Mitochondrial disorders in the South African context: A clinical and biochemical approach

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Mitochondriale toestande in die Suid-Afrikaanse konteks: ’n Kliniese en biochemiese benadering

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ABSTRACT

In the past, patients with suspected mitochondrial disorders (MDs) were identified only phenotypically in South Africa. However, the specific population-related characteristics were unknown and not documented. Up to as late as 1998, no facility was available in South Africa to confirm the diagnosis of MDs. The diagnosis of MDs is challenging under the best of circumstances, and thus it posed an opportunity to develop imaginative diagnostic strategies in a developing country such as South Africa with other major health-related issues. In order to develop a comprehensive service in a country burdened by tuberculosis and human immunodeficiency virus, it was important firstly to define the patient population. It was found that the MD phenotype in the South African population was unique compared with described populations in other countries. African patients predominantly presented with a myopathy and combined enzyme deficiencies in contrast to Caucasian patients, who predominantly presented with an encephalopathy or encephalomyopathy and tended to have more single enzyme deficiencies.

Interesting case presentations were identified, including a young adult male patient who presented with Kearns–Sayre Syndrome (KSS). A novel deletion of 3,431 base pairs (bp) between positions 7,115 and 10,545, flanked by a five bp direct repeat sequence, was found in 80% of this patient‘s muscle mitochondrial DNA (mtDNA). It was also demonstrated in this case that the absence of mtDNA-encoded *ATPase6* and *ATPase8* genes resulted in the aberrant synthesis of adenosine triphosphate (ATP) synthase.

Obtaining muscle biopsies in children was extremely difficult and was also complicated by patients living in remote areas with limited access to health care facilities. Consequently, an alternative, less invasive option of analyzing urine was investigated.

A metabolomics approach was evaluated by firstly investigating the organic acid-containing section of the metabolome, obtaining urine of patients with respiratory chain disorders (RCDs). It was possible to compile the first comprehensive list of 24 metabolites associated with RCDs, which were, both statistically and practically, significantly elevated. Secondly, a global metabolic profile involving carbohydrate, amino acid and fatty acid catabolism was also constructed. It clearly illustrated the diversity and complexity of the complex biochemical consequences in RCDs and that there was no single characteristic organic acid biomarker profile to distinguish between the complex I (CI), CIII and multiple complex deficiencies. Thirdly, amino acid and carnitine analyses were added to the metabolic profile to assist further in the development of an explorative biosignature. Finally, a biosignature comprising of six organic acids, six amino acids and one other marker was constructed. It included succinic acid, lactic acid, 3-OH-isobutyric acid, 3-OH-valeric acid, 3-OH-3-Me-glutaric acid, 2-OH-glutaric acid, α-aminoadipic acid, glutamic acid, alanine, glycine, serine, tyrosine, and creatine.

Differences between population groups, as observed in the clinical study, were not observed in the metabolomics studies, but the statistical processes in variable and case selection aimed to have complete separation between controls and patients. This resulted in a more homogenous patient group, a prerequisite to identify markers for RCDs. A limited number of Caucacian patients was finally included in the two different metabolomics studies, 11/39 (28.2%) and 5/20 (25.0%) respectively. Except for one Caucasian patient with a pure encephalopathy, all the others had muscle involvement as well. The clinical differences observed between African and Caucasian patients therefore remain to be investigated on a biochemical level in a follow up study.

This study was a multi-disciplinary project, with a clinical-biochemical approach, since 2009, which successfully described the South African RCD patient profile and which can lead to the development of a refined diagnostic service in South Africa. In addition, a significant

outcome of the study was the development of a potential biosignature in urine to assist in and simplify the diagnostic process in future.

The scientific contributions of this study resulted in five publications: two articles were published and one was submitted for publication in the Journal of Inherited Metabolic Disease, one article was published in Metabolomics and one was published in the South African Paediatric Review.

Keywords: mitochondrial disorders; respiratory chain disorders; South Africa; metabolomics; biosignature.

OPSOMMING

Pasiënte met moontlike mitochondriale defekte (MDe) was in die verlede alleenlik fenotipies in Suid-Afrika geïdentifiseer, maar die spesifieke populasie-verwante eienskappe was egter onbekend en nie gedokumenteer nie. Selfs so laat as in 1998, was daar geen fasiliteit in Suid-Afrika beskikbaar om die diagnose te bevestig nie. Aangesien die diagnose van MDe 'n groot uitdaging is onder die beste omstandighede, het dit 'n geleentheid geskep om verbeeldingryke diagnostiese strategieë te ontwikkel in 'n ontwikkelende land met ander belangriker gesondheidsverwante probleme. Om 'n omvattende diens te vestig in 'n land wat geteister word met tuberkulose en menslike immuungebrek virus, was dit belangrik om eerstens die pasiëntpopulasie te definieer. Wat MDe betref was daar gevind dat die Suid-Afrikaanse populasie uniek is in vergelyking met populasies beskryf in ander lande. Die Swart pasiënte presenteer predominant met 'n miopatie en 'n gekombineerde ensiemdefek teenoor die Kaukasiese pasiënte wat hoofsaaklik presenteer met enkefalomiopatie en neig om eerder enkel ensiemdefekte te hê.

Interessante gevallestudies was geïdentifiseer, onder andere 'n jong volwasse manlike pasiënt met Kearns–Sayre Sindroom (KSS). 'n Nuwe delesie van 3,431 basispare (bp) tussen posisies 7,115 en 10,545 met vyf bp direkte sekwensherhaling aan die kante was in 80% van hierdie pasiënt se spier- mitochondrial DNA (mtDNA) gevind. Daar was verder aangetoon dat die afwesigheid van die mtDNA-gekodeerde *ATPase6* en *ATPase8* gene tot abnormale sintese van ATP sintase gelei het.

Om spierbiopsies in kinders te bekom was uiters moeilik en verder gekompliseer deur pasiënte wat in afgeleë areas woon en beperkte toegang tot gesondheidsorgfasiliteite het. n Minder indringende alternatiewe opsie om die uriene te analiseer was ondersoek. 'n

Metabolomika-benadering was gevolg, deur eerstens die organiese suur-bevattende gedeelte van die metaboloom in uriene van pasiënte met respiratoriese kettingdefekte (RKe) te beoordeel. Dit was moontlik om die eerste omvattende lys van 24 metaboliete saam te stel wat statisties en prakties, betekenisvol verhoog was. Tweedens was 'n globale metaboliese profiel van koolhidraat-, aminosuur- en vetsuurkatabolisme daaruit gekonstrueer. Dit illustreer duidelik die diversiteit en kompleksiteit van die ingewikkelde biochemiese konsekwensies in RKe. Daar was egter geen enkele karakteristieke organiese suur biomerkerprofiel om tussen kompleks I (KI), KIII en veelvuldige kompleksdefekte te onderskei nie. Derdens was aminosuur- en karnitienanalises bygevoeg tot die metaboliese profiel om verder by te dra tot die ontwikkeling van 'n eksploratiewe bioteken. Daar was in die finale fase 'n bioteken gekonstrueer wat uit ses organiese sure, ses aminosure en een ander merker bestaan het. Dit sluit suksiensuur, melksuur, 3-OH-isobottersuur, 3-OH-valeriaansuur, 3-OH-3-Me-glutaarsuur, 2-OH-glutaarsuur, α-aminoadipiensuur, glutamiensuur, alanien, glisien, serien, tirosien en kreatien.

Die verskille wat tussen populasiesgroepe opgemerk was in die kliniese studie, was nie waargeneem in die metabolomika studie nie, maar die statistiese proses om die veranderlikes en gevalle te kies was ten doel om 'n volledige skeiding tussen die kontroles en pasiënte te bewerkstellig. Dit het tot 'n meer homogene groep gelei, wat 'n voorvereiste was om merkers vir RKe te identifiseer. 'n Beperkte aantal Kaukasiese pasiënte, onderskeidelik 11/39 (28.2%) en 5/20 (25.0%) was in die twee onderskeie metabolomika studies ingesluit. Slegs een van die Kaukasiese pasiënte het 'n suiwer enkefalopatie gehad, terwyl al die ander ook wel spierbetrokkenheid getoon het. Die kliniese verskille tussen Swart en Kaukasiese pasiënte moet op 'n biochemiese vlak ondersoek word in 'n opvolgstudie.

Die studie was 'n multidissiplinêre projek, met 'n klinies-biochemiese aanslag, sedert 2009, wat daarin geslaag het om die pasiëntprofiel van RKe te beskryf en tot die ontwikkeling van

'n diagnostiese diens in SA gelei het. 'n Verdere belangrike uitkoms was die ontwikkeling van 'n moontlike bioteken in uriene wat 'n bydrae kan lewer tot die diagnose en vereenvoudiging van die diagnostiese proses in die toekoms.

Die wetenskaplike bydraes van die studie het gelei tot die publikasie van vyf artikels: twee was gepubliseer en een was ingedien vir publikasie in die "Journal of Inherited Metabolic Disease", een artikel was in "Metabolomics" en een was in die "South African Paediatric Review" gepubliseer.

Sleutelwoorde: mitochondriale defekte; respiratoriese kettingdefekte; Suid-Afrika; metabolomika; bioteken

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LIST OF ABBREVIATIONS

$[M^+H^+]$	protonated molecules
°C	degree centigrade
°C/min	degree centigrade per minute
12S-rRNA	12S ribosomal RNA
16S-rRNA	16S ribosomal RNA
^{1}H-MRS	proton magnetic resonance spectroscopy
^{31}P-MRS	phosphorous magnetic resonance spectroscopy
A	African
A	alanine
AA	amino acids
ABR	auditory brainstem response
AC	acylcarnitines
AcCoA	acetyl coenzyme A
AD	autosomal dominant
ADL	activities of daily living
ADP	adenosine diphosphate
AD-PEO	autosomal dominant progressive external ophthalmoplegia
ALS	amyotrophic lateral sclerosis
ANT	adenine nucleotide translocator
ANT1	adenine nucleotide translocator isoform1
AR	autosomal recessive
AR-PEO	autosomal recessive progressive external ophthalmoplegia
ASD	Autism spectrum disorders
ATP	adenosine triphosphate
ATP6	ATP synthase subunit 6

ATP8	ATP synthase subunit 8
ATPase	adenosine triphosphatase
AV	atrio-ventricular
BAER	Brainstem auditory-evoked responses
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pairs
C	Caucasian
C	cysteine
CACT	carnitine-acyl-carnitine translocator
CI to IV	respiratory chain enzyme complexes I to IV, respectively
CI to V	complexes I to V respectively of the entire OXPHOS system
CK	creatine kinase
CMT	Charcot-Marie-Tooth disease
CNS	central nervous system
CoA	coenzyme A
CoQ	coenzyme Q
CoQ_{10}	coenzyme Q_{10}
COX	cytochrome *c* oxidase
COXI	cytochrome *c* oxidase subunit I
COXII	cytochrome *c* oxidase subunit II
COXIII	cytochrome *c* oxidase subunit III
CPEO	chronic progressive external ophthalmoplegia
CPT-I	carnitine palmitoyltransferase
CPT-II	carnitine-acylcarnitine translocase
Cr	creatinine
Crea	creatine
CS	citrate synthase
CSF	cerebrospinal fluid

CT scan	computerised tomography scan
CuSOD	copper superoxide dismutase
CXR	chest X-ray
Cyt b	cytochrome b
Cyt *c*	cytochrome *c*
Cyt	cytochrome
D	aspartic acid
d	effect size
DD	developmental delay
DDP	deafness dystonia protein
DGUOK	deoxyguanosine kinase
DI	diabetes insipidus
DIC	dicarboxylate carrier
D-loop	displacement loop
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
e^-	electrons
E	glutamic acid
ECM	encephalomyopathy
EEG	electroencephalogram
EFG1	elongation factor G1
EGTA	ethylene glycol tetra-acetic acid
EMG	electromyography
ESI	electrospray ion
ESI-MS/MS	electrospray ionisation tandem mass spectrometry ESI-MS/MS
ETC	electron transport chain
ETF	electron-transfer flavoprotein
ETF-DH	electron-transfer dehydrogenase

eV	electrovolt
F	female
F	phenylalanine
FAD	flavine adenine dinucleotide
$FADH_2$	reduced flavine adenine dinucleotide
FBSN	familial bilateral striatal necrosis
Fe-S	iron-sulphur
FGF-21	fibroblast growth factor 21
FID	free induction decay
FP	flavoprotein fraction of complex I or II
FTT	failure to thrive
G	glutamine
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GIT	gastrointestinal tract
GPX	glutathione peroxidase
GRACILE	growth retardation, aminoaciduria, iron overload, lactic acidosis, early death
H	histidine
H^+	proton
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hypochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
HP	complex I hydrophobic protein
hrs	hours
HSP	hereditary spastic paraplegia
I	Indian
I	isoleucine
I-IV	complexes I-IV respectively in illustrations

IMM	inner mitochondrial membrane
IMS	inter membrane space
IP	iron-sulphur protein fraction of complex I or II
IQ	intelligence quotient
IUGR	intra uterine growth retardation
K	lysine
K^+	potassium ion
KSS	Kearns–Sayre Syndrome
L	lactate
L	leucine
L:P	lactate to pyruvate
LA	lactic acidosis
LC	liquid chromatography
LFT	liver function test
LHON	Leber‘s hereditary optic neuropathy
LIMD	lethal infantile mitochondrial disease
LS	Leigh syndrome
LS,FC	Leigh syndrome French-Canadian type
M	male
M	methionine
MA	mixed ancestry
MD	mitochondrial disorder
MDC	Mitochondrial Disease Criteria
MELAS	mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy with ragged red fibres
mg	milligram
MILS	maternally inherited Leigh syndrome
MIMyCa	cardiomyopathy and myopathy
min	minute

ml	millilitre
ml/min	millilitre per minute
MLASA	mitochondrial myopathy and sideroblastic anaemia
mM	milimolar
MNGIE	mitochondrial neuro-gastrointestinal encephalomyopathy
MnSOD	manganese superoxide dismutase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
mtDNA	mitochondrial DNA
N	asparagine
Na^+	sodium ion
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NARP	neuropathy, ataxia and retinitis pigmentosa
NCS	nerve conduction studies
ND	NADH dehydrogenase
nd	Not done
ND1	NADH-ubiquinone oxidoreductase subunit 1
ND2	NADH-ubiquinone oxidoreductase subunit 2
ND3	NADH-ubiquinone oxidoreductase subunit 3
ND4	NADH-ubiquinone oxidoreductase subunit 4
ND4L	NADH-ubiquinone oxidoreductase subunit 4L
ND5	NADH-ubiquinone oxidoreductase subunit 5
ND6	NADH-ubiquinone oxidoreductase subunit 6
nDNA	nuclear DNA
NDUFS7	NADH-ubiquinone oxidoreductase Fe-S protein 7
NDUFS8	NADH-ubiquinone oxidoreductase Fe-S protein 8

NH_3	ammonia
NIDDM	non-insulin dependent diabetes mellitus
NMR	nuclear magnetic resonance
NO	nitric oxide
nt	nucleotide
O_2	molecular oxygen
O_2^-	superoxide
OA	organic acids
O_H	origin for replication of the heavy strand
OH^-	hydroxyl ion
O_L	origin for replication of the light strand
OMM	outer mitochondrial membrane
$ONOO^-$	peroxynitrite
OS	oligosaccharides
OXPHOS	oxidative phosphorylation
P	proline
P	pyruvate
PC	pyruvate carboxylase
PCA	principal component analysis
PDH	pyruvate dehydrogenase
PDHc	pyruvate dehydrogenase complex
PEO	progressive external ophthalmoplegia
PLS	partial least squares
PLS-DA	partial least squares discriminant analysis
VIPs	variables important in projection
POLG	polymerase gamma
PPK	palmoplantar keratoderma
pt	point/points
PUS1	pseudouridine synthase 1

Q	glutamine
Q-TOF-LC/MS	Quadrupole Time-of-Flight liquid chromatography mass spectroscopy
R	arginine
RC	respiratory chain
RCD	respiratory chain disorder
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP	retinitis pigmentosa
rpm	revolutions per minute
RRF	ragged red fibres
rRNA	ribosomal RNA
S	serine
SA	South Africa
SANDO	sensory ataxic neuropathy, dysarthria, ophthalmoplegia
SCAD	short-chain acyl-coenzyme A dehydrogenase deficiency
SCAE	spinocerebellar ataxia and epilepsy
SD	standard deviation
SDH	succinate dehydrogenase
sec	seconds
SIDS	sudden infant death syndrome
SNHL	sensorineural hearing loss
SPE	solid phase extraction
S_x	standard deviation
T	threonine
TCA	tricarboxylic acid
TK	thymidine kinase
TMS	tandem mas spectrometry
TNF	tumour necrosis factor
tRNA	transfer RNA

TSP	tetradeuteropropionic acid sodium salt
V	complex V or F_1F_0-ATPsynthase in illustrations
V	valine
VEP	visual evoked potentials
W	tryptophan
Y	tyrosine
Zn^{2+}/Cu^{2+}SOD	zinc/copper superoxide dismutase
α	alpha
β	beta
µl	micro litre
µmol/mg	micromole per milligram

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CHAPTER 1

INTRODUCTION AND AIMS

I was appointed as a consultant in the Department of Paediatrics in 1997 with the instruction to develop a paediatric neurology service at the H.F. Verwoerd Hospital being the academic hospital of the Medical Faculty of the University of Pretoria at that time. I discovered very soon that I was provided with an empty page and an entire new narrative was about to evolve. I did not have an appropriate area available to consult patients and I had to arrange for a temporary office in the occupational therapy department about 1.0 km from the main building, with the waiting area for the patients on the veranda of the building. There were no facilities, not even a fridge was available. It was clear right from the start that there was a very interesting cohort of patients without diagnoses and the diagnostic facilities were limited. I was fascinated by the concept of energy metabolism in man and I considered mitochondrial disorders (MDs) in many of those patients. I initiated a long-term study of MDs in the South African context in 1998.

1.1 PROBLEM STATEMENT

A mitochondrial disorder (MD) is either confirmed genetically or through enzyme analyses of the oxidative phosphorylation system (OXPHOS). The initial approach of our study was clinical-genetic, as enzyme analyses were not available at that stage and we had limited access to genetic studies. Only a single known point mutation (m.3243A>G) was found in a group of 90 patients. Numerous polymorphisms and novel mutations were detected, but the significance was unknown and there was no opportunity to pursue any further studies to determine pathogenicity. The relative high cost involved for a very low yield of confirmed genetic diagnosis was unrealistic in our circumstances and we had to change our approach.

After this period, in 2004 a clinical-biochemical orientated approach was adopted. The confirmation of MDs then relied on enzyme analyses requiring fresh biopsy tissue samples, e.g. muscle. Although the problem might not necessarily be confined to South Africa alone, obtaining muscle biopsies in children was extremely challenging: patients sometimes had to wait four months or longer to have a muscle biopsy done and apart from the normal risks and costs of hospitalisation, anaesthetics and trauma, logistic problems of dealing with patients in remote areas of our country prevented many patients of being diagnosed properly.

In 2006 we moved into the newly built academic facility, since 2009 known as the Steve Biko Academic Hospital, Pretoria. It was a major milestone and we got access to a small laboratory on site which was used for sample preparation and storage. Initial sample preparation and adequate storage of specimens were standardised and the methods utilised for the determination of enzymatic activities of the various complexes of the respiratory chain (RC) were standardised and validated at the Division for Biochemistry of the North-West University (NWU), Potchefstroom. It was therefore decided that, for the purpose of the biochemical part of the study, it would be preferable to focus on the patients included in the study since June 2006 for comparative reasons.

