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The Clinical Utility of NextGen Sequencing

The Future Is Already Here: Successful Use of Next-Generation Sequencing in One Clinical Practice;

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Woburn, Massachusetts
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Dysautonomia International; 18-July, 2015
Herndon, Virginia

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Disclosure: Dr. Boles wears many hats

Dr. Boles is a consultant for Courtagen, which provides diagnostic testing.

- Medical Director of Courtagen Life Sciences Inc.
  - Test development
  - Test interpretation
  - Marketing

Courtagen LIFE SCIENCES, INC.

- Researcher with prior NIH and foundation funding
  - Studying sequence variation that predispose towards functional disease
  - Treatment protocols

Children's Hospital LOS ANGELES We Treat Kids Better

USC UNIVERSITY OF SOUTHERN CALIFORNIA

- Clinician treating patients
  - Interest in functional disease (CVS, autism)
  - Geneticist/pediatrician 20 years at CHLA/USC
  - In private practice since 2014

Mitochondrial and Molecular Medicine
Richard G. Boles, M.D.
Medical Genetics
Pasadena, California

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Disclosure:
Off-label Indications

There are no approved treatments for mitochondrial disease.

Everything is “off label”

UNITED MITOCHONDRIAL DISEASE FOUNDATION®
HOPE. ENERGY. LIFE.

Mitochondrial Medicine
The Spectrum of Mito

Brain
- Developmental delays
- Dementia
- Neuro-psychiatric disturbances
- Migraines
- Autistic Features
- Mental retardation
- Seizures
- Atypical cerebral palsy
- Strokes

Nerves
- Weakness (may be intermittent)
- Absent reflexes
- Fainting
- Neuropathic pain
- Dysautonomia - temperature instability

Muscles
- Weakness
- Cramping

- Gastrointestinal problems
- Dysmotility
- Irritable bowel syndrome
- Hypotonia
- Muscle pain
- Gastroesophogeal reflux
- Diarrhea or constipation
- Pseudo-obstruction

Kidneys
- Renal tubular acidosis or wasting

Heart
- Cardiac conduction defects (heart blocks)
- Cardiomyopathy

Liver
- Hypoglycemia (low blood sugar)
- Liver failure

Ears & Eyes
- Visual loss and blindness
- Ptosis
- Ophthalmoplegia
- Optic atrophy
- Hearing loss and deafness
- Acquired strabismus
- Retinitis pigmentosa

Pancreas & other glands
- Diabetes and exocrine pancreatic failure (inability to make digestive enzymes)
- Parathyroid failure (low calcium)

Systemic
- Failure to gain weight
- Fatigue
- Unexplained vomiting
- Short stature
- Respiratory problems

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20 "Functional" Disorders:

- Attention deficit hyperactivity disorder
- Anxiety disorder
- Autistic spectrum disorders
- Chronic fatigue syndrome
- Complex regional pain syndrome
- Cyclic vomiting syndrome
- Depression (MDD)
- Fibromyalgia
- Functional abdominal pain
- Interstitial cystitis
- Insomnia (chronic, severe)
- Irritable bowel syndrome
- Migraine
- Panic disorder
- Post-traumatic stress disorder
- Postural orthostatic tachycardia syndrome
- Restless legs syndrome
- Temporomandibular disorder
- Tinnitus
- Vulvovaginitis syndrome

