Failing organelles and storage diseases: Update on diagnosis, treatment and pathogenesis.

Moderator: Guillermo A. Herrera MD, Saint Louis University, St. Louis, MO

8:30 am - Introduction- Guillermo A. Herrera

8:35-9:15 am – Animal models to study lysosomal storage disease: What we have learned from them. Nancy Galvin, Saint Louis University, School of Medicine, St. Louis, MO

9:20-9:55 am - Lysosomal storage disease: Making a diagnosis and finding a treatment. Carole Vogler, Cardinal Glennon’s Children Hospital and Saint Louis University, School of Medicine, St. Louis, MO

10:00 am- Break

10:30-11:05 am- Glycogen storage diseases: An update. John Hicks, Texas Children’s Hospital and Baylor School of Medicine, Houston, Texas

11:10-11:50 am- Mitochondriopathies: A current view of diagnosis. Kevin E. Bove, Cincinatti Childrens’s Hospital and University of Cincinnati College of Medicine, Cincinnati, Ohio
Murine Mucopolysaccharidosis (MPS) VII shares many clinical and pathologic features with human MPS VII (Sly Disease) and is a very attractive model for study of the pathophysiology and treatment of lysosomal storage disease (LSD) (Vogler et al 1990, Vogler et al 2001). Affected mice have beta-glucuronidase (GUS) deficiency and progressive visceral, skeletal and CNS lysosomal storage of glycosaminoglycans (GAG); dysmorphism with dysostosis; growth retardation; shortened life span; cardiac, visual, auditory and behavioral dysfunction (Birkenmeier et al 1989, Vogler et al 2001, Liu et al 2007).

CNS dysfunction can be significant in MPS patients; MPS VII mice have impaired spatial learning and memory. The mechanisms underlying CNS dysfunction in MPS are unclear (Liu et al 2007) but ultrastructural evaluation may provide clues to the etiology. MPS VII mice have small brains and lysosomal storage in neurons, glia, and meninges. In neurons, membranous, lamellar “zebra body”, and fibrillogranular material accumulate while glial, meningeal and perivascular cells have fibrillogranular storage. Purkinje cells have altered dendrite ultrastructure; their decreased number suggests cell loss or abnormal neuronal development. (Levy et al 1996). The “zebra body” storage is due to secondary accumulation of gangliosides GM2 and GM3; gangliosides don’t require GUS for degradation, and their storage may be due to inhibition of other lysosomal enzymes by GAGs. Alternatively, or in addition, altered ganglioside synthesis or trafficking may underlie their storage and neuron ganglioside accumulation may be linked to reinitiation of dendrite growth and altered synaptic connectivity, characteristic of many storage diseases (McGlynn R et al 2004). Gangliosides are constituents of specialized membrane microdomains important for cell signaling called “rafts”. Defects in composition, trafficking, and/or recycling of raft components -- a “raft log jam”, may lead to neuronal dysfunction in MPS disorders and altered signal transduction may be secondary to raft accumulation in endosomes/lysosomes (McGlynn et al 2004, Walkley 2007). Deciphering relations between CNS morphology and behavioral defects in MPS VII mice is an important challenge.

MPS VII mice have a constellation of skeletal abnormalities similar to dysostosis multiplex seen in children with MPS. Bone disease may reflect altered GAG-collagen interactions as GAGs influence collagen fibrillogenesis and excess GAG may alter collagen formation or turnover. Long bones are shortened with increased density and thickness. GAG distends osteoblasts and chondrocytes, and a wide, hypercellular growth plate with distorted chondrocyte columns has decreased primary spongiosa trabecular bone (Vogler 1990). MPS mice have a slower rate of epiphyseal bone matrix deposition and ultrastructural analysis shows that the osteoclasts fail to form ruffled border membranes and appear detached from the bone surface. In vitro, osteoclasts form smaller and fewer pits than those formed by normal osteoclasts (Monroy et al 2002).

MPS VII mice and many MPS patients have reduced hearing. External auditory canal occlusion by cerumen, thick tympanic membrane and middle ear mucosa, otitis media, ossicle deformation, sclerosis of the tympanic bulla, loss of hair cell
stereocilia and storage throughout the middle and inner ear are seen (Berry et al 1994, Ohlemiller et al 2001, Ohlemiller et al 2002).

**Vision is also impaired** in the MPS VII mice; retinal function decreases with age. Corneal stromal fibroblasts and retinal pigment epithelium are distended with GAG storage; photoreceptor outer segments shorten with progressive photoreceptor loss, likely related to altered interphotoreceptor matrix proteoglycans. The matrix alterations likely alter its cell support function leading to degeneration (Lazarus et al 1993).

**Cardiac failure** is a major cause of death in patients with MPS. Hearts are enlarged in MPS VII mice and ECG abnormalities include bradycardia. The aortic root is distended and aortic elastic laminae are irregular with zones of thinning and abundant storage in intimal, medial, and adventitial cells. The aortic valves are thickened with stromal cell storage. Conduction tissue myocytes are vacuolated and interstitial cells have abundant small vacuoles (Schuldt et al 2004).

**Therapy for Murine MPS VII**

Measurement of changes in the phenotype and clearance of lysosomal GAG in MPS VII mice allows evaluation of potential therapies for LSD. Arresting progressive damage and recovery of function in brain and skeleton are key challenges for potential therapy.

**Transplantation** of hematopoietic stem cells and neural progenitor cells has been tested. Early therapeutic intervention without a toxic preparative regimen offers the most successful outcome. Although fixed tissue macrophage storage improves in adult mice treated with BMT, neither bone nor brain responds. However, neonates with minimal clinical and pathologic disease at time of BMT have improved survival, hearing, and growth but no improvement in neuronal storage or behavioral abnormalities, possibly reflecting delay between BMT and engraftment. Neural progenitor cells transplanted into CNS integrate in a cytoarchitecturally appropriate manner and differentiate *in vivo* into neurons or glia. When implanted into lateral ventricles of newborn MPS VII mice, they engraft throughout the neuroaxis and decrease neuronal and glial storage (Snyder et al 1995).

**Viral-mediated gene therapy** holds promise for long-term therapy if safety issues can be resolved (Ponder and Haskins 2007). A virus carrying the cDNA for GUS can stably integrate into the host genome in a broad range of cell types, providing a permanent source of deficient GUS. In experiments using this model, i.v. injection of many vectors (e.g., adeno-associated virus (AAV), adenovirus, and retrovirus) carrying GUS cDNA corrects visceral storage, but CNS correction requires high circulating GUS activity. Alternative approaches are to transduce hematopoietic or other stem cells or to inject vector directly into brain or CSF, an invasive strategy that circumvents the blood-brain barrier (BBB). The results of these experiments provide hope that if sufficient enzyme crosses the BBB in the adult, it can reduce established storage and reverse established CNS functional deficits (Taylor and Wolfe 1997, Cearley and Wolfe 2007, Brooks et al 2002, Liu et al 2007).

**Enzyme replacement therapy (ERT)** is used to treat several LSD. Delivery of corrective enzyme to lysosomes depends on cell surface receptors that recognize phosphate-containing sugar moieties [mannose 6-phosphate (M6PR), and mannose receptor (MR)]. ERT reduces visceral storage; established CNS storage has been more refractory because of the blood-brain barrier (BBB). However, ERT in newborns prevents CNS and bone storage, normalizes the phenotype, prolongs life, and improves hearing and behavior (Sands et al 1997, Vogler et al 1996, O’Connor et al 1998). The BBB is not fully developed at birth and when phosphorylated enzyme
crosses it, it is taken up by the M6PR and reduces neuronal storage until age 2 wks in the mouse. Access to M6PR-mediated route of enzyme delivery is limited after age 2 wks in MPS VII mice because this receptor-mediated delivery is down-regulated by age 2 wks (Vogler et al 1999, Urayama et al 2004). However, recently we found that multiple infusions of high doses of GUS can reduce CNS storage in adult MPS VII mice (Vogler et al 2005). Perhaps saturation of relatively sparse M6PR in adult mouse brain over a long time drives enzyme into CNS. Alternatively, GUS may gain access to brain storage by another route such as phagocytic cell uptake or extracellular pathways (Banks 2004).

We recently tested GUS with carbohydrate-dependent receptor-mediated uptake inactivated by chemical modification. This treatment eliminated M6PR and MR-mediated uptake and greatly slowed plasma clearance. This long-circulating GUS, which targets no known receptor, was remarkably effective in clearing storage from neurons. This suggests a novel delivery system across the BBB that might be exploited therapeutically. Identifying the mechanism of delivery of GUS to brain may suggest strategies to make ERT for CNS storage a more realistic goal. The impact of high dose ERT and modified GUS on behavioral abnormalities in adult MPS VII mice requires investigation.

**In summary**, Murine MPS VII has allowed much progress in understanding potentials therapies for LSD. Results of therapeutic trials in animal models may be generalizable to treatment of human LSD. Definition of the ultrastructural morphology in this model has provided insight into the pathophysiology of LSD and has provided a benchmark for assessing therapies.
References


Lysosomal Storage Disease: Diagnosis and Role of Animal Models in Evaluation of Treatment
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The lysosomal storage diseases (LSDs) are a heterogeneous group of inherited disorders that affect 1 in 7,000 children. Around 50 different disorders have been identified. Most are the result of a change in an allele coding a specific lysosomal acid hydrolase, but defects in an enzyme coactivator, a membrane transporter, the targeting mechanism for protein localization to the lysosome, or intracellular vesicular trafficking can also cause LSD (Scriver et al, 2001). The defect in LSD results in failure to completely degrade one or more macromolecules and an accumulation of the undegraded substrate within lysosomes. Eventually the build up of material in lysosomes leads to cell and organ dysfunction. The particular tissue involved and the age of onset depends on which degradation pathway has been interrupted and what its importance is to the tissue.

