

Department of Clinical Chemistry

Metabolic Disease Newsletter

Number 30

Spring 2011

Contents

Page No

3. Editorial

Dr Jim Bonham and Dr Simon Olpin

5. Protocol for investigation of Featureless early developmental impairment.

Dr Simon Olpin

7. CSF Investigations for patients with seizures.

Dr Simon Olpin

13. Mitochondrial Respiratory Chain Disease.

Dr. Shamima Rahman

29. The investigation of Sudden Unexpected Death in Infancy (SUDI): The Sheffield's protocol.

Dr Marta Cohen

32. Ketogenic Diets.

Dr Mark Sharrard

Reviews

47. Peroxisomal disorders

Camilla Scott and Dr Simon Olpin

58. Diagnosis list 2010

Editorial

It is encouraging that Leicester have agreed funding for a metabolic physician post and that Nottingham are planning to make provision within the job plan of a consultant paediatrician to develop a responsibility for inherited metabolic disease. These are welcome developments to address long standing need and should help improve access to high quality services for patients and their families in East Midlands. The un-met need arising from “Rare disorders”, defined as conditions with a prevalence of fewer than 1:2,000 was stressed at various events around the country as part of Rare Diseases day on 28th February earlier this year. Within the EU it is believed that 1 in 17 people suffer from a rare disease, so 3.5m in the UK alone, 80% having a genetic origin and of course inherited metabolic disorders are an important group within these conditions. It is in everyone’s interest that we work together to recognise and treat these patients and their families effectively. A recent publication, produced by Genetics UK, as part of Rare Diseases day initiative highlights some of the issues and makes some useful recommendations (<http://www.raredisease.org.uk/documents/RD-UK-Strategy-Report.pdf>).

It is also helpful that a new Specialised Medical Definition Set, Number 36, has been agreed and paves the way for the development of a more coherent model for commissioning these services in England, this can be found at http://www.specialisedservices.nhs.uk/library/26/Specialised_Services_for_Metabolic_Disorders_all_ages.pdf Of course the mechanics of translating this definition into specialist services around the country has yet to be determined but will be the responsibility of the new NHS Commissioning Board rather than GP Consortia, and this is probably sensible.

The news on expanded screening is slightly less encouraging. The already fully funded project to screen more than 500,000 babies over two years in Trent, Yorkshire, the West Midlands and the North West was not, as we had hoped, agreed at the recent meeting of the National Screening Committee

(NSC) held on 10th November 2010. The NSC did however accept the pressing need to conduct further work in relation to the case for screening for the five conditions (IVA, GA1, LCHAD, MSUD and Hcys) identified in the proposed study. The NSC have discussed this need with the HTA and intend to commission a series of linked studies to cover the aspects already outlined in the study submitted for their consideration in November. It is hoped that by working together between our proposed study organised as part of the Collaboration for Leadership in Applied Health Research and Care (CLAHRC) initiative and HTA we can begin a screening study later in 2011/12. Such a study could be shortened to one year by increasing the number of centres involved, bringing us back on track to achieve the planned production of a report in 2013. In the interests of the families affected it would be good if we could achieve this timescale.

Back in our own laboratory we plan to continue to expand and develop the service that we provide for the detection of inherited metabolic disorders in sick children and to integrate this even more closely with molecular genetic investigation for the patients concerned. Notably we are working closely with Great Ormond Street Hospital to provide an enhanced service for the laboratory identification of patients with glycogen storage disease tackling this by bringing together enzymology, metabolite and molecular genetic components. It is our belief that this multidisciplinary approach can result in a more definitive and reliable service for patients and a more user friendly service for clinicians.

We hope that you enjoy this edition of the Newsletter and our thanks go to the authors who contributed the articles, some of which were the subject of lectures at our 8th Annual Trent Metabolic Study Day held on 22nd November in Sheffield and organised by Dr Mark Sharrard on the subject of *Seizures and Epilepsy in inherited metabolic diseases*. We acknowledge the kind support of Orphan Europe in arranging this event.

Dr JR Bonham & Dr S E Olpin, April 2011

Investigation of featureless early developmental impairment

A protocol for the investigation of “featureless early developmental impairment” has been implemented following discussions between our paediatric neurologists, consultant paediatricians, metabolic paediatrician and the Department of Clinical Chemistry.

Guideline:

The term “global developmental delay” should be avoided: “early developmental impairment” is preferable.

All children with developmental impairment shall have a full clinical assessment (history and physical examination) as outlined in the article “Assessment and investigation of the child with disordered development” by Karen Horridge Arch Dis Child Educ Pract Ed 2011;96:9-20.

Following this, if there is no clear cause of their developmental impairment the following investigations are recommended:

Karyotype

Full blood count with ZPP

U&E's, LFT's, Bone profile including calcium.

Thyroid function testing

Plasma and urine amino acids

Urine organic acids

Biotinidase

Urine GAG screening (not below 1 month of age)

Creatine kinase (males only)

DNA for storage

Fragile X - if suggested by family history

Lead level (if there is a risk factor)

Refer to audiology

Ophthalmology and orthoptics are not routinely indicated but should always be considered.

CSF Investigations for patients with seizures

Dr Simon Olpin - Department of Clinical Chemistry, SCH

Epileptic seizures are frequently encountered in patients with a wide range of inherited metabolic disease but are particularly common in those with disorders of cerebral grey matter. It is important to undertake a metabolic work-up of all infants and children with epilepsy in conjunction with additional symptoms such as impaired early development, mental retardation or neurological abnormalities. Before we concentrate on the CSF there are also a number of other initial tests that should be undertaken in such patients: see table 1.

Table 1

	Urine	plasma
U&E, LFT's, calcium, magnesium		√
Glucose		√
Ammonia		√
Blood gases		√
Biotinidase		√
Lactate		√
Organic acids	√	
Amino acids	√	√
homocysteine		√
ketostix	√	

In a few cases the results of these initial investigations may prove diagnostic but in most cases results will at least provide valuable information on which to base further investigations.

CSF investigations

There are a number of investigations on CSF that should also be undertaken in this group of patients. However some of these investigations should be paired to a plasma measurement in order to facilitate meaningful interpretation see table 2.

Table 2

	CSF	plasma
glucose	√ (fluoride)	√ (fluoride)
lactate	√ (fluoride)	
glycine	√	√
amino acids :- serine, threonine, alanine, proline	√	√

When obtaining a paired CSF and plasma sample it is important to take the plasma sample first as the stress of lumbar puncture will increase the plasma glucose concentration. A CSF/plasma glucose ratio is used to exclude deficiency of the GLUT1 glucose transporter that transports glucose into the CNS. A ratio of >0.6 excludes GLUT1 deficiency. However a ratio of <0.5 is suggestive of GLUT1 deficiency particularly if the CSF glucose is <2.5 . However normal neonates can sometimes have a ratio of <0.4 . Where repeated equivocal results are obtained it may be necessary to go to *SLC2A1* gene mutation analysis.

CSF lactate

CSF lactate is also a very useful measure for the investigation of patients with possible respiratory chain defects or pyruvate dehydrogenase deficiency PDH. It is often useful to also measure CSF alanine in such patients, as an increased CSF alanine ($>60 \mu\text{mol/L}$) in the presence of increased lactate is an additional marker for underlying respiratory chain disease. Increased CSF lactate in this group of patients is a more reliable indicator for respiratory chain disease than an increased plasma lactate and remains a good predictor even in patients who have had a brief single epileptic episode

within a 3 hours period prior to the lumbar puncture. In general most patients with epilepsy, (unless seizures are frequent and/or sustained), and who do not have respiratory chain disease, have a normal CSF lactate and alanine. The exceptions are patients with bacterial meningitis where increased CSF lactate is a consistent finding.

CSF pyruvate

A CSF lactate/pyruvate ratio is almost exclusively only useful in the differential diagnosis of PDH vs respiratory chain disease and even then only in patients where the CSF lactate is raised. In this context, a normal ratio (<20) is obtained in most patients with PDH while a raised ratio (>30) is indicative of respiratory chain disease.

CSF Glycine

CSF and plasma glycine concentrations plus the CSF/plasma glycine ratio are used in the investigation of patients suspected of suffering from non-ketotic hyperglycinaemia see Table 3. It is important to stress that a paired sample is required and that the CSF must not be contaminated with blood. Routinely we measure CSF proline (CSF reference values <5 µmol/L) as this is a good marker for uncontaminated CSF, as proline is present in blood at relatively high concentration (66-333 µmol/L).

Table 3

	Plasma glycine µmol/L	CSF glycine µmol/L	CSF/plasma glycine ratio
Term newborn	56-308	3-10	0.012-0.04 (usually <0.02)
Neonatal NKH	920-1827	83-280	0.09-0.25
Atypical NKH	447-	42, 72	0.06-0.10

CSF serine

Low CSF serine is a marker for defects of serine biosynthesis. In such disorders fasted plasma serine concentration is also low and plasma and/or CSF glycine concentrations may also be reduced. Such patients usually have severe neurological disease including seizures, microcephaly, psychomotor retardation and spastic tetraparesis. There is a steady but small reduction in the concentration of CSF serine with age and it is important to bear this in mind when interpreting CSF serine concentrations in neonates or young infants¹.

CSF threonine

CSF threonine along with glycine is invariably elevated in patients with pyridoxal phosphate dependency i.e. (pyridox(am)ine 5'-phosphate oxidase PNPO deficiency)² and in this respect is a useful investigation in all patients under investigation for seizures where this may be part of the differential diagnosis.

CSF pipercolate

CSF pipercolate is raised in pyridoxine responsive seizures and is the specimen of choice, in that it is the most reliable marker for this condition, remaining elevated even in patients already on pyridoxine treatment.

Neurotransmitters

Monogenic disorders of neurotransmission have become recognised as a cause of severe early-onset progressive encephalopathies and although it is important to mention them here, many do not have epilepsy as a primary feature of the disorder and in general they present as a spectrum of disorders from focal dystonia to “hereditary spastic diplegia” and “cerebral palsy” to severe infantile encephalopathies. Diagnosis is primarily based on the quantitative determination of neurotransmitters or their metabolites in CSF including the amino acids glutamate, GABA and the metabolites of biogenic amines and pterins.