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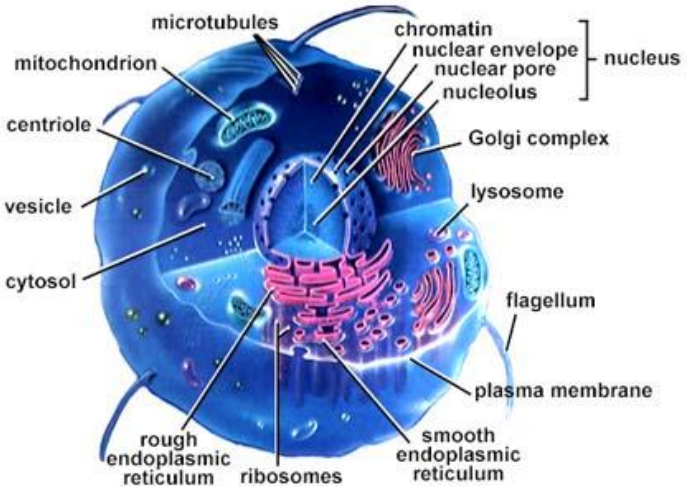
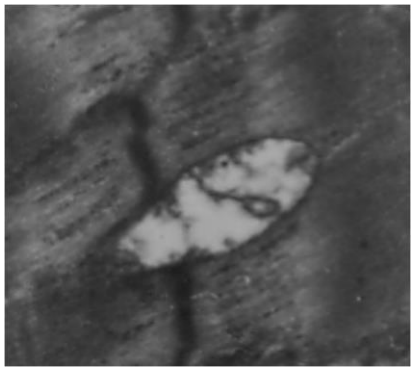
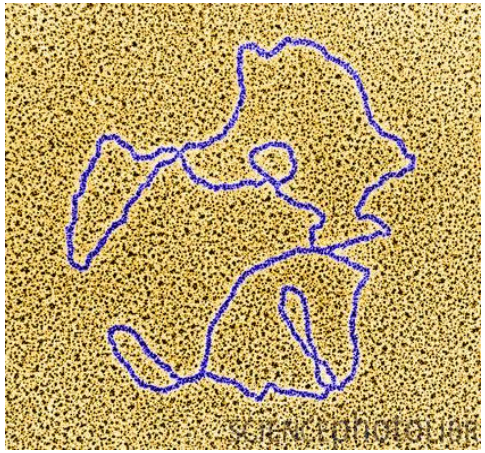
Mitochondrial Genetics
The Basics



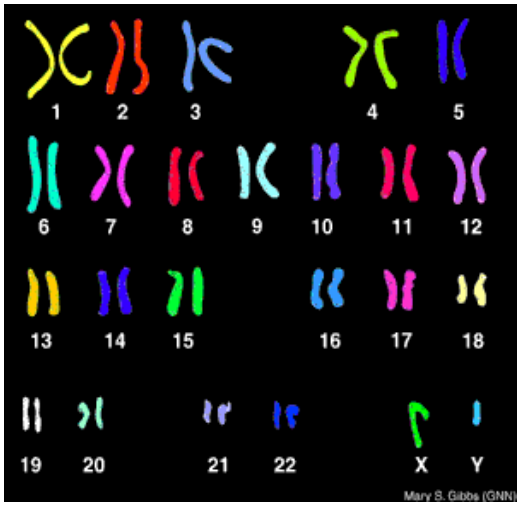


Mitochondrial DNA



37 genes
16,000 base pairs
- Maternal inheritance

Nuclear DNA



~22,000 genes
3,000,000,000 base pairs
1,013 genes encode mitochondrial proteins
- Autosomal recessive
- Autosomal dominant
- X-linked

