in cases of suspected peroxisomal disorders. Almost all known peroxisomal defects can be detected if all 4 parameters are measured. Plasma sterol analysis can also be used to detect inherited defects of sterol metabolism e.g. S-L-O syndrome.

Vitreous humour

Organic acid analysis of eye fluid may be useful in the absence of urine
e.g. 7-hydroxyoctanoate is elevated in cases of MCAD, as is octanoylcarnitine.
Vitreous humour glucose, provided the sample was taken peri-mortem into a fluoride tube will reflect plasma glucose levels at that time e.g. a normal glucose result in an acute admission (provided the patient was not administered glucose prior to death) excludes hypoglycaemia as a cause of collapse.

CSF

Octanoylcarnitine as measured by electrospray ionisation tandem mass spectrometry may be usefully measured in CSF in cases of suspected MCAD.[32]

Molecular Investigations

Mutation analysis may be used to confirm a diagnosis where enzyme confirmation is not possible e.g. organ specific disease expression OTC, disorders of structural, receptor or membrane proteins e.g. sarcomeric proteins in familial cardiomyopathy, [44] detection of mutations in OI or in diseases with single common mutations (MCAD, long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency. DNA for molecular analysis can be most easily obtained from EDTA whole blood. Guthrie card blood spots, frozen tissue biopsies and fibroblasts will also yield DNA. If RNA is required this can be obtained from cultured fibroblasts.

Histochemistry and ultrastructure

The histological or ultrastructural appearance of many tissues can be a good guide to the final diagnosis but is often non-specific. Staining for micro and macrovesicular fat and /or glycogen deposition in muscle, heart, liver and kidney at an early stage of the post mortem process may give an early indication of which lines of investigation to pursue. Evaluation of organ / tissue biopsy using both light and electron microscopy often provides complimentary information. Lipid inclusions suggest altered fatty acid oxidation or impaired mitochondrial function. Excess glycogen, determined by staining or quantitative analysis, suggests altered glycogen metabolism. Increased lipids are also present in some glycogen storage diseases e.g. GSD type I. Increased membrane-bound (i.e., lysosomal) glycogen is typical of Pompe disease. Electron microscopy may show abnormalities in mitochondriopathies such as increased size and number of mitochondria. Giant mitochondria with concentric lamellar tubular reticular or dissociated cristae are characteristic. The mitochondrial matrix may be swollen and display large spherical dense bodies, vacuoles or crystals. Rectangular crystals may be arranged in blocks of parallel crystals with “parking lot” configuration. However such changes are not in isolation diagnostic of respiratory chain disease and can only be taken as indicators. Often, particularly in the case of respiratory chain disease, a final diagnosis is a “balance of probabilities” based on cumulative clinical, histological and biochemical / molecular evidence.

Tissue assays

Cultured fibroblasts

Many of the enzymes known to be deficient in inborn errors of metabolism are expressed in skin fibroblasts with the notable exception of some urea cycle and some glycogen storage enzymes. For this reason obtaining a skin biopsy should be a routine procedure at post mortem. Homogenates or sonicates of cultured fibroblasts are suitable for many specific enzyme assays e.g. glutaryl-CoA dehydrogenase, or alternatively in some instances, assays using crude mitochondrial preparations may be more appropriate e.g. the investigation of respiratory chain disorders by polarography.[45] Fibroblasts taken from infants with mitochondrial respiratory chain defects will often express the defect and low complex(es) II/III of IV may be demonstrated.[46, 47] In some instances diagnosis can be confirmed by mutation analysis of known nuclear genes involved in the assembly of respiratory chain complexes e.g. *SURF1* in systemic cytochrome oxidase (COX) deficiency presenting as Leigh Disease.[48, 49]

One particular advantage of using intact living cells is that the uptake or incorporation of various substrates into the cell or the flux of metabolites through an entire pathway can be measured.[50] Additionally cultured cells have the advantage that they are not subject to deterioration of enzyme activity or secondary loss of function, which is so frequently a problem in biopsies and particularly other post mortem samples. An example of the use of intact cells is in the diagnosis of fatty acid oxidation disorders by tritiated release assay in cultured fibroblasts.[51] More recently cultured fibroblasts have been incubated with deuterium labelled fatty acids or other suitable substrates and the resultant acylcarnitine profiles analysed by MS/MS.[52] This latter technique has proved to be particularly informative in fatty acid oxidation disorders, but also has the potential for use in a wide range of other inherited metabolic diseases.

Other tissues

Fresh frozen muscle is often the tissue of choice for the diagnosis of mitochondrial respiratory chain disorders. Provided the muscle has been collected prior to death (or within two hours of death) complexes I, II, III and IV of the respiratory chain can be measured.[45] Other tissue such as heart and liver can also be used where specific involvement of these tissues is indicated e.g. mutation analysis of cardiac mitochondrial tRNAs in the case of isolated cardiomyopathy [53] or specific enzyme assay in fresh liver for some urea cycle and glycogen storage disorders.

