Towards a more useful morphological approach to Mitochondrial Disorders

Are morphologists players or handmaidens?

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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

Human mtDNA
16,569 bp

Aminoglycoside-induced deafness
Myopathy
Respiratory deficiency
Cyt b
ND 6
ND 5
ND 4L/4
COX III
ATPase 8/6
COX II
COX I
S
D
ND 3 R
G
L
SH
E
ND 6
LT
PT
12S
F
V
L"
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


<table>
<thead>
<tr>
<th>Major criteria</th>
<th>Minor criteria</th>
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<tbody>
<tr>
<td>• Clinical presentation, ↑lactate</td>
<td>• Clinical presentation, +/-</td>
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<tr>
<td>• Histology</td>
<td>• Histology</td>
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<tr>
<td>– &gt;2% RRF</td>
<td>– &lt; 2%RRF age 30-50y</td>
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<tr>
<td>– 2-5% COX-negative fibers</td>
<td>– &gt;2%SSMA (&lt;16y)*</td>
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<tr>
<td>• Enzymology</td>
<td>– Abnormal mitochondria (EM)*</td>
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<tr>
<td>– &lt;20% RC in a tissue or &lt;30% RC &gt;=2 tissues</td>
<td>• Enzymology</td>
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<tr>
<td>– &lt;30% RC in a cell line</td>
<td>– 20-30% RC in a tissue or 30-40% RC &gt;=2 tissues</td>
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<tr>
<td>• Functional</td>
<td>– 30-40% RC in a cell line</td>
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<td>– Fibroblast ATP synthesis rates &gt;3 SD below normal</td>
<td>• Functional</td>
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<tr>
<td>• Molecular</td>
<td>– Fibroblast ATP synthesis rates 2-3 SD below normal</td>
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<tr>
<td>– Nuclear or mtDNA mutation of undisputed pathogenicity</td>
<td>• Molecular</td>
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<tr>
<td>– Nuclear or mtDNA mutation of probable pathogenicity</td>
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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: micromacroversicular 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: Cryostat 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)

Ragged Red Fibers

Ragged Blue Fibers
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.

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 ∆
Mitochondriopathies: heart

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

Probable mitochondriopathy
Cardiomyopathy in Kearn-Sayres syndrome; arrythmogenic
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

## Not disorders of energy metabolism
- Viruses
- Autoimmune disease
- Metabolic diseases
  - Tyrosinemia
  - Galactosemia
  - Fructose intolerance
  - Bile synthetic defects
- Drugs (tylenol)
- **Idiopathic (most common)**

## Disorders of energy metabolism
- Alper syndrome
- Navajo hepatopathy
- mtDNA depletion
  - POLG defect
  - MPV17 defect
- Complex IV defect
- FAO disorder (episodic)
- Reye syndrome (transient)
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
    • Deoxyguanidin 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)
- POLGmma* (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

<table>
<thead>
<tr>
<th>Single Deletion</th>
<th>Multiple deletions</th>
<th>Depletion</th>
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<td><strong>C</strong></td>
<td><strong>P</strong></td>
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<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Deletion</td>
<td>Deletions</td>
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- **Sporadic K-S**
- **AD**
- **PEO**
- **MNGIE** Syn
- **Alpers** Syn
- **Cong myopathy**

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- **mtDNA**
- **nDNA**
Navajo neurohepatopathypathy*

• 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

H& E

COX

TRI

SDH

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

Wong L-J et al. Hepatol 2007

MPV17 defect
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 mitochondrialopathy (ALF) MPV17 defect*

Granular red hepatocytes

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

- Megamitochondria
- Mixed mito changes
- ? 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-Assemby 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.
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
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?

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* mitochondrial 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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