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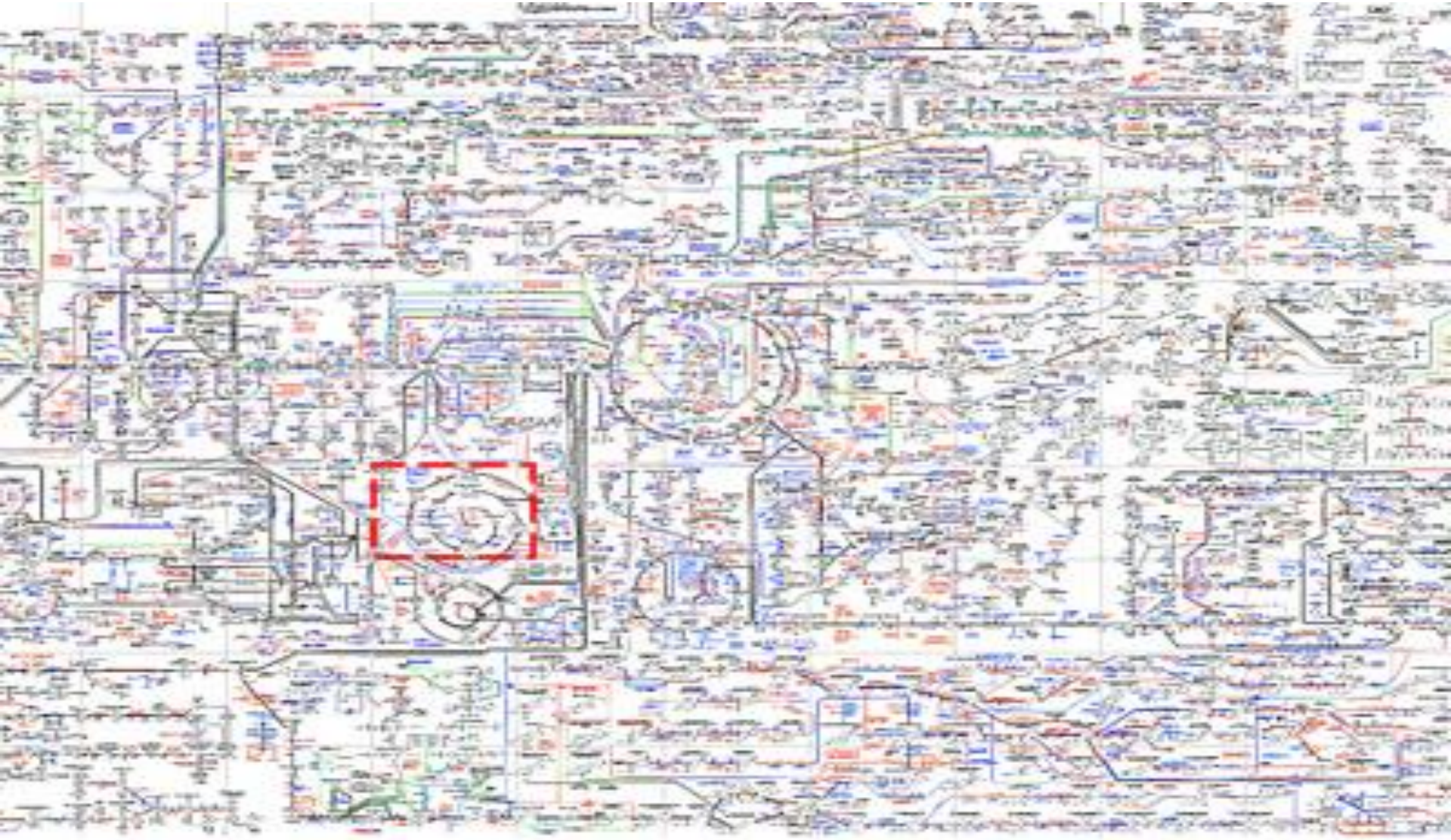
Metabolic Pathways



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Beyond the Metabolic Pathways

- There are hundreds of known causes of mitochondrial disease
- The mitochondria are composed of 1,200 proteins
- re are perhaps more causes of secondary mitochondrial dysfunction
- In most patients the underlying gene is not obvious even by an expert.
- The complexity lends itself to massive parallel sequencing = “NextGen sequencing”.

Not on this slide:

- Transcriptional elements
- Translational elements
- Chaperones
- Glycosylation
- Assembly factors
- Other post-translational elements
- Mitochondrial import
- Cofactor metabolism
- Antioxidant pathways
- Many others
- Causes of secondary mitochondrial dysfunction
  - Ion channels
  - Peroxisomal biogenesis factors
- Many others
- Phenocopies

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“Any sufficiently advanced technology is indistinguishable from magic.”

Clarke’s Third Law

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Next Generation Sequencing
Illumina MiSeq

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Courtagen mtSEEK

Sequences the entire mtDNA

- 16,569 base-pairs, 37 genes – from saliva
- Includes:
  - 13 protein-encoding genes
  - 22 transfer-RNA genes
  - 2 ribosomal-RNA genes
  - Control region
- Very high coverage of the entire mtDNA.
- All variants are looked at by an expert (not a computer).
- Reports prevalence, conservation, computer algorithm predictions.
- Reports all variants with MitoMap-listed possible to confirmed disease.
- Report is clinical orientated.
- Results in 4-6 weeks, not months.
- Option for email or telephone consultation with expert.