Patients with LSD present with a broad spectrum of phenotypes characterized by progressive morbidity and mortality. Most have progressive neurological degeneration, mental retardation and deafness. A variety of musculoskeletal abnormalities includes dysostosis multiplex with short stature, coarse facial features and stiff joints. Visceral abnormalities with storage in fixed tissue macrophages lead to hepatosplenomegaly and corneal clouding.

In diagnosis, clinical findings which warrant investigation for LSD include nonimmune hydrops, progressive organomegaly, skeletal abnormalities and joint stiffness, coarse facial features, progressive dementia or loss of developmental milestones, and unexplained neuropathic extremity or bone pain. As inherited diseases, LSD are present from conception in affected patients and early diagnosis is becoming more important as effective disease specific therapy becomes available. Ideally, patients should be identified before serious mental or physical impairments have developed so newborn screening programs based on lysosomal enlargement and increased lysosomal proteins are useful. Biochemical analysis of cultured fibroblasts, leukocytes or plasma for the deficient enzyme provides a definitive diagnosis, but the clinical presentation may not provide sufficiently specific information for the clinician to identify which enzyme or gene to test for abnormality. Tissue biopsy with ultrastructural evaluation is a sensitive and relatively inexpensive tool to screen for LSD (Alroy and Ucci, 2006; Prasad et al, 1996). Many LSD have characteristic ultrastructural alterations in rectal mucosa, skin, conjunctiva and peripheral blood leukocytes that provide criteria for diagnosis. Chorionic villous samples have been used for prenatal diagnosis of LSD and bone marrow, liver, muscle and peripheral nerve biopsies may also yield diagnostic material. Electron microscopy can exclude more than 90% of suspected cases of LSD. It is particularly useful for those disorders with incompletely characterized biochemical defects and when DNA studies fail to be informative (Ceuterick-de Groote and Martin, 1998).

Ultrastructural morphology of distended lysosomes and their tissue distribution are useful characteristics for diagnosis. In some cases morphology of stored lysosomal material is characteristic for a single disorder. For example, neuronal ceroid lipofuscinosis has curvilinear and granular storage material; acid lipase deficiency/Wolman disease has cholesterol clefts and neutral lipids; Farber disease has
banana-shaped lysosomes containing small vesicles. A finding of fine fibrillogranular storage material and/or zebra bodies suggests a group of disorders which includes MPS, mucolipidoses, mannosidosis and sialadosis. In ganglioside storage diseases, glycolipids appear as lamellated membrane bound finger prints or zebra bodies.

Treatment of LSD demands arrest or reversal of established bone, visceral and CNS disease. Advances in treatment over the last 10 years has included bone marrow or hematopoietic stem cell transplantation, gene therapy with gene transduction in CNS by adenovirus (Shen et al, 2004), enzyme replacement therapy, substrate reduction therapy, and chemical chaperone therapy. Enzyme replacement is an established therapy for a number of LSDs, including Gaucher, Fabry, Pompe, MPS I, II and VI.

Animal models of LSD are useful in evaluating treatment strategies because they allow controlled therapeutic trials without the clinical and genetic heterogeneity inherent in human studies. Biochemical and morphologic correction of the phenotype forms the basis of evaluation of therapy. A number of animal models have been developed for investigation of the pathophysiology of LSDs and efficacy of treatment. Two of these will be discussed in detail, a murine model of infantile neuronal ceroid lipofuscinosis (INCL) and a murine model of mucopolysaccharidosis Type VII.

INCL, also called Santavuori-Haltia disease, is caused by a mutation in the gene encoding the lysosomal enzyme palmityl-protein-thioesterase (PPT1). In this disorder fatty acid modified proteins are not degraded and accumulate as granular osmiophilic deposits (GRODS) identified by electron microscopy. Incidence of GRODS is most pronounced in the CNS. Patients present with rapidly progressing disease at around 1.5 years of age resulting in blindness, motor and mental decline, and seizures. There is brain atrophy with cortical and hippocampal neuronal loss associated with astrogliosis. Death in childhood is due to neurodegenerative disease.

A murine model of INCL with a knockout mutation in the gene that encodes PPT1 shares many clinical features with the disease seen in humans. Affected mice have neurological abnormalities evident by 8 months of age and usually die by 10 months of age. The clinical features include myoclonic seizures, progressive spastic motor abnormalities (Gupta et al, 2001), and retinal degeneration (Griffey et al, 2004). Ultrastructural analysis of lysosomal storage in these mice indicates that GROD accumulates progressively in CNS neurons with widespread astrocytosis and cortical atrophy. Lysosomal storage in the form of GRODS accumulates to a lesser extent in other organs, including kidney, spleen, bone, liver, eye and heart. Storage within smooth muscle cells from the media of the aortic arch correlates with structural alterations in luminal diameter of the aorta. Gene therapy experiments with this murine model of INCL have used a rAAV vector delivered directly to the brain parenchyma by injection. The therapy resulted in reduction of storage material in CNS, increased cortical thickness relative to control, reduced seizure activity, and improved motor function (Griffey et al, 2006).

References:


Glycogen Storage Diseases: A Brief Review and Update on Clinical Features, Genetic Abnormalities, Pathologic Features and Treatment

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Key Words: Glycogen storage disease, pathology, ultrastructure, genetics, molecular
Introduction

Glycogen storage diseases (GSD) affect primarily the liver, skeletal muscle, heart and sometimes the central nervous system and the kidneys. These unique diseases are quite varied in age of onset of symptoms, morbidity and mortality. Glycogen storage diseases are classified according to their individual enzyme deficiency. Each of these enzymes regulates synthesis or degradation of glycogen. Interestingly, there is great phenotypic variation and variable clinical courses even when a specific enzyme is altered by mutation. Depending upon the specific mutation in an enzyme, a GSD patient may have a favorable or unfavorable prognosis. With neonatal or infantile forms, some GSDs lead to death within the first year of life; whereas, other glycogen storage diseases are relatively asymptomatic or may cause only exercise intolerance.

The principle storage depots for glycogen are the liver and skeletal muscle. With GSDs, hypoglycemia is the primary indicator of liver involvement. Muscle cramps, exercise intolerance, muscle weakness (hypotonia) and fatigue are typical of glycogen accumulation in skeletal muscle. In addition, the peripheral and central nervous systems, myocardium and renal tubules may also suffer from aberrant glycogen accumulation.

Early diagnosis and treatment are important for improving quality of life, reducing the damaging effects on organs that become engorged with glycogen, and extending the patient’s lifespan. More recently, enzyme replacement and gene therapy have been explored using animal models and skeletal muscle cells obtained from affected patients. Clinical trials have led to licensing of recombinant enzyme replacement therapy for GSD Type II (Pompe disease). In addition, adenovirus vectors for recombinant and transgenic enzymes to replace defective or missing enzymes have shown great promise in well controlled laboratory studies. The following is a brief review and update of glycogen storage diseases, with respect to clinical features, genetic abnormalities, pathologic features and treatment.

Tissue Triaging

Perhaps, the most important aspect of providing an accurate diagnosis is appropriate triaging of tissue to allow for optimal evaluation. With a suspected metabolic disease, it is important that adequate tissue is obtained to perform all necessary tests for an appropriate diagnosis to guide future therapy and to avoid repeat biopsy. For glycogen storage diseases, there are many different studies that need to be completed.
Tissue should be obtained for routine histology (formalin fixation), histochemical stains (frozen in optimal cryomatrix material [OCT] at -20C and/or alcohol fixation), electron microscopy (glutaraldehyde), and genetic/molecular evaluation (frozen at -70C). It is especially important with glycogen storage diseases to maintain optimal preservation of glycogen. With formalin fixation, up to 70% of glycogen is lost due to the soluble nature of the predominant form of glycogen in the cytoplasm. Glycogen can be preserved with freezing and/or alcohol fixation, allowing for quantitative evaluation by analytical techniques (frozen tissue) and qualitative assessment by histochemical staining (PAS, PAS-diastase). Quantitative analysis of the suspected enzyme responsible for a specific glycogen storage disease must be done on frozen tissue. Assessment of gene mutation and sequencing of the gene responsible for the enzyme defect associated with a specific glycogen storage disease requires frozen tissue. The preservation of the enzyme, enzyme activity, DNA and RNA requires cryopreservation at -70C, and maintaining this temperature until the tissue reaches the appropriate reference laboratory. Depending upon the testing that is required for a definitive diagnosis, the tissue requirements may dictate an open biopsy of the liver or skeletal muscle. The current trend in surgical and interventional radiology practice has been toward needle core biopsies for diagnosis. The pathologist should be aware of what tissue requirements (grams of tissue) are necessary for appropriate testing to be completed. A single tissue core of 20 mm in length from a 16-gauge needle with a 1.5 mm diameter yields about 45 mg of tissue. With some tests, 100 mg or more of tissue will be needed. This may necessitate numerous tissue cores, or an open biopsy to obtain adequate tissue for all tests. This emphasizes the importance of communication of the healthcare team with the pathologist. Because tissue will be preserved in a steady state with cryopreservation (-70C), comprehensive workup (histopathology, histochemistry, electron microscopy) by the pathologist to determine which additional testing is most appropriate can be completed prior to performing specialized testing on the frozen tissue.