Although the enzyme analyses could be done successfully, obtaining muscle specimens in children remained a hurdle in the South African context. The practical issues, in addition to the complex nature of the disease prompted us to revisit our strategy again. It was crucial to explore other alternatives to assist and simplify the diagnostic process relying more on clinical and less invasive biochemical markers. Urine was an attractive alternative because it was readily available, easy to store and transport. The use of a metabolomics approach in the investigation and unravelling of MDs was an additional enticing novelty. The comment of Smeitink *et al.* (2006): *–Understanding its [mitochondrial disease] metabolic consequences thus requires a multidisciplinary approach combining in vitro assays with in vivo studies in patients and animal models. Global analytical tools for profiling RNA, protein and metabolite*

levels are helping to piece together metabolic changes to OXPHOS defects…" inspired us to pursue a metabolomics approach.

The Department of Science and Technology (subdivision BioPAD) from the South African government, invested in the development of a Metabolomics Platform at the North-West University, Potchefstroom in 2006. This institution had an established knowledge base of more than 20 years in inherited metabolic disorders in Southern Africa. One of the main objectives of developing this platform was to investigate its possible application in metabolic disorders that were difficult to identify, especially MDs. It was a unique opportunity to extend the existing MDs diagnostic facility by including a metabolomics investigation.

In view of these developments, the existing mitochondrial programme was expanded to include sample collection, storage and transport of not only muscle samples from selected patients but also urine, which was done according to international recommendations of the Metabolomics Standards Workshop (Castle et al 2006). Correct handling and storage of samples were crucial aspects in South Africa as there was only one diagnostic facility available to perform the analyses for the entire country and transport of specimens was problematic.

In 2009 it was decided that the more comprehensive clinical-biochemical approach including metabolomics could form the basis of this PhD thesis.

This thesis did not focus on the genetic aspects and diagnosis of MDs as it was not routinely available, but it is acknowledged that for the success and sustainablitiy of the mitochondrial program emphasis should be put on mitochondrial genetics. With novel and more readily available techniques like second generation sequencing and exome sequencing to better investigate the aetiology of MD in the South African population, it is crucial to develop the research and service in this area as well.

1.2 RESEARCH AIMS AND OBJECTIVES

This study was part of a multi-disciplinary project which involved collaboration with a number of scientists from various disciplines in order to investigate whether the combined clinical and urine metabolic profile of patients suffering from respiratory chain disorders (RCDs) can be used to distinguish them from healthy controls and be used for the development of a biosignature to simplify the diagnostic process. In order to achieve this aim, the initial objective was to document the clinical profile of mainly paediatric patients with MDs in South Africa. The subsequent objective was to analyze the biochemical profile of selected patients by using a gas chromatographic-mass spectrometric (GC-MS) and a limited nuclear magnetic resonance (NMR) metabolomics approach including organic acid, amino acids, carnitines and other metabolites to identify a putative biosignature for RCDs.

1.3 STRUCTURE OF THE THESIS

This thesis was compiled in article format and consisted of four published papers and one manuscript submitted for publication. Chapter two included the relevant literature review.

Chapter three addressed the clinical and experimental investigation and consisted of two papers. The first was an overview of a cohort of South African patients with mitochondrial disorders. The second paper was discussing the metabolomics of urinary organic acids in respiratory chain deficiencies in children.

In Chapter four applications were discussed and included a unique case presentation of an African patient with Kearns–Sayre syndrome in whom a novel deletion in mtDNA was found and the absence of both the *ATPase6* and *APTase8* genes resulted in aberrant ATP synthase synthesis. The development of a biosignature was discussed in Section 4.3 and the fourth paper in Section 4.4 was to increase the awareness of mitochondrial disorders amongst clinicians in South Africa during the course of the study. Chapter five was the final chapter including the discussion and conclusions.

The reference style of the unpublished parts off this thesis was according to the style of the Journal of Inherited Metabolic Diseases, because two of the articles were published in it and a third submitted was submitted to the same journal for publication. The additional references occurring in chapters not appearing in the articles are listed at the end of each chapter. The references for the articles were listed at the end of each article in the required format of the specific journals. The thesis ended with supplementary material including copies of the informed consent and assent, mitochondrial disease criteria and a list of scientific contributions since the onset of the mitochondrial project in South Africa, copyright licences of the different journals and permission as well as the specific contributions of the various co-authors and finally the instructions to authors from the different journals. These addenda are prescribed material of the NWU for a thesis including scientific papers.

1.4 ETHICAL CONSIDERATIONS

Ethical approval was initially obtained from the Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (UP) in 1998 with the protocol number of 91/98. The protocol was updated regularly. All the amendments were approved by the ethics committee of the University of Pretoria. The protocol number at NWU was 02M02.

Patients and controls were included in this study only after the parent, legal guardian or the patient him/herself signed informed consent and, if appropriate, assent. All the patients had muscle biopsies done and urine was collected for the metabolomics study since 2006. Two control groups used for different purposes were included in this study. The first control group consisted of 24 patients with no clinical features of MDs that underwent routine orthopaedic procedures. Muscle specimens obtained from these surgical procedures were utilized as controls for the enzyme analyses only. The second group was a different group of apparently healthy children from whom urine was obtained for the metabolomics study.

1.5 REFERENCES

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CHAPTER 2

LITERATURE REVIEW

In the development of a service for the comprehensive management of patients with mitochondrial disorders (MDs) it is crucial to have a thorough understanding of the complexity of the mitochondrial structure, function and clinical manifestations influencing the diagnostic abilities and associated limitations. Only then can novel approaches be explored, refined and applied.

2.1 MITOCHONDRIAL STRUCTURE, FUNCTION AND CLINICAL APPLICATION

2.1.1 Introduction

Primary mitochondrial disorders are a heterogeneous group of genetically inherited conditions resulting in impaired oxidative phosphorylation (OXPHOS) affecting energy metabolism. The diagnosis of patients suffering from these disorders are usually suspected clinically and confirmed with biochemical analyses and/or molecular evaluations, as reviewed by Haas et al (2008). Koenig (2008) also mentioned in her review that mitochondrial dysfunction may be attributed to multiple other reasons due to the numerous metabolic pathways taking place in mitochondria, e.g. pyruvate dehydrogenase complex (PDHc), beta-oxidation, the carnitine cycle and the Krebs cycle. There are also numerous conditions, referred to as secondary MDs, in which mitochondrial dysfunction as a secondary phenomenon is implicated, e.g. diabetes, bipolar disease, schizophrenia, transient ischaemic attacks, stroke, epilepsy, fibromyalgia and neuropathic pain. Different classes of medications, e.g. anticonvulsants, analgesics, anti-depressants, antipsychotic, cholesterol medication, diabetic medication and anti-retroviral drugs, emerge as significant causes of mitochondrial damage, which may explain the adverse effects of specific drugs (Neustadt and Pieczenik 2008). For the purpose of this review the focus will be on primary MDs.

2.1.2 Epidemiology

Mitochondrial disorders were always regarded to be very rare, but it has become clear that they are much more common than originally estimated. They account for up to 30% of the aetiology in children with neurometabolic disorders (Zeviani et al 1996). According to Munnich et al (1996) 44% of children with MDs have neuromuscular symptoms. The prevalence of mitochondrial myopathies in the North East of England is stated to be at least 1 in 15,217 adults (Chinnery et al 2000) and mitochondrial encephalomyopathies within children under the age of 16 years have a prevalence of 1 in 21,277 (Darin et al 2001). Schaefer et al (2004) concluded, after combining epidemiological results of children and adults, that MDs have a minimum birth prevalence of 1 in 5,000, which is comparable to Duchene's muscular dystrophy with a prevalence of 1 in 5,000 (Darin and Tulinius 2000). In a follow-up study by Schaefer et al (2008) the high prevalence of MDs was confirmed and it was documented that 9.2 adults per 100,000 (1 in 10,870) have clinical MDs. Adults and children at risk to develop MDs were found to be 1 in 6,060 (Schaefer et al 2008). The susceptibility to develop MDs was illustrated in the study reported by Elliott et al (2008) where it was revealed that a pathogenic mtDNA mutation at various levels of allele frequencies (heteroplasmy) was detected in more than 1 in 200 live births. The prevalence of specific mutations are also common as illustrated by Rahman et al (2011) in their report on mt1555A>G with a prevalence of 1 in 385 in the 1958 British birth cohort. Biner-Glindzicz et al (2009) reported in a study done in children between the ages of 7 and 9 years of age a population prevalence of 1 in 520 for the mt1555A>G mutation. Vandebona et al (2009) reported an almost identical prevalence of 1 in 500 of the same mutation in the Australian population with European descent. The prevalence of MDs in South Africa (SA) is still unknown, but the clinical phenotypes are recognised in all the different populations of SA. Findings from elsewhere can also not be applied directly due to the ethnic diversity of the patient population.

2.1.3 Mitochondrial biology

Mitochondria are exceptionally important organelles in the cell. The main function is to convert chemical energy obtained from food (including fats, carbohydrates and amino acids) or body reserves into adenosine triphosphate (ATP), the „currency of energy" in the cell. This is achieved by utilizing oxygen through the process of OXPHOS on the inner membrane, via a complex chain of oxidoreductases known as the respiratory chain (RC) and the final step trough ATP synthase, (Spinazzola and Zeviani 2009). Mitochondria are responsible for the production of about 90% of the required cellular energy (Chance et al 1979) and the number of mitochondria per cell, which is controlled by the specific tissue's energy requirements, can range from hundreds to thousands per somatic cell (Scheffler 1997). The metabolic active tissue, e.g. skeletal muscle, brain, heart and liver usually has the larger numbers of mitochondria and the only cells without mitochondria are erythrocytes.

Apart from energy production, mitochondria are also involved in numerous other processes and have recently been reviewed eloquently in several publications (Psarra and Sekeris 2009; Spinazzola and Zeviani 2009). Mitochondria host a variety of metabolic processes including the Krebs cycle, β-oxidation, haeme biosynthesis and nucleoside precursor production. They are responsible for the maintenance of the Ca^{2+}, Fe^{2+} and Mg^{2+} pool and play an important role in the production of heat as well as reactive oxygen species (ROS) and apoptosis (Psarra and Sekeris 2009; Spinazzola and Zeviani 2009). They receive and integrate multiple regulatory signals, with steroid and thyroid hormones being major role players by stimulating mitochondrial messenger RNA (mRNA) synthesis (Enriquez et al 1999; Psarra and Sekeris 2009). Mitochondria consequently regulate metabolic processes, growth, development and, as mentioned before, apoptosis (Psarra and Sekeris 2009).

2.1.3.1 Metabolic processes in mitochondria involved in energy production

Glucose is metabolised through glycolysis producing two pyruvate molecules that enter the mitochondrion through the double membrane. One of two enzymes, pyruvate carboxylase

(PC) or the PDHc will then be activated according to the energy status of the cell. Pyruvate carboxylase is activated if there is a relatively high concentration of ATP in the cell, and the pyruvate molecules are rerouted in the direction of gluconeogenesis. If ATP is required, PDHc is activated and converts the two pyruvate molecules into two acetyl-coenzyme A (AcCoA) molecules which enter the Krebs cycle producing nine intermediates, six reduced nicotinamide adenine dinucleotide (NADH) and four reduced flavine adenine dinucleotide ($FADH_2$) molecules (Neustadt and Pieczenik 2008). Fatty acid oxidation contributes to the pool of AcCoA molecules: the fatty-acyl-CoA enters the mitochondrial matrix requiring carnitine, carnitine palmitoyltransferase (CPT-I) and carnitine-acyl-carnitine translocase (CACT). A spiral of β-oxidation follows, releasing AcCoA which enters the Krebs cycle (Figure 2.1) (Di Mauro and Schon 2003; Neustadt and Pieczenik 2008). Ketogenic amino acids (AA) are catabolised to AcCoA and enter the Krebs cycle. The NADH and $FADH_2$ carry the electrons to the RC with NADH, entering at complex I (CI) and the reduced flavins at complex II (CII) or complex III (CIII) (Figure 2.1) (Nicholls and Ferguson 2002a).

2.1.3.2 The oxidative phosphorylation system

The OXPHOS system consists of five multi-subunit complexes, which comprise the respiratory chain (RC) complexes I-IV, complex V or F_1F_0-ATP synthase, as well as two electron carriers, coenzyme Q_{10} (CoQ_{10}) and cytochrome *c* (cyt *c*). All of these are associated with the inner mitochondrial membrane (IMM). A detailed discussion on the different components is beyond the scope of this review, but they are well described by Nicholls and Ferguson (2002a). The characteristics of the OXPHOS system are illustrated in Figure 2.2 and summarised in Table 2.1. Complex I (CI, NADH: ubiquinone oxidoreductase or NADH dehydrogenase or NADH-Coenzyme Q reductase, E.C. 1.6.5.3) is the largest of the complexes. For bovine complex I it is established to have 45 subunits (Carroll et al 2006) of which only seven (ND1, -2, -3, -4, -4L, -5 and -6) are encoded by mtDNA and the rest by nDNA. Complex II (CII, succinate:ubiquinone oxidoreductase or succinate dehydrogenase (SDH), E.C. 1.3.5.1) has four subunits and is the only complex encoded solely by nDNA.

Complex III (CIII, ubiquinol:ferricytochrome *c* oxidoreductase or ubiquinol cytochrome *c* reductase, E.C. 1.10.2.2) has one mtDNA and ten nDNA encoded subunits. Complex IV (CIV, cytochrome *c* oxidase (COX) or ferrocytochrome-*c*:oxygen oxidoreductase, E.C. 1.9.3.1) has three mtDNA and ten nDNA encoded subunits. Finally, complex V (CV, ATP phosphohydrolase or F_1F_0-ATP synthase, E.C. 3.6.1.3) consists of two mtDNA and ~14 nDNA encoded subunits (Table 2.1; Figure 2.2) (DiMauro and Schon 2003; Zeviani and Di Donato 2004). Electrons derived from NADH and $FADH_2$ are transported along these complexes and ultimately form water and ATP.

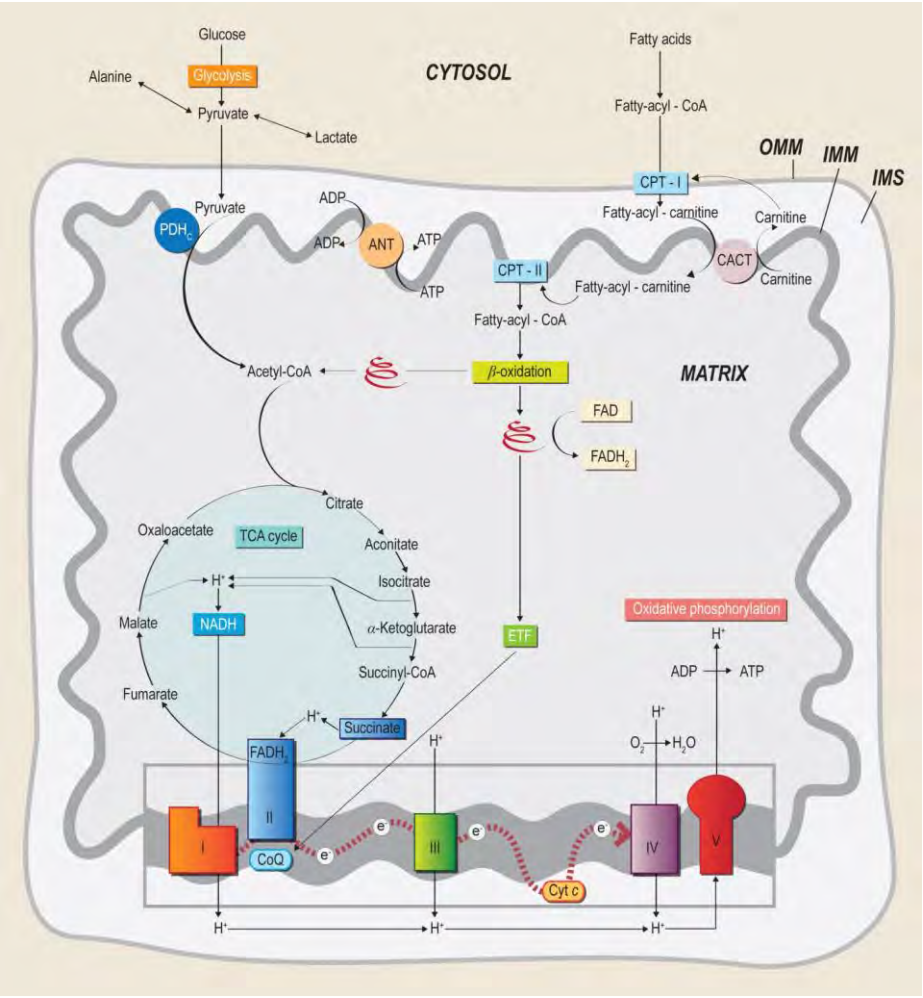

Figure 2.1 Selected metabolic pathways in mitochondria involved in energy production

The spirals indicate *β*-oxidation resulting in the production of acetyl-coenzyme A. Roman numbers I to IV, respiratory chain enzyme complexes I to IV, respectively; V, Complex V or F_1 F_0- ATPase; ADP, adenosine diphosphate; ATP, adenosine triphosphate ; ANT, adenine nucleotide translocator; CACT, carnitine-acyl-carnitine translocator; CoA, Coenzyme A; CoQ, coenzyme Q; CPT-I, carnitine palmitoyltransferase; CPT-II, carnitine-acylcarnitine translocase; Cyt *c,* cytochrome *c*; ETF, electron-transfer flavoprotein; FAD, flavine adenine dinucleotide; $FADH_2$, reduced flavine adenine dinucleotide; IMM, inner mitochondrial membrane; IMS, inter membrane space; NADH, reduced nicotinamide adenine dinucleotide; OMM, outer mitochondrial membrane; PDHc, pyruvate dehydrogenase complex; TCA, tricarboxylic acid. Adapted from DiMauro and Schon 2003.

NADH donates its electrons via CI and $FADH_2$ via CII to the RC. This occurs via a chemiosmotic process where protons are pumped simultaneously across the inner mitochondrial membrane by CI, III and IV into the mitochondrial matrix to form an electrochemical gradient across the IMM (Mitchell 1961). ATP is then generated if these protons enter the mitochondrion again via CV and to form ATP. Alternatively, the electrochemical gradient can be disrupted by uncoupling proteins which are also situated in the IMM. The energy status and metabolic needs of the cell and mitochondrion are responsible for the balance between the two components of OXPHOS namely respiration (oxidation) and phosphorylation (ATP synthesis) (Spinazzola and Zeviani 2009). Coenzyme Q_{10} acts as an electron carrier from CI and CII to CIII while cytochrome-*c* shuttles the electrons from CIII to CIV (Nicholls and Ferguson 2002b).