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William, age 10 years

- William presented to my clinic at age 6 years.
- Chronic pain, including pain in the eyes, head and abdomen.
- Limb-girdle myopathy; chronic fatigue.
- Constipation, obstipation and encopresis.
- He is an excellent student.
- Body fluid biochemical testing and electron microscopy on a muscle biopsy specimen suggested mitochondrial disease.
- Pedigree: probable maternal inheritance, with multiple manifestations of functional disease in the mother, including chronic pain, fatigue, and bowel dysfunction.
- mtSEEK (NextGen sequencing of the mtDNA) revealed 14960G>A at 55% in the mitochondrially-encoded *CYTB* gene encoding a subunit of complex III of the respiratory chain. His mother has 78% heteroplasmy for that nucleotide.
- The "mitochondrial cocktail" and has shown much improvement in energy level and, pain including the essential resolution of headache, muscle cramps and abdominal pain.

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Elizabeth, age 6 years
William's little sister

- William presented to my clinic at age 6 years.
- Anxiety: Became severe at age 5 years. She cannot get a teeth cleaning, attend birthday parties, or participate in gymnastic or scouting. Randomly cries over half the day (regarding various fears), especially with any changes in the routine. Severe separation anxiety and cries for hours when mother is not present, even if with other relatives.
- Pain: Developed chronic right ankle pain, occurring every day.
- mtSEEK (NextGen sequencing of the mtDNA) revealed 78% heteroplasmy for 14960G>A in the *CYTB* gene. There are 5 different sequence changes from that of mother and/or brother.
- Placed on "mitochondrial cocktail" and sertraline (Zoloft, 30 mg/day; her weight is 17 kg)
- Anxiety and pain resolved.

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Courtagen nucSEEK

Sequences the entire nuclear-encoded mitome

- 1,207 genes – from saliva
- Includes:
  - All MitoCarta genes (encoding proteins located in the mitochondria)
  - All peroxisomal genes
  - Metabolic enzymes in other cellular compartments
  - Ion channels and other phenocopies of “mitochondrial disease”
- Very high coverage of almost all included genes.
- Computer scored to remove variants assumed to be benign due to prevalence (>3% in population), position (outside the exome and splice sites) and effect (those that do not change the protein sequence).
- All other variants are carefully considered:
  - Monogenic interpretation only (“nucSEEK standard”)
  - Monogenic + polygenic interpretation (“nucSEEK plus”)
- Report is clinical orientated.
- Results in 4-6 weeks, not months.
- Option for email or telephone consultation with expert.

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Payton, 15-year-old

- Presented to my clinic at age 11 years.
- Cyclic vomiting syndrome from ages 1-10 years, with 2-day episodes twice a month of nausea, vomiting and lethargy.
- Episodes had morphed into daily migraine.
- Chronic pain throughout her body.
- Chronic fatigue syndrome = chief complaint.
- Substantial bowel dysmotility/IBS
  Multiple admissions for bowel clean-outs.
- Excellent student
- Pedigree: probable maternal inheritance

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TRAP1-Related Disease (T1ReD) *Mitochondrion*, 2015

- NextGen sequencing at age 14 years revealed the p.Ile253Val variant in the *TRAP1* gene.
- *TRAP1* encodes a mitochondrial chaperone involved in antioxidant defense.
- This patient is one of 26 unrelated cases identified by Courtagen to date who have previously unidentified disease associated with mutations in the ATPase domain.
- The common feature recognized at present is chronic pain, fatigue and GI dysmotility.
- Tachycardia/palpitations and dizziness may also be common.
- That variant comes from Payton's father, who himself has frequent pain, fatigue and diarrhea.
- In these patients, chronic pain and fatigue improved greatly on aggressive antioxidant therapy.
- On aggressive antioxidant therapy, all manifestations of disease in Payton were substantially improved. Issues remaining included chronic abdominal pain and moderate fatigue. She became functional in life, but still on a shortened school schedule.