When investigation these disorders it is vital to include full clinical details and drug history of the patient and to collect the following CSF samples in order to allow meaningful interpretation of results.

Collection Instructions

- **Tube 1** 0.5 ml for HVA and 5HIAA measurements.
- **Tube 2** 0.5 ml for 5MTHF (folate) determination.
- **Tube 3** 1.0 ml (contains 1mg of dithiothreitol as preservative) for pterin (neopterin, dihydrobiopterin and tetrahydro-biopterin) analysis.

The 3 CSF samples must be placed in Liquid Nitrogen *immediately* after collection. Return the liquid nitrogen container with a request form to Clinical Chemistry.

Test	Tick if required	Result	Units	Reference Range
HVA*			nmol/l	
5HIAA*			nmol/l	
HVA:5HIAA ratio				1.0-3.7
5MTHF* (folate)			nmol/l	
Neopterin			nmol/l	7-65
Dihydrobiopterin			nmol/l	<0.4-13.9
Tetrahydrobiopterin*			nmol/l	

*Age Related Reference Intervals

Cerebral folate deficiency

CSF 5-methyltetrahydrofolate MTHF is turning out to be a useful marker for not only primary defects of folate metabolism but also as a secondary marker for a range of other disorders see table 4. However peripheral folate status should also be assessed in these patients as this will be important in the differential diagnosis of these conditions. It is particularly important to recognise these disorders as a number are amenable to treatment with folic acid³.

Table 4**Causes of low CSF 5-MTHF**

Primary defects of folate metabolism	Secondary causes
<i>FOLR1</i>	Dihydropteridine reductase DHPR deficiency
<i>FOLR2</i>	Aromatic L-amino acid decarboxylase AADC deficiency
MTHFR deficiency	3-Phosphoglycerate dehydrogenase deficiency
	Rett syndrome
	Acardi Goutieres
	Mitochondrial respiratory chain defects
	L-dopa treatment
	Methotrexate
	Anticonvulsants
	Steroids
	Co-trimoxazole

**FOLR* = folate receptor

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Mitochondrial disease and epilepsy

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Abstract

Mitochondrial respiratory chain disorders are relatively common inborn errors of energy metabolism, with a combined prevalence of 1 in 5000. These disorders typically affect tissues with high energy requirements, and cerebral involvement occurs frequently in childhood, often with seizures.

Mitochondrial diseases are genetically heterogeneous; mutations have been reported to date in all 37 mitochondrially encoded genes and over 80 nuclear genes. The major genetic causes of mitochondrial epilepsy are mitochondrial DNA (mtDNA) mutations (including those typically associated with the MELAS and MERRF syndromes); mutations in *POLG* (classically associated with Alpers syndrome but also presenting as the MIRAS and SCAE syndromes in older patients) and other disorders of mtDNA maintenance; complex I deficiency; disorders of coenzyme Q₁₀ biosynthesis; and disorders of mitochondrial translation such as *RARS2* mutations. It is not clear why some genetic defects are particularly associated with seizures and not others. Epilepsy may be the presenting feature of mitochondrial disease but is often part of a multisystem clinical presentation. Mitochondrial epilepsy may be very difficult to manage, and is often a poor prognostic feature. At present there are no curative treatments for mitochondrial disease. Patients with mitochondrial epilepsy are frequently prescribed multiple anticonvulsants, and the role of vitamins and other nutritional supplements and the ketogenic diet remain unproven.

Introduction

Mitochondria are extremely dynamic subcellular organelles with a multitude of functions. The most well-known of these functions is of course ATP generation by the oxidative phosphorylation (OXPHOS) system, but

mitochondria also have important roles in intracellular calcium homeostasis, generation of reactive oxygen species (ROS), regulation of apoptosis (programmed cell death) and cell-specific functions, such as neurotransmitter synthesis in neuronal cells. The term 'mitochondrial disease' refers to any disorder affecting the respiratory chain and OXPHOS system, a series of five multisubunit enzyme complexes (complexes I-V) embedded in the inner mitochondrial membrane (1). Mitochondrial disorders are common, with an estimated birth prevalence of 1 in 5000 (2), although recently we have demonstrated that 1 in 500 children has a pathogenic mitochondrial DNA mutation (3). Mitochondria are unique amongst cellular organelles in that they contain their own genetic material, the small (~16.6 kilobases) circular mitochondrial DNA (mtDNA) molecule. This small genome is exclusively maternally inherited and is present inside the mitochondria of cells in multiple copies. The 37 mitochondrial genes encode 13 proteins (all components of the mitochondrial respiratory chain and OXPHOS system) and 24 RNA molecules necessary for the intramitochondrial synthesis of these 13 proteins. Correct co-ordinated expression of the 13 proteins encoded by the mitochondrial genome is essential for efficient mitochondrial energy production, and also requires the contribution of many of the ~1500 nuclear-encoded proteins that comprise the mitochondrial proteome. In this review I will discuss the clinical epilepsy phenotypes observed in mitochondrial disease, together with the biochemical classification, molecular genetics and management of mitochondrial epilepsies.

Epilepsy phenotypes in mitochondrial disease

Few studies have systematically examined epilepsy phenotypes in the context of mitochondrial disease (4-6). The group of Isabelle Desguerre at the Hôpital Necker in Paris studied a series of 56 children with mitochondrial disease and seizures and classified the types of epilepsy they observed into 6 groups, according to age at onset and major seizure type: 1) Neonatal refractory status and multiorgan failure; 2) Neonatal myoclonic epilepsy; 3) Infantile spasms; 4) Refractory/recurrent status epilepticus; 5) Epilepsia partialis continua; and 6) Myoclonic epilepsy (4). This group reported that in >80% of their cases the first seizures were preceded by *other* symptoms,

such as failure to thrive, developmental delay, ataxia, and evidence of multiorgan involvement (4). 60% of their patients had several seizure types, emphasising the complexity of this group of disorders.

Clinical recognition of mitochondrial epilepsy is difficult. Elevation of lactate in blood and/or CSF may be a clue, but normal values do not exclude mitochondrial disease. Elevated alanine in the plasma amino acid profile may reflect persistent lactic acidosis. Involvement of other organs, such as sensorineural hearing loss, pigmentary retinopathy, cardiomyopathy or cardiac conduction defects, diabetes mellitus, liver disease and renal tubulopathy, may also be useful pointers to an underlying mitochondrial disorder. There is no single gold-standard diagnostic test for mitochondrial disease, and definitive diagnosis usually requires muscle (or sometimes liver) biopsy, with subsequent histological, biochemical and genetic analysis of the affected tissue. In very specific cases, such as Alpers syndrome, it may be possible to make a genetic diagnosis (*POLG* mutations) in DNA extracted from blood, without the need for tissue biopsy.

The genetic basis of mitochondrial epilepsy

Mitochondrial diseases may be classified by the biochemical defect identified in skeletal muscle (or another affected tissue): isolated deficiency of a single respiratory chain complex (most commonly complex I or complex IV), or multiple defects affecting several complexes. Another classification system is according to the underlying genetic defect. However, at present genetic classifications are inevitably incomplete since the responsible mutation is identified in only 20-25% of childhood cases using routine diagnostic tests, although in specialised research laboratories focussing on highly selected subgroups of patients and using the latest next generation sequencing technologies the diagnostic rate may approach 50% (7). Here I will review the types of epilepsy associated with specific biochemical or genetic defects of the respiratory chain.

1. Isolated Complex I deficiency

This is the most commonly identified biochemical defect in most centres, representing 25-30% of all mitochondrial disease presenting in childhood.

The relative proportion of complex I deficiency may be even higher in mitochondrial epilepsy (5). Mutations have been reported in all 7 mtDNA-encoded subunits of complex I, associated with seizures in many cases, for example we have observed infantile spasms in a patient with the m.13513A>G mutation in the ND5 subunit, which is frequently associated with Leigh syndrome (subacute necrotising encephalomyelopathy) or MELAS (**m**itochondrial **e**ncephalomyopathy with **l**actic **a**cidosis and **s**troke-like episodes) syndrome (8). Mutations in mitochondrial transfer RNA (tRNA) genes and large-scale rearrangements of the mtDNA may also lead to isolated complex I deficiency, and overall changes in mtDNA account for 20-25% of cases of complex I deficiency. Mutations have been reported in 13 of the 38 nuclear-encoded subunits of the enzyme in another 20-25% of patients with complex I deficiency. Of these nuclear subunits, epilepsy has been associated with mutations in NDUFV1 and NDUFA1 (9;10). The remaining ~50% of complex I deficiency is believed to be caused by mutations in proteins needed for assembly and/or proper functioning of the enzyme. Nine assembly genes have so far been associated with complex I deficiency, including 3 new genes reported in the last few months of 2010 (7;11;12). Epilepsy is a feature of mutations in 5 of the 9 known complex I assembly factors: NDUFAF2, NDUFAF3/C3orf60, NDUFAF4/C6orf66, C8orf38 and FOXRED1 (11;13-15).

2. Isolated Complex II deficiency

This is a rare disorder and usually presents as Leigh syndrome caused by mutations in the SDHA subunit, although recently two assembly factors have been identified. Epilepsy appears to be unusual in complex II deficiency, but may occur (16).

3. Isolated Complex III deficiency

This is also rare. Mutations have been reported in cytochrome b, the only mtDNA encoded subunit of the enzyme, and in two of the ten nuclear-encoded subunits. Only one complex III assembly factor is known, BCS1L, and mutations are associated with a broad spectrum of disease, ranging from the severe neonatal-onset GRACILE (**g**rowth **r**etardation,

aminoaciduria, cholestasis, iron overload, lactic acidosis and early death) syndrome to the milder Björnstad syndrome (congenital sensorineural hearing loss with pili torti). Seizures have occasionally been reported in children with *BCS1L* mutations (17), but are not recognised to be a major feature of complex III deficiency.