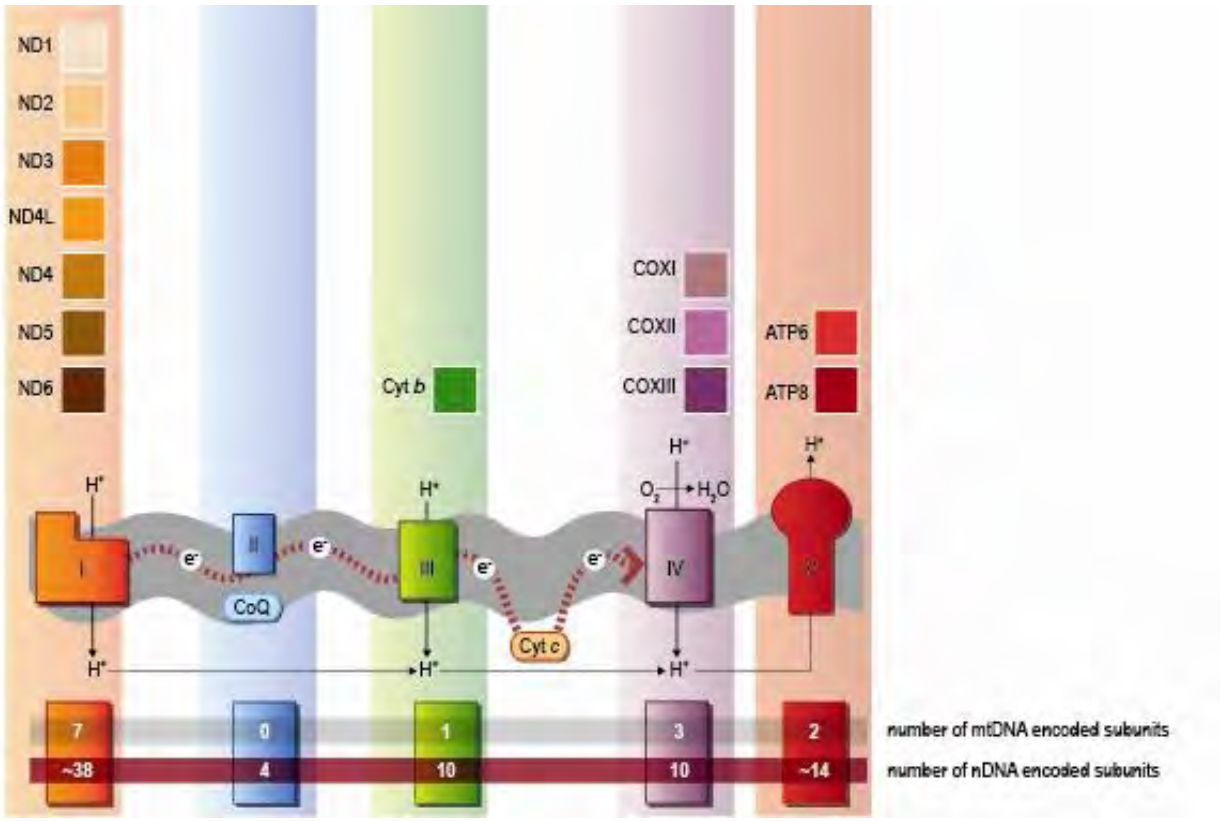

Figure 2.2 The OXPHOS system

Roman numbers I to IV, respiratory chain enzyme complexes I to IV, respectively; V, complex V or F_1F_0–ATP synthase; mtDNA-encoded genes are indicated in the top part of the figure. ATP6, ATP synthase subunit 6; ATP8, ATP synthase subunit 8; CoQ, coenzyme Q; COXI, cytochrome *c* oxidase subunit I; COXII, cytochrome *c* oxidase subunit II; COXIII, cytochrome *c* oxidase subunit III; Cyt *b*, cytochrome *b;* Cyt *c,* cytochrome *c;* mtDNA, mitochondrial DNA; nDNA, nuclear DNA; ND1-6, NADH dehydrogenase subunits 1-6 respectively. The OXPHOS system comprises of complexes I to IV of the respiratory chain and complex V or F_1F_0–ATP synthase. The electrons (e^-) are transferred from complex I to complex IV, via CoQ and Cyt *c.* Complex IV transfers the electrons then to oxygen (O_2), the final electron acceptor. A proton gradient builds up as a result of CI, CIII and CIV that pump protons (H^+) across the membrane. Complex V finally produces ATP. Adapted from DiMauro and Schon 2003.

Table 2.1 Components of the OXHOS system

	Systematic and alternative names	E.C. number	Number of subunits	nDNA encoded subunits	mtDNA encoded subunits
CI	NADH:ubiquinone oxidoreductase NADH:coenzyme Q reductase NADH:ubiquinone reductase (H^+-translocating)	1.6.5.3	~45	~38	7 (ND1, 2, 3, 4, 4L, 5, 6)
CII	succinate:ubiquinone oxidoreductase succinate dehydrogenase succinate dehydrogenase (ubiquinone)	1.3.5.1	4	4	0
CIII	ubiquinol:ferricytochrome-*c* oxidoreductase ubiquinol-cytochrome-*c* reductase cytochrome *c* reductase	1.10.2.2	11	10	1 (cytochrome *b*)
CIV	ferrocytochrome- *c*:oxygen oxidoreductase cytochrome *c* oxidase	1.9.3.1	13	10	3 (COX I-III)
CV	ATP phosphohydrolase adenosinetriphosphatase (ATPase) ATP synthase	3.6.1.3	~16	~14	2 (ATPase 6 and 8)
CoQ_{10}	Coenzyme Q_{10} Ubiquinone	*	*	*	*
Cyt *c*	Cytochrome *c*	*	*	*	*

CI-V, OXPHOS complexes I-V respectively; CoQ_{10}, Coenzyme Q_{10}; NADH, Nicotinamide adenine dinucleotide; Cyt *c,* cytochrome *c*; ATP, adenosine triphosphate; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; COX, cytochrome oxidase; ATPase, adenosine triphosphatase. Compiled from DiMauro and Schon 2003; Zeviani and Di Donato 2004; OMIM http://www.ncbi.nlm.nih.gov accessed on 26 August 2009; http://www.chem.qmul.ac.uk/iubmb/enzyme/ and http://www.brenda-enzymes.org/index.php4, both accessed on 18 Oct 2011.

2.1.3.3 The mitochondrial genome

Mitochondria are the only organelles containing their own unique DNA in the form of maternally inherited mitochondrial DNA (mtDNA). The mitochondrial genome in man (GenBank NC_012920.1) consists of 16,569 base pairs with 37 genes, encoding 13 structural subunits of the RC, 22 transfer-RNAs (tRNAs), two ribosomal RNAs (rRNAs), and do not contain any introns (Anderson et al 1981) (Figure 2.3). The Cambridge reference sequence was revised by Andrews et al (1999) and they advised that the ten simple substitutions in the original sequencs should be corrected, the rare polymorphisms should be retained and that the revised Cambridge reference sequence should be a true reference and not be regarded as a consensus sequence only.

The mitochondrion has the ability to produce and process its own RNA as well as proteins, but the remainder of the 1,000-2,000 proteins constituting the mitochondrial proteome are nuclear encoded. They include factors required for protein importation, folding and assembly factors of the RC and ancillary proteins required for mtDNA replication, transcription, translation and maintenance factors (Neupert and Herrmann 2007; Mokranjac and Neupert 2009). Each cell contains two to ten mtDNA copies and some of these genomes may contain nucleotide variations of variable frequencies per copy, which is a phenomenon called „heteroplasmy" (Zeviani and Di Donato 2004). A specific number of these mutations (when it's a pathogenic variation) should be present, or reach a tissue specific threshold, before clinical signs become evident (DiMauro and Schon 2003).

2.1.4 Consequences of OXPHOS dysfunction

Impairment of the OXPHOS results in numerous upstream as well as downstream effects is a specific area of research, and a comprehensive description of it is beyond the scope of this review. The most important aspects have been reviewed by Koopman et al (2004). To summarize: electrons accumulate due to ineffective shuttling through CI-CIV and the electron carriers, ubiquinone and cyt *c*. The electrons leak and react with oxygen to form

ROS. Complexes I and III are the main contributors to the pool of superoxide radicals (O_2^-), but other sources are α-ketoglutarate dehydrogenase from the Krebs cycle and the subunit SdhB of CII. Of the O_2^- not scavenged by manganese superoxide dismutase (MnSOD), zinc / copper superoxide dismutase (Zn^{2+} / Cu^{2+} SOD), antioxidants, metallothioneins or

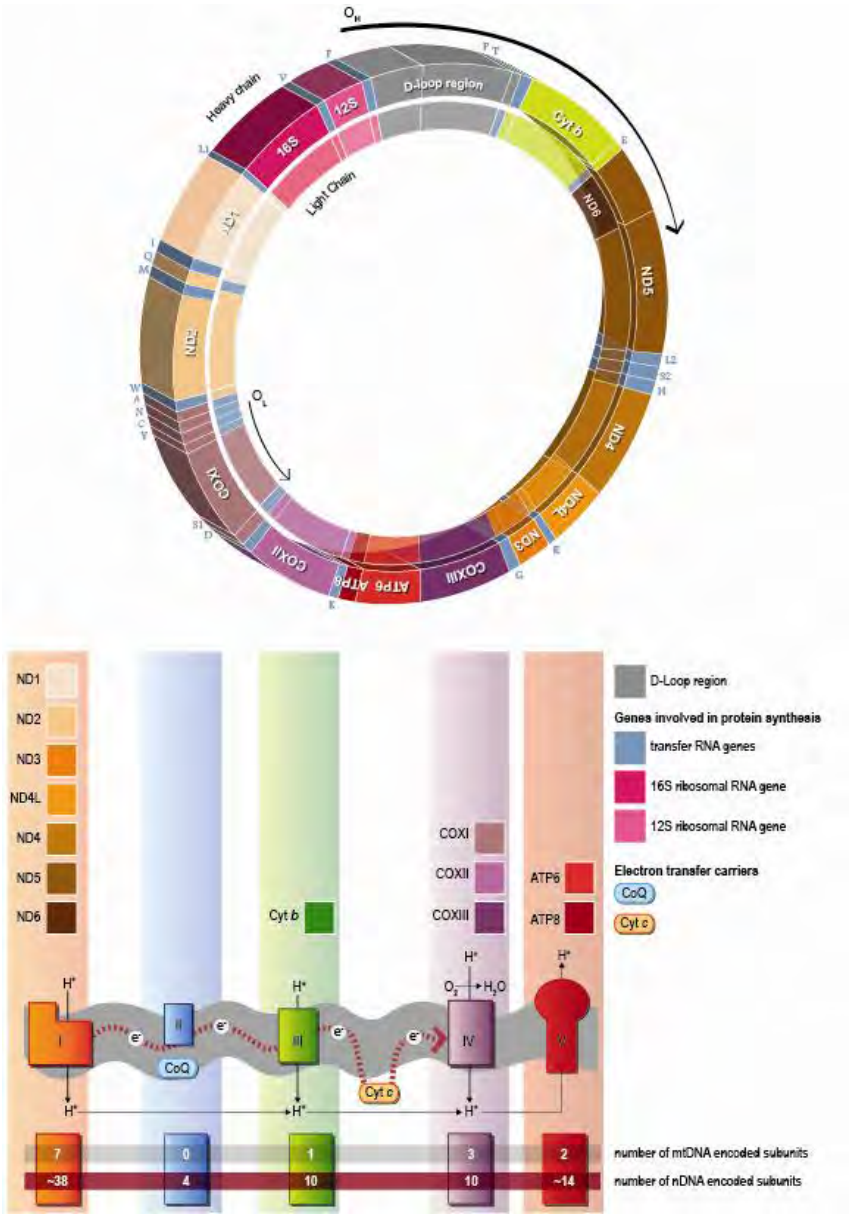

Figure 2.3 The mitochondrial genome

The heavy chain encodes all genes indicated on the outer circles and the light strand the tRNA genes coloured blue on the inner circle and ND6. All the tRNA genes are indicated with blue and by the single letter amino acid abbreviation. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glutamine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; ATP6, ATP synthase subunit 6; ATP8, ATP synthase subunit 8; CI to IV, respiratory chain enzyme complexes I to IV, respectively; CoQ, coenzyme Q; COXI, cytochrome *c* oxidase subunit I; COXII, cytochrome *c* oxidase subunit II; COXIII, cytochrome *c* oxidase subunit III; Cyt *b*, cytochrome *b;* Cyt *c,* cytochrome *c;* D-loop, displacement loop; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; ND1-6, NADH dehydrogenase subunits 1-6 respectively. O_H, origin for replication of the heavy strand; O_L, origin for replication of the light strand. Adapted from DiMauro and Schon 2003.

converted into water by glutathione peroxidase (GPX), are further converted into other ROS such as hydrogen peroxide (H_2O_2), hydroxyl ion (OH) and reactive nitrogen species (RNS), e.g. nitric oxide (NO) and peroxynitrite ($ONOO^-$). ROS and RNS damage macromolecules, alter protein function, act as messengers to induce genes involved in maintenance and restoration of the cell‘s redox balance (Reinecke et al 2009).

It is often assumed that deficient OXPHOS will lead to decreased ATP production and therefore explain the mechanism of symptoms related to MDs. However, ATP synthesized extra-mitochondrially can be transported into the mitochondria to supply ATP-dependent reactions within the mitochondria (Cabrera et al 2005).

Another consequence of an altered redox state of the cell is the accumulation of pyruvate, which is converted into lactate by lactate dehydrogenase. This may result in lactic acidosis and disturbed cellular pH (Munnich et al 1992). Pyruvate is further converted to alanine by alanine aminotransferase resulting in an elevated level of alanine, often found in patients with an OXPHOS related disorder. Proline may be elevated as a result of decreased oxidation due to an elevated lactate level and the citrulline concentrations may be very low (Rabier et al 1998).

The altered $NADH/NAD^+$ and ADP/ATP ratios modulate the regulation of several dehydrogenases involved in energy metabolism and, amongst other metabolic processes, may result in an increased ketone body ratio (β-hydroxybuterate:aceto-acetate) (Munnich et al 1992). Furthermore, mitochondria are important in the maintenance of Ca^{2+} pool and homeostasis which, if disrupted along with increased ROS, may result in the opening the mitochondrial transition pore and initiation of apoptosis (Nicholls 2005).

Apoptosis may be induced as a consequence of mitochondrial dysfunction or, on the contrary, be inhibited by dysfunctional OXPHOS resulting in cells that die too early or fail to die when they should. Intramitochondrial signalling of apoptosis is complex and multiple factors are involved, including the electrochemical proton gradient, ROS production,

metabolites and ion concentrations as well as ATP levels (Smeitink et al 2006). There are adaptive responses to these changes. In addition to the well described antioxidant defence system within and outside of the mitochondrion, transcriptional responses of mtDNA and nDNA also include OXPHOS and other genes. These responses, which have been studied in a limited number of disease models, are highly diverse and often inconsistent as reviewed by Reinecke et al (2009).

2.1.5 Classification of mitochondrial disorders

2.1.5.1 Introduction

The number of MDs associated with specific mutations has increased dramatically since the first disorder was described by Luft et al (1962), especially after the discovery of the mitochondrial genome (Anderson et al 1981). The fact that mitochondrial function is under dual genetic control implies that the genetic defect of MDs may be caused by mutations in nDNA or mtDNA. The number of mutations is rapidly growing and around 825 different mutations or candidate mutations have already been reported on MITOMAP (http://www.mitomap.org) by September 2011 as summarised in Table 2.2. Although not all of these reported mutations might be pathogenic, it clearly reflects the amount of data generated and highlights the complex, but crucial issue of proving pathogenicity in order to form a clearer understanding of the genetic control of MDs. It is further important to mention that a comprehensive review of the genetic origin of MDs is beyond the purpose of this thesis, which focusses on the biochemical and clinical aspects. However, it remains important to have general understanding of the molecular concepts in order to develop an approach in the management of MDs. Accordingly, only the most essentialgenetic aspects will be presented here. Experimental models, including transmitochondrial cybrids, yeats and mouse models as reviewed by Tuppen et al (2010), have contributed considerably in the understanding of molecular mechanisms of MDs, but it falls beyond the purpose of this review to be discussed in detail as the focus of the study is on clinical and biochemical aspects.

Table 2.2 Overview of mutation types as reported in MITOMAP

mtDNA
Reported base substitutions (including confirmed, reported, unclear, polymorphism or possible haplogroup defining mutations)
Coding and control region point mutations
rRNA and tRNA
mtDNA deletions
Multiple deletions
Pathogenic inversion
Simple insertions
Complex rearrangements
nDNA
Structural nuclear genes
Non-structural genes involved in
Complex assembly
mtDNA stability
Mitochondrial import
Mitochondrial protein synthesis
Iron homeostasis
Chaperone function
Mitochondrial integrity
Mitochondrial metabolism

Compiled from MITOMAP http://www.mitomap.org accessed on 10 September 2011

Although numerous mutations have been reported that may direct molecular genetic investigations, it is still an enormous task to characterize patients genetically. It was suggested that only ~50% of adults and 10-20% of paediatric patients will have a clear genetic basis identified for their specific MDs (Zeviani and Di Donato 2004). In a more recent high-throughput molecular investigation of a relatively large cohort of patients with CI deficiency, which is the most frequently occurring RC deficiency, it was also found that only ~50% of the cases could be resolved genetically (Calvo et al 2010). Swalwell et al (2011) have reported that mtDNA mutations were found in 29% of their patients with CI deficiencies and that nuclear genes were involved in 38% of CI deficient patients. Taking into account that pathogenicity of mtDNA variations is not consistently proven (Montoya et al 2009), mtDNA point mutations account for up to 40% of MDs in adults, but only ~ 10-25% in paediatric cases (McFarland et al 2004; Munnich and Rustin 2001; Zeviani and Carelli

2003). Bernier et al (2002) reported that mtDNA mutations were found in only 5% of paediatric patients and that nuclear DNA mutations are responsible for up to 90% of mutations found in children (DiMauro and Hirano 2005; Lamont et al 1998). Table 2.3 summarises the genetic classification of MDs and the complicated interplay between nDNA, mtDNA and the OXPHOS is further illustrated in Figure 2.4.

Table 2.3 Generic genetic classification for mitochondrial disorders

mtDNA mutations affecting:	**nDNA mutations affecting:**
Protein synthesis tRNA rRNA	RC subunits
RC subunits	Assembly proteins
	Intergenomic communication *Mitochondrial genome maintenance* Large scale deletion of mtDNA mtDNA depletion *Mitochondrial protein synthesis* Ribosomal protein Impaired mtDNA translation Initiation, elongation and release factors
	Post transcription modifying of mitochondrial tRNA
	Enzymes for lipids and cofactors biosynthesis
	Mitochondrial motility, fission, fusion

Adapted from DiMauro and Hirano 2005; Smeitink et al 2006; Spinazzola and Zeviani 2009; Zeviani and Di Donato 2004.

2.1.5.2 Mitochondrial disorders associated with mtDNA mutations

Mitochondrial disorders may arise from a variety of genetic variants in the mitochondrial genome which may be either sporadic or maternally inherited. They include mtDNA rearrangements, point mutations in the tRNA, rRNA or polypeptide encoding genes. Table 2.4 summarises a suggested classification as proposed by Mancuso et al (2007) and Table 2.5 summarises the mtDNA genes with known diseases causing mutations affecting the RC subunits (OMIM http//www.ncbi.nlm.nih.gov 2011). These tables are an attempt to organize a rapid expanding wealth of information for a clinician in order to develop at least a limited

degree of understanding. It will always have to be correlated with the latest literature. The use of well-established acronyms may also be confusing and not only have a wide genetic heterogeneity, but also an extensive intra- and inter-familial phenotypic heterogeneity (Filosto and Mancuso 2007; Montoya et al 2009). These conditions include examples like mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERRF), maternally inherited Leigh syndrome (MILS), Leber‘s Hereditary Optic Neuropathy (LHON), Lethal Infantile Mitochondrial Disease (LIMD), Leigh Syndrome (LS), Kearns–Sayre Syndrome (KSS), neuropathy, ataxia and retinitis pigmentosa (NARP) and progressive external ophthalmoplegia (PEO), are referred to as classical MDs or syndromic MDs and are summarised in Figure 2.5 (DiMauro and Schon 2003; Zeviani and Di Donato 2004).