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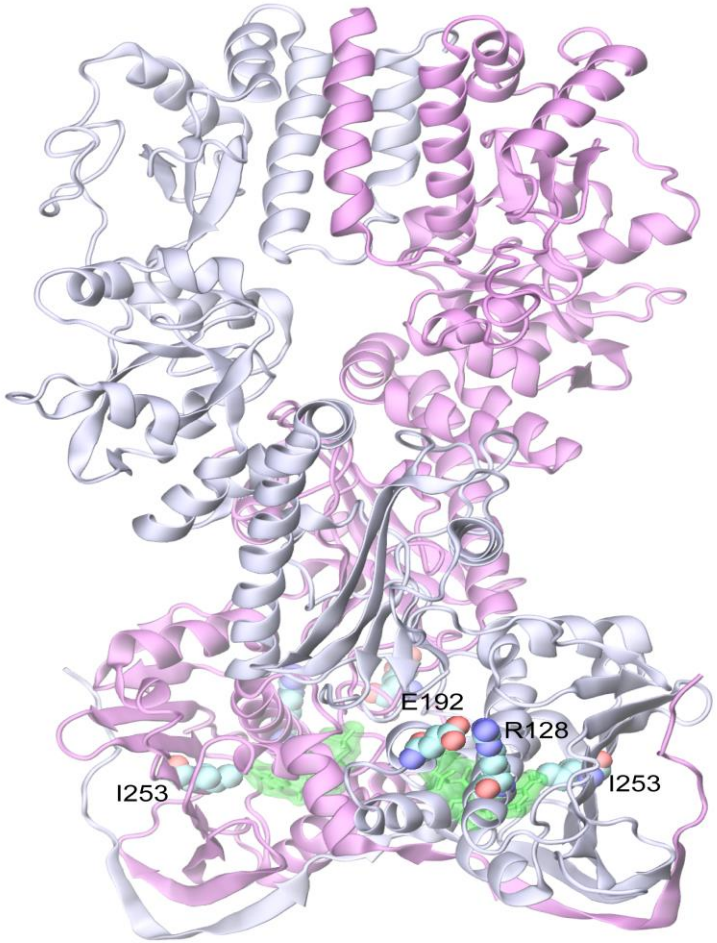
Molecular structure of TRAP1 *TRAP1*-Related Disease (T1ReD)



- 1. An ATPase domain hydrolyze the energy-rich triphosphate bond of ATP to convert into mechanical work of folding proteins.
- 2. The two homodimers of TRAP1 are shown in grey and pink.
- 2. ATP bound in its pocket is shown in green, in each dimer.
- 3. The “common mutation” p.Ile253Val is labeled in each dimer.
- 4. The “salt bridge” mutations, R128H (p.Arg128His) and E192K (p.Glu192Lys), are labeled in one dimer.

Can we design a therapy that blocks ATP entrance into mutant TRAP1, but not normal TRAP1?