**Glycogen Storage Disease Types**

**Glycogen Storage Disease Type 0 (Aglycogenosis)**

GSD Type 0 is an autosomal recessive disease that is due to a deficiency in glycogen synthase (chromosome 12p12.2). Deficiency in glycogen synthase leads to a marked reduction in liver glycogen stores. This results in dietary carbohydrate being
converted to lactate rather than being stored as glycogen in the liver. During the postprandial period, there is hyperglycemia, glycosuria and hyperlactic acidemia, which alternate with hypoglycemia and hyperketonemia during fasting. Due to the hyperglycemia and hyperuricemia, the child may be thought to have an early stage of diabetes, especially considering that the liver is not enlarged. The symptoms of GSD type 0 are those associated with hypoglycemia and include lethargy, pallor, nausea, vomiting and rarely seizures in the early morning before breakfast. Developmental delay may be seen in some children. Short stature and osteopenia are also features noted.

Definitive diagnosis can be provided by performing a liver biopsy. The liver biopsy will demonstrate small amounts of glycogen and moderate steatosis. This biopsy allows quantitative analysis of glycogen, which will be quite low (about 0.5% vs. 1.6% for normal wet liver weight). Enzymatic evaluation of the liver tissue will reveal low to absent glycogen synthase activity. Genetic/molecular testing can be performed to further confirm that there is mutation of the glycogen synthase gene (chromosome 12p12.2). Currently, gene mutational analysis is performed to provide a definitive diagnosis.

Symptoms are rapidly ameliorated with protein-rich meals every 4 hours and bedtime feeding of uncooked cornstarch in low fat or skim milk. Increased protein during meals provides the substrate for gluconeogenesis, and a lower carbohydrate diet reduces postprandial hyperglycemia, glycosuria and hyperlactic academia.

Glycogen Storage Disease Type I (von Gierke’s Disease, Hepatorenal Glycogenosis)

GSD Type I occurs in an autosomal recessive pattern and is composed of 3 subtypes depending upon the deficient enzyme involved – type Ia (glucose-6-phosphatase, chromosome 17q21), type Ib (glucose-6-phosphatase transporter, chromosome 11q23), and type Ic (phosphatase transporter, chromosome 11q23-24.2). The incidence of GSD type Ia is 1 per 100,000 to 400,000 births per year in Caucasians. GSD types Ib and Ic are even less frequent. Ashkenazi Jews have an incidence of 1 in 20,000. The presenting symptoms are due to impaired glycogenolysis and gluconeogenesis, resulting in severe hypoglycemia and increased lactic acid, triglycerides and uric acid shortly after birth. The hypoglycemia does not respond to glucagon or epinephrine treatment. Typically symptoms, such as tremors, irritability, hyperventilation, cyanosis, apnea, convulsions, sweating and pallor begin to appear when the infant begins to sleep through the night without nocturnal feeds or when illness
interrupts normal feeding. The children also have a tendency for epistaxis due to impaired platelet function. The liver may be enlarged at birth or become enlarged later on in life. Older children may develop eruptive xanthomas, rickets, anemia, chronic renal disease due to hyperuricemia, and renal stones. Short stature is common and gout may be present in affected children and adults.

GSD type Ib affected children have similar symptoms, with the addition of persistent or cyclic neutropenia. This results in recurrent bacterial infections, usually before 1 year of age. These children are susceptible to recurrent oral mucosal ulceration, gingivitis, rapidly progressive periodontal disease and otitis. Recombinant human granulocyte colony stimulating factor improves neutrophil function and increases their numbers. A unique finding is the occurrence of inflammatory bowel disease (Crohn’s-like) with fever, diarrhea, and oral and perioral ulcers.

Liver biopsy demonstrates distension of liver cells with a uniform distribution of glycogen. The glycogen stains with PAS and digests with diastase. The liver cells are arranged in a mosaic pattern. There are glycogenated nuclei and steatosis with both small and large lipid droplets. Fibrosis may also be present. In glycogenoses in general, hepatocytes tend to have a plant cell-like appearance, with thickened cell membranes, peripheral displacement of organelles and a mosaic tile pattern. Electron microscopy shows abundant and uniform distribution of glycogen within enlarged hepatocytes. The organelles tend to displaced by the abundant glycogen. The glycogen particles are mainly of the monoparticulate variety, and can be found associated with smooth endoplasmic reticulum membranes. Glycogen particles may be seen within large lipid droplets. Mitochondrial tend to be enlarged, but overall reduced in numbers. Skeletal muscle biopsies show no increase in glycogen. The tissue type affected is different with the GSD Type I subtypes: type Ia – liver, kidney, intestine; type Ib – liver; and type Ic – liver.

Especially troubling is the development of hepatic adenomas in the majority of patients by the second and third decades of life. Hepatocellular carcinoma arising from hepatic adenoma is known to occur. Serum alpha-fetoprotein may not be helpful in following these patients, because it tends not to be elevated in hepatic adenomas, and is elevated in only some hepatocellular carcinomas.

Treatment consists of continuous dietary nasogastric infusion of glucose or frequent oral uncooked cornstarch at 3-6 hour intervals during the day and at night. If hypoglycemia and hyperlactic academia are prevented, the liver tends to decrease in
size, growth improves and serum levels of uric acid, triglycerides and cholesterol decrease to near normal. In those not compliant with dietary restrictions, with refractory hyperglycemia or with hepatocellular carcinoma, liver transplantation may be necessary. Renal transplantation may be necessary in those with end-stage kidney disease.

Gene therapy using adenovirus vectors has been evaluated in murine models of GSD type I. Enzyme levels have been restored in affected organs with reversal of symptoms. These animal model findings hold promise for similar gene therapy for those affected in the future.

Glycogen Storage Disease Type II (Pompe Disease)

GSD Type II is caused by a deficiency in alpha-1-4-glucosidase (acid maltase) mapped to chromosome 17q25.2-q25.3, which is transmitted in an autosomal recessive manner. In contrast to the other GSDs, this type is a lysosomal storage disease. Pompe disease has also been classified as a neuromuscular disease, a metabolic myopathy and a cardiac disorder. This is due to the fact that it affects all tissue types, with the liver, skeletal muscle, leukocytes, fibroblasts and amniocytes being target tissues for diagnosis. The incidence ranges from 1 in 14,000 to 300,000 births, depending upon geographic region and ethnicity. Overall, the incidence is considered to be 1 in 40,000 births. The incidence tends to be higher in African-Americans, Southern Chinese and Taiwanese. There are several GSD Type II subtypes. The classic (infantile-onset) subtype has enzyme deficiency in all organ systems with the liver rarely enlarged, except secondary to cardiac failure. Hypoglycemia and acidosis does not occur. Cardiomyopathy and muscular hypotonia are the hallmarks with death during the first year of life. The infantile subtype involves primarily skeletal muscle with less severe cardiomyopathy and lack of left ventricular outflow obstruction. There is some residual enzyme activity present. The juvenile and adult (late onset) subtypes show progressive muscular disease overtime. Those with late onset disease may be misdiagnosed with muscular dystrophy, polymyositis, spinal muscular atrophy, or scapuloperoneal syndrome.

The classic (infantile-onset) subtype presents with feeding difficulties, poor weight gain, respiratory difficulties with superimposed infections, and delayed milestones. There is profound generalized muscular weakness with floppiness (hypotonia) and head lag. Macroglossia and moderate hepatomegaly may be present.
Cardiomegaly is present with elevated creatine kinase, aldolase, ALT (muscle fraction), AST (muscle fraction) and LDH.

Glycogen accumulation is noted within the heart, skeletal and smooth muscle, liver, kidney tubules, endothelial cells, anterior horn cells, and motor neurons in the brainstem. Biopsy of the liver shows marked distension of the liver cells with delicate microvacuolization due to glycogen accumulation. Lipid is usually not present. The liver cells lack a mosaic pattern. Electron microscopy demonstrates membrane bound vesicles variably filled with monoparticulate glycogen (lysosomes) and glycogen rosettes in the cytoplasm. The glycogen-containing vesicles may vary in size from <1 to 8um. Skeletal muscle shows glycogen free in the cytoplasm, as well as within vesicles. There is loss of myofibrils. Biopsy of the heart reveals central cytoplasmic clearing of myocardial cells. The loops of Henle and collecting ducts demonstrate glycogen accumulation with kidney biopsies. One should be alert to the possibility that many of the membrane-bound glycogen inclusions may be ruptured. Careful ultrastructural study needs to be performed to search for glycogen-filled membrane bound vesicles. Similarly with skeletal muscle in other GSDs, a frequently observed phenomenon is glycogen that “leaks into” non-lysosomal cytoplasmic vesicles – usually dilated sarcoplasmic reticulum. This closely mimics the pathognomonic feature (glycogen-filled lysosomes) of Pompe disease. This potential pitfall can be avoided by bearing in mind that Pompe disease is a systemic disease. Examination of other nearby cell types, such as endothelial cells, may provide the confirmatory evidence necessary for diagnosis.