The rearrangements of mtDNA can be deletions or duplications. The latter is less common and the former is usually the result of impaired intergenomic signalling orchestrated by genomic DNA (see Section 2.5.3.3). The length and location for the deletions can vary from 1.1 to 9.6 kilobases with an average of 5.1±1.6 (Yamashita et al 2008). Mita et al (1990) found that the position of the most common deletions fall between nucleotide 5835 to 12112 (Mita et al 1990). More recently Yamashita et al (2008) reported the most common positions for deletions to be between nt 5834 to nt 13911 and nt 9519 to 16123. The three well-known syndromes associated with large scale deletions include Pearson syndrome, KSS, and CPEO. The age of onset varies from early infancy for Pearson syndrome to the second decade for KSS and a later onset for CPEO. Pearson syndrome is very rare and presents in early infancy with anaemia and pancreatic insufficiency (Gillis and Kaye 2002; Larsson et al 1990). The anaemia disappears after one year of age (Yamashita et al 2008) and the survivors may develop KSS at an older age (Larsson et al 1990).

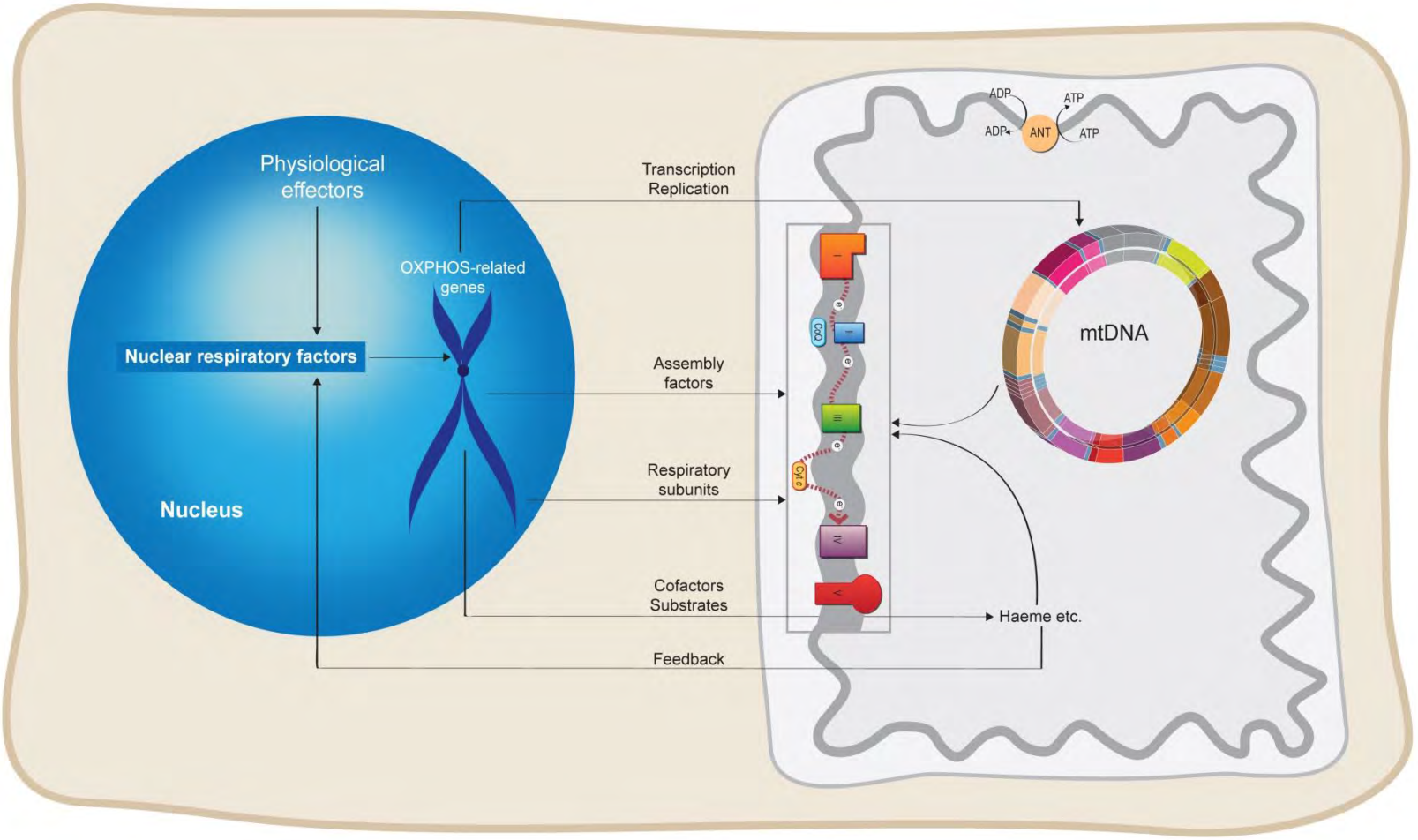

Figure 2.4 The interplay between nDNA, mtDNA and the OXPHOS system.

Both the nDNA and mtDNA encode the various components of the OXPHOS system and regulate through various factors including assembly factors, cofactors and substrates. Roman numbers I to IV, respiratory chain enzyme complexes I to IV, respectively; V, Complex V or F_1 F_0- ATPase; ADP, adenosine diphosphate; ATP, adenosine triphosphate ; ANT, adenine nucleotide translocator; CoQ, coenzyme Q_{10} ; Cyt c, cytochrome c; mtDNA mitochondrial DNA, nDNA, nuclear DNA. Adapted from Zeviani et al. 2003.

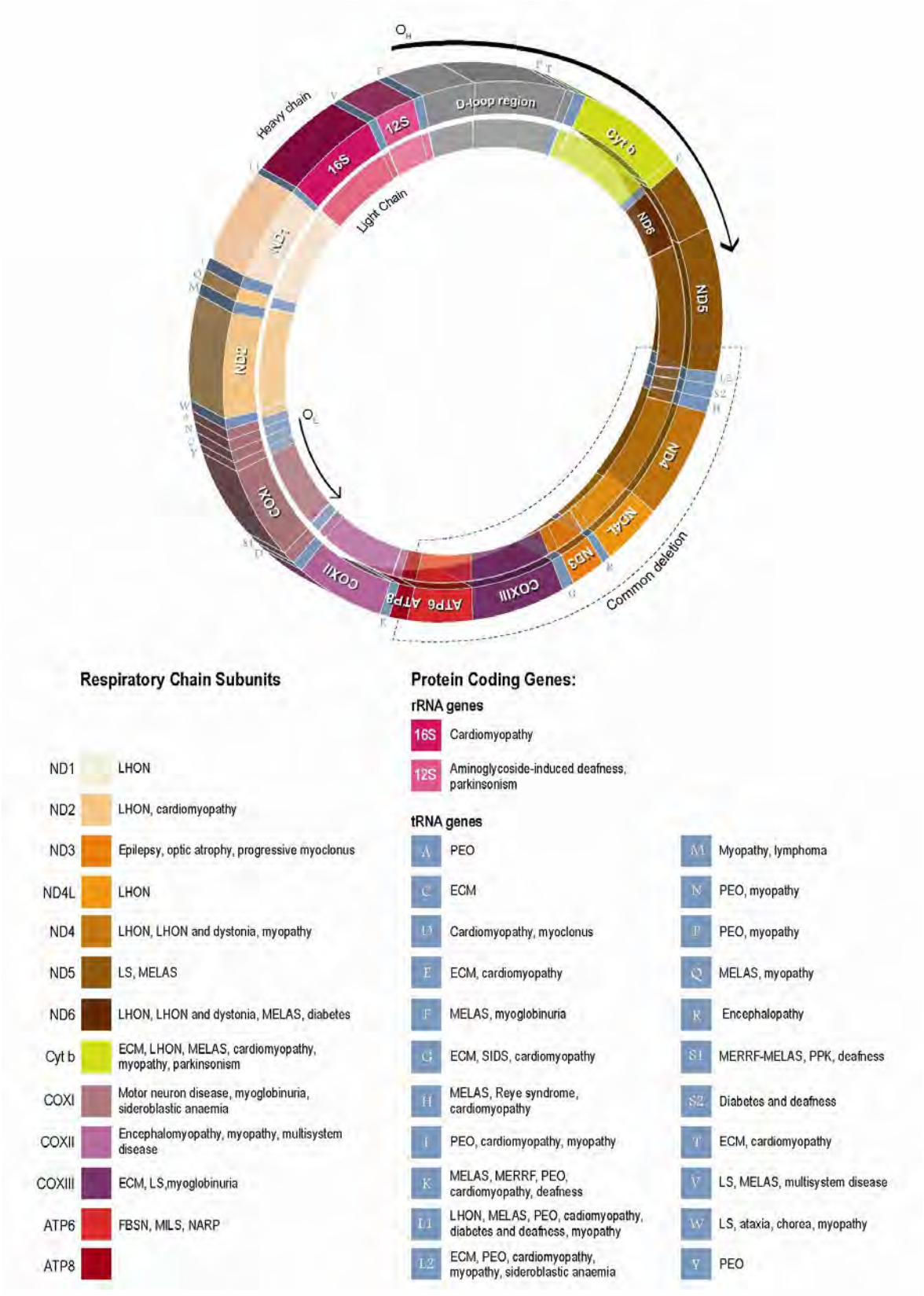

Figure 2.5 Schematic representations of well-known mitochondrial disorders due to mtDNA mutations

ECM, encephalomyopathy; FBSN, familial bilateral striatal necrosis; LHON, Leber's hereditary optic neuropathy; LS, Leigh syndrome; MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; MILS, maternally inherited Leigh syndrome; NARP, neuropathy, ataxia and retinitis pigmentosa; PEO, progressive external ophthalmoplegia; PPK, palmoplantar keratoderma; SIDS, sudden infant death syndrome. Adapted from Danks et al 1988; DiMauro and Schon 2003; Shin et 2000; Taylor et al 2004; Uusimaa et al 2004, Zeviani and Di Donato 2004.

Table 2.4 Classification of mtDNA mutations

Defect	Maternally inherited	Sporadic
mtDNA rearrangements		
	CPEO	KSS
	Multisystemic syndromes	Pearson syndrome
		CPEO
		Diabetes and deafness
mtDNA point mutations		
	Point mutations in polypeptide encoding genes	CPEO
	LHON	MELAS
	NARP	Exercise intolerance
	LS	Isolated myopathy
	Point mutations in tRNA encoding genes	
	MELAS	
	MERRF	
	MIMyCa	
	CPEO	
	Isolated myopathy	
	Diabetes and deafness	
	Hypertrophic cardiomyopathy	
	Tubulopathy	
	Point mutations in rRNA encoding genes	
	Aminoglycoside-induced non syndromic deafness	
	Hypertrophic cardiomyopathy	

CPEO, chronic progressive external ophthalmoplegia; KSS, Kearns–Sayre Syndrome; LHON, Leber's Hereditary Optic Neuropathy; LS, Leigh Syndrome; MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; MIMyCa, cardiomyopathy and myopathy; NARP, neuropathy, ataxia and retinitis pigmentosa; rRNA, ribosomal RNA. Adapted from Mancuso et al 2007.

Kearns-Sayre syndrome presents with progressive external ophthalmoplegia, retinitis pigmentosa, cardiac conduction defects, ataxia, myopathy, diabetes, short stature, and the CSF protein may be elevated (Kearns and Sayre 1958; Rowland 1983). Chronic progressive external ophthalmoplegia is depicted more as a muscle-specific disease, in contrast to KSS that is regarded as a systemic disorder (Rowland 1983; Yamashita et al 2008). Yamashita et al (2008) reported that KSS patients have significantly longer mtDNA deletions with more tRNAs involved than CPEO patients. They further reported that the patients with more tRNAs involved have an earlier onset of disease and deletions in *ND1* are more often associated with CPEO. Patients with earlier onset of symptoms, have involvement of areas including the COX or ATPase rather than ND and/or Cytb genes. It is also interesting that

the levels of heteroplasmy vary from 18% to 90% (52.2 ± 16.8%) and that there is no correlation between the age of onset, phenotype and heteroplasmy (Yamashita et al 2008).

Leber's hereditary optic neuropathy is the main phenotype due to point mutations in the polypeptide-encoding genes of the mtDNA. It is characterised by vision loss in one eye followed by the other eye within weeks to months in mostly young men due to optic neuropathy occasionally associated with cerebellar ataxia, cardiac conduction defects and peripheral neuropathy (Carelli et al 2004). The three point mutations in CI subunits are in nt 3460 of *ND1*, 11778 of *ND4* and 14484 of *ND6* and account for 95% of all cases (Mancuso et al 2007).

Maternally-inherited Leigh syndrome (MILS) has a highly variable phenotype, but one of the key features is subacute necrotizing encephalomyopathy presenting in the first year of life, associated with the mt8993T>C mutation in the ATPase6 gene (De Vries et al 1993). Other mutations in *ND1*, *ND3, ND4, ND5* and *ND6* have been reported as well (MITOMAP http://www.mitomap.org 2011).

Point mutations in tRNA encoding genes are very important and responsible for the majority of mitochondrial disease (Yarman et al 2010). Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) is an excellent description of the phenotype. The disorder is characterised by migraine-like headaches, seizures, short stature, vomiting, exercise intolerance and lactic acidosis, and the stroke-like episodes involve the posterior cerebral areas not irrespective of vascular distribution (Hirano et al 1992). The point mutations often associated with MELAS are the mt3243A>G and mt3271T>C mutations in the tRNA $^{Leu\ (UUR)}$ genes and in the subunits of CI (Servidei 2003). Myoclonic epilepsy with ragged red fibres (MERRF) includes myoclonus, ataxia, epilepsy and ragged-red fibres in the muscle. They may also have deafness, exercise intolerance, short stature and neuropathy (DiMauro et al 2002). The mutations are mostly in the $tRNA^{Lys}$ gene with the mt8344A>G mutations responsible for 80-90% of all the cases (DiMauro et al 2002; Jaksch

et al 1998). The other tRNAs involved are $tRNA^{Asp}$, $tRNA^{Val}$, $tRNA^{His}$ and $tRNA^{Phe}$ (Mancuso et al 2004).

The best-known point mutations in rRNA genes are the mt1555A>G and mt1292T>C mutations responsible for deafness after exposure to aminoglycosides (Bitner-Glindzicz et al 2009; Hutchin et al 1993; Rahman et al 2011). It is important, because these conditions can be prevented through neonatal screening programmes (Bitner-Glindzicz et al 2009).

The point mutations in mtDNA tRNA genes were usually functionally considered to be recessive, but the concept of dominance was introduced in the description of a mt5545C>T mutation in a boy. The pathogenic threshold for the cybrinds was only between 4 and 8% (Sacconi et al 2008).

In addition to qualitative changes in mtDNA as described above, quantitative changes or mt depletion may occur. It is the result of primary nuclear mutations responsible for mtDNA biosynthesis which lead to a reduction in the copynumber of mtDNA (Spinazolla and Zeviani 2009; Wong 2010)

Table 2.5 Examples of mtDNA genes involved in known disease causing mutations affecting the RC complexes

Genetic classification	Gene(s) involved	Phenotype
Mutations in RC subunits		
CI	*MTND1*	LHON; MELAS; NIDDM
	MTND2	LHON; MELAS
	MTND3	LHON + dystonia; LS
	MTND4	CPEO; LHON; LS Progressive dystonia
	MTND4L	CPEO; LHON; MELAS Sporadic myopathy
	MTND5	LHON; MELAS; MERRF Ataxia and PEO
	MTND6	LHON, MELAS, SNHL
	MTTS2	CPEO Deafness Myopathy
CIII	*MTCYB*	LHON ALS-like syndrome Encephalomyopathy Exercise intolerance Sporadic anaemia Sporadic myopathy
CIV	*MTCO1/COXI*	Prostate cancer Motor neurone disease Myopathy
	MTCO2/COXII	LHON; SNHL Myopathy
	MTCO3/COXIII	LHON; LS Alzheimer's disease Myoglobinuria Myopathy
	$tRNA^{Ser}$ */MTTS1*	CPEO; SNHL
	$tRNA^{Leu}$ */MTTL1*	CPEO; KSS; MELAS
CV	*ATPase 6*	LHON; MIDD; NARP
	ATPase 8	MILS FBSN

ALS, amyotrophic lateral sclerosis; ATPase, adenosine triphosphatase; CI to V, complexes I to V respectively of the entire OXPHOS system; COXI-III, cytochrome *c* oxidase subunit I-III respectively; CPEO, chronic progressive external ophthalmoplegia; FBSN, familial bilateral striatal necrosis; KSS, Kearns-Sayre syndrome; LHON, Leber's hereditary optic neuropathy; LS, Leigh syndrome; MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; MILS, maternally inherited Leigh syndrome; NARP, neuropathy, ataxia and retinitis pigmentosa; NIDDM, Non insulin dependent diabetes mellitus; PEO, progressive external ophthalmoplegia; SNHL, sensorineural hearing loss; $tRNA^{Leu}$, transfer RNA for leucine; $tRNA^{Ser}$, transfer RNA for serine. Compiled from DiMauro and Hirano 2005; MITOMAP http://www.mitomap.org accessed on 10 September 2011; OMIM http://www.ncbi.nlm.nih.gov/sites/entrez accessed on 19 October 2011.