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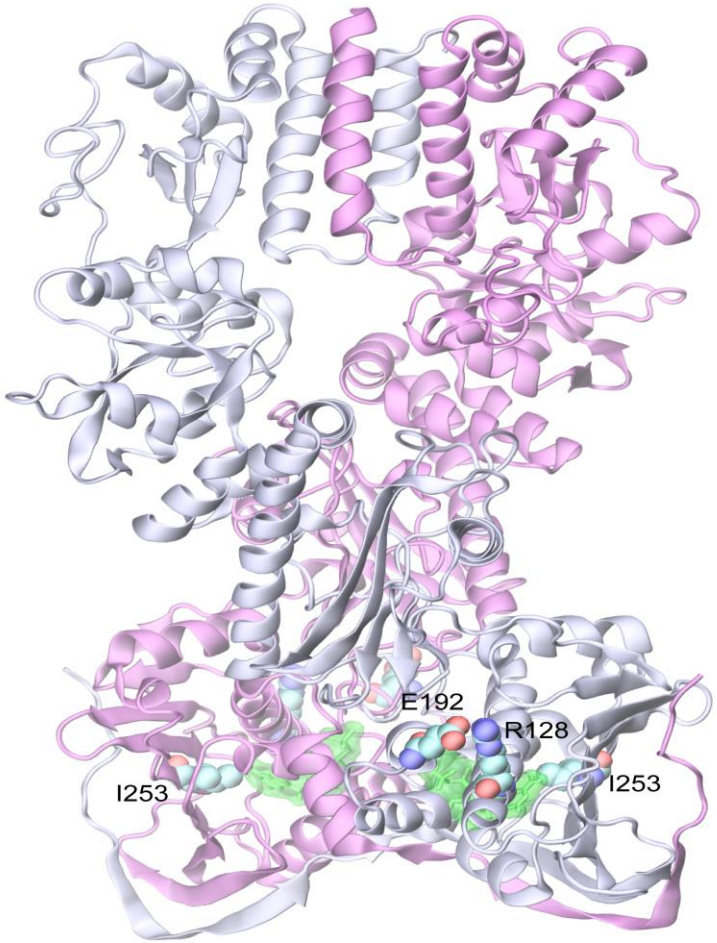
Molecular structure of TRAP1
TRAP1-Related Disease (T1ReD)



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Computer modeling was performed based on the human TRAP1 crystal structure by Jeffrey Skolnick at the Georgia Institute of Technology.

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TRAP1 variants greatly increase the prevalence of functional disease

TRAP1 variants	Pain syndromes	Chronic fatigue	Gastro-intestinal dysmotility	Triad of pain, fatigue & GI	Total number of patients
All conserved in ATPase domain	17 (65%) 4.9 (2.2-11) P = 0.001	16 (62%) 3.3 (1.5-7.3) P = 0.004	14 (54%) 3.1 (1.4-6.8) P = 0.005	12 (46%) 6.4 (2.9-14) P < 0.0001	26
Conserved elsewhere in protein	3 (7%) 0.19 (0.06-0.61) P = 0.005*reverse*	10 (22%)	11 (24%)	2 (4%)	45
p.Ile253Val	11 (69%) 5.7 (2.0-17) P = 0.001	10 (63%) 3.4 (1.2-9.4) P = 0.02	9 (56%) 3.4 (1.2-9.2) P = 0.02	8 (50%) 7.5 (2.8-20) P = 0.0001	16
Conserved in ATPase excluding p.Ile253Val	7 (64%) 4.6 (1.3-1.6) P = 0.02	7 (64%) 3.6 (1.0-1.2) P = 0.04	6 (55%) *P = 0.06*	5 (45%) 6.2 (1.9-21) P = 0.003	11
Salt bridges: p.Arg128His, p.Glu192Lys	5 (71%) 6.5 (1.3-34) P = 0.03	5 (71%) *P = 0.053*	5 (71%) 6.6 (1.3-34) P = 0.02	5 (71%) 18 (3.6-100) P = 0.0005	7
None	224 (28%)	266 (33%)	222 (27%)	95 (12%)	808

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Audrey, 3-years-old
p.Ile253Val in *TRAP1*

- GI dysmotility: on full TPN cannot tolerate any enteral intake, including jejunal drips -> gut is dead, no improvement
- Chronic pain: severe leg pain and headache -> substantially improved
- Chronic fatigue – sleeping 22 hours a day -> 12 hours/day
- Hypoglycemia, even on 24-hour drip feedings -> improved on hydrocortisone for adrenal insufficiency.
- Anemia – received multiple blood transfusions -> unchanged.
- Dysautonomia: tachycardia, temperature instability, hypoxia
- Neurogenic bladder (in and out catheter every 1.5-2 hours through umbilicus) -> resolved on targeted tx.
- TRAP1 is a mitochondrial disorder:
  - Generalized organic aciduria, including mild elevations in lactate, pyruvate, and 3-methylglutaconate.
  - Carnitine levels: Total 17 and free <4
  - Rotenone-sensitive NADH-cytochrome c reductase deficiency = 7%.