Diagnosis can be made by measuring enzyme activity in affected tissues. The most common tissues used for this assay are skeletal muscle and skin fibroblasts. It is also possible to provide prenatal diagnosis with chorionic villus sampling. Enzyme activity is usually <1% for infantile-onset, 1-10% for childhood/juvenile-onset, and 2-40% for adult-onset disease. It is possible to measure glucose oligosaccharides or acid maltase enzyme concentrations in urine, plasma or blood spots as a diagnostic tool. Additionally, gene mutation studies can also be done to confirm the clinical suspicion.

Treatment is directed toward symptomatic relief in those affected. Dietary therapy includes high protein, low carbohydrate diets or a diet high in L-alanine. More recently, intravenous enzyme replacement therapy with human recombinant and transgenic acid-alpha-glucosidase (acid maltase) has become available and approved by the Food and Drug Administration. This replacement enzyme therapy has proven particularly beneficial for those with some residual enzyme levels and late onset disease. There
have been marked improvement in outcome with reduce cardiac disease and increased lifespan compared with infants who lack enzyme activity. Some patients have shown declining glycogen stores within affected organs in clinic trials of 52 to 72 weeks in length. Those with less severe disease tend to fair better with enzyme replacement therapy.

There are certain limitations to enzyme replacement therapy. Variability of response with different tissues is known to occur. Heart muscle responds better than skeletal muscle, due to the presence of higher numbers of mannose-6-phosphate receptors which are needed for enzyme internalization. Type I skeletal muscle fibers (slow twitch) are more responsive than Type II skeletal fibers (fast twitch). The formation of neutralizing antibodies is of concern, especially in those children with minimal to no endogenous enzyme. The enzyme dose for GSD Type II is quite high due to the large muscle mass that requires enzyme replacement. The high dose increases the likelihood of a humoral immune response to the recombinant enzyme.

Adenovirus-mediated transfer of the acid-alpha-glucosidase gene into fibroblasts, myoblasts and myotubes from patients with early onset (infantile) GSD type II has demonstrated promise within the laboratory setting. Markedly elevated enzyme levels (10 to 100-fold) are produced and clearance of glycogen from the affected cells has been shown by quantitative analysis and by electron microscopy.

It would appear that gene therapy may be of benefit in the future. It is also encouraging to learn that there are several spontaneous animal models and gene knockout murine models that are available to allow for testing of novel current and future therapies.

**Glycogen Storage Disease Type III (Cori Disease, Forbes Disease, Limit Dextrinosis, Debranching Enzyme Disease)**

GSD Type III occurs when there is a deficiency in glycogen debranching enzyme (amylo-1-6-glucosidase, chromosome 1p21) and account for about 25% of all GSD cases. The incidence of GSD III in the United States is 1 in 100,000 births. Release of glucose from glycogen requires the action of both glycogen phosphorylase and glycogen debranching enzyme. When there is absence or a deficiency in the debrancher enzyme, glycogenolysis is halted at the outermost branch points. Accumulation of abnormal glycogen (phosphorylase limit dextrin) occurs in affected organs (liver, heart, skeletal muscle, leukocytes in GSD Type IIIa; liver in GSD Type IIIb). There are 4 major
isoforms of the debranching enzyme: isoform 1 predominantly in liver, and isoforms 2, 3 and 4 exclusively in skeletal muscle and heart. There are extremely rare cases of selective loss of glucosidase activity (GSD IIIc) and transferase activity (GSD IIId). In the United States, 85% of affected patients have GSD Type IIIa.

The symptoms include hepatomegaly, hypoglycemia, short stature and dyslipidemia. Muscle weakness tends to be minimal during childhood, but may become the predominant feature during adulthood with slowly progressive weakness and distal muscle wasting. Interesting, liver symptoms tend to improve with age and may resolve after puberty. Elevated liver function profiles (AST, ALT, LDH) noted during infancy also improve during puberty. Osteoporosis is noted, but is secondary to poor nutrition, lactic acidosis and hypogonadism. Of note is the fact that hepatic adenomas occur in 25% of patients with malignant transformation to hepatocellular carcinoma considered to be rare.

Liver biopsies show uniform distension of hepatocytes secondary to glycogen accumulation. The glycogen stains with PAS and can be digested with diastase. There are glycogenated nuclei, and the hepatocytes are arranged in a mosaic pattern. There is often septal formation, periportal fibrosis, reticular fibrosis, fine microsteatosis, and less frequently micronodular cirrhosis without inflammation or interface hepatitis. Electron microscopy show dispersed glycogen and small lipid droplets. There is extensive multiparticulate glycogen accumulation with markedly distended hepatocytes with peripheral placement of organelles. The glycogen may be associated with the vesicles of the smooth endoplasmic reticulum. A starry-sky pattern of glycogen distribution may be seen with some hepatocytes. Reduction in size and number of peroxisomes can be noted. Collagen fibers (fibrosis) within the intercellular spaces and space of Disse tend to be present. Skeletal muscle shows subsarcolemmal glycogen accumulation.

Diagnosis may be made with finding abnormal glycogen (limit dextrin with short outer branches) on quantitative analysis or a deficiency of the branching enzyme in liver and/or skeletal muscle. Gene mutation analysis may also be performed with liver, skeletal muscle, heart and cultured fibroblasts.

Treatment is dietary with frequent meals high in carbohydrates and uncooked cornstarch supplements. In those patients with myopathy, a high protein/amino diet is necessary. Liver transplantation may be performed with GSD IIIb patients (hepatic form), in those with cirrhosis or in those with hepatocellular carcinoma. The prognosis of GSD
IIIb (pure hepatic form) is quite favorable. GSD IIIa with hepatic disease, as well as cardiomyopathy and myopathy is less favorable, especially with progressive myopathy.

**Glycogen Storage Disease Type IV (Glycogen phosphorylase deficiency, Andersen Disease, Brancher Deficiency; Amylopectinosis, Glycogen Branching Enzyme Deficiency)**

GSD IV is caused by a deficiency in amylo-1,4 to 1,6-transglucosidase (branching enzyme) located on chromosome 3p12. This is a rare autosomal recessive disease and accounts for less than 1% of GSDs. With branching enzyme loss, glycogen can not undergo branching and resembles an amylopectin-like structure – polyglucosan. Such polyglucosan bodies (PAS positive amylopectin-like material) accumulate in many tissue types, including liver, skeletal muscle, amniocytes, fibroblasts and leukocytes.

Clinical manifestations are quite varied. In the classic form, affected children appear to be normal at birth and present by 18 months of age with failure to thrive, portal hypertension, hepatosplenomegaly and cirrhosis. Typically, the disease progresses rapidly, leading to death by 3 to 5 years of age. Central nervous system involvement may also occur. A perinatal form may present with hydrops fetalis, polyhydramnios and arthrogryposis. There have been adult females that are heterozygotes with cardiomyopathy. There are also adult forms that present with progressive myopathy that resemble muscular dystrophy with difficulty walking and proximal limb weakness.

Liver biopsy shows amphophilic to slightly eosinophilic, ground-glass hyaline bodies that are deeply PAS positive and resistant to diastase digestion, but digest with pectinase and amylase (alpha and beta types). These inclusions stain with colloidal iron (green), Best’s carmine (red) and Lugol’s iodine (mahogany brown). These round to oval to bean-shaped bodies are more prominent in periportal hepatocytes. Globular PAS-positive inclusions with Maltese-cross birefringence have been noted with congenital GSD Type IV cases. Of note is that the cytoplasm of the hepatocytes stain deeply with PAS. The hepatocytes may also be vacuolated. There is prominent periportal fibrosis with progression to cirrhosis. Electron microscopy shows fibrillar material that resembles amylopectin. This fibrillar material is composed of undulating, randomly oriented, delicate fibers of about 5nm in diameter. Tubular structures (10nm in diameter) with ill-defined glycogen rosettes may also be seen. These structures are similar to those found in Lafora’s disease. The similarity of Lafora bodies (intraneuronal polyglucosan inclusions) to the amylopectin-like structures in GSD IV is of interest. It
has been found that glycogen branching enzyme and glycogen synthase imbalance may be responsible for the production of Lafora bodies. Laforin protein appears to interact with and inhibit glycogen synthase. In Lafora’s disease, this protein is mutated and nonfunctional, and may allow glycogen synthase to remain in an active state and continue to produce glycogen unabated. This results in amylopectin-like bodies (polyglucosan). Glycogen branching enzyme had been shown to have “milder” mutations in adult polyglucosan body disease, which has identical inclusions as those seen in Lafora’s disease.

Diagnosis can be made with histologic and ultrastructural examination. Enzyme deficiency or gene mutation analysis for the branching enzyme can be performed on muscle, liver, fibroblasts, amniocytes (chorionic villus sampling) or leukocytes.

Treatment for GSD IV is liver transplantation for those with progressive liver disease. Transplantation may improve muscular disease due to systemic microchimerism. It has been shown by HLA typing and PCR analysis that donor cells are present at distant sites from the liver transplant. Organ donor lymphocyte-macrophages appear to serve as migrating enzyme carriers. It has been suggested that the donor cells can transfer enzyme to the native enzyme-deficient cells. This may be responsible for the resultant decrease in amylopectin in other organ systems in patients who have undergone liver transplantation.