4. Isolated Complex IV deficiency

Complex IV (cytochrome oxidase, COX) deficiency is another relatively common cause of mitochondrial disease, representing ~25% of childhood-onset cases. Mutations affecting subunits of this enzyme are relatively rare, and only occasionally associated with epilepsy (18). One of the most frequent presentations of COX deficiency is Leigh syndrome, ~50% of which is caused by mutations in the SURF1 assembly factor. Although seizures may occur in other causes of Leigh syndrome, they appear to be a rare feature of SURF1 deficiency. Mutations in seven other assembly factors for COX have been linked to human disease (19), only occasionally associated with seizures. For example, epilepsy is a feature of *FASTKD2* mutations (20) and has been reported in some patients with hypertrophic cardiomyopathy and encephalopathy caused by mutations in the SCO2 assembly factor (21), which is part of the molecular system responsible for inserting copper prosthetic groups into the COX holoenzyme.

5. Isolated Complex V deficiency

Activity of the mitochondrial ATP synthase (complex V) can only be reliably assayed in fresh tissue. This assay is offered in only a few specialised centres, and so complex V deficiency is probably underdiagnosed. Maternally inherited Leigh syndrome is caused relatively frequently by mutations in the mtDNA-encoded ATP6 subunit of complex V, such as the m.8993T>G mutation, which may be associated with seizures (22;23). Nuclear-encoded defects of complex V have so far been associated primarily with hypertrophic cardiomyopathy phenotypes, although seizures have occasionally been reported (24;25).

6. Multiple respiratory chain defects

Approximately 25% of children with mitochondrial disease present with multiple OXPHOS defects, involving two or more of the enzyme complexes. Combined OXPHOS deficiencies may occur for a number of reasons, including disorders of mtDNA maintenance, disorders of mitochondrial translation and defective biosynthesis of coenzyme Q₁₀.

Disorders of mitochondrial DNA maintenance

The mtDNA depletion syndrome (MDDS) is characterised by a severe quantitative reduction of the mtDNA copy number. Residual mtDNA levels in affected tissues may be as low as 1-2% of those observed in controls (26). MDDS is most frequently caused by mutations in the *POLG* gene, encoding the catalytic subunit of the DNA polymerase gamma, the only polymerase able to replicate mtDNA. In our cohort of 26 patients with MDDS who had residual mtDNA <35% of control values, we found *POLG* mutations in 19 cases, many of whom had seizures [(27;28) and unpublished data]. The clinical spectrum of hepatocerebral MDDS caused by *POLG* mutations overlaps that of the Alpers syndrome of progressive neuronal degeneration with epilepsy. Patients with Alpers syndrome typically present with focal clonic and complex-focal seizures. Epilepsia partialis continua is also frequently seen. Initial EEG often shows unilateral occipital rhythmic high-amplitude delta with superimposed (poly)spikes, although subsequently discharges tend to generalise (28). Older patients with *POLG* mutations may have multiple mtDNA deletions rather than depletion. Patients with milder or heterozygous mutations may present with progressive external ophthalmoplegia without cerebral involvement, but two clinical syndromes associated with recessive *POLG* mutations are characterised by epilepsy, namely SCAE (spinocerebellar ataxia with epilepsy) and MIRAS (mitochondrial recessive ataxia syndrome) (29).

Eight other genes have also been reported to cause MDDS (26) and seizures are often associated with mutations in these genes. For example, we reported seizures in the first patient with mutations in *SUCLA2*, encoding the beta subunit of the Krebs cycle enzyme succinylCoA ligase which is required for mtDNA maintenance (30).

Disorders of mitochondrial translation

The most frequent disorders of mitochondrial translation affect the mitochondrial tRNA genes, either as point mutations such as m.3243A>G and m.8344A>G, causing the MELAS and MERRF (myoclonic epilepsy with ragged red fibres) syndromes respectively, or by large-scale deletion involving several tRNA genes, as in the Pearson marrow pancreas and Kearns-Sayre syndromes. Epilepsy is a defining feature of MERRF syndrome and also occurs relatively frequently in MELAS syndrome. More recently, nuclear-encoded defects of mtDNA translation have been reported (31), and these may also be associated with epilepsy. Mutations of RARS2, required for aminoacylation of the mitochondrial tRNA for arginine, appear to be particularly associated with intractable epilepsy (32).

Coenzyme Q₁₀ deficiency

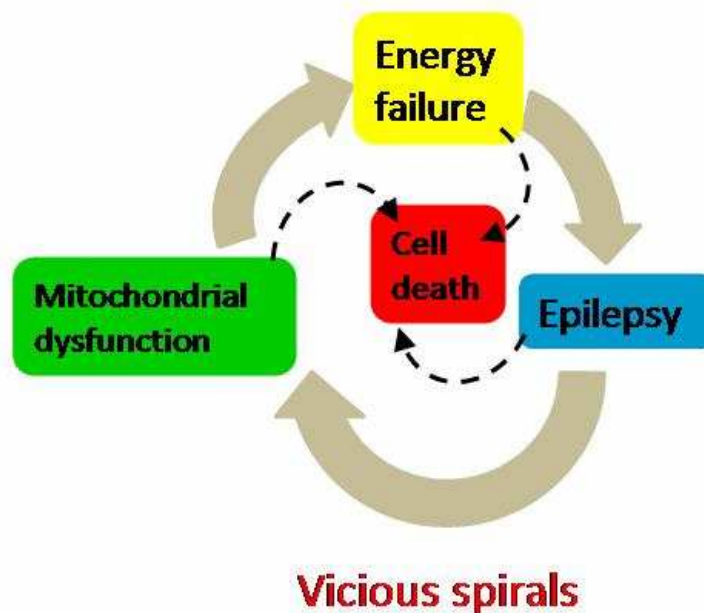
Defective biosynthesis of the lipophilic electron carrier coenzyme Q₁₀ (CoQ₁₀) typically presents with combined deficiency of complexes I+III or II+III (33) and is frequently associated with seizures. The first phenotype reported to be caused by CoQ₁₀ deficiency was of recurrent rhabdomyolysis associated with an encephalopathy with prominent seizures (34). Seizures are also a feature of other phenotypes of CoQ₁₀ deficiency, such as the multisystem disorder of infancy (35-38) and the syndrome of ataxia with seizures caused by mutations in the kinase ADCK3 (39;40). Although rare, it is important to recognise this group of disorders, since at present CoQ₁₀ deficiency represents the only treatable subgroup of mitochondrial disease. To this end, we have recently published a diagnostic algorithm to aid in the clinical diagnosis of CoQ₁₀ deficiency (33).

Pathogenesis of mitochondrial epilepsy

The pathomechanisms leading to epilepsy in mitochondrial disorders are not clear. Energy failure undoubtedly plays a role but does not explain the phenotypic variability of mitochondrial epilepsy, nor why epilepsy is not a feature of all mitochondrial disorders. Other aspects of mitochondrial dysfunction, such as ROS production, abnormal calcium handling and increased apoptosis, are also likely to contribute to seizure generation.

Furthermore, there is some evidence that seizures themselves can trigger mitochondrial dysfunction (41), implicating a vicious spiral in the aetiology of mitochondrial epilepsy (Figure 1). It is also possible that mitochondrial epilepsy may be triggered by an autoimmune response, as has been suggested for *POLG* mutations (42). Finally, in occasional patients seizures may be secondary to electrolyte disturbances arising from severe renal tubulopathy.

Figure 1: Pathogenesis of mitochondrial epilepsy



Management of mitochondrial epilepsy

Mitochondrial epilepsy can be very difficult to manage. It is important to identify and treat disorders of CoQ₁₀ biosynthesis, since these remain the only treatable causes of mitochondrial epilepsy. Recognition and treatment of electrolyte disturbances caused by renal tubulopathy is also critical. In all other patients, symptomatic treatment remains the mainstay of management. The choice of antiepileptic drug (AED) depends on the seizure type, but it is important to avoid valproate in mitochondrial disease if at all possible, particularly in patients with *POLG* mutations, since valproate may trigger fatal

hepatic failure in these patients (43). Levetiracetam is the first choice for myoclonus in MERRF syndrome (44), whilst lamotrigine may exert a neuroprotective effect (45). In many cases multiple AEDs are needed to achieve seizure control. Vigilant monitoring for and treatment of multisystem disease manifestations is essential. Interventions which may be required include hearing aids, insulin, thyroxine and drugs for cardiomyopathy. Arginine may reduce the severity and frequency of stroke-like episodes in patients with MELAS syndrome (46), and folinic acid may lead to symptomatic benefit in patients with low CSF 5-methyltetrahydrofolate (47). The role of other vitamins and nutritional supplements is not clear, and so far has not been supported by clinical trials (48). However, the prognosis for mitochondrial epilepsy is extremely poor, and there is clearly an urgent need for novel treatments for these patients. A fatal outcome was observed in 45% [22/56] of the Paris cohort, and 50% of these cases died within 9 months of epilepsy onset (4).

Ketogenic diet: what's the evidence in mitochondrial disease?

The ketogenic diet (KD) is a high fat, low carbohydrate diet that aims to stimulate fatty acid utilisation by mitochondrial beta-oxidation, with subsequent formation of ketone bodies to provide an alternative energy source for brain and other tissues. Ketone bodies are metabolised to acetylCoA, which feeds into the Krebs cycle and thence to the respiratory chain to generate ATP, and may at least partially bypass complex I. Preliminary preclinical studies have suggested that KD *may* be beneficial in a subgroup of mitochondrial disease, namely those with mtDNA deletions. A study in which cultured cells (transmitochondrial cybrids) harbouring a heteroplasmic mtDNA deletion were grown in a ketone-rich culture medium devoid of glucose demonstrated that the ketogenic diet favoured wild type over mutant mtDNA, leading to a reduction in the proportion of deleted mtDNA and functional rescue of the respiratory chain defect in these cells (49). The authors proposed that KD treatment might be beneficial for patients with heteroplasmic mtDNA deletions. A more recent study investigated the effects of KD in the 'Deletor' mutant mouse (50). This is a transgenic mouse harbouring a mutation in the Twinkle helicase, leading to

accumulation of multiple mtDNA deletions and a late onset myopathy (51). This study showed reduction in *some* features of mitochondrial disease in the mice, particularly with presymptomatic initiation of the KD treatment. A number of case reports have described KD in the treatment of children with mitochondrial disease (52), but few studies have examined the effects of KD in a more systematic way. One retrospective study reported the use of KD in 24 children with respiratory chain defects and reported that 50% became seizure free on KD (5). However duration of follow-up and long-term outcome for these patients was not reported. Some patients benefited from KD in the Paris study, but no dramatic improvements were observed (4). Overall, there is a suggestion that whilst seizure frequency may reduce on a KD, the diet does not appear to influence the relentlessly progressive course of mitochondrial disease in many patients. It is not clear whether some subgroups of mitochondrial disease may respond better than others to a KD. Formal clinical trials are needed, with the aim of determining which patients are likely to benefit from a KD.