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Clinical NextGen Sequencing Overview and Goals

- Most human disorders are polygenic.
- Targets for sequencing include both monogenic and polygenic disorders
- Goal is often to identify potential genetic factors that can be targeted with specific therapy.
- More targets = more chances to successfully intervene
- Common variant, common disease model
- Combines traditional and personalized medicine

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Big Zach, age 22 years

- Autism – early infancy
  - Lost language skills acquired at 18 months.
  - Diagnosed with "autism" at age 2 yrs
- Cyclic vomiting syndrome – age 6 years
  - Episodes of nausea, vomiting and lethargy lasting from a few days to a week or more
- Bowel dysmotility
  - Hospitalized many times for "clean-outs"
  - Multiple procedures to place tubes in different bowel segments
- Complex regional pain syndrome – age 12 yrs
  - Episodes in which right foot becomes cold, purple, tender, allodynia, unable to bear weight; wheelchair bound for months
- Other chronic intermittent symptoms
  - Headache, muscle pain, photophobia, ptosis, tics, hours-long episodes of hiccups.
- Severe exercise intolerance

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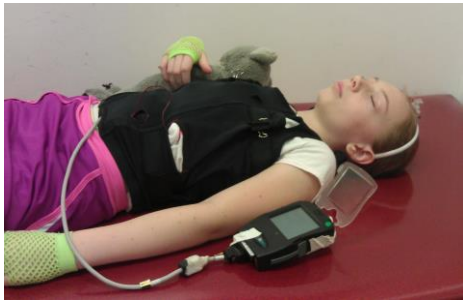
CHoline O-AcetylTransferase (CHAT) Haploinsufficiency

- Encodes for the enzyme catalyzes the synthesis of acetylcholine from choline and acetyl-CoA in cholinergic neurons
- Four cases, each with apparent maternal inheritance
- Distinct manifestations are :
  - Episodic mental status changes w/o known triggers
  - POTS/dysautonomia
  - Severe reactions to anticholinergic medications
- Parasympathetic deficiency
- Anecdotal, yet dramatic, improvement with anticholinesterase inhibitors (Aricept)
- Digenic: mtDNA + *CHAT* "polymorphism"



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Multiple functional conditions
Kelly, 28-year-old

- Presented to my clinic as a teenager
- CVS with episodes once a month for several hours, some prolonged and requires ER
- Main issue is continuous migraine headache
- Constant severe nausea and vertigo.
- Chronic fatigue syndrome – severe, disabling.
- Bowel dysmotility/IBS
- Dysautonomia, including tachycardia and POTS
- Depression and anxiety – severe, disabling
- Autistic spectrum disorder – high functioning
- Probable maternal inheritance.

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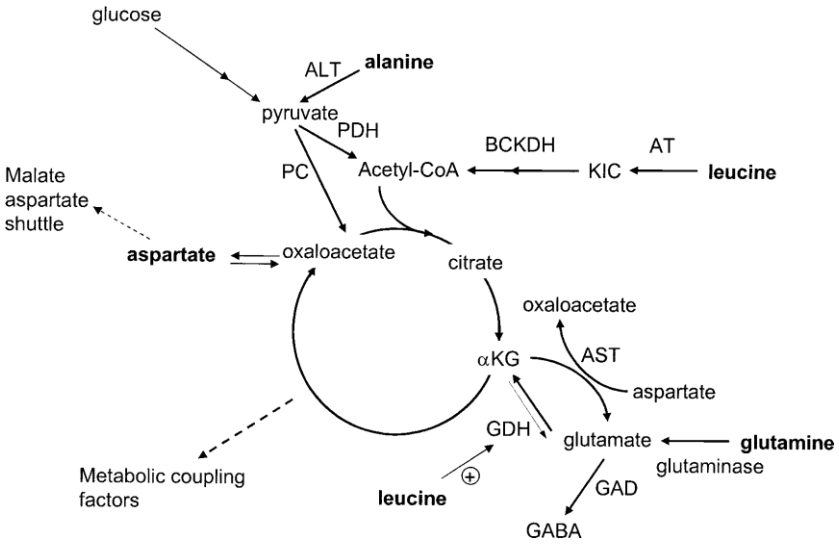
GLS2
Two predicted mutations

- Glutaminase 2
- Encodes an enzyme that converts glutamine to glutamate
- *GLS2* regulates cellular energy metabolism by increasing production of glutamate and alpha-ketoglutarate, which in turn results in enhanced mitochondrial respiration and ATP generation.