Glycogen Storage Disease V (McArdle Disease)

GSD Type V is caused by a deficiency in muscle glycogen phosphorylase (myophosphorylase) mapped to chromosome 11q13, and is transmitted in an autosomal recessive pattern. Myophosphorylase initiates glycogenolysis in skeletal muscle. The clinical symptoms begin in early adulthood and consist of muscle pain, muscle cramps, tenderness in masticatory muscles and weakness after exercise (exercise intolerance). These patients also experience myoglobinuric which can lead to acute renal failure. Creatine kinase is elevated. There is a certain degree of heterogeneity with GSD Type V. In fact, GSD Type V is comprised of 3 distinct forms: rapidly fatal neonatal form, a mild form with congenital myopathy, and a benign classic form with myalgia and dark colored urine. No correlation has been found among the phenotypes and the various genetic mutations.

Muscle biopsy shows atrophy of type I fibers, and subsarcolemmal glycogen and less frequently intermyofibrillar accumulation on PAS staining and electron microscopy.
The liver has no pathologic abnormalities. The muscle biopsy material can also be analyzed for the enzyme levels and gene mutations.

Treatment is directed toward avoiding strenuous exercise. High protein diet with branched chain amino acids (leucine, isoleucine, valine) and Vitamin B6 supplementation are suggested to rebuild damaged muscle. Animal models (Marino sheep, Charolais cattle) have a similar metabolic defect. An adenovirus vector with recombinant myophosphorylase cDNA has been able to restore normal phosphorylase activity in primary myoblasts from phosphorylase-deficient human and sheep muscle in the laboratory. It is anticipated that gene transfer may be possible in the future.

**Glycogen Storage Disease Type VI (Hers disease)**

GSD Type VI is a rare autosomal recessive disease with a deficiency in liver glycogen phosphorylase E (chromosome 14q21-q22). The most commonly affected group is the Mennonite community. This disease is manifest in infants with asymptomatic hepatomegaly and growth retardation. It usually has a benign course with mild to moderate hypoglycemia. Liver biopsy shows a mosaic pattern and irregular distension of the hepatocytes due to glycogen deposition. The periportal hepatocytes tend to be more commonly affected. Microsteatosis, mild periportal fibrosis and septal formation may be present. Electron microscopy reveals large pools of monoparticulate glycogen interspersed with glycogen rosettes and lipid vacuoles with glycogen embedded. The hepatocytes have irregularly shaped aggregates of low density granular material scattered within the glycogen, giving a starry-sky appearance. Skeletal muscle is normal appearing. Diagnosis can be made with enzyme assay using liver, leukocytes and erythrocytes. Gene mutation analysis can also be performed. Treatment is directed toward avoiding prolonged fasting and ingestion of a bedtime snack to avoid early morning hypoglycemia.

**Glycogen Storage Disease Type VII (Tarui Disease)**

GSD Type VII is caused by phosphofructokinase enzyme deficiency (chromosome 12q13) and has clinical features that closely resemble those ascribed to GSD Type V (McArdle’s disease). Phosphofructokinase catalyzes the phosphorylation of fructose-6-phosphate at position 1. There are 3 isoforms – muscle (chromosome 12q12), liver (chromosome 21q23) and platelet/fibroblast (chromosome 10p15.2-p15.3) isoforms. Those affected have exercise intolerance, muscle cramps, myoglobinuria,
mild hyperbilirubinemia and reticulocytosis. Serum markers for creatine kinase, LDH and AST (muscle) are elevated. Only about 90 cases have been reported in the literature, with Ashkenazi Jews being over represented. Muscle biopsy shows glycogen accumulation in the subsarcolemmal space. There is also variation in myofibril size. In some case, polyglucosan bodies are noted.

Treatment is directed toward avoiding strenuous exercise. High protein diet with branched chain amino acids (leucine, isoleucine, valine) and Vitamin B6 supplementation are suggested to rebuild damaged muscle. There are 2 canine models for GSD Type VII disease that exist and these may provide insight into novel therapy.

**Glycogen Storage Disease Secondary to Phosphorylase Activation System Defects**

Several GSDs are caused by phosphorylase kinase system defects (GSD VIII, IX) and these will be discussed together. Phosphorylase kinase is comprised of 4 subunits (alpha, beta, gamma, delta) with each subunit gene mapped to different chromosomes. The alpha and beta subunits have regulatory functions. The gamma subunit has a catalytic function. The delta subunit binds calcium. The alpha subunit has both a muscle isoform and a liver isoform that are encoded by separate genes on the X chromosome (Xp22.2-22.1). The beta subunit gene is located at 16q12-q13. The gamma subunit (16p12.1) occurs as a muscle isoform and a testis-liver isoform.

**X-linked Liver Phosphorylase Kinase (alpha subunit) Deficiency** is considered to be the mildest GSD with low phosphorylase activity in the absence of adenosine monophosphate. This GSD represents 25% of all GSD cases. The most common signs and symptoms present between 1 and 5 years of age and are hepatomegaly (92%), growth retardation (68%), motor skill delay, hypotonia, and increased AST (56%), ALT (56%), cholesterol (76%) and triglycerides (70%). There is fasting hyperketosis and hypoglycemia. Overall, the clinical course tends to be benign with adult patients being asymptomatic. With aging, the clinical and serological findings gradually decrease. Hepatomegaly and growth retardation usually resolve during puberty. Splenomegaly and cirrhosis are very rare.

Liver biopsy shows irregular distension of hepatocytes with glycogen. The hepatocytes are organized into a mosaic pattern, and there may be some septal formation. Microsteatosis may be seen. Electron microscopy shows extensive monoparticulate glycogen, glycogen rosettes and frequent lipid vacuoles containing glycogen particles. Glycogen rosettes arranged in parallel arrays associated with
endoplasmic reticulum membranes are seen. This GSD has a starry-sky pattern attributed to scattered areas with finely granular, organelle-free clear zones alternating with densely packed glycogen particles. Mitochondria tend to decreased in size and numbers. Skeletal muscle is normal. Diagnosis can be made with enzyme analysis of liver tissue and by gene mutation analysis.

**Autosomal Liver and Muscle Phosphorylase Kinase (beta subunit) Deficiency** is caused by a deficiency in the beta subunit of phosphorylase kinase and is autosomal recessive in inheritance. It is characterized by hepatomegaly with abdominal distension, mild growth retardation and lipidemia. Most cases have mild or absent symptoms. When symptoms are present, the liver is primarily affected with the potential for severe liver disease and cirrhosis. There are reported cases with progressive neurologic deterioration. Both liver and muscle biopsies show glycogen accumulation by light and electron microscopy. There are also lipid vacuoles with glycogen embedded. Diagnosis can be made with enzyme analysis of liver tissue and by gene mutation analysis.

**Autosomal liver phosphorylase kinase (gamma subunit) deficiency** is due to a deficiency in the gamma subunit of liver phosphorylase and has an autosomal recessive pattern. This GSD is associated with liver cirrhosis. Renal tubular acidosis and/or neurologic disorders (peripheral sensory neuropathy) may be seen. Diagnosis can be made with enzyme analysis of liver tissue and by gene mutation analysis.

**Glycogen Storage Disease Type X**

GSD Type X is caused by a deficiency in cyclic 3',5' AMP-dependent kinase (chromosome 17q23-24) and is inherited in an autosomal recessive pattern. This disease is characterized by asymptomatic hepatomegaly. There is no glucose increase following glucagon or epinephrine administration. Liver biopsy shows a mosaic pattern with the hepatocytes and irregular distension of the hepatocytes due to glycogen deposition. Microsteatosis and septal formation may be present. Electron microscopy reveals glycogen rosettes and lipid vacuoles with glycogen embedded. The amount of glycogen varies vastly from near normal amounts to well-defined dense deposits. Lysosomal monoparticulate glycogen may be seen in addition to cytoplasmic glycogen rosettes. The lysosomes also contain cell membranes, lipofuscin and other cell components, as well as monoparticulate glycogen. This is helpful in distinguishing GSD Type X from GSD Type II (Pompe disease). Skeletal muscle shows sarcolemmal
glycogen deposits. The prognosis is considered to be good. Diagnosis can be made with enzyme and gene mutation analysis on liver and skeletal muscle.

**Glycogen Storage Disease Type XI (Fanconi-Bickel Syndrome, GLUT2 Deficiency)**

GSD Type XI is caused by a deficiency in the GLUT 2 (glucose transporter 2) localized to 3q26.1-q26.3 and has an autosomal recessive inheritance pattern. GLUT2 is the most important glucose transporter in hepatocytes, pancreatic beta-cells, enterocytes and renal tubular cells. Both glucose and galactose utilization are impaired, because both of these are dependent upon GLUT2 for exportation from affected cells. Impaired exportation results in hepatorenal glycogen accumulation and proximal tubule dysfunction. The affected patients typically present between 3 to 10 years of age with fasting hypoglycemia, postprandial hyperglycemia and hypergalactosemia, hypophosphatemic rickets with osteoporosis, and marked growth retardation. Puberty is delayed. There is hepatomegaly with a protuberant abdomen. The patients have moon facies and fat deposits in the abdomen and shoulders. Pancreatitis occurs due to hypercholesterolemia and hyperlipidemia. Renal Fanconi syndrome (hypophosphatemia, hyperuricemia, hyperaminoaciduria, albuminuria) occur as a result of tubular nephropathy. There is also hyperglucosuria. Cataracts are reported in a few cases owing to hypergalactosemia. Diagnosis can be made due to galactose intolerance and gene mutation analysis.