Conclusion

The application of electrospray ionisation tandem mass spectrometry to clinical chemistry offers the opportunity for detailed biochemical analysis in tiny samples of blood, plasma and bile. This technique coupled with recent advances in molecular genetics now greatly improves the chances of accurate post mortem diagnosis for a wide range of inherited metabolic diseases. This process relies on the timely collection of suitable post mortem samples and careful review of any pre-mortem laboratory results that may be helpful in the subsequent selection of further laboratory testing. In many cases where there is a suspicion of an underlying metabolic cause of death it is strongly advised that, wherever possible, the neonatal screening card should be analysed for acylcarnitines and if indicted for amino acid analysis also. These analyses carried out on the neonatal Guthrie card blood spot generally provide a more reliable indicator of many metabolic diseases than analyses performed on post-mortem samples.

Table 2 Samples and containers for biochemical / molecular analysis

Sample	Means of preservation	Storage	Type of analysis	Examples of disorders detected
Whole blood	Guthrie card	Dry at room temperature	AC, AA	FAO, OAs AAs/UC
Bile	Guthrie card	Dry at room temperature	AC	FAO, OAs
Urine	Sterile vial	-20^{o}C or +4^{o}C	OA	OAs, FAO
Whole blood	Li/hep	Plasma at +4^{o}C	AC, AA, VLCFAs, sterols	FAO, AAs Peroxisomal defects, S-L-O
Whole Blood	EDTA	-20^{o}C	DNA	OI, MCAD, LCHAD
Skin (punch biopsy) or 3mm x 3mm	Sterile medium	Do NOT freeze +4^{o}C or room temperature	Flux / enzyme assay, DNA, RNA	FAO, RES, OAs, PDH GSD II & IV, GA I, GA II Niemann-Pick type C
Muscle (fresh)	Liquid nitrogen	-80^{o}C	Enzyme assay Respiratory chain Complexes I-IV	GSD II & IV, RES
Liver (fresh)	Liquid nitrogen	-80^{o}C	Enzyme assay Respiratory chain Complexes I-IV	OTC, CPS 1, NKH, GSD I, IV, 0 RES

Table 2 abbreviations
AC – acylcarnitine profile
AA – amino acids
AAs/UC – aminoacidopathies / urea cycle defects
OA – organic acids
OAs – organicacidurias
FAO – fatty acid oxidation defects
RES – mitochondrial respiratory chain defects
NKH – non-ketotic hyperglycinemia
GA II – Glutaric aciduria type II (multiple acyl-CoA dehydrogenase deficiency)
GA I – glutaric aciduria type I (glutaryl-CoA dehydrogenase deficiency)
GSD – glycogen storage disease
OTC – ornithine transcarbamylase deficiency
CPS I – carbamylphosphate synthase I deficiency
MCAD – medium chain acyl-CoA dehydrogenase deficiency
LCHAD – long chain 3-hydroxyacyl-CoA dehydrogenase deficiency
PDH – pyruvate dehydrogenase deficiency

OI – osteogenesis imperfecta

Table 1.
Clinical patterns of deterioration / presentation associated with metabolic disorders*

Hypoglycaemia	Acid-Base Disorders	Cardiac Disorders	Neurological deterioration	Liver Disease	Dysmorphology

Disorders of fatty acid oxidation Fructose 1,6-bisphosphatase deficiency Glycogen storage disease type 1 (GSD 1) Respiratory chain disorders Organic acidaemias Hereditary fructose intolerance	Metabolic acidosis: Organic acidaemias Congenital lactic acidosis Fructose 1,6-bisphosphatase deficiency 3-Ketothiolase deficiency Respiratory alkalosis: hyperammonaemia - urea cycle defects	Disorders of fatty acid oxidation Congenital disorders of glycosylation (CDG) Pompe disease (GSD II) Respiratory chain disorders	Hyperammonaemia Organic acidaemias Maple syrup urine disease Disorders of fatty acid oxidation Congenital lactic acidosis Peroxisomal disorders Non-ketotic hyperglycinaemia (NKH) Molybdenum cofactor deficiency	Galactosaemia α-1-antitrypsin deficiency Respiratory chain disorders Neonatal haemochromatosis Disorders of fatty acid oxidation Tyrosinaemia type 1 Niemann-Pick type C Hereditary fructose intolerance	Polycystic kidneys GA II (severe) CPT II (severe) Zellweger Agenesis of the cor callosum - PDH Craniofacial abnormalities - Zellweger, S-L-O Respiratory chai disorders Hydrops fetalis lysosomal storag disease Cataract - Senger disease Galactosaemia

*Adapted from J V Leonard and A A M Morris Inborn errors of metabolism around the time of birth.[6] Reprinted with permission from Elsevier.

Table 1 abbreviations

CPT II - carnitine palmitoyltransferase type II
GA II – glutaric aciduria type II (multiple acyl-CoA dehydrogenase deficiency)
NKH – non ketotic hyperglycinaemia
S-L-O – Smith-Lemli-Opitz
PDH – pyruvate dehydrogenase deficiency

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