Conclusions

In summary, seizures occur frequently in mitochondrial disease. They may be the presenting feature but are often part of a multisystem presentation. Mitochondrial epilepsies are biochemically and genetically heterogeneous, but some of the more common causes are mtDNA mutations and mutations in *POLG*. The pathogenesis of mitochondrial epilepsy remains poorly understood, contributing to the immense difficulties in treating this condition. Epilepsy is a poor prognostic sign in mitochondrial disease, and clinical trials of the ketogenic diet and novel therapeutic agents are desperately needed.

Acknowledgements

I am funded by the DH/HEFCE Best Research for Best Health Senior Lecturer scheme, and also receive support from Great Ormond Street Hospital Children's Charity. I currently receive grant funding from Ataxia UK, the Child Health Research Appeal Trust, the Children's Liver Disease Foundation, Climb, the Medical Research Council and Vitaflo.

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The investigation of Sudden Unexpected Death in Infancy (SUDI): The Sheffield protocol

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SUDI refers to the sudden death of a previously well infant. A specific cause of death is only identified in approximately 20% of cases after further investigation. It is only by adopting a systematic and multidisciplinary approach that this yield can be improved. In fact, a number of serious case reviews have highlighted the necessity of a locally co-ordinated approach as there is a lack of guidance for health professionals dealing with these cases. SUDI can occur in apparently completely well and healthy children, in infants and children who were considered to be mildly unwell but in whom death is unexpected and in others with a known serious but stable condition who suddenly die. The unifying theme of all of the above situations is the occurrence of a rapid deterioration that culminates in death.

Sudden Infant Death Syndrome (SIDS) is the largest single category of death in babies over one month old in developed countries. SIDS is defined as the sudden death of an infant under one year of age which remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history. In the UK SIDS declined rapidly after the introduction of the back to sleep campaign in 1991 and since then remains at a rate of 0.44 per 1,000 live births.

The Kennedy Report "Sudden Unexpected Death in Infancy" (2004) was the result of the working group convened by the Royal College of Pathologists and the Royal College of Paediatrics and Child Health. The multiagency protocol guides the investigation and care of families after the sudden an unexpected death of an infant or child. The key elements of the Kennedy protocol include: 1) the staff involved should retain an open mind (natural death versus neglect or abuse); 2) the Coroner has jurisdiction over the body; 3) babies found dead at home are always taken to the A&E department (not to the mortuary); 4) after the baby has been declared dead a

paediatrician takes a clinical history from the parents; 5) the baby is examined carefully; 6) the presence of bruises, injuries, or retinal haemorrhages should raise suspicion; 7) the police would lead these cases that will be done jointly by the forensic and paediatric pathologists; 8) a standard set of samples, which include metabolic investigations, are taken after death is confirmed (unless unnatural death suspected). Ideally within 24 hours a police officer trained in SUDI cases and the on-call SUDI paediatrician visit the home to talk with the parents and examine the place with the baby died. The SUDI paediatrician inquiries about the clinical history, including details of “the last sleep” and prepares a report for the Coroner and the pathologist. The autopsy is performed as soon as possible and using a systematic procedure. A multidisciplinary meeting chaired by the SUDI paediatrician takes place after the post mortem report is issued. This meeting should be attended by all agencies involved: police, pathologist/s, social services, health visitor, the general practitioner of the baby and the coroner’s officer. The purpose of the meeting is to share information, agree the cause of death and plan future care for the family. Relevant information concerning the circumstances of the death, the infant’s history, family history, risk factors for SUDI and subsequent investigations are reviewed. An explicit discussion of the possibility of abuse or neglect happens and, if no evidence is identified to suggest maltreatment, this should be documented as part of the report of the meeting. The death is classified according to the Avon classification of SUDI (Fleming et al 2004). After the meeting, the SUDI paediatrician discusses the findings and future care with the parents; the Coroner holds the Inquest and registers the cause of death. Using this approach we have been able to identify the cause of death in 50% of SUDI. Metabolic causes of death represent approximately 1-3% of the cause of death. Also important, the families received better care and bereavement support.

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The Ketogenic Diet in Seizures and Metabolic Disease

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The ketogenic diet is a high fat low carbohydrate diet which contains sufficient calories to meet energy requirements, and sufficient protein and micronutrients for repair and growth. Biochemically the ketogenic diet emulates starvation in that fat is used as the major energy source. In many tissues, notably muscle including heart, β -oxidation generates acetyl-CoA for use through the tricarboxylic acid (TCA) cycle. The liver generates ketones which can be exported and used as an energy source through ketolysis, the TCA cycle and respiratory chain in most tissues, but notably in brain which is not able to effectively use the β -oxidation of fat as an energy source. During normal feeding, brain uses glucose as an energy source. Glucose is transported across the blood brain barrier by the glucose transporter GLUT1 but ketones, when present during relatively short fasts do not cross the blood brain barrier. On longer fasts, as the concentration of ketones in plasma rises, the ketone transporter MCT1 is induced in cerebral capillary endothelial cells which allows passage of ketones into the brain where they can be freely used as an energy source.

The biochemical effect of the ketogenic diet is to replace glucose with fatty acid and ketones as the major energy source. Dietary fat rather than body fat is used as a source of fatty acids and hence ketones. The brain, which in the young child is the major consumer of glucose is able to adapt to the ketotic state and switch from glucose to ketone consumption (figure 1).

Types of ketogenic diet

There are several variations on the ketogenic diet. The classical (4:1) form of the diet has 80% of dietary calories as fat with 20% of energy as protein and carbohydrate. Other forms are the 3:1 or 2:1 diets (75% or 66% of energy as fat), carbohydrate restricted diet (10-20g per day of carbohydrate or modified Atkins diet) which can induce ketosis or a low glycaemic index diet in which fruit, bread and starchy foods are not allowed. A form of the diet uses medium chain triglycerides (MCT) as the fat source, which because

of the increased ketogenicity of MCT allows more carbohydrate in the diet. However this MCT rich diet is associated with more adverse events. Nevertheless, addition of some MCT to the diet can reduce some of the complications of the classical diet.

Adverse effects

The diet cannot be tolerated by all individuals and the relatively low palatability of the high fat content may make it impossible for some to continue the diet. Initial adverse effects include acidosis, which may respond to a reduction in the degree of ketosis, hypoglycaemia, which is often asymptomatic and inconsequential, abdominal pain, nausea, vomiting and constipation. Electrolyte abnormalities and dyslipidaemia may respond to alteration in the diet composition. Increased plasma urate may cause/promote renal stones or gout and can be treated with allopurinol. Late onset complications include renal stones (secondary to hypercalciuria, urinary acidity and hypocitraturia), osteopenia, cardiomyopathy, liver disease, optic neuropathy and basal ganglion disease. Ten percent of patients on a ketogenic diet will experience an adverse event requiring diet modification. However in some societies, a ketogenic diet is the normal form of intake which is maintained lifelong without adverse effects.

Ketosis, either through starvation or through use of the ketogenic diet has a very long history for the treatment of a wide variety of conditions and is currently still being investigated as a treatment strategy for some more recently described conditions. Here the use of ketosis in epilepsy will be briefly described followed by a discussion of the use of the ketogenic diet in treating inherited metabolic disease.

Epilepsy

Starvation has been used as a treatment for epilepsy since the time of ancient Greece. Hippocrates in the fifth century BC described a man whose epileptic convulsions were effectively cured by complete abstinence from food and drink. In the Gospel of St Mark, a boy possessed by demons (epileptic seizures) is presented to Jesus who says "This kind [of demon] can

come out only by prayer and fasting.” Jesus recognised the value of fasting in treating seizures.

The potential of treating epilepsy by fasting came to the attention of Bernarr Macfadden in the United States at around the start of the 20th century.

Macfadden was a physical fitness guru who claimed that almost any disease could be cured by diet and exercise and that fasting from 3 days to 3 weeks could amongst other things cure epilepsy. Macfadden’s assistant, osteopath Hugh Conklin developed the practice of fasting to treat epilepsy. Patients were fasted for between 18 and 25 days or as long as the fast was tolerated and ‘cure’ rates for epilepsy were reported as 90% for children under 10 years, 80% for adolescence 10-15 years, 65% for those 15-25 years and 50% for those 25-40 years.

In 1921, Wilder from the Mayo clinic commented that ketogenesis could be produced by feeding a diet rich in fat and poor in carbohydrate and proposed a trial of ketogenic diet in epilepsy. The first three patients in whom the diet was tried experienced a dramatic improvement in seizure control. Wilder then speculated that the ketogenic diet could be a more acceptable treatment for epilepsy than the previously used starvation therapy. In fact, use of the ketogenic diet to treat epilepsy became widespread over the next 20 years as an effective and non-sedating alternative to the only available pharmacological agents, bromides and phenobarbitone.

The discovery of phenytoin in 1938 led to a decline in the popularity of the ketogenic diet and by 1995, with a substantial increase in the number of available antiepileptic drugs, it was felt that use of the diet was no longer justified. However, around the same time, a Hollywood producer, Jim Abrahams used media connections to promote the ketogenic diet. His son Charlie had epilepsy which did not respond to multiple antiepileptic drugs. Charlie’s seizures were completely controlled by the ketogenic diet and television film “First Do No Harm” led to a multicentre study of the ketogenic diet.