Liver biopsy shows increased glycogen deposition and steatosis. There are typical glycogen granules of increased number on electron microscopy. Renal biopsy will demonstrate glycogen deposits in tubular epithelial cells.

Treatment tends to be symptomatic with an attempt to stabilize glucose, replace renal loss of solutes and supplementation with Vitamin D. Small frequent antiketogenic meals with adequate calories and uncooked cornstarch are instituted. Galactose restriction is important. Following such dietary restrictions result in liver size reduction with decreased glycogen content. Even untreated, GSD Type XI may be compatible with survival into adulthood.

**Glycogen Storage Disease Type XII (Aldolase A Deficiency)**

This GSD has been recently described and results from a deficiency in aldolase A (chromosome 16q22-q24) which phosphorylates fructose 6-P to fructose 1,6-P. This glycolytic enzyme catalyzes the reversible conversion of fructose-1,6-bisphosphate to
glyceraldehyde 3-phosphate and dihydroxyacetone. Aldolase A deficiency blocks terminal glycolysis. Aldolase A is primarily found in skeletal muscle and erythrocytes. The clinical features are myopathy with exercise intolerance and nonspherocytic hemolytic anemia. Creatine kinase serum levels tend to be moderate, and myoglobulinuria may be minimal to mild in affected individuals. These patients tend to have proximal muscle wasting. There are exacerbations that occur with febrile illness, ascribed to the abnormal thermolability of mutated Aldolase A. Muscle biopsy shows atrophy of type I fibers, subsarcolemmal glycogen, and less frequently intermyofibrillar accumulation of glycogen, as noted on PAS staining and electron microscopy. Enzyme levels can be assessed on muscle biopsy tissue. Genetic evaluation for mutation in Aldolase A can also be performed.

Treatment is directed toward avoiding strenuous exercise. High protein diet with branched chain amino acids (leucine, isoleucine, valine) and Vitamin B6 supplementation are suggested to rebuild damaged muscle.

**Future Directions**

GSDs are complex, rare and varied diseases that until the last decade were managed symptomatically. Current treatment is still directed toward lessening symptoms, but there is an effort to prevent the effects or partially ameliorate the sequela of aberrant glycogen storage. The introduction of recombinant enzyme replacement for infants with GSD type II (Pompe disease) has shown promising results. Several systematic studies have documented the effects of enzyme replacement therapy on GSD type II-affected skeletal muscle, as assessed by periodic biopsy, for up to 72 months after beginning treatment. Using a well-designed grading system of muscle involvement at the ultrastructural level, it has been shown that dramatic improvement with clearing of glycogen can occur in these patients. Concomitantly, clinical improvement in respiratory function, muscle function and motor development were found. Perhaps, the most important finding was the necessity of assessment of the degree of muscle injury at the time of initiation of enzyme replacement therapy. Those children that had primarily lysosomal glycogen versus cytosol (free) glycogen and minimal disruption of myofibrils on electron microscopic examination of their muscle biopsies had the greatest degree of improvement with enzyme replacement therapy. Glycogen was cleared primarily from type 1 muscle fibers (slow twitch). This indicated that the muscle biopsy may be a decision point in deciding how successful enzyme
replacement therapy will be. It is also a means to determine how effective the therapy is at designated points in time.

Transfer of acid alpha-glucosidase via an adenovirus vector to fibroblasts, myoblasts, and myotubes from children with GSD Type II (Pompe disease) has shown promise in cell culture studies. It is possible to transduce enzyme levels in GSD affected cells from nondetectable to 10 to 100-fold higher than that expected for normal cells. Of interest was the observation that clearing of lysosomal accumulations of glycogen were noted on electron microscopic examination. This correlated quite well with the increasing enzyme expression by the transduced cells. The major issues to workout for clinical trials are delivery of the adenovirus vector for direct infection of the GSD affected skeletal and cardiac muscle, and avoidance of an inflammatory reaction. Currently, such gene transfer technology is being tested in murine models of GSD Type II.

Therapeutic liver repopulation has been studied extensively in animal models of tyrosine kinase deficiency. Hepatocyte cell transplantation has been suggested for metabolic diseases. This type of cell transplantation has several advantages: 1) less invasive with lower morbidity and cost; 2) single donor liver can be used for several recipients; 3) cells can be cryopreserved and stored for later use; and 4) cell suspensions may be less immunogenic than solid organ transplants. Animal models have shown that two conditions need to be met to achieve successful liver repopulation. The transplanted cells must have both selective proliferation and survival advantage over that for native hepatocytes. The native hepatocytes need to be eliminated, either acutely or chronically, to accommodate the space for the transplanted cells. This information from animal models for liver repopulation indicates that it is required to block the cell cycle of the native liver cells (retrosine, radiation or chemotherapy) to induce cell death over time and to provide a mitotic stimulus for the transplanted cells (partial hepatectomy). The major problem in liver repopulation with humans is that acute hepatic failure with induction of native hepatocyte cell death needs to be avoided, because liver repopulation may take several weeks. Currently, effort is directed at understanding how apoptosis can be controlled to minimize premature native hepatocyte cell death while waiting for donor hepatocyte repopulation of the liver to occur. Fortunately, animal models of GSD exist which will facilitate progress with hepatocyte transplantation for persons afflicted with GSDs.
Simplified pathway of glycogen synthesis and degradation.
References


Nicolino MP, Peuch J-P, Kremer EJ, Reuser AJJ, Mbebi C, Verdiere-Sahuque M, Kahn A, Poenaru L. Adenovirus-mediated transfer of the acid-alpha-glucosidase gene into fibroblasts, myoblasts and myotubules from patients with glycogen storage disease type
II leads to high level expression of enzyme and corrects glycogen accumulation. Human Molecular Genetics 1998;7(11):1695-702.


Towards a more useful morphological approach to Mitochondrial Disorders

Are morphologists players or handmaidens?

Kevin E. Bove M.D.
Cincinnati Children’s Hospital
A tale of two interacting genomes in good times and bad

**Disorders of energy metabolism**

- Overlapping phenotypes
- Expanding genotypes

What is the role of the morphologist?
Definition: disorders of energy metabolism

- Fatty acid oxidation disorders
  - Transport of lipid into cell
  - Transfer of substrate into mitochondria
  - Lipid substrate specific disorders
- Deficiency of pyruvate pathway
- Deficiency of Krebs cycle pathway
- Respiratory chain disorders*
Mitochondriopathy: definition

- Cellular energy deficit resulting from insufficient production of ATP to meet metabolic/functional needs
- Due to mutations in mtDNA or nDNA affecting activity or assembly one or more respiratory chain enzymes, or overall production of mtDNA or BOTH
- Maternally inherited, AR, X-linked or sporadic.
- Clinically heterogeneous: overlapping, evolving, single or multiple organ systems.
Mitochondrial genome map
Prevalence of Mitochondrial disease

- Minimum prevalence of pathogenic mtDNA mutations, Newcastle (UK) region is 7/100,000 *
  - LHON point mutation: 3.29/100,000
  - Non-LHON point mutation: 1.96/100,000
  - Deletions: 1.33/100,000
  - Abnormal mtDNA at risk: 7.59/100,000
  - Total prevalence: 12.48/100,000 (1/8000)

- Large scale mtDNA deletions, northern Finland: 1.6/100,000 adults**

Prevalence of Mitochondrial disease in children

- Mito encephalomyopathy < 6 years old (Sweden): 1/11,000*
  - Leigh syndrome, 1/32,000
  - Alper syndrome, 1/51,000
    (compared to COX negative infantile myopathy, 1/51,000)
- Mito encephalomyopathy, 16 yrs old, point prevalence (1999): 1/21,000
  (32 pts in 16 years; 4 mDNA point mutations, 2 mtDNA deletions, 2 SURF1 nuclear mutations)
- Birth prevalence of Resp Chain Disease (Australia): 6-13/100,000**

Understanding of Mitochondrial pathobiology is evolving rapidly

• Normal mitochondrial protein translation
  – Mainly coded by nuclear genes

• Abnormal mitochondrial protein translation
  – due to mutant mtDNA
  – or to nDNA mutations that determine amount of mtDNA, or assembly / integrity of ETC components

• New classes:
  – Nuclear genes that affect mtDNA levels
    • POLG; MPV17, EFG1
  – Nuclear genes that affect mito protein assembly
    • SURF1
Mitochondria

• **Dynamic** evolving population of ancient symbionts with a high mutation rate.

• **Functional Status** determined by status of genomes (2), metabolic demand and internal/external environment. May be
  – Sufficient
  – Stressed
  – Incompetent

• **Challenge to morphologists**: can we develop a comprehensive, clinically useful pathology of mitochondrial disease ??
## Diagnostic Criteria in Adults and Children


### Major criteria
- Clinical presentation, ↑lactate
- Histology
  - >2% RRF
  - 2-5% COX-negative fibers
- Enzymology
  - <20% RC in a tissue or <30% RC >=2 tissues
  - <30% RC in a cell line
- Functional
  - Fibroblast ATP synthesis rates >3 SD below normal
- Molecular
  - Nuclear or mtDNA mutation of undisputed pathogenicity

### Minor criteria
- Clinical presentation, +/-
- Histology
  - < 2%RRF age 30-50y
  - >2%SSMA (<16y)*
  - Abnormal mitochondria (EM)*
- Enzymology
  - 20-30% RC in a tissue or 30-40% RC >=2 tissues
  - 30-40% RC in a cell line
- Functional
  - Fibroblast ATP synthesis rates 2-3 SD below normal
- Molecular
  - Nuclear or mtDNA mutation of probable pathogenicity
Tools for study of disorders of energy metabolism

- Workable clinical definition
  - Family history
  - Multi-system disease (one-organ may dominate)
    Muscle > heart > Liver > Brain + LHON
  - Progressive (ETC) or episodic (FAO)
  - Imaging studies: brain MRI and MRS

- Laboratory methods
  - Lactate, Pyruvate, L/P ratio, alanine
  - Urine acylcarnitine profile; organic acids

- LM & EM: MUSCLE, also liver, heart
  - Must Correlate LM & EM!
Tools for study of disorders of energy metabolism, continued.

