Diagnosis of Mitochondrial Disease in the South African Context
(a laboratory perspective)

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Focus Area for Human Metabolomics
"Energy metabolism disorders"

- Disorders involving energy metabolism:
  - *Rare (monogenic) disorders of*
    - OXPHOS, fatty acid oxidation, TCA cycle, creatine biosynthesis, ketolysis, enolase, gluconeogenesis, glucophosphate isomerase, phosphoglycerate kinase kinase 1, pyruvate metabolism, triose phosphate isomerase
  - "Common" diseases (incl. non-communicable/infectious/toxin induced)
    - Neurodegenerative, cardiovascular, type 2 diabetes, autoimmune, cancers, AIDS/HIV-treatment, and many more.

"Mitochondrial disorders" vs. "mitochondrial disease" vs. "mitochondrial respiratory chain disease" vs. "mitochondrial DNA disease"; “primary/secondary”
Why the heterogeneity?

1. Central role of proton-motive force ($\Delta \rho$)

Adapted from *Bioenergetics 4*, Nicholls & Ferguson
2. Genetics of OXPHOS and the “Threshold Effect”

Why the heterogeneity?
Why the heterogeneity?

Mitochondrial Threshold Effect

Phenotype

% mtDNA mutated
(OXPHOS dysfunction)
Why the heterogeneity?

3. Mitochondrion-nucleus communication
Clinical Selection

Mitochondrial disease syndrome? (or mutation in family)

Clinical phenotype suggestive of mitochondrial disorder?

Yes

Tissue biopsy (+/- Fibroblasts)

Biochemical evaluations
Enzyme/functional/structural analyses/histopathology

Molecular genetics

Screen for specific mtDNA/nDNA mutations

mtDNA sequence

mtDNA depletion

mtDNA integrity

nDNA genes sequencing

Exome sequencing

Biochemistry

Yes

+/- Metabolic investigations
Populations of African origin carry up to 3x as many rare variants as European or East Asian populations.

Nature DOI: 10.1038/nature11632
Census 2011: South African population groups

Demographics
- African (78.4%)
- White (9.1%)
- Coloured (8.9%)
- Indian/Asian (2.6%)
- None dominant
Genetic diversity

Where to find information when accessing genetic variation?

• 1000 Genomes Project
• Southern African Human Genome Project
• Other & “in house” data

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</table>

Should we investigate (considering no treatment as yet)?

How should it be structured considering separated capacities?

How do we approach – from which side?
MD (NWU/UP) study cohort

Steve Biko Academic Hospital (Pretoria, since 2006)
- ~6000 paediatric referrals to neurology clinic
- Clinical evaluations, including MD scoring
- 200 patients undergo muscle biopsy, urine collection

Cohort (200):
- Mpumalanga, Gauteng, Limpopo (from 8.4 million children)
- Black African 61%, White/Caucasian 32%, other 7%
- Equal gender

Muscle Biochemistry
- Muscle RC enzyme kinetics + PDHc
- Respirometry (pilot)
- Native PAGE (selected)
- Histochemistry (selected)

Molecular genetics
- mtDNA copy nr (muscle)
- mtDNA sequencing
- nDNA, CI – CIV, CoQ structural & associated genes

Metabolomics
- Mass spectrometry & NMR
- Aim: to investigate value of urine metabolites to better select and diagnose patients

Poster: Maryke Schoonen
Lecture: Roan Louw
Poster: Karien Esterhuizen
Diagnostic & Research strategy

Clinical Selection

Mitochondrial disease syndrome? (or mutation in family)

Clinical phenotype suggestive of mitochondrial disorder?

• Clinical scoring criteria (NCMD)
• Metabolic biosignature in urine

+/- Metabolic investigations

Biochemistry

Muscle biopsy (+/- Fibroblasts)

Biochemical evaluations

• RC single enzymes activities & PDHc
• Structural analyses (Native/denaturing PAGE)
• CoQ10 levels

Histopathology - NHLS

• Muscle morphology
• Subsarcolemmal mitochondrial accumulation (ragged red fibres)
• COX/SDH differential staining

Molecular Genetics

Screen for specific mtDNA/nDNA mutations in blood/urine

mtDNA sequence
• Full-length mtDNA sequencing
Variant detected
• Family study to investigate mutation segregation
• Specialized investigations to determine pathogenicity

mtDNA depletion
• Real-time PCR
Depletion detected
• Screening for mutations or sequencing of genes associated with mtDNA depletion

mtDNA integrity
• Long-range PCR
Deletion detected
• Screening for mutations or sequencing of genes associated with mtDNA deletion

nDNA genes investigations

• Screening for mutations or sequencing of selected nuclear genes
• Specialized investigations to determine pathogenicity