In 1998 (Freeman JM *et al* 1998) the results of a prospective study of 150 children treated with the diet for up to 12 months were published, with the results of 3 and 6 year follow up of the same children published in 2001. Prior to the diet the children had failed to respond to multiple antiepileptic

drugs. After 12 months of treatment, 7% were seizure free, 20% had 90-99% reduction in seizure frequency and 23% had 50-89% reduction in seizure frequency. At 3-6 year follow up, 13% were seizure free and 43% had greater than 50% seizure reduction with most children being off the diet and on fewer or no drugs.

Subsequently there have been a number of international studies of the efficacy of the diet. Similar results have been obtained from these studies which appear to be broadly concordant with those obtained from the early studies in the 1920s. Generally 10-15% of children are seizure free after one year of diet, 30% have a better than 90% reduction in seizure frequency and 40-50% found the diet to be ineffective or could not be tolerated. Despite the introduction of new antiepileptic drugs, the ketogenic diet is still found to be effective in many children whose seizures are unresponsive to medication. A broad range of seizure types respond to the ketogenic diet including both partial and generalized seizures. Certain refractory disorders respond well to the diet, including infantile spasms, myoclonic-astatic seizures, tuberose sclerosis, Rett and Dravet syndromes. On the other hand, focal seizures respond less effectively. Vagal nerve stimulation appears to be effective in combination with the ketogenic diet (Freeman *et al* 2007).

There is no clear indication as to which type of ketogenic diet is the most effective in epilepsy with ability to maintain ketosis perhaps being one of the main determinants of efficacy.

The mechanism by which the ketogenic diet exerts its effect in epilepsy remains unclear. The diet has an effect in preventing seizures which may persist long term after cessation of the diet indicating that the antiepileptic properties must be in part at least more than a simple alteration in brain biochemistry. Ketone bodies themselves (acetoacetate and acetone, but not 3-hydroxybutyrate) have antiepileptic properties, and the antiepileptic properties of the diet do correlate with the degree of ketosis. The ketogenic diet increases flux through the γ -aminobutyric acid (GABA) pathway. GABA is an important inhibitory neurotransmitter and increase in GABA flux may suppress epileptic activity. However these observations would not explain the continuing antiepileptic effect after discontinuation of the diet.

The ketogenic diet may have an antiepileptic effect modulated through neuroprotection. The ketogenic diet appears to enhance ATP production. Further the diet, in mice at least, increases uncoupling proteins which reduce the mitochondrial membrane potential and protect against free radical damage. Also the diet increases glutathione peroxidase which prevents membrane damage by lipid peroxidation. The diet also appears to prevent cells entering the apoptotic pathway (Freeman *et al* 2007).

More recently, 2-deoxyglucose, an inhibitor of glycolysis has been shown to protect from seizures. The action of the ketogenic diet may in part be from inhibition of glycolysis.

The ketogenic diet in inherited metabolic disease

The ketogenic diet shifts the mode of energy production in practically all cells from glycolysis and glucose dependency to fat oxidation and ketolysis. As individuals can tolerate the ketogenic diet for years, as seen in the treatment of epilepsy, or for a lifetime, as seen in the Inuit population of Northern Canada, it would seem logical that those individuals with inherited defects in glucose metabolism would benefit from long term treatment with the ketogenic diet. Such defects would include disorders of glucose transport, most specially GLUT1 deficiency, glycogen storage disorders, disorders of glycolysis and pyruvate dehydrogenase deficiency. In addition, there is a growing body of evidence to suggest that the energy block in mitochondrial complex I deficiency may be partially circumvented by ketosis.

GLUT1 deficiency

GLUT1 is a passive glucose transporter that transports glucose across the membranes of the endothelial cells of the brain capillaries and therefore across the blood brain barrier. GLUT1 also transports glucose into glial cells and neurons. GLUT1 deficiency result in impaired transport of glucose across the blood brain barrier and cerebral glycopenia.

In the fed state, brain consumes a substantial portion of body glucose (up to 80% in the young child). Brain has very limited capacity to store glucose or to undertake gluconeogenesis, and cannot use fat as an energy source. Individuals with GLUT1 deficiency will rapidly become cerebrally glycopenic

as plasma glucose levels fall from the postprandial state resulting in the cells of the CNS becoming energy depleted, as initially there will be insignificant ketosis and there is an inability to transport ketones into the brain before MCT1 is induced. However, with prolonged ketosis and induction of MCT1 the brain can avidly use ketones as an energy source (figure 1).

Clinically GLUT1 deficiency usually presents as a refractory epileptic encephalopathy during the first year of life. Later seizure may be generalized tonic clonic, myoclonic or absences. There is developmental delay and a motor disorder with hypotonia and ataxia and sometimes dystonia which may be episodic. Occasionally patients may not have epilepsy. Diagnosis is suggested by finding a low CSF: plasma glucose ratio and confirmed by molecular analysis of the *SLC2A1* gene.

The mainstay of treatment is the ketogenic diet. Most patients have been treated with classical 4:1 or 3:1 diets although MCT based and modified Atkins diets have also been shown to be effective. Most patients achieve seizure control on the diet but some need additional anticonvulsants. In Klepper's series of 15 GLUT1 deficient children (Klepper 2005), 12 became seizure free and 10 could discontinue anti epileptic drugs. Alertness, activity movement disorder and ambulatory skills may improve but effect on development is variable. Whilst it is generally recommended that the diet is continued through childhood, as cerebral glucose consumption is high, it is not clear if the diet should be continued in to adult life or if a more relaxed version of the diet can be used.

Pyruvate dehydrogenase (PDH) deficiency

The pyruvate dehydrogenase complex is the final enzyme in the pathway in which glucose is converted through glycolysis to acetyl-CoA for energy provision through the TCA cycle. The biochemical hallmark of the disorder is hyperlactataemia with increased plasma pyruvate, and a relatively normal lactate: pyruvate ratio. Whilst biochemical abnormalities are present in plasma, they are often more pronounced in CSF and the manifestations of the disorder are essentially those of cerebral energy depletion.

The PDH complex is composed of the E1 α , E1 β , E2 and E3 binding proteins (exclusive to PDH) and components common to other dehydrogenases.

Most cases of deficiency are caused by E1 α defects which are X-linked, with the less common forms being recessively inherited.

There are four groups of presenting features (Barnias *et al* 2009): neonatal encephalopathy with lactic acidosis which is fatal, infantile encephalopathy with developmental delay hypotonia and seizures, infantile onset progressive Leigh disease and recurrent ataxia. Symptoms are essentially related to failure or cerebral energy provision through glycolysis.

Pharmacological treatment is available for some PDH deficient patients. A minority will respond to pharmacological doses of thiamine. Lactic acidosis may respond to treatment with sodium dichloroacetate which acts by stimulating residual PDH activity.

An early report of using a ketogenic diet to treat PDH deficiency was published in 1992 (Wijburg *et al*). A one year old boy with a Leigh disease phenotype benefited clinically, biochemically and radiologically from the diet. The ketogenic diet is a logical candidate for the treatment of PDH deficiency (figure 1). Firstly energy can be provided by fat oxidation or by ketogenesis bypassing the PDH step and accessing the brain. Secondly the low glucose intake of the diet reduces flux through glycolysis and reduces the lactic acidosis. In practice, the diet has been shown to benefit some patients with PDH deficiency. Typically those with childhood onset epilepsy or paroxysmal dystonia benefit from the diet (Barnias *et al* 2009). Those with severe neonatal onset or Leigh disease forms appear to benefit less. Wexler *et al* 1997 treated seven boys with E1 α deficiency. Those with severe early onset presentations died early, but the longest survivor with best developmental outcome was placed on the most carbohydrate restricted diet. A group with later presentation responded more favourably to the diet with longer survival and better developmental outcomes.

There have been several further case reports of the benefits of the diet in PDH deficiency especially for paroxysmal manifestations – epilepsy and movement disorders. However, as indicated by Weber *et al* 2001 there is lack of uniformity in application of the diet to PDH deficient patients.

In our practice, we have found that the ketogenic diet has benefited some PDH deficient patients in terms of developmental outcome, alertness and weight gain even in the absence of epilepsy or paroxysmal movements, and with resolution of lactic acidosis. Conversely not all patients with seizures show a response to the diet.

Mitochondrial Disease

Epilepsy is a frequent complication of disorders of energy metabolism. Disorders of the mitochondrial respiratory chain are amongst the most frequently encountered disorders of energy metabolism. In some cases, a specific molecular defect can give rise to a defined mitochondrial disease phenotype associated with specific types of epilepsy. An example would be mutations in *POLG* resulting in Alpers disease causing epilepsia partialis continua. However in most cases of neurologically presenting mitochondrial cytopathy there is a non-specific encephalopathy with progressive neurological dysfunction and often epilepsy.

Lee *et al* 2008 studied 48 children with mitochondrial respiratory chain disease and epilepsy. Most (73%) were deficient in complex I alone, 2% had combined complex I and IV deficiency the remainder having complex IV deficiency alone 23% of complex II deficiency (2%). Clinically, 21% presented as Leigh disease, 2% as myopathy, encephalopathy lactic acidosis and stroke like episode (MELAS), 2% as Alpers's disease and 75% as an uncategorised encephalopathy. The epilepsy diagnosis was Ohtahara syndrome in 4%, West syndrome in 21%, Lennox-Gastaut syndrome in 25%, Landau-Kleffner syndrome in 4%, generalised epilepsy in 29% and partial epilepsy in 17%. Thirty-one children had intractable epilepsy and of these 24 went on to be treated with a ketogenic diet. Three of these patients had to discontinue the diet because of infection or persistent acidosis. Twelve of the diet treated patients became seizure free on diet, 2 had over 90% reduction of seizures and 4 had seizure reduction to 50-90%. So of the 24 children initially diet treated, 75% experienced significant benefit in terms of seizure control.