- Assay of ETC enzyme activity
  - Muscle, Fibroblast Culture
- Screen for known mutations in mtDNA
- Quantify mtDNA/nDNA (Southern blot or copy #)
- Assess for specific nuclear mutations in genes that control assembly or directly code for ETC enzymes
  - POLG
  - MPV17
  - Etc.
Mitochondria: ultrastructure in health and disease

- Fixation issues
- Stress changes (ethanol, NAFLD, portal vein obstruction, urea cycle defects etc)
- Changes associated with disorders of the mitochondrial and nuclear genomes
EM: Proper fixation isn’t the only thing; it’s everything!

Poor fixation is a formidable obstacle to progress
Mitochondrial stress changes
Fatty acid oxidation defects

- Long chain CoA dehydrogenase (LCAD)
- Medium chain (MCAD)
- Carnitine palmitoyltransferase I and II
- Carnitine acylcarnitine translocase
- Fatty acid transport defects
- Electron transfer flavoprotein deficiencies
Fatty Acid Oxidation Defects: eg MCAD or CPT

Heart: vacuolar change
Liver: micromacrosvesicular steatosis
Kidney-ORO stain
Muscle-Sudan Black stain
Fatty acid oxidation disorders

- Metabolic crises: fasting, infection
  - progressive cardiac and liver disease (LCACD)
- Non-ketotic hypoglycemia, sudden death
- Organs affected: liver, heart, kidney, muscle
  (neutral lipid accumulates)
- EM: mitochondrial changes are inconstant,
  not specific for FAO disorder, non-dramatic
  and presumably reversible (non-progressive)
Muscle morphologists laid the foundation for mitochondrial diseases by applying new technical methods to tissue specimens for light and electron microscopy.
Phase 1: muscle biopsy revolution

Modified Trichrome  SDH  COX
Trichrome stain: Cryosat sections normal muscle

Chromotrope2R stains normal/abnormal mitochondria red. Highlights distribution in cell. Highlights aggregates. Highlights “ragged red fibers”

Could be used to assess numeric density but has not!
Succinic dehydrogenase histochemistry

SDH is marker for distribution of mitochondria; identifies SSA and IMFA and ↑ numbers ("ragged blue fibers")

Small SS aggregates in 0-25% of myofibers is normal.

Large aggregates >3 microns, are unusual except in MM.

>2% large SSA is minor criterion for Dx of mitochondriopathy.
Cytochrome Oxidase Histochemistry

Reaction product roughly proportionate to activity but influenced by substrate conditions, section thickness.

Useful to detect marked reduction COX activity in all fibers or in single fibers.

Isolated COX negative myofibers are uncommon in children, and prevalent in RRF in adults.
Ragged red fibers (MM)

- Result from heteroplasmy (usually)
- Mutated mtDNA accumulates
  - Maternally-inherited
  - Age-related
- RRF are uncommon in infants with MM
- Deficient production of ATP
- RRF are single myofibers in crisis
- RRF are a source of COX negative myofibers
Low-Hanging Fruit

• Diseases with “ragged red fibers”
  – Kearn-Sayres syndrome (large mtDNA deletion/duplications)
  – CPEO (multiple mtDNA deletions)
  – MNGIE (multiple mtDNA deletions/depletion)
  – MELAS (point mutation: A3243G: tRNA)
  – MERRF (point mutation: 8344: mtDNA)
  – Generalized mitochondrial cytopathy
  – Isolated Skeletal muscle mitochondriopathy
Non-uniform mito proliferation in Kearns-Sayres syndrome (high heteroplasmy)
Rare RBF and SSA in child with ptosis (early Kearns-Sayre S): low heteroplasmy

Concept of threshold!
Kearn-Sayres syndrome: EM correlates of heteroplasmy

Many areas contain normal mitochondria

Focal abn mitochondria

Pale matrix
Displaced cristae
Paracrystalline inclusions
Pearson pancreas bone-marrow syndrome*

- Pancreas: acinar atrophy, fibrosis, ↓islets
- Bone-marrow: vacuolated leukocytes, ring sideroblasts
- Muscle: RRF
- Liver: +/- fatty change, fibrosis

*mtDNA deletion similar to KS syndrome
Tool: two large mtDNA probes designed to detect large common deletion of KSS

Normal control fibroblasts
Heteroplasmy in Pearson syndrome: + deletion demo; two mtDNA designer probes

Probe 1

Pearson syndrome fibroblasts; van de Corput et al. J Histo Cyto 1997
Ragged Red Fibers: product of mtDNA or nDNA defects

• Kearn-Sayres syndrome: muscle weakness, cardiomyopathy, retinitis pigmentosum (sporadic large mtDNA deletions)
• MNGIE syndrome: neuro-gastrointestinal-encephalomyopathy (AR nuclear mutations in thymidine phosphorylase affect mtDNA production and ETC activity
• Both conditions have RRF

*nDNA defects influence mtDNA
Mitochondriopathy: no RRF

- Nuclear gene defects
- Muscle, Muscle-brain, brain-liver, viscera alone (heart, liver)
- Examples: Leighs encephalopathy; infantile hypertrophic cardiomyopathy; acute liver failure in infants, lethal infantile MM
Cytochrome C oxidase (Complex IV) deficiency:

- No RRF in most cases
- M, MK, MH, H, MLK, Liver+ Brain, Brain only
- Autosomal recessive
- Multiple gene defects described
  - Mito tRNA (lethal in infancy)
  - Nuclear gene: SCO2 (rare cause of HCM)
  - Nuclear gene: COC17 (rare cause of HCM)
  - Nuclear gene: SURF1 (Leigh encephalopathy)
Lethal COX deficiency: infants

Focal RRF-like change

Pleomorphism, budding, pathological Δ
Mitochondriopathy: heart

- Hypertrophic cardiomyopathy
- Kearn-Sayres syndrome
- X-linked cardiomyopathy (Barth)
- Cardiomyopathy in Leigh syndrome
Mitochondrial proliferation: idiopathic hypertrophic cardiomyopathy

Probable mitochondriopathy
Cardiomyopathy in Kearn-Sayres syndrome; arrhythmogenic
X-linked Cardiomyopathy: large aggregates and excess inner membranes

Bissler et al. Lab Invest 2002
X-linked Cardiomyopathy (Barth syndrome)

Original description: Harry Neustein, 1979
Gene defect at Xq28: Bolhuis, 1991
“Taffazins”: mutant acyl transferases. that cause ↑ fatty acid saturation during assembly of mitochondrial membranes.
Diagnosis based upon mutational analysis. Heart EM is distinctive.

Three dimensional study of Barth lymphocyte mitochondria shows multifocal fusion of inner-membranes. Cause: altered membrane fluidity ?
Result: compensatory membrane excess and progressive cardiac dilatation

Acehan et al. Lab Invest 2007
Causes of liver failure in infants/children

<table>
<thead>
<tr>
<th>Not disorders of energy metabolism</th>
<th>Disorders of energy metabolism</th>
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<tbody>
<tr>
<td>• Viruses</td>
<td>• Alper syndrome</td>
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<td>• Autoimmune disease</td>
<td>• Navajo hepatopathy</td>
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<tr>
<td>• Metabolic diseases</td>
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<td>– Tyrosinemia</td>
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<td>– Fructose intolerance</td>
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<td>– Bile synthetic defects</td>
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<td>• Drugs (tylenol)</td>
<td>• Reye syndrome(transient)</td>
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<tr>
<td>• <em>Idiopathic</em> <em>(most common)</em></td>
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Mitochondriopathy: virus-associated, ASA-linked (Reye Syndrome)
Mitochondriopathy: virus-associated, ASA-linked (Reye Syndrome)

First hepatic mitochondriopathy. Acute, transient, ASA-linked.

Decreased mitochondrial volume fraction implies mitochondrial loss. (Daugherty et al, AJPath. 1987)

MPT (mitochondrial permeability transition) is Ca++ mediated, and pro-apoptotic.