2.1.5.3 Mitochondrial disorders associated with nDNA mutations

The nDNA mutations known at present to be associated with MDs are extremely important, especially in children, where it is expected that ~75-90% of genetic mutations not found in mtDNA might be in the nDNA as previously mentioned in Section 2.5.1 (DiMauro and Hirano 2005; Lamont et al 1998). According to Calvo et al (2006) there are around 1,500 nuclear encoded genes involved in mitochondrial function. This provides a great number of possibilities for mutations to occur that involves mitochondrial function and by 2010 Wong (2010) already reported in her review that diagnostic laboratories have identified around 150 nuclear mutations. Known mutations with well known phenotypes are summarised in Table 2.6, but novel mutations and associated phenotypes are discovered on a daily basis and resources e.g. MITOMAP http://www.mitomap.org and OMIM http://www.ncbi.nlm.gov/sites/entrez are constantly updated.

i. Mutations in RC subunits

Since the 1990s the emphasis has shifted from mtDNA mutations to the search for nDNA mutations contributing to the defects in the RC subunits. With CI having ~38 subunits encoded by nDNA, it is expected that numerous mutations in these would be responsible for CI deficiency. The Nijmegen group identified several mutations in *NDUFS2, NDUFS4, NDUFS7, NDUFS8* and *NDUFV1* genes (Schuelke et al 1999; Ugalde et al 2004; van den Heuvel 1998). Other mutations were also identified, including *NDUFS1* and *NDUFV2* (Benit et al 2001; Benit et al 2003; Martin et al 2005). These patients usually had an early onset soon after birth with a rapid, progressive encephalopathy, LS-like lesions on the MRI and they died before the age of three years. It may also have an adult-onset associated with neurodegenerative disorders (OMIM http//www.ncbi.nlm.nih.gov 2011). Cardiomyopathy was found in association with *NDUFS2* (DiMauro and Hirano 2005). Other mutations followed including *NDUFS3, NDUFS6, NDUFA2, NDUFA11, and NDUFAF3* and are summarised in Table 2.6 (Berger et al 2008; Kirby et al 2004; Saada et al 2009). Except for *NDUFA1* that has an X-linked dominant

Table 2.6 Categories and examples of nDNA mutations involved in mitochondrial disorders

	Gene	Locus	Function	Phenotype examples
Mutations in RC subunits				
CI	*NDUFS1*	2q33.3	IP fraction	LS
	NDUFS2	1q23.3	IP fraction	Encephalopathy, cardiomyopathy
	NDUFS3	11p11.11	IP fraction	LS
	NDUFS4	5q11.2	IP fraction	LS
	NDUFS6	5p15.33	IP fraction	LS
	NDUFS7	19p13.3	HP fraction	LS
	NDUFS8	11q13	HP fraction	LS
	NDUFA1	Xq24	HP fraction	Infantile LS, DD, myoclonic epilepsy
	NDUFA2	5q31.2	HP fraction, proton translocation	LS
	NDUFA11	19p13.3	*	Fatal infantile metabolic acidosis
	NDUFAF3	3p21.31	Shares a domain of unknown function	Macrocephaly, hypotonia, pale optic disks, seizures, leukomalacia
	NDUFV1	11q13.2	FP fraction	LS, leukodystrophy, myoclonus
	NDUFV2	18p11.31-p11.2	FP fraction	Cardiomyopathy, hypotonia, encephalopathy
CII	*SDHA*	3q29; 5p15	FP subunit	LS
	SDHB	1p36.1-p35	IP subunit	Paraganglioma, phaeochromocytoma
	SDHC	1q21	Membrane subunit	AD paraganglioma type 3
	SDHD	11q23	Membrane subunit	AD paraganglioma type 1, phaeochromocytoma
CIII	*UQCRB*	8q22	Electron transfer	Hypoglycaemia, LA
	UQCRQ	5q31.1	*	Mental retardation, Extrapyramidal signs
CIV	*COX6B1*	19q13.1	*	Infantile encephalopathy
Mutations in ancillary proteins				
CI	*ACAD9*	3q26	Assembly	Hypertrophic cardiomyopathy, hearing loss, excercise intolerance
	C20orf7	20p12.1	Assembly	IUGR, dysmorphisms, absent corpus callosum, diaphragmatic hernia, LA, adrenal insufficiency

Table 2.6 Continue…

	Gene	Locus	Function	Phenotype
	C6orf66/HRPAP20	6q16.1	Assembly	Metbolic acidosis, encephalopathy, scoliosis
	C3orf60/NDUFAF3	3p21.31	Impaired translation	LA, spasticity, myoclonic seizures
	C8orf38	8q22.1	Assembly	LS, LA, ataxia,rigidity, weakness
	HRPAP20/NDUFAF4	6q16.1	Assembly	Infantile encephalopathy, antenatal cardiomyopathy
	NDUFAF1/CIA30	15q13.3	Chaperone	FTT, LA,hypotonia, cardiomyopathy, RP, osteopetrosis
	/NDUFA2L/B17.2L	5q12.1	Assembly, chaperone	Early onset encephalomyopathy, LA
CII	*SDHAF1*	19q12-q13.2	Assembly	Psychomotor regression, spastic quadriplegia
	SDHAF2/SDH5	11q22.2	Assembly	Hereditary paraganglioma
CIII	*BCS1L*	2q33-37	Assembly	GRACILE syndrome, LS
	TTC19	17p12	Assembly	Neurodegenrative disorder
	UQCRQ	5q31.1	Ubiquinone binding protein	Encephalopathy
CIV	*COX10*	17p12-p11.2	Assembly, haeme A farnesyltransferase	LS-like, tubulopathy, leukodystrophy/tubulopathy
	COX15	10q24	Assembly, haeme A synthesis	Hypertrophic cardiomyopathy
	COX6B1	19q13.1	Assembly	Myopathy, regression, ataxia visual disturbance, leukodystrophy
	FASTKD2	2q33.3	Translation and control of apoptosis	Encephalomyopathy with asymmetric brain atrophy
	LRPPRC	2p21-p16	Assembly, mRNA binding protein	LS,FC
	SCO1	17p13-p12	Assembly, copper transport	Neonatal hepatic failure, encephalopathy
	SCO2	22q13	Assembly, copper transport	Cardioencpehalomyopathy
	SURF1	9q34	Assembly	LS
	TACO1	2q33.3	Translational activator	Late-onset LS
CV	*ATPAF2/ATP12*	17p11.2	Assembly	Early fatal onset encephalomyopathy, LA
	TMEM70	8q21.11	ATP synthesis	3-methyl glutaconic aciduria, cardiomyopathy
Impaired intergenomic communication				
mtDNA deletions	*ANT1*	4q34	mtDNA stability, adenine nucleotide translocator isoform1	AD-PEOA2, muscle weakness, ataxia, depression, hypogonadism, hearing loss, peripheral neuropathy
	C10orf2	10q24	Twinkle helicase	AD-PEOA3, SANDO

Table 2.6 Continue…

	Gene	Locus	Function	Phenotype examples
	ECGF1	22q13.32-qter	mtDNA stability, thymidine phosphorylase	MNGIE
	POLG	15q25	mtDNA stability, mtDNA replication	Alpers, AD-PEOA1, AR-PEO, male infertility, SANDO, SCAE
	TP	22q13.32-qter	Thymidine phosphorylase, regulates intra mitochondrial nucleotide pool	MNGIE
mtDNA depletion	*C10orf2*	10q224.31	*	Hepatocerebral mtDNA depletion syndomre type 7, ataxia, SCA
	DGOUK	2p13	mtDNA stability, deoxyguanosine kinase, mitochondrial dNTP pool maintenance	Hepatocerebral mtDNA depletion syndrome
	MPV17	2p23.3	Inner membrane protein	Infanitle hepatic failure
	POLG	15q25	mtDNA stability, mtDNA replication	Alpers, AD-PEOA1, AR-PEO, male infertility, SANDO, SCAE
	RRM2B	8q22.3	*	Leigh-like, MNGIE-like
	SUCLA2	13q14.2	*	Myopathy
	SUCLG1	2p11.2	Succinyl CoA Ligase GDP-bindin protein subunit alpha	Myoapthy, encephalopathy and lactic acidosis
	TK2	16q22	mtDNA stability, thymidine kinase, dNTP pool maintenance	Myopathy
	TWINKLE	10q24.31	DNA helicase	AD-PEO
	TYMP	22q13.33	*	*
Import	*DDP*	Xp22	Protein import	Deafness-dystonia or Mohr-Tranebjaerg syndrome
Lipid milieu				
	G4.5 or TAZ	Xq28	Mitochondrial integrity, defective cardiolipin	Barth syndrome, X-linked dilated cardiomyopathy
Mitochondrial biogenesis				
Motility	*KIF5A/SPG7*	16q24.3	Paraplegin ATPase protease, impaired chaperone function	HSP
Fission	*			None
Fusion	*OPA-1*	3q28-q29	Mitochondrial integrity, dynamin related protein	AD-Optic atrophy
	MNF2		Mitochondrial integrity, mitofusin	CMT type 2A

Table 2.6 Continue…

	Gene	Locus	Function	Phenotype examples
nDNA mutations involved in mitochondrial structure and function				
tRNA modifying genes and tRNA synthetase genes	*AARS2*	6p21.1	Alanyl tRNA synthetase	Cardiomyopathy
	DARS2	1q25.1	Aspartyl-tRNA synthetase	Leukoencephalopathy
	HARS2	5q31.3	Histidyl- tRNA synthetase	
	YARS2	12p11.21	Tirosyl- tRNA synthetase	
	RARS2	6q15	Arginyl-tRNA synthetase	Pontocerebellar hypoplasia
	TRMU	22q13.31	Translation	Reversible hepatopathy
Ribosomal protein genes	*MRPS16*	10q22.1	Defective translation	Dysmorphisms, hypotonia, severe LA. Elevated liver enzymes, limb oedema, CII normal, CI, CIII and CIV affecred
	MRPs22	3q23	*	Skin oedema, hypotonia, cardiomyopathy, tubulopathy
Mt tRNA processing	*PUS1*	12q24.33	Posttranscriptional modification of tRNA	MLASA
Elongation factors	*EFTu*	16p11.2	Translation	Infantile macrocystic leukodystrophy with polymicrogyria
	EFTs	12q13-q14	Translation	Encephalomyopathy, hypertrophic cardiomyopathy
	EFG1	3q25.1-q26.2	Translation	Severe hepatoencephalopathy and LA
	C12orf65	12q24.31	Release of protein from mt ribosome	LS, optic atrophy, ophthalmoplegia, optic atrophy
Iron homeostasis	*FRDA*	9q13	Frataxin, trinucleotide repeat	Friedreich's ataxia
Iron transport	*ABC7*	Xq13.1-q13.1	Impaired iron transport	X-linked sideroblastic anaemia with ataxia
Mitochondrial metabolism	*PDHA1*	Xp22.2-p22.1	Pyruvate dehydrogenase E1-α subunit	X-linked LS
	ETHE1	19q13.32	Ethylmalonic acid metabolism	Encephalopathy, ethylmalonic aciduria
mtRNA processing	*RMRP*	9p21-p12	Mitochondrial integrity, RNAse	Metaphyseal chondrodysplasia
Krebs cycle enzymes				
	FH	1q42.3-q43	Fumarate hydratase	Multiple cutaneous and uterine leiomyoma

AD, autosomal dominant; AD-PEO, autosomal dominant progressive external ophthalmoplegia; CI to IV, respiratory chain enzyme complexes I to IV, respectively; CMT, Charcot-Marie-Tooth disease; dNTP, deoxynucleotide triphosphate; GRACILE, growth retardation, aminoaciduria, iron overload, lactic acidosis, early death; HP, complex I hydrophobic protein; HSP, hereditary spastic paraplegia; IP, complex I or II iron-sulphur protein fraction; IUGR, intra uterine growth retardation; KSS, Kearns–Sayre Syndrome; LA, lactic acidosis; LS, Leigh syndrome; LS,FC, Leigh syndrome French-Canadian type; mRNA, messenger RNA; MLASA, mitochondrial myopathy and sideroblastic anaemia; MNGIE, mitochondrial neuro-gastrointestinal encephalomyopathy; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; RC, respiratory chain; SANDO, sensory ataxic neuropathy, dysarthria, ophthalmoplegia; SCAE, spinocerebellar ataxia and epilepsy. Compiled from Diaz et al 2011; DiMauro and Schon 2003; Zeviani and Di Donato 2004; DiMauro and Hirano 2005; Kemp et al 2011; MITOMAP http://www.mitomap.org accessed on 10 September 2011; OMIM http://www.ncbi.nlm.nih.gov/sites/entrez accessed on 19 October 2011 and 22March 2012, Wong 2010; Zhu et al 2009.

inheritance pattern, all the others are autosomal recessively inherited (OMIM http//www.ncbi.nlm.nih.gov 2011).

The first nDNA mutation described affecting the largest of the four subunits of CII was in the *SDHA* gene and the patients had LS (Bourgeron et al 1995). Rustin and Rötig (2002) comprehensively reviewed the inborn errors of CII, discussing the spectrum of clinical presentation including encephalopathy, myopathy, tumours, optic atrophy and hypertrophic cardiomyopathy. Mutations in the *SDHB, SDHC* and *SDHD* are associated with paragliomas and phaeochromocytomas (Astuti et al 2001).

The first mutation reported in the nDNA encoded subunits of CIII is the *UQCRB* gene and it was associated with hypoglycaemia and lactic acidosis (Haut et al 2003; MITOMAP http://www.mitomap.org 2011). A mutation in the *UQCRQ* gene was associated with mental retardation and extrapyramidal signs (Barel et al 2008). The first nDNA mutation affecting a subunit of CIV was described by Massa et al (2008) in patients with severe infantile encephalomyopathy.

ii. Mutations in ancillary proteins

Ancillary proteins are involved in the assembly or stability of the different RC complexes, and play a translational role or act as different chaperones. Mutations reported in ancillary genes involved in CI assembly are *B17.2L, HRPAP20 and C20ORF7* (Table 2.6) (OMIM http//www.ncbi.nlm.nih.gov 2011; MITOMAP http://www.mitomap.org 2011). The *C20ORF7* gene specifically affects an early CI assembly intermediate containing ND1 (OMIM http//www.ncbi.nlm.nih.gov 2011). It resulted in severe intra-uterine growth retardation (IUGR) of a boy, with a diaphragmatic hernia, abnormal hair pattern and toes, a sacral pit, agenesis of the corpus callosum and adrenal insufficiency. He also had a severe LA and died at day seven due to cardiorespiratory arrest (Sugiana et al 2008). Many more mutations in respiratory chain assembly factors have now been identified as illustrated by Diaz et al (2011) and shown in Table 2.6.

The SDHAF1 is an essential assembly factor for CII and the patients present with psychomotor regression, spastic quadriplegia, loss of postural control and leukodystrophy (Ghezzi et al 2009).

Although patients with CIII deficiency usually have a mtDNA mutation in the apoprotein of cytochrome b, two mutations in ancillary proteins have been reported: a mutation in the *BCS1L* gene that is associated with severe encephalomyopathy in early childhood, renal tubulopathy, liver failure, psychomotor retardation, LA, and LS-like brainstem lesions (De Lonlay et al 2001) or growth retardation, aminoaciduria, iron overload, LA and early death (GRACILE) (Fellman 2002); and a mutation in the *UQCRQ* gene that affects the ubiquinone binding protein resulting in encephalopathy (Barel et al 2008). More recently, a mutation in *TTC19* was identified by Ghezzi et al (2011). It caused CIII deficiency associated with neurological impairment in humans and flies. They also discovered that TTC19 is embedded in the inner mitochondrial membrane as part of two other complexes which coincide with CIII (Ghezzi et al 2011).

Complex IV, or also known as cytochrome c oxidase (COX) deficiency, has different phenotypes associated with nDNA mutations affecting its assembly: LS may be caused by mutations in *SURF1* (Tiranti et al 1998; Zhu et al 1998), *COX15, TACO1* and *LRPPRC* genes (OMIM http//www.ncbi.nlm.nih.gov 2010); infantile cardioencephalopathy is associated with a mutation in *SCO2* and a variety of symptoms in combination with encephalopathy is related to mutations in *COX10, COX6B1, SCO1* and *FASTKD2* (OMIM http//www.ncbi.nlm.nih.gov 2011).

iii. Impaired intergenomic communication

Impaired intergenomic communication is the result of abnormalities in the nDNA affecting the integrity, stability, amount and expression of mtDNA (Spinazzola and Zeviani 2009). If the quality of the mtDNA is seriously affected, multiple deletions may be found and depletion is

encountered if the quantity is affected (Spinazzola and Zeviani 2009). Four broad groups of clinical manifestations are described in Spinazzola and Zeviani (2009):

1. The adult-onset encephalomyopathy with PEO that could be AD or AR inherited and in which molecularly multiple mtDNA deletions are found. The nDNA genes held responsible are *ANT1, C10ORF2* and *POLG*;
2. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) with multiple deletions and partial depletion of mtDNA. The genes involved are *TP* and *ECFG1*;
3. An AR inherited spectrum of neurological syndromes including Alpers-Hunterlocher syndrome with an infantile onset, to sensory-ataxic neuropathy, dysarthria and ophthalmoplegia (SANDO) having a juvenile onset, to spinocerebellar ataxia with epilepsy, (SCAE) occasionally accompanied by PEO. The genes involved are *C10ORF2* and *POLG*;
4. Organ-specific AR inherited syndromes with an early onset myopathy, myo-nephropathy, encephalomyopathy and hepatocerebral forms. Genes at present reported are: *TK2, DGOUK* and *EFG1*.

iv. Defective lipid milieu

Cardiolipin is an abundant acidic phospholipid in the IMM that hosts the RC (Schlame et al 2000). A mutation in the gene *G4.5* leads to a marked decrease in the synthesis and alters the quality of cardiolipin. It is associated with Barth syndrome characterised by cardiomyopathy, cyclic neutropaenia and mitochondrial myopathy (Barth et al 1983; Vreken et al 2000). It is postulated that the defective cardiolipin is synthesised due to a mutant acyltransferase encoded by *G4.5* known to encode a family of tafazzins that share conserved regions with the acyltransferases of diverse organisms (DiMauro and Hirano 2005).

v. Impaired mitochondrial motility, fission and fusion

Mitochondria can fuse, divide and move within cells and form tubular networks (Bossy-Wetzel et al 2003). Novel concepts in the pathology of MDs describe defective motility,

fission and fusion of mitochondria. The first disorder associated with this defect is autosomal dominantly inherited hereditary spastic paraplegia (HSP). The mutation is in a gene called *KIF5A* that encodes a kinesin that is protein assisting in the propelling of mitochondria on microtubular rails (Fichera et al 2004). Mitochondrial fission has been linked to a GTPase named dynamin-related prot1 (DRP-1) (Bossy-Wetzel et al 2003). It has not been associated with a specific disorder yet. OPA-1 embedded in the inner membrane and mitofusin 1 and 2 (MNF 1, MNF2), two GTPases on the outer membrane, are involved in the fusion of mitochondria (Bossy-Wetzel et al 2003). Two disorders have been associated as yet with abnormal fusion, namely autosomal dominant optic atrophy due to a mutation in the *OPA-1* gene, responsible for blindness in young adults and Charcot-Marie-Tooth (CMT) type 2a due to a mutation in *MNF2* (Alexander et al 2000; Delettre et al 2000; Zuchner et al 2004).

vi. nDNA mutations indirectly involved in defective OXPHOS

Kemp et al (2011) summarised these concepts and previously reported patients with mutations in nuclear genes affecting mitochondiral protein synthesis, in addition to their comprehensive study on a cohort of patients with combined respiratory chain deficiencies and suspected defects in these genes.

Defective protein synthesis in mitochondria may be due to several mechanisms:

i. Impaired mitochondrial translation due to a mutation in the mitochondrial ribosomal protein subunit 16 (MRPS16), resulting in dysmorphisms, hypotonia, severe LA, deranged liver enzymes, limb oedema and decreased CI, CIII and CIV enzyme activities (Miller et al 2004);

ii. Defective pseudouridilation of tRNA genes due to a mutation in the gene *PUS1* encoding for pseudouridine synthase 1. It is associated with myopathy, LA, and sideroblastic anaemia (MLASA) and the onset may be juvenile or in childhood (Bykhovskaya et al 2004);

iii. Mutations if *EFG1* is associated with impaired mitochondrial protein synthesis and a clinical presentation of severe hepatoencephalopathy with LA (Coenen et al 2004).

2.1.5.4 Impaired electron carriers

Coenzyme Q_{10} or ubiquinone is a mobile electron carrier in the inner mitochondrial membrane, responsible for the transfer of electrons from CI and CII to CIII, and plays a role in the regulation of dehydrogenases and anti-oxidant defence (Duncan et al 2009; Nicholls and Ferguson 2002c; Rötig et al 2007).

Primary CoQ_{10} deficiency is an autosomal recessive condition with all the enzymes involved in the biosynthesis encoded by nDNA. There are six genes implicated in CoQ_{10} deficiency, namely *COQ2, APTX, PDSS1, PDSS2, CABC1* and *CoQ9* summarised in Table 2.7 (OMIM http//www.ncbi.nlm.nih.gov 2011). Five major phenotypes have been described and are summarised in Table 2.8 (Montero et al 2007). These patients often have deficient CI+III and CII+III (quinone-dependent activities) and they respond to CoQ_{10} supplementation (Rötig et al 2007). Secondary CoQ_{10} deficiency can be found in association with mevalonic aciduria due to mevalonate kinase deficiency and also in statin myopathy. The latter might be due to the inhibition of mevalonate caused by statins, and mevalonate is an obligatory intermediate in the synthesis of CoQ_{10} (DiMauro and Hirano 2005).

Table 2. 7 Examples of genes implicated in CoQ_{10} deficiency

Gene	Locus	Alternative titles	E.C. number
COQ2	4q21-q22	4-hydroxybenzoate nonaprenyltransferase parahydroxybenzoid-polyprenyltransferase	2.5.1.39
APTX	9p13.3	aprataxin FLJ20157	*
PDSS1	10p12.1	decaprenyl diphosphate synthase subunit 1 prenyl diphosphate synthase subunit 1 trans-prenyl transferase	*
PDSS2	6q21	decaprenyl diphosphate synthase subunit 2 prenyl diphosphate synthase subunit 2	*
CABC1	1q42.2	chaperone activity of BC1 complex-like AARF domain containing kinase 3	*
COQ9	16q13	C16ORF49 Chromosome 16 open reading frame	*

* Information not available. Compiled from OMIM http://www.ncbi.nlm.nih.gov/sites/entrez accessed on 19 October 2011.