Kang *et al* 2007 studied 14 children with epilepsy and respiratory chain complex disease. Seizures were not controlled by three or more anti

epileptic drugs. With the ketogenic diet, 7 children became seizure free and 3 children experience a 50-90% reduction in seizures, and all but one patient experienced some reduction in seizure frequency. Most patients had isolated complex I deficiency. One child with isolated complex II deficiency experienced a 100% reduction in seizure frequency and as did one patient with isolated complex IV deficiency, and two other complex IV deficient patients achieved some seizure reduction. Eight patients also showed improvement on cognitive and behaviour function tests following the diet, although it is not clear to what extent the improvement was independent of seizure reduction.

Interestingly Joshi *et al* 2009 described the efficacy of the ketogenic diet in a girl with Alpers disease and epilepsy partialis continua who became seizure free on the diet.

The data from both the Kang and Lee studies indicate that the ketogenic diet is safe to use in respiratory chain disease and it appears to be as affective for epilepsy in these patients as it is in epilepsy in general. Unlike the application of the ketogenic diet for PDH and GLUT1 deficiencies, the biochemical role of the diet in respiratory chain disorders is not clear. There is evidence *in vitro* that ketones can cause heteroplasmic shifting, with apparent benefit to normal mitochondria rather than those respiratory chain defects (Santra *et al* 2004). Initially it was felt unsafe to use the diet in respiratory chain disorders (Nordli and de Vivo 2001) as it may impose oxidative stress, which provokes seizures. However, in ketotic conditions, mitochondria produce lower quantities of oxidative species. In the hippocampus, the diet stimulates glutathione peroxidase. Mitochondrial biogenesis is stimulated and apoptosis decreased. Through a mechanism involving increased GABA synthesis, neuronal excitability is increased. Also the efficiency of ATP production is increased. Thus there are a variety of mechanism by which the diet could be neuroprotective and anticonvulsant. Some effects of the diet may be modulated by polyunsaturated fatty acids present in increased amounts in the high fat diet.

The mechanism by which the ketogenic diet enhances ATP synthesis is uncertain. However some interesting observation from research into amyotrophic lateral sclerosis (ALS) gives some indication of the mechanisms

involved. ALS results from the death of motor neurons. Some cases are caused by defective Cu/Zn superoxide dismutase I (SOD1). Mice expressing SOD1 experience progressive weakness mimicking that seen in humans with ALS. SOD1 mice fed a ketogenic diet showed less disease progression and relative preservation of motor neurons (Zhao *et al* 2006). *In vitro* studies of mitochondria from SOD 1 mice showed that 3-hydroxybutyrate prevented rotenone mediated inhibition of mitochondrial complex I but not malonate inhibition of complex II. The inference is that ketones generated by the ketogenic diet can supply electrons to the respiratory chain bypassing complex I and hence have a beneficial effect in complex I disease (figure 1). The mechanism of entry into the respiratory chain at a point other than complex I is not known, and this mechanism would not explain the benefits of the ketogenic diet in respiratory chain complex disease other than complex I disease.

Other metabolic diseases

The ketogenic diet provides an energy source as an alternative to that derived from glucose and the glycolytic pathway. Potentially defects of glycogen storage and glycolysis could be treated with a ketogenic diet. However for most disorders of glycogen storage there is a simple and effective treatment in the form of regular carbohydrate provision as starch, which can effectively prevent the major manifestations of these disorders, hypoglycaemia and liver disease. However, in the muscle glycogen storage diseases McArdle disease (deficiency of muscle glycogen phosphorylase) and phosphofructokinase deficiency, the problem is deficiency of the rate of provision of energy through glycolysis leading to exercise intolerance, myalgia, stiffness and myolysis (figure 2). In severe forms of phosphofructokinase deficiency there is compromise in the newborn period with myopathy which may affect respiration and arthrogyrosis. There are case reports of the ketogenic diet benefiting patients with both these conditions. Busch *et al* demonstrated that the ketogenic diet improved a McArdle disease patient by improving exercise tolerance and reducing blood creatine kinase concentrations. Swoboda *et al* demonstrated significant

improvement in muscle strength and development in a patient with severe phosphofructokinase deficiency when treated with a ketogenic diet. Non-ketotic hyperglycinaemia is an inherited defect in the glycine cleavage system which results in excessive glycine accumulation in brain with a result intractable epileptic encephalopathy, severe hypotonia and paucity of movement and severe developmental delay. Bzduch treated an 18 month old child affected by non-ketotic hyperglycinaemia with a ketogenic diet. The effect was not only to reduce brain glycine levels but to improve alertness, tone and muscle strength and prevent seizures. The mechanism for this effect has not been fully determined and may in part be through the anti-epileptic effect of the diet. However there may also be an effect of reducing glycine synthesis by reducing glycolysis and hence reducing one of the substrates for glycine synthesis, namely 3-phosphoglycerate.

Conclusions

The ketogenic diet is a well established treatment for a variety of conditions including intractable epilepsy and some inherited metabolic disease. With supervision by an experienced dietician and careful monitoring the diet is safe. It would appear that the diet can generate beneficial effect through a variety of mechanisms. In inherited metabolic disease such as GLUT1 deficiency and PDH deficiency the mechanism involves an alternative provision of energy, especially in the brain. However in mitochondrial disease and epilepsy the mechanism is less clear and involves more than just the provision of ketones as an energy source. It would appear that improved efficiency of the respiratory chain, and reduced oxidative damage to mitochondria are an important factor. Stabilization of mitochondria and reduction of apoptosis may result in a significant neuroprotective effect. Further research into the mechanism of the ketogenic diet is clearly warranted and it is expected that the neuroprotective effect of the diet will lead to a widening of application of the diet.

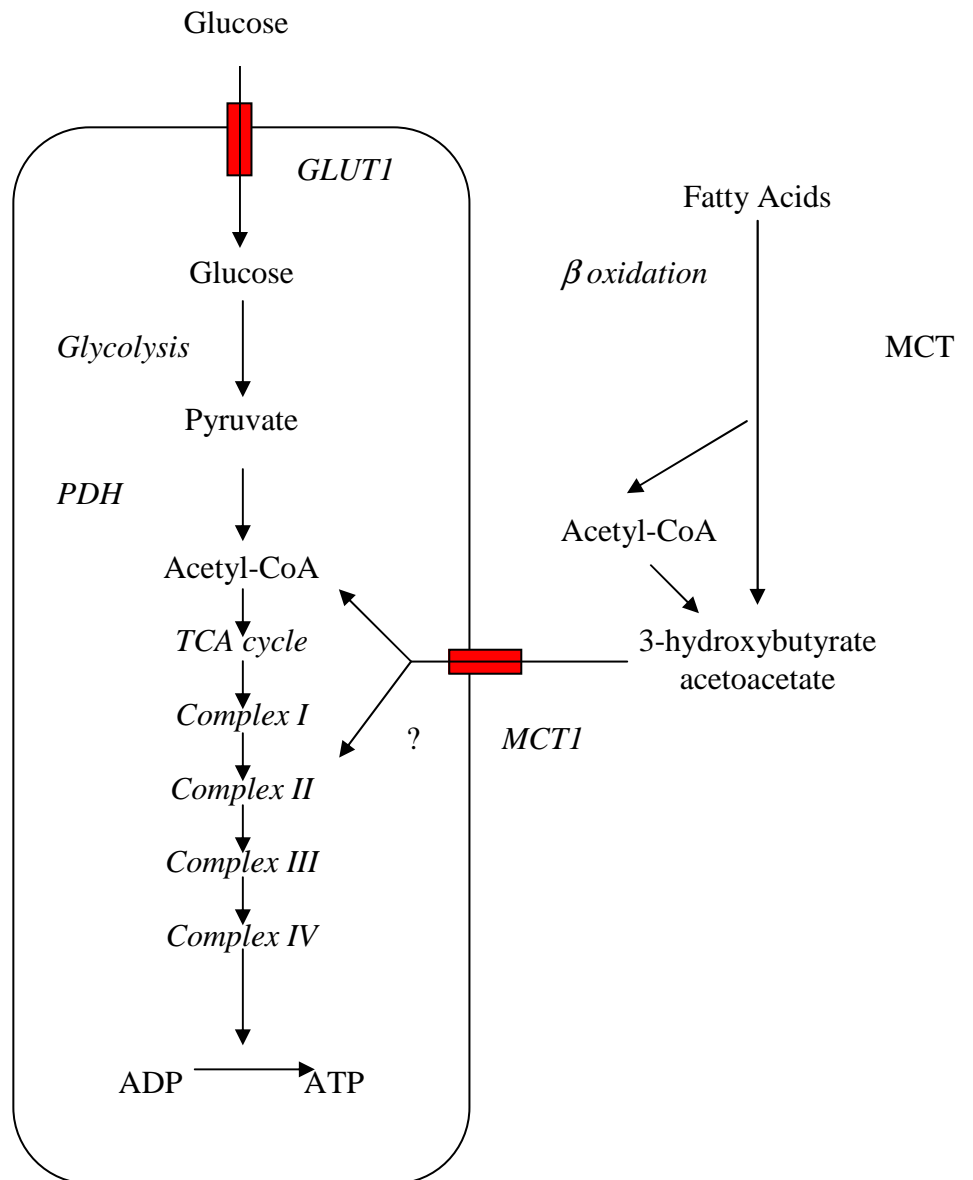


Figure 1. Use of ketosis to provide energy as an alternative to glucose in GLUT1 deficiency, PDH deficiency and possibly mitochondrial complex I deficiency.