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Two predicted mutations

- Glutaminase 2
- Encodes an enzyme that converts glutamine to glutamate
- *GLS2* regulates cellular energy metabolism by increasing production of glutamate and alpha-ketoglutarate, which in turn results in enhanced mitochondrial respiration and ATP generation.
- Started on alpha-ketoglutarate
- *"The supplements seem to have made a remarkable difference! Her symptoms are much better controlled and mostly manageable & she has been able to resume some of her daily activities & a small amount of local travel. This has lifted her spirits greatly. It hasn't "cured" it all but is is quite a miracle all the same."*

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Molecular (DNA) Diagnosis

- Mitochondrial DNA (mtDNA)
  - Standard mtDNA analysis
    - PCR for common point mutations (3243A>G, 8344A>G, 8993T>G or C)
    - PCR or Southern blotting for large rearrangements
  - Full mtDNA sequencing
- Nuclear DNA testing
  - Single gene (MNGIE)
  - Small Panel (few-several genes: e.g. COX deficiency, mtDNA depletion)
  - Mito-exome (1,100 genes)
  - Exome (22,000 genes)
  - Genome (all of the DNA)

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LARGE PANEL

- Pre-selected “relevant exome” gene list
- Misses the dx if you are wrong
- Many VUS
- Occasional incidentals
- Expert interpretation
- Data to mine later

EXOME

- Gene list selected at time of interpretation
- Misses very little, but will you notice it?
- True exome = buried alive in VUS
- Many incidentals
- Perhaps less-than-expert
- Tremendous data to mine

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The VUS

VUS = Variant of Uncertain Significance

- The VUS is a mysterious creature best handled with extreme care.
- Most are harmless polymorphisms.
- Some are disease causing or related.
- Whatever they are, they are very numerous
- What do you do with them?

There is no consistent criteria for calling a variant a VUS other than the person cannot make the call of benign versus disease related. The ones of greatest interest are both:

- Possibly deleterious
- Possibly relevant to the provided phenotype

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When to Suspect Mitochondrial Disease?

Suspect mitochondrial/metabolic disease if there are two or more of the following "**Red Flags**":

- Autistic spectrum disorder/pervasive developmental disorder
- Loss of milestones/regression
- Movement disorder (including ataxia, dystonia, chorea, tics)
- Stroke or stroke-like episodes
- Myopathy, especially ocular or cardiac
- Chronic bowel dysmotility (especially if severe or at more than one level)
- Cyclic vomiting
- Dysautonomia (including POTS, frequent tachycardia, unexplained fevers)
- Chronic pain condition (including migraine, myalgia)
- Chronic fatigue
- Mood disorders
- Waxing and waning clinical course (including altered mental status or psychosis)
- Hypoglycemia
- Metabolic acidosis (either renal tubular loss and/or anion gap)
- Elevated liver transaminases (including only trace elevated, if frequent)

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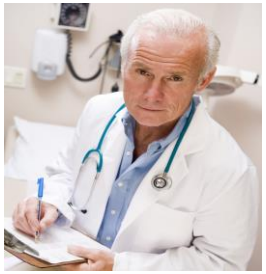
What Do I Need To Get Started?

www.courtagen.com

- 1. An order for the test from any physician.

- 2. Authorization from your insurance company, followed by a financial survey completed by the family.



- 3. Clinical information, either a sub-specialist physician note and/or completed checklist.

- 4. A collection kit will be sent by mail for the saliva sample. Results are not affected by diet, treatment, or time.