Table 2.8 Features of primary and secondary CoQ_{10} deficiency

Presentation	Onset	Associated features	Special investigations	CoQ_{10} response
Primary CoQ10 deficiency				
Encephalomyopathic form	Childhood	CNS	Lactate↑	Excellent
		Learning disabilities	Muscle biopsy	
		Seizures	CoQ_{10} levels ↓↓	
		Dysarthria	RRF	
		Ataxia		
		Myopathy		
		Myoglobinuria		
		Exercise intolerance		
		Muscle weakness		
		Growth retardation		
Leigh syndrome	Neonates	Visual loss	Muscle biopsy	
	Infants	Failure to thrive	CoQ_{10} levels ↓–↓↓	
		Liver failure		
Myopathic form	Childhood	Exercise-related myoglobinuria	Muscle biopsy	Dramatic
	Adulthood	CNS involvement with	CoQ_{10} levels ↓	
		Seizures		
		Ataxia		
		Mental retardation		
Ataxic form	Infancy	Ataxia	Muscle biopsy	Less dramatic
	Adulthood	Cerebellar atrophy	Normal	
		Frequent	Non-specific changes	
		Seizures	CoQ_{10} levels ↓–↓↓	
		Pyramidal signs		
		Mental retardation		

Table 2.8 Continue...

Presentation	Onset	Associated features	Special investigations	CoQ_{10} response
Ataxic form		Rare Deafness Mild spasticity MRI: features of LS Late-onset ataxia Hypergonadotrophic hypogonadism		
Multisystem form	Neonates Infancy	CNS Ataxia Dystonia Renal disease: Nephritic syndrome Cardiomyopathy Visual loss Deafness Fatal if untreated	Muscle biopsy CoQ_{10} levels ↓–↓↓	Life-saving
Secondary CoQ10 deficiency				
Mevalonic aciduria		Ataxia Cerebellar atrophy Muscle-CoQ_{10} levels ↓		
Statin myopathy		Serum-CoQ_{10} levels ↓		

↓, mildly decreased;↓↓, markedly decreased; ↑, elevated; CNS, central nervous system; CoQ, coenzyme Q; LS, Leigh syndrome. Compiled from DiMauro and Hirano 2005; Montero et al 2007; OMIM http://www.ncbi.nlm.nih.gov/sites/entrez accessed on 19 October 2011; Rötig et al 2007.

2.1.6 Heterogeneity of mitochondrial disorders

Mitochondrial disorders are extremely heterogeneous in terms of the phenotype, biochemistry and genotype. The latter has been addressed in Section 2.1.5 in the discussion of the genetic classification and different aspects involved. The biochemical heterogeneity is discussed in Section 2.2.2. The clinical and biochemical profiles are equally variable, e.g. LS can occur in a number of isolated deficiencies of the OXPHOS system, pyruvate carboxylase, PDHc or impaired CoQ synthesis (Debray et al 2008).

2.1.6.1 Clinical heterogeneity

It is generally accepted that adults are more, and children less, likely to present with the classical mitochondrial syndromes. In the previous sections phenotypes associated with well-defined molecular defects were described, but for those patients where a specific genetic cause is unknown or if they have limited access to facilities, an alternative approach should be followed (Koenig 2008). A mitochondrial disorder should also be considered if two or more unrelated systems are involved (Koenig 2008). Debray et al (2008) reiterates the validity of the dictum: "any tissue, any symptom, any age", but the dilemma is that the full clinical spectrum of systems affected may not be present during their initial presentation at the physician (Haas et al 2008). The clinical heterogeneity is influenced by many different factors, including the dual genetic control, affected gene, type of the mutation, haplogroup, level of heteroplasmy, the tissue affected and environmental influences (Di Mauro and Schon 2003; DiMauro and Hirano 2005; Mancuso et al 2007; Zeviani and Di Donato 2004). The difference in the expression of disease as a consequence of the influence of either the nuclear or mitochondrial genes regulating OXPHOS is demonstrated in the study by Rubio-Gozalbo et al (2000). It was found that nuclear mutations lead to a much earlier onset of disease even in infancy and early childhood, with a much more progressive course, opposed to mitochondrial mutations in which the disease may manifest later in life with a less progressive course. Mitochondrial disorders in children can present in many different ways.

i. *Neuromuscular manifestations*

In a study done by Munnich et al (1996) it was found that 44% of patients with RC disorders presented with neuromuscular symptoms. The neuromuscular symptoms can include hypotonia, muscle weakness, peripheral neuropathy, ataxia and leukodystrophy (Zeviani et al 1996). Both the involvement of the peripheral and central nervous system is common and the degree can vary from occasional migraine to devastating encephalopathy, stroke or just mild deafness (Turnbull 2011).

- *Neurological manifestations*

The pure neurological involvement includes ataxia, bulbar signs, ophthalmoplegia, ptosis, stroke-like episode, spasticity, dystonia, tremor, chorea, migraine-like headaches, epilepsy, myoclonus (specifically mentioned), and peripheral neuropathy (Munnich et al 1996). Neurological symptoms associated with hepatic involvement, are called the Alpers syndrome and may have an early or later onset (Morris 1999). It is also important to understand that certain common mutations e.g. mt3243A>G can cause severe stroke-like episodes or be asymptomatic in another (Turnbull 2011).

Patients with unexplained infantile spasms should also be investigated for a mitochondrial disorder (Sadleir et al 2004). El Sabbagh et al (2010) investigated the epilepsy profiles of paediatrics patients with confirmed MDs. They found that myoclonic seizures and status epilepsy were clearly the most common form of epilepsy, nut infantile spasms, focal, tonic and tonic clonic seizures were found. Different seizure types were found in more than 60% of patients during the course of epilepsy and monotherapy was effective in only 5% of the cases. It was also very difficult to control the epilepsy and a mean of four antiepileptic drugs were given in combination or at different times (El Sabbagh et al 2010).

- *Muscular manifestations*

Muscle as high-energy demand tissue is often involved in MDs as a single entity or as part of a multi-system disorder. The myopathy may develop in infancy or later in life. It may be localized to specific muscle groups e.g. extraocular muscle or it may evolve in a progressive myopathy (Turnbull 2011). Other manifestations include progressive proximal weakness with the arms usually affected more than the legs, myalgia, exercise intolerance and rhabdomyolysis (Chinnery and Turnbull, 1999; McFarland et al 2002; Taylor et al 2004). The serum creatine kinase (CK) will be normal or only slightly elevated (< 2x normal) and the electromyogram (EMG) may also be normal, implying that if these findings are associated with a patient having significant weakness, a mitochondrial disorder should be considered (Munnich et al 1996).

ii. *Vision and hearing*

Retinitis pigmentosa, cataracts and strabismus may develop, but children may still have vision (DiMauro et al 1999). The two most common inherited optic neuropathies are LHON and AD-optic atrophy. The most common mutations in LHON are mt3460G>A, mt11778G>A and mt14484T>C. Yu-Wai-Man et al (2011) reviewed the disease mechanisms and therapeutic strategies of mitochondrial optic neuropathies in depth and he also discussed the role of haplogroups in the expression of LHON, environmental factors e.g. smoking, alcohol, hormonal influences and light exposure. The other mitochondrial optic neuropathies include CMT disease type VI, HSP, Freidreich ataxia, autosomal recessive non-syndromal optic atrophy, mitochondrial protein-import disorders e.g. Mohr-Tranebjaerg syndrome and numerous overlapping phenotypes. Classical mitochondrial syndromes e.g. MERFF, MELAS, CPEO, KSS, MILS and MNGIE may also develop optic neuropathy (Yu-Wai-Man et al 2011). Sensori-neural deafness is a red flag sign of MDs (DiMauro et al 1999).

iii. *Psychiatric disorders*

In a meta-analysis it was found that 5% of patients with autism spectrum disorder (ASD) had a MD. It is however unclear if mitochondrial dysfunction is an epiphenomenon or whether it contributes to the pathogenesis of ASD. The authors concluded that a reasonable approach in the management of newly diagnosed patients with ASD would be to investigate them for MDs until further studies are available (Rossignol and Frye 2011).

Hroudová and Fišar (2011) reviewed the role of mitochondrial dysfunction associated with major depressive disorder, bipolar disorder and schizophrenia and it was mentioned that mental diseases are more matrilineal that may support the maternal inheritance pattern of MDs.

iv. *Cardiac manifestations*

Cardiac involvement is present in up to 33% of patients and mostly consists of conduction defects, but cardiomyopathy may also be present (Sciacco et al 2001).

v. *Renal manifestations*

Renal involvement seems to be more prevalent in children with MDs than in adults. Tubular dysfunction is directly implied and the reason is that ATP is essential for the sodium-potassium-ATPase channel to function and maintain an electrical gradient across the proximal tubular cell. This gradient is important for cellular function. The absorption of sugars, amino acids and phosphate is *via* co-transporters (Niaudet 1998).

Renal manifestation includes proximal tubulopathy that may present as Toni-Debré-Fanconi syndrome (Majander et al 1991; Matsutani et al 1992; Moraes et al 1991; Morris et al 1995; Niaudet et al 1994; Ogier et al 1988; Rötig et al 1992; Emma et al 2011). These patients have aminoaciduria, glucosuria, proteinuria, as well as losses of calcium, phosphate, bicarbonate, potassium, and water. It is not uncommon that rickets develop in these patients.

Tubulointerstitial nephritis leads to a concentrating defect with polyuria as consequence. It progresses in to renal failure. These patients usually do not have any tubular defects. The histological lesions include diffuse interstitial fibrosis with tubular atrophy and sclerotic glomeruli. The patients usually have other systems involved as well (Niaudet 1998).

Glomerular disease with nephrotic syndrome has also been described in association with MDs. The histological picture is usually that of focal glomerular sclerosis and resistance to treatment (Niaudet 1998). Proximal renal tubular acidosis was initially described as part of a partial deficiency of cytochrome *c* oxidase deficiency (Matsutani et al 1992). It was previously believed that renal symptoms are always associated with extrarenal manifestations including myopathy, neurological symptoms, Pearson's syndrome, diabetes mellitus and cardiac abnormalities (Niaudet 1998), but Ueda et al (2004), described a boy who presented with asymptomatic proteinuria for years. He subsequently developed cataracts and should be closely monitored for the development of any other symptoms.

Renal biopsy findings are non-specific including of the tubular epithelium with dilation and obstruction via casts, de-differentiation or atrophy (Niaudet 1998).

vi. *Gastrointestinal manifestations*

A very common phenomenon in gastrointestinal presentation is failure to thrive. Other symptoms include chronic diarrhoea, frequent vomiting, villous atrophy and adults may present with chronic pseudo-obstruction (Hirano et al 1994). A MD should be considered in a child with valproate-induced deranged liver functions (Kranenbuhl et al 2000). MDs are associated with a wide range of chronic liver disease (Grattagliano et al 2011).

vii. *Endocrine manifestations*

The endocrine presentation may include diabetes mellitus, hypoglycaemia, short stature, hypoparathyroidism, central diabetes insipidus and precocious puberty (Nissenkorn et al

1999). It is estimated that 1-2% of patients with diabetes may have a mitochondrial cytopathy (Niaudet 1998). Ketotic hypoglycaemia may be the only initial symptom of children with MDs (Mochel et al 2005).

viii. *Haematological manifestations*

The haematological symptoms may include sideroblastic anaemia or pancytopaenia. It is, however, difficult to recognise a mitochondrial disorder in patients solely on haematological symptoms (Munnich and Rustin 2001).

ix. *Skin manifestations*

The skin manifestations can be divided into six categories including: lipomas, erythema and disorders of pigmentation, abnormalities of the hair shaft and alopecia, acrocyanosis, hypertrichosis and a diverse group including dermatomyositis (Birch-Machin 2000).

2.1.7 Clinical basis of diagnosis

The diagnosis of these disorders, which present with a wide-ranging phenotype, requires a combination of clinical modalities, biochemical assessment of RC/OXPHOS function and enzymes, histochemical, as well as molecular genetic studies (Taylor et al 2004; Jameson E and Morris 2010; Rodenburg 2011). The biochemical aspects of the diagnostic process are discussed in Section 2.2.

2.1.7.1 Clinical scoring systems

The diagnosis of MDs is extremely complicated and expensive and the choices of specific investigations are most of the time dictated clinically. Different centres have different protocols. It is essential to standardise the diagnosis of these disorders. The diagnostic criteria in infants and children (The Mitochondrial Disease Criteria, MDC) by Wolf and Smeitink (2002) are helpful, but still difficult to apply directly in specific circumstances with restricted resources. The criteria proposed by Bernier et al (2002) provide a comprehensive

way to finalise a MD, but as many of the criteria rely on histological findings, functional analyses and molecular data, it is very difficult to utilize these as a screening tool to select patients for further invasive investigations.

2.1.7.2 Histological assessment of mitochondrial function

Muscle biopsies form a central part in the diagnosis of MDs. The indications to perform a muscle biopsy are when the genetic analyses in the case of a classical syndrome is negative, or when the clinical suspicion is very high, but the patients do not fit a classical syndrome. The histochemistry and biochemistry are done first. Molecular studies will follow if the phenotype is still very suspicious, but the histochemistry and biochemistry only suggest a mitochondrial disorder, or if the clinical suspicion is still very high despite a normal biopsy. Further studies are rarely continued if clinical suspicion is very low and the biopsy, as well as the biochemistry, is normal (Taylor et al 2004.)

Muscle is usually removed from the quadriceps, and frozen for histochemical analyses. The different stains include the Gomori trichrome stain that demonstrates the subsarcolemmal collection of mitochondria. It appears red with an irregular outline and is referred to as ragged red fibres. More specific enzyme studies (as tissues stains) include enzyme reactions for mitochondrial enzymes namely succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX). The SDH also demonstrates the subsarcolemmal collection of mitochondria. It is useful in patients with complex II deficiencies, but patients with complex I and III deficiencies may have normal biopsies, as no stains are available to assess these complexes. COX staining is useful in myopathies as it contains subunits encoded by the nuclear as well as mitochondrial genomes. Normal muscle contains oxidative or type I and glycolytic or type II fibres. COX reacts stronger with the type I fibres. In patients with a heteroplasmic mtDNA disorder a mosaic pattern of COX activity arises due to the deficient COX fibres. The outline of these fibres is difficult to detect. Varying COX activity may suggest different genetic aetiologies (Taylor et al 2004, see Table 2.9). In a mosaic pattern

with a low percentage of COX deficient fibres it might be indicated to do sequential COX-SDH histochemistry to identify abnormal fibres (Sciacco et al 1994).

Table 2.9 Cytochrome *c* oxidase activities in muscle and possible genetic aetiology

COX activity in muscle	Suggested molecular defect
Normal with mitochondrial proliferation	MELAS
	Point mutations in *ND* genes
	Point mutations in *ND* genes
Mosaic pattern: majority of fibres COX deficient	Heteroplasmic mtDNA mutations involving tRNA genes
	Mutations in mtDNA-encoded structural *CO* genes
Global decrease	nDNA mutation in assembly genes for COX e.g. *SURF1*
	Pathogenic homoplasmic mitochondrial tRNA mutations

MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; *ND,* genes encoding for NADH dehydrogenase subunits; *cyt b,* genes encoding for cytochrome b; COX, cytochrome *c* oxidase, mtDNA, mitochondrial DNA; tRNA, transfer RNA; *CO,* genes encoding for cytochrome *c* oxidase subunits; *SURF,* gene encoding for the assembly of cytochrome *c* oxidase. Compiled from Andreu et al 1999a; Andreu et al 1999b; McFarland et al 2002; Pavlakis et al 1984; Sciacco et al 1994; Taylor et al 2003; Tiranti et al 1998; Zhu et al 1998.

Although electron microscopy (EM) can demonstrate characteristic abnormalities like absent cristae, paracrystalline inclusions or enlarged mitochondria, it is not contributing to the finalisation of a specific diagnosis (Taylor et al 2004). Koenig (2008) reviewed the use of EM and concluded that 30-44% of children will have ultra-structural changes, making EM an important diagnostic modality. It should be kept in mind that these changes may also occur in muscular dystrophies, long-term steroid use, neurogenic atrophy, inflammatory myopathies, antiretroviral therapy, aging and other metabolic disorders (Koenig 2008).

Other non-specific histology findings associated with MDs are internal nuclei, atrophic or hypertrophic fibres, fibre-type grouping of type I or II fibres, lipid droplets, evidence of inflammation and glycogen as summarised by Koenig (2008).

2.1.7.3 Brain imaging

Despite the fact that MRI findings for many MDs are non-specific, Bianchi et al (2007) classified the changes in three main categories: Type I is the non-specific findings including

mild to moderate cerebral and cerebellar atrophy and high signal intensities in sub-cortical or paraventricular white matter. The predominant myopathic and non-syndromic encephalomyopathic phenotypes are included in this group. Type II includes the syndromic phenotypes and they may have more specific MRI changes, including cortical and subcortical grey matter involvement with the basal ganglia, cerebellar dentate, brainstem grey matter and colliculi more often affected than the thalami. KSS is included in this group. Type III involves primarily the white matter. Small cysts may appear in the white matter and signal changes in the brainstem and cerebellar white matter. The basal ganglia and nuclei in the brain-stem are seldom involved (Bianchi et al 2007).

2.1.7.4 Spectroscopy

Koenig (2008) reviewed the role of spectroscopy in the diagnosis of MDs. Lactate may be detected with proton magnetic spectroscopy (^{1}H) in the absence of lactic acidosis suggestive of a MDs, but other causes of raised cerebral lactate e.g. ischemia or infection should be excluded. Haas et al (2008) further add the implications of the different peaks in MDs and it is summarised in Table 2.10.

Table 2.10 Proton magnetic spectroscopy peaks contributing in the diagnosis of MDs

Peak	Chemical shift/ppm	Considerations	Condition in MD
Lactate	1.33	Sensitivity 18-27% for MDs	↑
N-acetyl-*L*-aspartate	2.02	Biomarker of neuronal integrity	↓
Succinate	2.40	Low concentrations present in normal brain	↑ in CII deficiency
Choline	3.22	Reflects membrane turnover and demyelination	May be ↓

↑, elevated;↓, decreased; CII, complex II of the respiratory chain; MD, mitochondrial disorder; ppm, parts per million. Compiled from Haas et al (2008)

Near-infrared spectroscopy can measure oxygen extraction in tissue. Patients with MDs exhibit a paradoxical reaction during exercise: they have increased oxygenation during exercise and rapid return to baseline whereas healthy controls would have prompt deoxygenating during exercise and then prompt reoxygenation afterwards. It implies that

diseased tissue will have increased oxygen supply during exercise, but impaired utilization (Koenig 2008).

Phosphorous magnetic resonance spectroscopy can quantify the utilization of energy in muscle and it is related to the ratio between phosphocreatine/inorganic phosphate. The ratio is high at rest of normal muscle, implying a low metabolic rate and high energy capacity. Patients with MDs will have lower ratios at rest, a rapid decline during exercise with a slower recovery than healthy controls after exercise (Koenig 2008).

2.1.8 Treatment

A Cochrane review of six randomised trials on treatments of MDs concluded that there is currently no established treatment for MDs (Chinnery et al 2006). However, anecdotal reports where patients have been treated with different degrees of success with oral supplements such as co-enzyme Q_{10} (ubiquinone) or other quinone derivatives, vitamins and metabolic supplements as well as other pharmacologic agents have been published. In adult patients, exercise therapy has also been tested (DiMauro and Mancuso 2007). All of these treatments demonstrated only varied improvement in isolated cases. Treatment of patients with MDs is problematic, but numerous creative novel approaches including alternative gene expression, enzyme replacement, alteration of the balance between mutated and wild-type mtDNA and modulation of cell signalling are currently being investigated (Koene and Smeitink 2009). The five principles of metabolic manipulation are recently reviewed and include prevention of damage caused by ROS, lipid peroxidation amelioration, regulation of aberrant transcription and correction of calcium homeostasis as well as altered membrane potential (Koene and Smeitink 2011).