Black – routinely done
Red – not routinely done
Enzyme analyses Muscle Biopsies

Frozen Muscle
• Frozen muscle (vastus lateralis), transported dry ice
• 600 x g homogenates
• CI-CIV, CII+III + markers: citrate synthase (CS) & protein
• Reference ranges (n = ~70), normalized to CS, CII & CIV
Clinical & Biochemical profile

- Heterogeneous clinical profile, few “classical”/syndromic phenotypes
- Black African patients predominantly muscle phenotype*

RC enzyme deficiencies:

- 129/200 (65%)
  - 65 Single enzyme (50%)
    - 42 CI (65%)
    - 4 CII (6%)
    - 15 CIII (23%)
    - 4 CIV (6%)
  - 64 combined enzyme (50%)
    - 40 CII+III (63%)
    - 24 other (37%)

*Smuts et al, 2010, J Inh Met Dis;
Molecular Genetics - strategy

**Patients**
Selection based on clinical & RC enzyme data

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**mtDNA sequencing (16.5 kbp)**
- 112 patients, two overlapping PCR fragments
- Bar coding, Roche 454 and Ion Torrent PGM sequencing
- Average base coverage: ~200

**Data analysis**
- Align to rCRS & identify variants (*Variant Caller/CLCBio*)
- Classify variants: High/low confidence
- Assign haplogroups (*Mitovariome/Phylotree*)
- Identify novel/previoulsy reported pathogenic variants using panel of databases (*dbSNP, MITOMAP, mtDB, mtSNP, Google*)
- Classify novel variants: damaging or less damaging – *Alamut, MitoTool*

**Assessing pathogenicity**
- Novel candidate pathogenic variants: Case-by-case, genetics, structural and functional (cybrids + Seahorse XF® 96) analyses

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**Nuclear DNA sequencing (550 kbp coding)**
- 128 patients (grouped based on deficiency)
- Target enrichment, Ampliseq (either CI, CII, CIII, CIV, CoQ10 panels); Ion Torrent PGM, 318 chip
- Average base coverage: ~250

**Data analysis**
- Torrent Suit (v5.0.2): alignment and variant calling
- Secondary data analysis: Ensemble variant effect predictor (VEP runner V85)
- Data mining: GEMINI (V0.18.)
  - Identify previously reported and novel variants
  - Identify previously reported pathogenic variants

**Assessing pathogenicity**
- Novel candidate pathogenic variants: Case-by-case, genetics, structural and functional analyses
Molecular Genetics – Results summary

• mtDNA investigations*:  
  □ General lack of common pathogenic mtDNA variants  
  □ 11 previously reported disease-associated variants  
  □ Conflicting reports on pathogenicity of previously reported mutations  
  □ Large number of novel variants (significantly more in African patients)  
  □ 20 candidate novel possible pathogenic variants in 39 cases  
  □ Pathogenicity evaluations key  
  □ **Two confirmed cases with mtDNA involvement (large deletion; m.14484 T>C), = ~1% prevalence**

• nDNA investigations (*Maryke Schoonen poster*):  
  □ At least 9 genes in 30 patients identified  
  □ Confirmation of these cases required

* van der Westhuizen et al, 2010, J Inh Met Dis 33, S55-62  
van der Walt et al, 2012, Eur J Hum Genet 20, 650-656  
van der Westhuizen et al, 2015, Human Mut 36, 569-571
Metabolome investigations in MD

Lecture: Roan Louw
Conclusions and Future directions

• MD diagnostics:
  - Sufficient local expertise available
  - Research environment required - fundamental understanding of bioenergetics, mitochondrial physiology & metabolism, and human genetics

• Paediatric cohort (UP neurology clinic):
  - Biochemical evaluations valuable, but can improve
  - Metabolomics: potential proven
  - Molecular genetics:
    - mtDNA involvement lower than norm (~1% cases)
    - nDNA wide ranging candidates (~15% cases)
    - Differs from data from NHLS referrals
    - More SA population genetic data required, and for patients a more extensive genomic approach at this time

• South Africa/Africa:
  - Patients access to specialized clinics very low
  - Collaboration & networks of specialized expertise
  - Consolidated national diagnostic strategy (processes, funding, research etc.)
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South Africa

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