Salicylate induces reactive oxygen species that cause MPT.*

* Battaglia et al. J Biol Chem 2005
Mitochondriopathy: virus-associated (Reye Syndrome)
Hepatic disorders of energy metabolism

• Primary Fatty Acid Oxidation defects
  – LCHAD
  – MCAD
  – Miscellaneous other disorders of FAO

• Primary Respiratory chain disorders
  – mtDNA depletion
    • Deoxyguanidinyl kinase (dGK)
    • DNA polymerase gamma (POLG)
    • MPV17
  – Cytochrome C oxidase deficiency (complex IV)
  – mtDNA deletion (Pearson syndrome)

• Unknown etiology: Reye syndrome
mtDNA depletion: a disorder caused by multiple nuclear genes, many with unknown functions

- Myopathic presentation
- Hepatocerebral presentation
  - Alper-Huttenlocher syndrome
  - Navajo neurohepatopathy
- Acute liver failure of infancy (R/O brain)
nDNA gene mutations determine total mtDNA and abn ETC activity

- Deoxyguanosine kinase* (Liver failure)
- POLG Gamma* (PEO, Liver failure)
- MPV17* (Alper syn, Navajo NH)
- Thymidine phosphorylase* (MNGIE syn)
- BCSIL (Complex III): Liver Failure
- SCO1 (Complex IV): Liver Failure

* mtDNA depletion: all complexes lo
The Southern blot
Navajo neurohepatopath*y

• Three phenotypes: liver failure predominates in infants
• Characteristic liver light microscopy
  – Steatosis, Cholestasis, Fibrosis
• Abnormal mitochondrial morphology
  – Mild pleomorphism, abnormal cristae and matrix

Navaho neurohepatopathy

- Originally peripheral neuropathy in older children; autosomal recessive
- Slowly progressive liver disease with episodic deterioration (Reye-Syndrome like)
- Infant form: acute liver failure
Navajo Neurohepatopathy
Navajo neurohepatopathy

Holve et al. J Pediatr 1999
Navajo neurohepatopathy: EM

Navajo neurohepatopathy: etiology

- Autosomal recessive trait*
- mtDNA depletion and reduced ETC activity in all complexes
- Mutation in MPV17, a nuclear gene that codes for a mitochondrial inner membrane protein (function unknown)

* Genetic bottlenecks X 3: Bering migration, contact with European viruses, post US civil-war attrition
mtDNA depletion due to nuclear genes: causes acute liver failure in infants, usually but not always with encephalopathy

MPV17 defect

Wong L-J et al. Hepatol 2007
Case #1: mtDNA depletion causes acute liver failure (ALF) in non-Navajo infants
Case #2: ALF in Alper syndrome due to Complex III deficiency or mtDNA depletion
Case # 3: ALF in infant with Alper syndrome + mtDNA depletion: No nDNA gene defect identified
Cases #4 & 5: Infant hepatic mitochondriopathy (ALF) MPV17 defect*

*Granular red hepatocytes

Cases # 4 & 5: Infant hepatic mitochondriopathy (ALF) MPV17 defect

? specific

megamitochondria

Mixed mito changes
Case # 5: Infant hepatic mitochondriopathy (ALF) MPV17 defect and mtDNA depletion: EM

? Typical for mtDNA depletion
Mitochondriopathy: Brain

- RRF+ : MELAS, MERRF, CPEO, K-S, Pearson syndromes,
- RRF- : Leigh, Navajo NH, LHON
- Leigh phenotype (at least 6 different genetic defects described)

**Brain involvement**: progressive or episodic
- symmetrical lesions, basal ganglia-brain stem
- multifocal stroke-like lesions, cortex
- diffuse leukoencephalopathy (rare and controversial)
Leigh Encephalopathy

Mutations: 124 cases, China*
- SURF1-Assembly gene (20%): COX deficiency
- Pyruvate dehydrogenase (2%)
- mtDNA (5%)
- No abn detected (74%)

Zhang Y et al. 2007 J Inherit Metab Dis
Leigh Encephalopathy: mutations: 100 pts (Japan)*

- SURf1, COX C deficiency, Complex IV (15)
- mtDNA, Complex VI, ATPase (18)
- PDH complex (4)
- Complex I, NADH-Coenzyme Q (1)
- Complex II, SDH-ubiquinone reductase (1)
- No abnormality detected (61%)

* Makino M et al. 2000; J Hum Genet
Leigh encephalopathy: Muscle usually morphologically normal but useful for chemical and genetic study.

Normal COX

Normal muscle

Leigh muscle, minority of cases

Low COX
What is Mitochondrial “proliferation”?

- Normal baseline: growth, exercise
- More numerous than expected?
- Synonym for pleomorphism?
- Signs of budding/fission?
- More or larger aggregates than usual?
- All of the above?
- “I know it when I see it”
To find a better approach to evaluate muscle ultrastructure

- Report results of LM and EM of muscle biopsy in suspected mitochondriopathy in 103 consecutive cases with ETC data
- Study properties of aggregates and compare to background non-aggregated mitochondria in normal muscle, in suspected but unproven mitochondriopathies and unequivocal mitochondriopathies*

* Miles L et al. Hum Pathol 2005
Patients and Methods

• 103 patients (60m/43f, 2 weeks-21 years, median 2.7 years, 2000-2002)
  – Group I: syndromic or classical mitochondriopathy (13, only 6 with ETC measured)
  – Group II: encephalomyopathy and elevated lactate (8)
  – Group III: encephalomyopathy (18)
  – Group IV encephalopathy (46)
  – Group V: myopathy (18)
Results- Light Microscopy

• Group I:
  – RRF and/or RBF, 7/13
  – COX ↓ or absent, 5/13

• No RRF, RBF or COX decreased fibers in the other groups
Results- Electron Microscopy

• Pathological mitochondria: 12/13 in group I, 2/8 in group II, 0/72 in other groups
• Mitochondrial branching and budding more common in groups I and II
• No significant difference in frequency of IMF or SS aggregates in any patient groups
Results- Electron Microscopy

- Size variation of mitochondria: no diagnostic significance, but greater in SS aggregates in all groups
  - Mitochondrial index: ratio of the largest and smallest mitochondria
Results-ETC

Abnormal ETC enzymes are most common in group I (83%), but are not different in groups II, III, IV and V.
Our study conclusions

• High frequency of normal ETC data in pts with low clinical suspicion: 73/82 (89%)
• Low frequency of positive EM studies in patients with low clinical suspicion: 0/72 (0%)
• Suggestions to modify Bernier criteria:
  – SS aggregates up to 25 % of fibers in normal bx
  – Only aggregates > 4 micrometers are pathologic
  – Pathological mitochondria in this clinical setting should be a major criterion
Mitochondrial aggregates: how best to assess?

Subsarcolemmal

Intermyofibrillar

Formal morphometry of mitochondrial clusters may have utility in diagnosis of mitochondriopathies
Mitochondrial image analysis vs “expert” analysis in COPD

- Low BMI
- ↓ response to endurance training
- low citrate synthase activity in muscle
- Reduced mito fractional area and number in skeletal muscle*

X2000; central zone; 3-5 fibers, minimum 275 mito analyzed; z-band width controlled

Rational investigation for mitochondrial disease

- Patient Class: Classical vs probable vs unexplained organ dysfunction (r/o mito disease)
- Algorithm for investigation: does one size fit all? Probably not!
- What is sufficient in each class?
- Is ultrastructure always necessary?
- What is the objective?
  - counseling
  - treatment
  - intellectual curiosity
Cost ($) of investigation for mitochondrial disease is $5-10,000

- Muscle, fibroblast culture, blood DNA, liver biopsy
  - Surgeon, anesthesia, OR: $1500
  - Histochemistry battery: $500
  - Ultrastructure: $750
  - ETC activity profile: $900
  - mtDNA depletion/ 3 common mutations: $300
  - mtDNA mutation screening panel: $800
  - nDNA mutation sequencing (5 known defects): $2000/gene
  - mtDNA whole genome sequencing: $2500
  - Fatty acid oxidation in fibroblast culture: $1500

Solution: Use clinical data, organ pattern and muscle bx findings to focus investigation!
Summary: organ distribution in mitochondriopathy

• Primary mtDNA defects:
  – Maternal transmission or new mutations
  – Gradual accumulation of mutant mitochondria to heteroplasmy threshold (clinical effect)
  – Any organ may be involved; muscle most often

• nDNA defects:
  – Autosomal recessive trait
  – Expressed in organs with highest demand for energy, as in FAO defects
  – Muscle, Brain, Liver, Heart, Kidney
Summary: diagnostic approach is multifaceted

- Clinical presentation determines approach
- Muscle biopsy, best tissue source
  - Correlate LM and EM
  - ETC activity
  - mtDNA for mutation analysis
- Liver biopsy, if liver involved
- Leukocyte DNA (nDNA mutations)
- Fibroblast culture (nDNA mutations and FAO investigation)
Notes for the pathologist

- Correlation of light and electron microscopy is critically important.
- Avoid handicap of poor fixation
- Mitochondrial ultrastructure changes may be subtle in early stages of disease
- Morphometry of mitochondrial populations is untapped for clinical use but has potential value, especially study of aggregates
- Pathological mitochondria exhibit a narrow range of changes (?) specific classes)
Mitochondrial ultrastructure in disease: 5 recognizable patterns

- **Stress changes**: mild pleomorphism, matrix crystalloids in normally dense matrix
- **Membrane permeability transition** (ischemia-reperfusion; Reye syndrome: amoeboid pleomorphism, uniform matrix swelling, displacement of cristae
- **mtDNA mutations/deletion**: mild pleomorphism, central pale matrix, rearrangements of cristae, crystalloids within inner cristal space, occ megamitochondria
- **mtDNA depletion**: ↑ number, ↓ or ↑ density matrix, expanding dense matrix, displacement or saccular dilatation of cristae, few megamitochondria
- **Barth disease**: extreme pleomorphism, ↑ cristae with stacks, whorls and fusion, many megamitochondria
A useful Morphological approach to Mitochondrial Disorders is within reach!

Too soon to give up!
General references

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