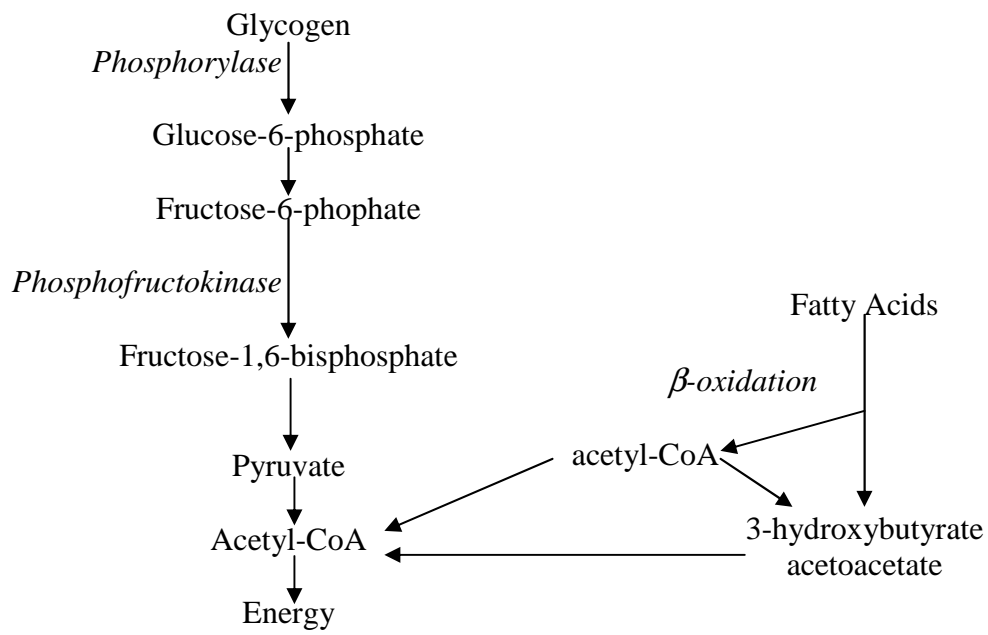


Figure 2. Ketogenesis in muscle: use in McArdle disease (muscle glycogen phosphorylase deficiency) and phosphofructokinase deficiency

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Peroxisomal Disorders

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Introduction

Peroxisomes are complex single-membrane cell organelles found in all cell types except erythrocytes. Peroxisomes have both catabolic and anabolic functions and these functions predominantly involve lipid metabolism.

Peroxisomal functions include the synthesis of plasmalogens which are important constituents of cell membranes and myelin. They are also involved in the formation of bile acids, polyunsaturated fatty acids, cholesterol and isoprenoids. Peroxisomes β -oxidise very long-chain fatty acids (VLCFA's), α -oxidise phytanic acid and catabolise lysine via pipecolic acid and glyoxylate to glycine. Importantly they also contain catalase which converts highly reactive hydrogen peroxide into oxygen and water.

Peroxisomes multiply by division of existing peroxisomes. Peroxisomal membranes are assembled and peroxisomal matrix proteins are targeted from the cytosol and then imported into the organelle by a highly complex process dependent on specialised proteins termed peroxins which are encoded by *PEX* genes. As a consequence peroxisomal biogenesis involves the correct expression of multiple *PEX* genes of which 16 have been identified in humans. There are also a large number of single enzyme functions within the peroxisome encoded by non-*PEX* genes and defects in these result in a range of disorders with single enzyme deficiency.

Peroxisomal disorders are broadly categorised into defects of peroxisomal biogenesis with deficiencies of multiple pathways e.g. Zellweger spectrum or defects affecting single enzymes such as D-bifunctional protein deficiency. Most disorders are autosomal recessive, however the commonest peroxisomal disorder X-linked adrenoleucodystrophy has an X-linked mode of inheritance.

Peroxisomal disorders present with a wide spectrum of clinical disease ranging from the severe neonatal Zellweger syndrome with dysmorphic features, neurological abnormalities, hepatorenal and gastrointestinal dysfunction with death typically occurring within the first 6 months of life to adult onset X-linked adrenoleucodystrophy which can be confined only to adrenal insufficiency.

Peroxisomal assembly

Peroxisomal biogenesis is complex and peroxisomes multiply by division of pre-existing peroxisomes. Peroxisomes do not contain any DNA and subsequently all of the proteins required for assembly and function are encoded by nuclear genes and synthesised on free polyribosomes in the cytosol before post-translational import into the peroxisome. Transportation is highly selective and requires the presence of specific import sequences known as peroxisomal targeting sequences (PTS). PTS1 is the C-terminal peroxisome targeting sequence and PTS2 is the N-terminal peroxisomal targeting sequence. PTS's are recognised by receptors (PTS1 receptor and PTS2 receptor) which direct the peroxisomal proteins to the peroxisomal membrane. The target protein then enters the peroxisome by a sequential multi-step process involving recognition, docking, translocation across the peroxisomal membrane and recycling.

All proteins (peroxins) involved in peroxisomal biogenesis are encoded by *PEX* genes. To date 16 *PEX* genes have been identified as essential for human peroxisomal formation. *PEX5* encodes for the PTS1 receptor and *PEX7* encodes for the PTS2 receptor. *PEX1*, *PEX6* and *PEX26* are required for matrix protein import and encode proteins involved in the recycling of the PTS1 and PTS2 receptors. *PEX2*, *PEX10* and *PEX12* encode proteins involved in matrix protein import. *PEX13* encodes a docking factor for PTS1 and is also required for matrix protein import. *PEX3*, *PEX16* and *PEX19* encode proteins involved in the production of peroxisomal biogenesis proteins. In addition to the assembly proteins, the peroxisome also contains over 50 matrix proteins and numerous membrane proteins.

Peroxisomal disorders

Peroxisomal disorders arise from either a defect in peroxisomal biogenesis (the peroxisomal biogenesis defects) or a defect in a single peroxisomal enzyme or protein (the single enzyme defects).

Clinical presentation

The peroxisomal biogenesis defects include the Zellweger spectrum which accounts for approximately 80% of patients, while rhizomelic chondrodysplasia punctata (RCDP) accounts for the remaining patients with peroxisomal biogenesis disorders. RCDP is clinically and genetically distinct from the Zellweger spectrum.

The clinical phenotype of Zellweger spectrum, also known as cerebrohepatorenal syndrome, consists of three overlapping phenotypes. The most severe phenotype being Zellweger syndrome (ZS) followed by an intermediate form, neonatal adrenoleucodystrophy (NALD), which is not to be confused with X-linked ALD, and the mildest form infantile Refsum disease (IRD). The overall frequency of ZS is approximately 1:50,000. ZS classically presents with characteristic craniofacial features including large anterior fontanelle, full forehead, shallow orbital ridges, epicanthal folds, high arched palate, broad nasal bridge and small nose with anteverted nares. Ocular abnormalities such as cataracts, glaucoma and corneal clouding are common. In addition there is encephalopathy, seizures, severe hypotonia, hepatorenal abnormalities including renal cysts and skeletal abnormalities. Patients usually succumb to the disorder within the first few months of life and survival is extremely rare beyond a year. Patients with the milder forms of the Zellweger spectrum have similar but less severe symptoms to ZS and survival varies from four months to several decades. For example, virtually all IRD patients have moderate dysmorphic features and sensorineural hearing loss with pigmentary retinopathy. Early hypotonia and deranged liver function are common. However most IRD patients learn to walk, although their gait is frequently ataxic and their mental function is in the severely retarded range as compared to profound retardation in NALD and ZS.

RCDP is clinically distinct from the Zellweger spectrum and also has severe classical presentations and milder phenotypes. Clinically, RCDP symptoms include characteristic proximal shortening of the limbs (rhizomelia), cataracts, facial dysmorphism, microcephaly, small stature, and psychomotor retardation. For all of the peroxisomal biogenesis disorders treatment is largely symptomatic and supportive.

Single enzyme defects

The single enzyme defects result in the loss of a single protein and subsequently the loss of a single peroxisomal function. Although over 50 peroxisomal matrix and numerous membrane proteins have been identified only about 10 disorders associated with single enzyme defects have been described, indicating that there are many more unrecognised disorders. The known single peroxisomal enzyme/protein defects are summarised in table 1, the more common/frequently encountered defects are summarised below.

Defective peroxisomal function	Disorder
β -oxidation of very long chain fatty acids	X-linked adrenoleukodystrophy Acyl-CoA oxidase deficiency Bifunctional protein deficiency Sterol carrier protein deficiency α -methyl-acyl-CoA racemase deficiency
α -oxidation of phytanic acid	Refsum disease
Hydrogen peroxide metabolism	Catalase deficiency
Glyoxylate metabolism	Hyperoxaluria type I
Etherphospholipid biosynthesis	DHAP-AT deficiency Alkyl-DHAP synthase deficiency

Table 1. Summary of the single peroxisomal protein/enzyme defects.

The most common single enzyme defect is X-linked adrenoleucodystrophy. The inheritance is X-linked with approximately 50% of female carriers eventually presenting with clinical symptoms. The clinical phenotypes vary from the severe childhood cerebral presentation through to a mild adult form. There is also an Addison only presentation. Severe childhood disease takes the form of a progressive demyelination of the cerebral neurones and adrenal insufficiency. This early onset male disease usually starts between 3 to 10 years of age with behavioural abnormalities. Initial referral is often to a psychiatrist or psychologist. There is further progression to dementia, speech difficulty with loss of hearing and vision and finally relentless progression to decorticate spastic quadriplegia, with pigmentation of the skin secondary to adrenal insufficiency. The most effective treatment is hematopoietic stem cell transplantation which is only effective if carried out in pre-symptomatic or early symptomatic patients. There is also late onset adolescent and adult cerebral forms of X-ALD which follow a similar but delayed course. The milder adult onset X-ALD presents with peripheral neuropathy and Addison disease (adrenomyeloneuropathy), with or without cognitive decline, may affect both males and female carriers. A small cohort of X-ALD patients will present with isolated adrenal insufficiency (Addison only X-ALD).

Refsum disease, which should not be confused with Infantile Refsum disease, is also a single enzyme defect and is due to defective phytanoyl-CoA hydroxylase. The enzyme is required for the α -oxidation of phytanic acid to pristanic acid. Patients with Refsum disease accumulate large amounts of phytanic acid in plasma and tissues. The clinical features include; pigmentary degeneration, peripheral neuropathy and cerebellar ataxia usually presenting before the second decade of life. However, the age of onset and clinical severity varies according to the degree of residual enzyme activity. Effective treatment can be achieved by strict avoidance of dietary phytanic acid and plasmaphoresis.

Bi-functional enzyme deficiency is a single enzyme defect due to defective bi-functional enzyme which is required for peroxisomal β -oxidation. Bi-functional enzyme deficiency is rare and classically presents with neonatal hypotonia, dysmorphic features, seizures, hepatomegaly and developmental delay. The degree of severity is however highly variable.