2.2 BIOCHEMICAL CHARACTERISATION OF MITOCHONDRIAL DISORDERS AND CONTEMPORARY APPROACHES

The confirmation of a diagnosis in patients with a mitochondrial disorder is challenging and requires a broad spectrum of laboratory tests including metabolite analyses, measurement of enzyme activities and molecular genetic testing (Bernier et al 2002, Haas et al 2008, Morava et al 2006, Rodenburg 2011, Thorburn and Smeitink 2001, Walker et al 1996, Wolf and Smeitink 2002).

2.2.1 Biochemical assessment of mitochondrial function

Biochemical assessment has always played an important role in the diagnostic process of mitochondrial disorder, but biochemical markers for MDs obtained with minimal invasion in urine, serum and CSF, lack specificity and has until recently been limited in number, due to the large number of proteins involved in the entire energy machinery. Wolf and Smeitink (2002) proposed more specific markers including elevated lactate in urine and cerebrospinal fluid associated with an increased lactate:pyruvate ratio, Krebs cycle intermediates, ethylmalonic acid, 3-methylglutaconic acid, and dicarbonic acids (adipic, suberic and sebacic acid) in the Nijmegen Mitochondrial Disease Criteria, but no specific ranges of abnormal values are specified. The current *status quo* on the diagnosis of MDs is reviewed in depth by Haas et al (2008) for the Mitochondrial Medicine Society‘s and the important aspects are discussed below. Table 2.11 summarises the important considerations about the biochemical markers and the differential diagnosis that is important in the interpretation of the results.

2.2.1.1 Lactate and pyruvate

Although lactate is traditionally associated with MDs, several special considerations should be taken into account: it is important to exclude the other causes of hyperlactatemia e.g. shock, sepsis, hypoxia, cardiac failure and other inborn errors of metabolism. Debray et al (2007) further advise that a child with an elevated lactate, but otherwise low index of suspicion for a MD should have lactate sampling at several occasions including pre- and

post prandial with an indwelling catheter. The results should be carefully interpreted together with those of the pyruvate, amino acids, specifically alanine, glucose and ketone bodies values. The cerebrospinal fluid lactate is more reliable than the blood level, especially with central nervous system involvement (Finsterer 2001).

2.2.1.2 Amino acid analyses

Although amino acid analyses are often underutilized in the diagnosis of MDs, they may be quite useful as reviewed by Haas et al (2008). True hyperalaninaemia can be distinguished from relative raised alanine, by comparing it to lysine and tyrosine plus phenylalanine. A ratio of alanine:lysine > 3.1 and alanine:tyrosine plus phenylalanine > 4.1 is strongly indicative of true hyperalaninaemia. The determination of amino acids in urine is of value if the serum bicarbonate is decreased. A generalized aminoaciduria in combination with glucosuria and renal tubular acidosis, known as renal Fanconi syndrome, is particularly known to be associated with mtDNA deletions.

A number of pitfalls in amino acid analyses should be remembered:

1. Improper storage causes artificial elevation of glutamate, aspartate and ornithine with a decrease in glutamine, cysteine, asparagine and homocysteine.
2. Haemolysis causes artificially low aspartate, glutamate, ornithine, phosphoserine and taurine.
3. Post-prandial specimens may have generalized aminoaciduria including branched amino acids and alanine.

The analyses are performed in different ways. The most accurate, but longest method is ion exchange chromatography with post-column derivitization. Tandem mass spectrometry offers a high throughput at relative low cost, but quantification can be problematic as the case also with reverse-phase HPLC (Haas et al 2008). It was also shown that MDs are associated with hypocitrullinaemia and glutathione deficiency (Atkuri et al 2009).

Table 2.11 The differential diagnosis of biochemical markers associated with mitochondrial disorders

Analyte	Comments	Differential diagnosis
Lactate and pyruvate (plasma, CSF, urine)		
↑plasma lactate	> 2.1 mmol/l Non specific marker for MDs	Shock Sepsis Struggling of patient Tourniquet use during sampling
↑CSF lactate	May be elevated without ↑plasma lactate in predominantly brain involvement	Stroke CNS infection Seizures
↑plasma pyruvate	↑Alanine marker of long-standing pyruvate accumulation	Postprandial
↑plasma and CSF pyruvate	Pyruvate is unstable, must be collected in 8% perchlorate and transported on ice	PDHc and PC deficiency Biotinidase deficiency
↑lactate:pyruvate	Reflects NADH:NAD^+ cytoplasmic redox state	Poor handling of specimens Thiamine
Amino acids (plasma, CSF)		
Alanine	>450µmol/l in plasma	Meningitis Epilepsy Schizophrenia Cardiac failure Leukaemia
Proline, glycine, sarcosine, tyrosine		Non-ketotic hyperglycinaemia
Creatine kinase (plasma)		
↑Creatine kinase		Other muscular dystrophies
Organic acids (urine)		
TCA intermediates		Renal immaturity in babies younger than 1 year of age
Ethylmalonic acid		SCAD deficiency Malnutrition Anorexia nervosa Malonyl-CoA decarboxylase deficiency
3-methyl glutaconic acid		3-methylglutaconic aciduria Smith-Lemli-Opitz syndrome 3-hydroxy-methylglutaryl-CoA lyase deficiency
Dicarboxylic acids		Medium chain triglycerides supplement in diet Prolonged fasting Drugs
Acylcarnitines (plasma)		
Low free carnitine ↑acyl:free carnitine ratio		Disrupted fatty acid oxidation

↑, elevated; CSF, cerebrospinal fluid; MDs, mitochondrial disorders, NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PC, pyruvate carboxylase; PDHc, pyruvate dehydrogenase complex; SCAD, short-chain acyl-coenzyme A dehydrogenase deficiency; TCA, tricarboxylic acid. Compiled from Haas et al (2008) and HMDB http://hmdb.ca/metabolites accessed on 17 October 2010.

2.2.1.3 Organic acid analyses

Organic acids are formed in the catabolism of carbohydrates, fats and proteins. Urine is the preferred specimen for organic acid analyses, and plasma organic analyses rarely provide additional diagnostic information. It may have a low sensitivity for the diagnosis of MDs during periods of clinical stability. Urinary lactate is also a poor marker of MD's, and other non-specific organic acids currently used are Krebs cycle intermediates, ethylmalonic acid, and 3-methyl glutaconic acid. Dicarboxylic aciduria is often associated with MDs and it results from impaired mitochondrial fatty-acid β-oxidation.

Organic acids are normalized to creatinine in urine resulting in false elevations of organics acids in patients with a decreased muscle bulk for various reasons (starvation and other muscle disorders) or defective creatine synthesis (Haas et al 2008).

2.2.1.4 Carnitine analyses

Carnitine is involved in numerous functions: it shuttles free fatty acids, plays a role in coenzyme A metabolism, and promotes urinary excretion of estrified intermediates. The value of carnitine analyses is mainly to exclude other disorders of fatty acid oxidation defects, aminoacidopathies and other organic acidurias. It is often difficult to interpret acyl-carnitine profiles, because the kidney produces different acyl-forms (Haas et al 2008).

2.2.1.5 Creatine

Shaham et al (2010) found that plasma creatine compared to alanine and lactate may add value in the diagnosis of MDs.

2.2.1.6 Creatine kinase

Creatine kinase (CK) may be elevated in MDs, but is also non-specific and may be elevated in other dystrophies (Haas et al 2008).

2.2.1.7 Enzyme assays

Gellerich et al (2004) ask why enzymatic measurements are still utilised in the genomic and proteomic era and they eloquently discuss the factors contributing to the complexity of the diagnostics of MDs. Mutations are responsible for changes in the transcriptome, proteome and metabolome, on the level of the mitochondrion itself and consequently on different tissues, and the individual ultimately experience disease. A definitive diagnosis still relies mainly on enzymatic or functional data with or without DNA mutation analysis. The biochemical analyses include single enzyme activity measurements that can be done on fresh or frozen tissue samples (mostly obtained from muscle biopsies, but liver, fibroblast or cardiac muscle can also be utilised) and functional analyses performed on either enriched mitochondrial preparations or crude homogenates from fresh tissue samples (Taylor 2004; Janssen et al 2003). The reason for using muscle rather than less invasively obtained cells such as fibroblasts or lymphocytes is that the disease is often not homogeneously expressed in the body and may not be detected in fibroblast or lymphocytes (Mazat 2001). Furthermore, the majority of patients with MDs have skeletal muscle involvement and muscle is rich in mitochondria providing an ideal opportunity to detect abnormal mitochondrial function (Rodenburg 2011). As described in Section 2.3.3 mitochondria contain several copies of maternally inherited mtDNA which are not homogeneously spread amongst different tissues. Therefore, a mutation in mtDNA may exist at a higher frequency among mtDNA copies in specific tissues compared to others. In addition, tissues having a higher energy demand, such as muscle and neurons, are more susceptible to the effect of mutations of both mitochondrial and nuclear encoded proteins (Mazat 2001).

The combination of single enzyme and functional analyses is complementary in identifying specific deficiencies. According to a leading diagnostic centre, some cases of MDs (±25%) may not be identified from single enzyme analysis only (Janssen et al 2003). The functional analyses include measurements of either substrate oxidations using radioactive labelled substrates, ATP production or respiration analyses (oxygen reduction) (Taylor et al 2004).

Apart from the intrinsic heterogeneous nature of MD's, a lack of standardisation further adds to the perplexing findings often encountered in these disorders. The problem of inter-laboratory variation in the enzyme measurements of MDs was illustrated by Gellerich et al (2004). Fourteen laboratories in eight countries were included and they determined the activities of CI, CI+III, CII+III, CIV, CV and CS on homogenates prepared from bovine muscle. The results for all the enzyme assays varied more than one order of magnitude. They concluded that the reason for the large variation in results was the differences in the protocols used by the different laboratories (Gellerich et al 2004). There is currently a huge drive to standardise and assure quality. The Mitochondrial Medicine Society has a dedicated committee on diagnosis and updated news and progress in this regard can be followed at www.mitosoc.org.

Low and behold the biochemical characterisation of patients will continue to be important in the diagnostic process of patients with MDs, as it will assist in the selection of groups of candidate genes and in the interpretation bioinformatic data of candidate genes (Rodenburg 2011).

2.2.1.8 Provocative tests

Loading tests and fasting have limited diagnostic usefulness in MDs as it may precipitate metabolic crises in some patients (Debray et al 2008).

2.2.1.9 Proteomic procedures

Blue native polyacrylamide gel electrophoresis (BN-PAGE), an alternative method to polarographic and spectrophotometric assessment of the OXPHOS system has been developed in recent years and discussed in detail by Diaz et al (2009). The protein complexes are separated by electrophoresis in the presence of Coomassie blue and then extracted from the gel. It has the additional advantage that it is less influenced by suboptimal storage conditions of the tissue than some of the other techniques (Debray et al 2007). The

disadvantage is that, although in-gel native enzyme activities can be measured as end-point assays, it does not provide accurate information on the activity and kinetic properties of the protein (Gellerich et al 2004).

2.2.2 Biochemical heterogeneity

2.2.2.1 Lactate

Lactate is known to be increased in MDs, but Debray et al (2007) illustrated that it may be absent in proven MDs and can be highly variable in individual patients. Patients may have normal basal lactate levels, but these levels are elevated during exercise or a metabolic crisis. Normal or minimally elevated levels of lactate are associated with POLG associated MDs namely KSS, LHON, LS and CI deficiencies.

2.2.2.2 Fatty acid oxidation defects

The clinical and biochemical profiles of fatty acid oxidation defects (FAOD) and MDs may overlap and complicate the diagnosis as eloquently reviewed by Sim et al (2002a). They also documented abnormal acylcarnitine profiles in fibroblasts of patients with MDs as well as those with fatty acid oxidation defects (Sim et al 2002b).

2.2.2.3 Enzyme deficiency and phenotypic heterogeneity

Specific enzyme deficiencies of the OXPHOS system may be associated with a variety of clinical symptoms and vice versa. Complex I is often associated with LS, but also leukoencephalopathy, cardiomyopathy and myopathy. Cardiomyopathy may also be associated with CIV or CoQ_{10} deficiency (Haas et al 2008).

2.2.3 Novel biomarkers

Due to the complexity of MDs and lack of accurate biomarkers there is huge drive amongst clinicians and biochemists to find ways to simplify the diagnostic process, but Mancuso et al (2009) concluded that a reliable marker is not available currently. Suomalainen (2011) also

concluded that less invasive biomarkers in serum are still absent, but metabolic fingerprints may be useful in future and that transcriptomics, proteomics and metabolomics approaches can helpful. An ideal biomarker should be easily detectable in specimens obtained with minimal intervention, e.g. urine, saliva or exhaled air and different routes can be explored including ~~-e~~ducated guesses", ~~-o~~mics" approaches, mice models, cultured cells or patient studies (Suomalainen 2010). There are however numerous efforts to find a biomarker. The most recent novel biomarker is fibroblast growth factor 21 (FGF-21), a hormone-like cytokine regulating lipid-metabolism and the starvation response, indentified in a multi-centre study. It can be measured in serum with an ELISA test and the sensitivity (92.3%), as well as the specificity (91.7%) was better than for lactate, pyruvate and CK (Suomalainen et al 2011; Turnbull 2011). The ~~-o~~mics" approach requires close collaboration with bioinformatics specialists to analyse the enormous amount of data and metabolomic data analyses are still lagging behind (Suomalainen 2011).

2.2.4 Metabolomics

Although a metabolomics approach was an important aspect of this investigation, it is beyond the purpose of a clinician to present a comprehensive review of this biotechnology. Accordingly, only a limited number of metabolomics aspects will be presented here.

Metabolomics is the systematic study of metabolites involved in biological systems and requires identification of the metabolites and subsequent quantitative and qualitative measurements (Oldiges et al 2007). The identification requires different analytical approaches and includes target analysis, metabolic profiling and metabolic fingerprinting. In a targeted approach, substrate and/or product metabolites of a target protein are quantified. If a set of pre-defined metabolites linked to a specific pathway or class of compounds is studied, it is then defined as metabolic profiling (Fiehn 2002). Metabolic fingerprinting is a semi-quantitative analysis of the intracellular metabolites or endo-metabolome (Fiehn 2001; Oldiges et al 2007). Allen et al (2003) introduced the terminology of metabolic footprinting in cases in which the extra-cellular metabolite pool is analysed. Wishart et al (2009) then

defined metabolomics as the characterisation of metabolites with rapid and high throughput techniques. The study of the metabolome is extremely important, as the metabolites are downstream of the regulatory structures of the genome and the proteome providing information about the regulatory and catalytic properties of the gene product (Oldiges et al 2007).

Dunn et al (2005) reviewed the metabolomics experimental process or pipeline critically, in light of the importance of the experiment requiring a trans-disciplinary approach. The different stages of the metabolomics experiment include the experimental design, sampling, sample preparation, sample analysis, data pre-processing and data processing. The experimental design is crucial to ensure that valid statistical interpretations can be drawn. The sampling conditions are important, as biological systems are in constant flux and perturbations observed may be unrelated to the specific question being studied. The samples size is influenced by many factors, including the biological organism, environmental influences and finances available. The selection of controls is crucial in the interpretation of the metabolomics data and it should be as homogeneous as possible. Sample preparation depends on the specific analytical technique and subset of the metabolome under investigation. Mass spectrometry (MS) is a valuable analytical tool in metabolomics and different types of instrumentation are used, e.g. gas chromatography-mass spectrometry (GC-MS). Nuclear magnetic resonance (NMR) spectroscopy is used widely for metabolomics research. It benefits from being specific yet non-selective and from providing structural information as well (Dunn et al 2005).

Madsen et al (2010) conducted a coherent and comprehensive review of the complicated task of data processing. The steps required to analyse data obtained with MS analysis include: alignment of data; baseline correction; peak-picking or deconvolution; peak-identification; normalization and scaling. The data obtained from NMR require phasing and baseline correction, alignment, normalization, bucketing or peak-picking and scaling. Statistical analyses follow and involve data overview with e.g. principal component analysis

(PCA), model building, model optimization, validation, predictions and identification of biomarkers. Biological interpretation follows and perturbed metabolic pathways are identified, disease mechanisms are explained and follow-up studies performed (Madsen et al 2010).

Barshop (2004) was the first to publish a metabolomic approach in MDs by investigating urine organic acids. However, this was a retrospective correlation study of randomly selected urine samples. He demonstrated the limitations in the use of organic acids to distinguish between patients with MDs and other sick or stressed patients. He concluded that, at best, 25–30% of patients with MDs can be identified with a 5% false positive rate if a cut-off value of 90 mmol/mol creatinine is used for fumarate and malate in infants, and a value of 25 for older patients is used (Barshop 2004).

Despite the limitations pointed out by this initial metabolomic study, the value of finding a biomarker or biosignature using a non-invasive biological fluid, such as blood or urine, cannot be disregarded. It probably presents, along with the recent developments in high throughput DNA sequencing and DNA bioinformatics, the most promising development towards improving diagnostics of MDs (Carrao et al 2009). Among the omic technologies, metabolomics is still in its infancy and its diagnostic value, as will be demonstrated in this thesis, has potential but needs to be developed fully.

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CHAPTER 3

CLINICAL AND EXPERIMENTAL INVESTIGATIONS

3.1 INTRODUCTION

Mitochondrial disorders (MDs) are frequently encountered diseases, with a minimum prevalence of 1 in 5,000 patients of all ages (Schaefer et al 2004). However, the prevalence of MDs in South Africa is unknown and has been complicated by the absence of a readily available comprehensive diagnostic service. This study was the first, to our knowledge, to report on the clinical and biochemical findings in South African patients. The patients were assessed at the Paediatric Neurology Unit at Steve Biko Academic Hospital, Pretoria, South Africa, over a period of 12 years (from 1999 until 2010). Some aspects of the assessment was undertaken in collaboration with the North-West University.

The first part of the study focused on the clinical description of the patient population. For the purpose of this part of the study, 63 patients diagnosed with a respiratory chain (RC) and/or pyruvate dehydrogenase complex (PDHc) deficiency in the period 1999 until June 2009 were included, and the clinical and relevant biochemical characteristics are discussed in Section 3.2. The most important clinical findings were that African patients predominantly presented with myopathy associated with CII+III deficiencies and Caucasian patients presented with encephalopathy or encephalomyopathy. Furthermore, Caucasian patients more commonly had single enzyme deficiencies in contrast with African patients, who presented with combined enzyme deficiencies.

In the period 1997 until 2010, a total of 194 biopsies were performed, including 24 muscle controls specimens, obtained from individuals without a suspected mitochondrial disorder who underwent routine orthopaedic surgery. The OXPHOS and PDHc activities were

determined on these specimens. Numerous problems were encountered, e.g. storage and transport of specimens, because the hospital and laboratory were 200km from each other. Solutions had to be created; and the methods for the analyses of the enzyme activities were refined over time and conditions for handling, storage and transport were optimised. These refinements included the sample preparation using frozen biopsies, optimization and standardization of reaction conditions, development of reference ranges using biopsies of healthy controls and, more recently, modifying the assays to be performed in a high throughput format. From July 2006 up to December 2010, the refined protocols were applied and a total of 122 patients received biopsies during that period. Obtaining muscle biopsies remained an extremely complicated and logistically challenging issue.