Diagnostic approach

As described in the clinical section, peroxisomal disorders can be grouped into two broad subgroups; the single enzyme defects and the peroxisomal biogenesis disorders. The initial diagnostic approach is similar for both groups for most disorders. Along with strong clinical suspicion and a panel of metabolites in plasma and urine, a likely diagnosis can be reached within a couple of weeks. Most specialist metabolic laboratories investigate three or more pathways to reach a diagnosis. The most commonly investigated pathways include:

- (1) β -oxidation of the very long chain fatty acids (VLCFA's)
- (2) α -oxidation of phytanic acid.
- (3) biosynthesis of ether phospholipids (plasmalogens)
- (4) bile acid synthesis

More detailed studies involve measuring specific enzyme activities including dihydroxyacetonephosphate acyltransferase (DHAP-AT) in blood platelets or fibroblasts and very long chain fatty acid oxidation and phytanic acid oxidation in cultured fibroblasts. A suspected or likely diagnosis from clinical and biochemical abnormalities is usually confirmed by molecular studies wherever possible.

The most frequently investigated pathway is peroxisomal β -oxidation of the VLCFA's. In plasma abnormal C26:0/C22:0 ratios are seen in both the peroxisomal biogenesis disorders and in X-ALD. These ratios are significantly raised in the peroxisomal biogenesis disorders and are moderately raised in males with X-ALD. In female carriers for X-ALD the ratios are more subtly raised and it is important to be aware that

approximately 10% of female carriers will have normal plasma VLCFA's. In symptomatic females it may be necessary to measure the VLCFA's in cultured fibroblasts, although these will still be normal in approximately 5% of patients. In this cohort diagnosis can only be achieved by molecular studies of the ALD gene.

When a peroxisomal disorder is suspected, the second common pathway to be investigated is the α -oxidation of phytanic acid. The loss of α -oxidation results in increased phytanic acid and if this is taken in combination with increased VLCFA's this strongly supports a diagnosis of a peroxisomal biogenesis disorder. Phytanic acid is raised in isolation in the single enzyme defect Refsum disease and clinicians should go straight to measurements of phytanic acid when suspecting Refsum disease on clinical grounds.

Other metabolites including red blood cell plasmalogens and urine and plasma bile acids can also be measured to complete the investigations for a suspected peroxisomal biogenesis disorder. Table 2 summarises expected results and investigations in the single enzyme defects and in the spectrum of generalised peroxisomal disorders.

Table 2. Summary of biochemical investigations for Peroxisomal Disorders

	VLCFA's (C22:C26) (plasma)	Phytanic acid (plasma)	Pristanic acid (plasma)	Bile acids (urine & plasma)	Plasmalogens (red blood cell)	DHAP-AT activity (fibroblasts & platelets)	Catalase expression (fibroblasts)
Zellwegers Syndrome	+++	N/+	N/+	+++	Low	Low	Low
Neonatal Adrenoleukodystrophy	++	N/+	N/+	++	Low	Low	Low
Infantile Refsum Disease	++	N/+	N/+	++	Low	Low	Low
RCDP type 1	N	N/+	N/Low	N	Low	Low	N
X-Linked Adrenoleukodystrophy	++	N	N	N	N	N	N
Bifunctional protein deficiency	++	N/+	N/+	N/+	N	N	N
α -methyl-acyl-CoA-racemase deficiency	N	N/+	+	++	N	N	N
Refsum Disease	N	+++	Low	N	N	N	N
Hyperoxaluria Type 1	N	N	N	N	N	N	N
Acyl-CoA oxidase deficiency	++	N	N	N	N	N	N
Catalase deficiency	N	N	N	N	N	N	Low
DHAP-AT deficiency	N	N	N	N	Low	Low	N
Alkyl-DHAP synthase deficiency	N	N	N	N	Low	Low	N

Genetic diagnosis

Peroxisomal biogenesis disorders

Genetic diagnosis for the peroxisomal biogenesis disorders is particularly important if future pre-natal diagnosis is to be considered. However because of the number of genes involved, a clear strategy for investigation must be employed. Rather than systematically working through the genes, complementation studies in cultured fibroblasts can be carried out. Complementation involves fusing cultured fibroblasts from the patient under investigation with fibroblasts from a cell line in which the defect is known. The formation of peroxisomes in the fused cell lines can be assessed by immuno-staining for the peroxisomal enzyme catalase using fluorescent labelled antibodies. If the patient has a defect in the same gene as the known cell line, peroxisomes will not be formed, and this gene can then be sequenced. If the patient has a defect in a different gene then peroxisomes will be formed and further complementation studies would need to be undertaken. These elegant studies allow identification of the defective gene in a fast and cost effective manor. Figure 1 demonstrates the principles of complementation.

Over 100 mutations in *PEX* genes have been described in the literature and although many mutations are private a few common mutations have been identified. Despite many mutations, the majority of patients have mutations in one of only four of the *PEX* genes. *PEX1* mutations account for 70% of the peroxisomal biogenesis defects, followed by 10% in *PEX6* and 5% in *PEX12* and *PEX26*.

RCDP is genetically distinct from Zellweger syndrome spectrum. All mutations associated with RCDP are in the *PEX7* gene which encodes the cytosolic PTS2 receptor, Pex 7.

Single enzyme defects

Of the single enzyme defects only X-ALD will be discussed in this review. X-ALD is caused by mutations in the *ABCD1* gene. This gene encodes for the protein ALDP which is a member of the ATP-binding cassette (ABC) transporter protein superfamily. ALDP is located on the peroxisomal membrane and although its function is not fully characterised it is strongly suspected that ALDP is involved in the transport of the VLCFA's across the peroxisomal membrane.

The overall incidence of X-ALD is 1:17,000 including both hemizygotes and heterozygotes. As previously mentioned, because not all female carriers have abnormal VLCFA's in plasma or fibroblasts, it is recommended that women at risk of X-linked ALD should be screened by mutation analysis of the *ABCD1* gene.

Genetic counselling is also recommended for families when a patient is newly diagnosed with X-ALD. De-novo mutations in the *ABCD1* gene are rare and account for less than 8% of mutations described. Genetic investigations of the extended family have the potential not only to identify hemizygote females but also to identify neurologically asymptomatic males with the potential for pre-symptomatic allogeneic haematopoietic stem cell transplantation. Early diagnosis can also help to avoid Addisonian crises. It is however important to note that there is no genotype/phenotype correlation and siblings with the same mutation may present with very different phenotypes.

Prenatal diagnosis

Prenatal diagnosis for all of the peroxisomal disorders is carried out using chorionic villus CV samples or cultured amniotic fluid cells.

The poor outcome and often early death seen in the peroxisomal biogenesis disorders and in particular in Zellweger syndrome makes prenatal diagnosis a particularly important service for families with previously affected children. Historically prenatal diagnosis has been carried out using biochemical

techniques; in the case of Zellweger syndrome this has involved measuring the activity of DHAP-AT on either direct CV or cultured CV cells. False negatives and positives have been reported using this strategy and the biochemical basis for prenatal diagnosis currently involves measuring both DHAP-AT activity and VLCFA concentrations in cultured fibroblasts. Although this has improved the sensitivity, there remains the disadvantages of the length of time taken to grow the cells, the potential for failure of cell growth altogether and the additional risk of maternal cell overgrowth. Increasingly now the preferred option is to identify the mutation in the index case and carry out molecular analysis on direct CV with cultured CV as a back up.

Conclusion

Much has been learned about peroxisomal diseases since the first description of a patient with X-linked ALD in 1923 & ZS in 1964. However it took some time before a fuller understanding of peroxisomal function and biogenesis was achieved. In the last 25 years there has been considerable advancement in our understanding of the biochemistry and more recently the genetics of these disorders, but much has still to be learned. To date there is no effective treatment for many of these disorders and this great challenge lies ahead.

Metabolic Diagnosis List 2010

Disorder	NUMBER
Aromatic Amino Acid Decarboxylase Deficiency.	1
Carnitine Acylcarnitine Translocase deficiency	1
Cobalamin D deficiency	1
Chodrodysplasia Punctata Type 2 (Conradi Hunermann Happle Syndrome)	1
Homocystinuria	3
Carnitine Palmitoyl transferase type 2	5
Cystinuria	2
fructose-1,6 biphosphatase	1
Glutaryl-CoA dehydrogenase deficiency type 1	3
Multiple acyl-CoA dehydrogenase deficiency	2
Glycogen storage disease type IV	1
Glycogen storage disease type III	1
HMG-CoA Lyase deficiency	1
Infantile Refsum's Disease	2
Isovaleric Acidaemia	3
L-2-Hydroxygluatric Aciduria	1
Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency	1
Medium chain acyl-CoA dehydrogenase deficiency	8
Methylcrotonylglycinuria	1
Methymalonic aciduria (unclassified)	2
Mucopolysaccharidosis Type 1	1
Maple syrup urine disease	1
Neutral Lipid Storage Disease with Myopathy	1
Non-ketotic hyperglycinaemia	2
Niemann Pick type C	2

Disorder	NUMBER
Ornithine aminotransferase deficiency	1
Ornithine transcarbamylase deficiency	2
Propionic acidaemia	1
Pyruvate dehydrogenase deficiency	2
Phenylketonuria	3
Ehlers Danlos type VI	1
POLG1	1
Primary Carnitine Deficiency	4
Primary cobalamin synthesis disorder or deficiency in B12 uptake	1
Pyridoxine dependent epilepsy	1
pyruvate carboxylase deficiency	1
Respiratory chain deficiency	6
Riboflavin responsive multiple acyl-CoA dehydrogenase deficiency	1
Smith Lemli Opitz	1
Trichthiodystrophy	1
Very long chain acyl-CoA dehydrogenase deficiency VLCAD	7
X-Linked Chondrodysplasia Punctata [CDPX2]	1
X-linked Adrenoleukodystrophy	4
X-linked Adrenoleukodystrophy [carrier]	1
Zellweger spectrum disorder	2
Total	90

NB Trimethylaminuria diagnoses not included (>100)