3.2 CLINICAL OVERVIEW OF A COHORT OF SOUTH AFRICAN PATIENTS WITH MITOCHONDRIAL DISORDERS

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RESEARCH REPORT

An overview of a cohort of South African patients with mitochondrial disorders

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Abstract Mitochondrial disorders are frequently encountered inherited diseases characterized by unexplained multisystem involvement with a chronic, intermittent, or progressive nature. The objective of this paper is to describe the profile of patients with mitochondrial disorders in South Africa. Patients with possible mitochondrial disorders were accessed over 10 years. Analyses for respiratory chain and pyruvate dehydrogenase complex enzymes were performed on muscle. A diagnosis of a mitochondrial disorder was accepted only if an enzyme activity was deficient. Sixty-three patients were diagnosed with a mitochondrial disorder, including 40 African, 20 Caucasian, one mixed ancestry, and two Indian patients. The most important findings were the difference between African patients and other ethnicities: respiratory chain enzyme complexes CI+III or CII+III deficiencies were found in 52.5% of African patients, being of statistical significance (p value=0.0061). They also presented predominantly with myopathy (p value=0.0018); the male:female ratio was 1:1.2. Twenty-five (62.5%) African patients presented with varying degrees of a myopathy accompanied by a myopathic face, high palate, and scoliosis. Fourteen of these 25 also had ptosis and/or progressive external ophthalmoplegia. No patients of other ethnicities presented with this specific myopathic phenotype. Caucasian patients (16/20) presented predominantly with central nervous system involvement. Of the South African pediatric neurology patients, Africans are more likely to present with myopathy and CII+III deficiency, and Caucasian patients are more likely to present with encephalopathy or encephalomyopathy.

Abbreviations

CI to IV	Respiratory chain enzyme complexes I to IV, respectively
CS	Citrate synthase
EGTA	Ethylene glycol tetra-acetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
MDC	Mitochondrial Disease Criteria

Introduction

Patients and parents of children with unexplained chronic disorders usually have a critical need to understand what is wrong and why they have the disorder. Smeitink (2003) concludes that a mitochondrial disorder should be considered in "every unexplained chronic, intermittent, or progressive disorder with single or multisystem involvement, even if the lactic acid is normal". With mitochondrial disorders now among the most frequently encountered inherited diseases, with a minimum prevalence of at least 1 in 5,000 affecting patients of all ages and mitochondrial encephalomyopathies affecting 1 in 21,000 children under the age of 16 years, it is crucial to have a center that is able to offer a comprehensive diagnostic service (Schaefer et al. 2004; Darin et al. 2001). Although previously recognized,

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the prevalence of mitochondrial disorders in South Africa is still unknown. Diagnosis of these disorders is complicated and requires a combination of clinical, histochemical, and biochemical assessment of oxidative phosphorylation and other related functions, as well as molecular genetic studies. Although screening for a limited number of point mutations is available in SA, a comprehensive service has not been available to confirm diagnoses. Logistical issues further complicate diagnosis; for example, patients living in remote rural areas with no transport or means of communication readily available and the scattered and distant locations for clinical and biochemical evaluations. This paper reports on the clinical and biochemical findings in South African patients obtained during a 10-year study on these disorders.

Methods

Ethical considerations

Ethical approval for the study was obtained from the University of Pretoria (No. 91/98 and amendments) and from the North-West University (02M02). Informed consent was obtained from the parents of patients and controls.

Patients

Patients with neuromuscular disorders, mostly children, were assessed at the Paediatric Neurology Unit at Steve Biko Academic Hospital, Pretoria, South Africa, which provides services for urban and rural communities. The area from which the majority of the patients were referred has 5.9 million children under the age of 15 years and primarily encompasses three provinces: Gauteng, Mpumalanga, and Limpopo (Statistics South Africa 2009). Assessment included a detailed history; clinical examination; baseline investigations, including lactate (L), pyruvate (P), creatine kinase (CK), and ammonia (NH_3); and appropriate patient-specific investigations according to clinical findings. A computed tomography (CT) scan or magnetic resonance imaging (MRI) of the brain was performed in cases in which the patient had clinical features of central nervous system (CNS) involvement. Nerve conduction studies (NCS) and electromyography (EMG) were performed in cases in which a neuropathy or myopathy was suspected. Brainstem auditory-evoked responses (BAER) and visual-evoked potentials (VEP) were requested in cases in which the patient had clinical hearing or visual impairment. Electrocardiography (ECG) and cardiac sonography were performed, and a chest X-ray (CXR) was taken in cases in which the patient had cardiomegaly, cardiac murmurs, or irregular pulses. The Mitochondrial Disease Criteria (MDC) score was calculated for every patient (Wolf and Smeitink 2002). Muscle biopsies on vastus lateralis muscle were performed on patients with an MDC score of six and higher or in cases in which the patient had a specific phenotype suggestive of a mitochondrial etiology. A total of 191 patients with possible mitochondrial disorders were assessed from 1999 until June 2009, and a total of 140 muscle biopsies were analysed, including 24 controls.

Biochemical analyses

Metabolic analyses of urine samples included analyses of amino acids (AA), organic acids (OA), acylcarnitines (AC), and oligosaccharides (OS) (Jooste et al. 1994; Loots et al. 2007; Sewell 2008; Van Rooyen et al. 1994). Mitochondrial respiratory chain (RC) enzymes (CI–IV; EC 1.6.5.3, EC 1.3.5.1, EC 1.10.2.2, EC 1.9.3.1, respectively), pyruvate dehydrogenase complex (PDHc, EC 1.2.4.1), and citrate synthase (CS, EC 2.3.3.1) activities were measured in muscle, essentially as described by Rahman et al. (1996), Janssen et al. (2007), and Shepherd and Garland (1969). PDHc activity reported in this paper was measured using the pyruvate dehydrogenase (PDH) Enzyme Activity Dipstick Assay Kit (MitoSciences®, Eugene, OR, USA). Analyses were performed using 600 x *g* supernatants that were prepared from homogenizing frozen muscle samples in an isotonic buffer [mannitol 210 mM; sucrose 70 mM; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 5 mM; ethylene glycol tetraacetic acid (EGTA) 0.1 mM; pH 7.2). Reference values for enzyme activities, normalized to CS, CII, and CIV activities, were developed using muscle samples obtained from healthy children, predominantly of ages 3–16 years, who were undergoing routine orthopedic surgery (n=24). With a limited number of controls, distribution of control values was estimated using the Transformation Kernel Density Estimation program (Sheather and Marron 1990). A diagnosis of a mitochondrial disorder was made in cases where an enzyme deficiency was identified as follows: when an enzyme activity was lower than reference values when expressed against at least two of three enzyme markers (CS, CII, or CIV), providing that these were not deficient.

Data analyses

Biochemical data distributions of control values were measured as described in the previous section. A Jaccard cluster analysis was performed on the clinical and biochemical data using Bionumerics version 5 (Applied Maths).

Results

Sixty-three patients were diagnosed with RC and/or PDHc enzyme deficiencies. Using enzyme analyses performed on

muscle, seven of 33 patients were diagnosed in the initial phase (January 1999 to December 2003), seven of 15 were diagnosed in the intermediate phase (January 2004 to June 2006), and 49 of 63 were diagnosed in the final phase (July 2006 to June 2009) when reference values for enzyme activities of healthy controls were available, as opposed to using retrospective patient data in the initial phase of the study. Forty African patients, 20 Caucasians, one of mixed ancestry, and two Indians were included. The overall male: female ratio was 1.2:1, with 34 male patients (54.0%) and 29 female patients (46.0%). The African male:female ratio was 1:1.2, with 18 male patients (45.0%) and 22 female patients (55.0%). The Caucasian population had a male: female ratio of 1.9:1, with 13 male patients (65.0%) and seven female patients (35.0%). The male:female ratio of the other ethnicities >was collectively 2.3:1, with 16 male patients (69.6%) and seven female patients (30.4%). . The three patients of other ethnicities were all male. The majority of the patients (42 of the 63; 66.7%) had early onset of symptoms, with 21 (33.3%) having symptoms presenting in the neonatal period and 21 (33.3%) with symptoms presenting in the first year of life. As can be seen in Fig. 1, 21 patients (33.3%) presented with symptoms after the first year of life, nine (14.3%) between 1 and 5 years, five (7.9%) between 6 and 10 years, five (7.9%) in the second decade of life, and one (1.6%) each in the third and fourth decades of life.

The main clinical manifestations of mitochondrial disorders are illustrated in Fig. 2, and Table 1 summarizes the clinical and biochemical manifestations of the individual patients investigated. Muscle involvement, including hypotonia, weakness, exercise intolerance, and myalgia, was found in 55 of the 63 patients (87.3%). Thirty-seven of the

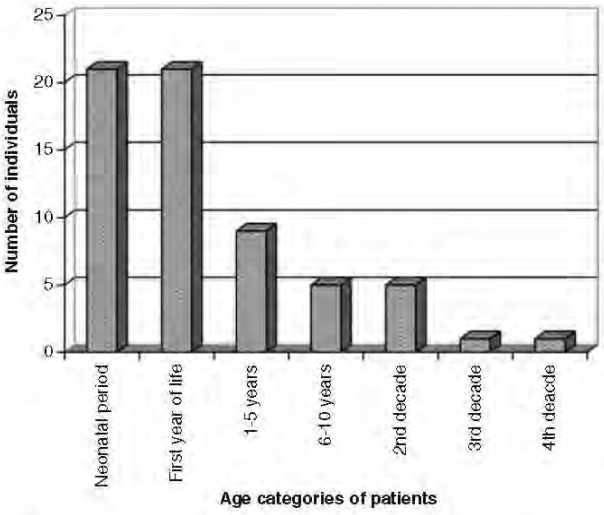

Fig. 1 Age categories depicting age of symptom onset of South African patients with confirmed mitochondrial disorders

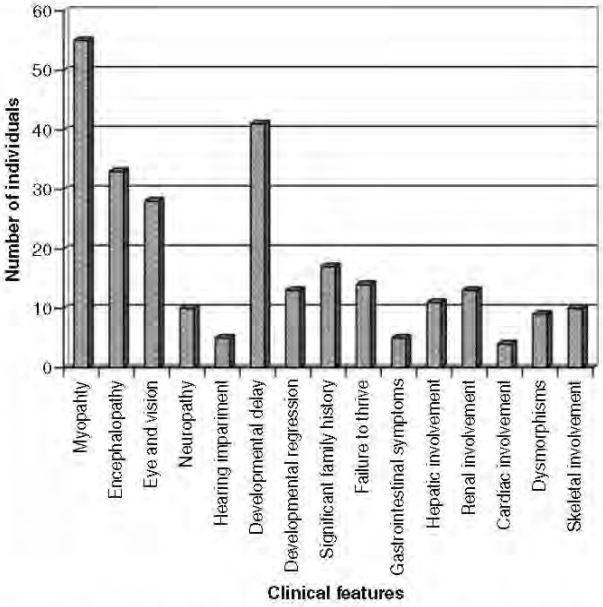

Fig. 2 Summary of major clinical findings in South African patients with confirmed mitochondrial disorders

40 African patients (92.5%) presented with muscular involvement (Table 2; Fig. 3a). The typical presentations of these patients (25 of 40; 62.5%) included varying degrees of myopathy accompanied by a myopathic face, high palate, triangular mouth, and scoliosis. Fourteen of these 25 patients (56.0%) also had ptosis and/or progressive external ophthalmoplegia. None of the Caucasian or patients of other ethnicities presented with this specific myopathic phenotype. Only four of the 20 Caucasian patients (20.0%) presented with predominant muscular symptoms. CNS involvement as the predominant symptom with or without muscular symptoms was the more common phenotype among Caucasian patients. It is interesting to observe that seven of the 20 (35.0%) older Caucasian patients presented with significant progressive myalgia and exercise intolerance (Table 2; Fig. 3b). None of these seven patients had presented with any symptoms in the first 2 years of life, and four of the seven (57.1%) presented with symptoms in the second decade of life and later. Four of the 20 Caucasian patients (20.0%) had significant encephalopathy, and 12 (60.0%) had encephalomyopathy (Table 2; Fig. 3b). Only three of the 40 African patients (7.5%) presented with predominant CNS involvement, and 12 (30.0%) presented with encephalomyopathy (Table 2; Fig. 3a). The z-test for the difference between two proportions (Table 2) yielded a p value of 0.0018 for the difference between proportions of Africans and Caucasians and those of other ethnicities with myopathy, indicating that significantly more African patients had myopathy than Caucasian and other ethnicities.

Table 1 Summary of clinical and biochemical data of South African patients with confirmed mitochondrial disorders

			Major clinical manifestations										Enzyme deficiencies								Biochemical abnormalities								
Patient number	Sex	Ethnicity	Muscle	CNS	Eye and vision	Hearing impairment	Neuropathy	GIT	Kidney	Liver	Skeletal involvement	MDC	CI	CII	CIII	CIV	CV*	CI+III*	CII+III	PDHc deficiency	Lactic acidosis	Raised L:P	Raised CK	Raised NH_3	Lactate in urine	OA	AC	AA	OS
1	F	C	■	■						■		7								■									
2	F	C	■		■	■			■		■	6				■									■	■		■	■
3	M	MA	■					■	■	■	■	4						■								■		■	■
4	M	A	■		■						■	7				■										■			■
5	M	C	■									6				■							■			■		■	
6	M	A	■								■	8						■					■			■			■
7	M	C	■	■								7					■									■		■	
8	F	A		■					■			3							■						-	-	-	-	-
9	M	A	■		■							5						■				■				■			
10	F	A	■						■			6	■											■		■			
11	M	A	■		■		■					7						■				■				■			
12	M	I		■	■							5	■													■			■
13	M	C		■								8				■						■				■			
14	M	C		■							■	3				■						■			-	-	-	-	-
15	F	C	■	■	■		■					8				■	-	-				■				■			■
16	F	A	■		■		■					8	■				-	-	■			■				■		■	
17	F	A	■		■							3		■			-	-	■	■			■			■			
18	F	A	■									3		■	■		-	-							-	-	-	-	-
19	F	A	■		■							6			■		-	-				■			-	-	-	-	-
20	F	A	■									5	■				-	-							-	-	-	-	-
21	M	A	■		■							3		■	■		-	-	■						-	-	-	-	-
22	M	C	■	■					■			8					-	-		■		■				■			■
23	M	I	■	■								6	■				-	-				■	■			■			
24	M	C	■	■								8	■		■		-	-								■			
25	M	C	■		■							2				■	-	-										■	
26	M	A	■	■	■							10	■			■	-	-	■			■	■	■		■	■	■	■
27	F	A	■		■				■			6			■		-	-								■			■
28	F	A	■	■	■							6	■				-	-	■							■			
29	M	C	■	■								3					-	-	■			■			-	-	-	-	-
30	F	A	■	■								5			■		-	-	■						-	-	-	-	-
31	M	A	■	■	■					■		5	■				-	-								■			■
32	M	A	■		■		■					8	■	■	■	■	-	-	■			■							
33	M	A	■	■						■		3		■			-	-							-	-	-	-	-
34	F	A	■									2	■	■	■	■	-	-	■	■			■			■			
35	F	C	■	■				■				7				■	-	-	■							■			
36	F	C	■	■	■	■						7	■				-	-	■			■							
37	M	A	■									2					-	-	■						-	-	-	-	-
38	M	C		■								8	■				-	-				■	■			■			
39	M	A		■						■		6	■		■		-	-								■			■
40	M	A	■					■	■	■	■	7	■		■	■	-	-	■			■	■			■			
41	M	C	■	■			■					7	■				-	-				■	■			■			
42	M	A	■		■					■		8					-	-				■	■			■			■
43	F	A		■	■							3	■		■	■	-	-	■						-	-	-	-	-
44	F	A	■		■							4					-	-		■						■			
45	F	A	■	■								4			■		-	-	■						-	-	-	-	-
46	M	A	■					■	■		■	6	■				-	-	■							■			■
47	F	A	■	■								7	■			■	-	-	■			■				■			■
48	M	A	■	■			■				■	7			■		-	-	■							■			
49	M	A	■						■			3		■			-	-	■										■
50	F	A	■				■					8	■				-	-					■			■			■
51	F	A		■	■							6			■		-	-								■			■
52	F	C		■		■				■		3	■			■	-	-							-	-	-	-	-
53	F	A	■						■			7	■				-	-			■					■			■
54	M	A	■		■		■				■	6				■	-	-								■			■
55	M	C	■			■	■					5				■	-	-							-	-	-	-	-
56	F	C				■		■				10	■				-	-								■			
57	F	A	■	■	■				■			8				■	-	-				■				■			■
58	F	A	■	■	■					■		8				■	-	-					■	■		■			■
59	M	A	■									4				■	-	-					■			■			■
60	F	A	■	■							■	8				■	-	-					■			■			
61	F	A	■	■	■							8				■	-	-				■	■	■		■			■
62	M	C	■	■						■		6	■			■	-	-	■				■			■			
63	M	C	■	■						■		7	■			■	-	-					■			■		■	
TOTAL			55	33	28	5	10	5	13	11	10		23	6	27	20	1	4	23	5	24	14	10	5	7	44	28	35	27

◀ Grey cells indicate presence of clinical or biochemical involvement. Blocks containing hyphens (-) indicate absence of information (not done). *Analyses performed using enriched mitochondria in initial samples only. Abbreviations: AA, amino acids; AC, acylcarnitines; A, African; C, Caucasian; CI to IV (respiratory chain enzyme complexes I to IV, respectively); CV, complex V; CK, creatine kinase; CNS, central nervous system; F, female; GIT, gastrointestinal tract; I, Indian; L:P: lactate to pyruvate; M, male; MDC, Mitochondrial Disease Criteria; MA, mixed ancestry; NH_3, ammonia; OA, organic acids; OS, oligosaccharides; PDHc, pyruvate dehydrogenase complex.

Ophthalmologic involvement was found in 28 of the 63 patients (44.4%). Symptoms included nonparalytic strabismus, external ophthalmoplegia, ptosis, and retinitis pigmentosa. None of the Caucasian patients presented with external ophthalmoplegia or ptosis, but 17 of the 22 African patients with ophthalmologic involvement (77.3%) had external ophthalmoplegia and/or ptosis. Five of the 63 (7.9%) patients had hearing impairment and ten (15.9%) had neuropathy. Developmental delay was present in 41 of the 63 patients (65.0%), with 27 of the 40 African patients (67.5%) and 14 of the 23 Caucasian patients and patients of other ethnicities (60.1%). Thirteen patients (20.6%) experienced developmental regression.

Thirteen of the 63 (20.6%) patients had renal involvement ranging from asymptomatic aminoaciduria to rickets and de Toni-Debré-Fanconi syndrome. Gastrointestinal symptom was dysmotility and was found in five (7.9%). Failure to thrive was documented in 14 (22.2%) and hepatomegaly or deranged liver functions were in 11 (17.5%). A detailed family history was present in 17 (27.0%). Only four patients (6.3%) had documented cardiac involvement; nine (14.3%) had minor dysmorphisms; ten (15.9%) had skeletal involvement, including rickets and hypermobile joints, but scoliosis secondary to muscle weakness was excluded. Mean MDC score was 5.9. Lactic acidosis was found in 24 (38.1%) and a raised lactate–pyruvate ratio (>18) in 14 (22.2%). Creatine kinase was raised in ten (15.9%) and ammonia in five (7.9%). Abnormal organic acid profiles with elevated Krebs's cycle metabolites and/or dicarboxylic acids were found in 24 (38.1%). No OA data was available for 14 of the 63 patients (22.2%). Abnormal amino acid profiles were found in 28 (44.4%) and amino acid data was unavailable for 14 (22.2%). Abnormal acylcarnitines were found in 22 (34.9%), and 27 (42.9%) had abnormal oligosaccharide profiles. Radiological changes were found in 34 (54.0%); nonspecific atrophy in 17; white matter involvement in 12; basal ganglia in five; cerebellar atrophy in five; absent or hypoplastic corpus callosum in two; and findings resembling recovered stroke episodes in two.

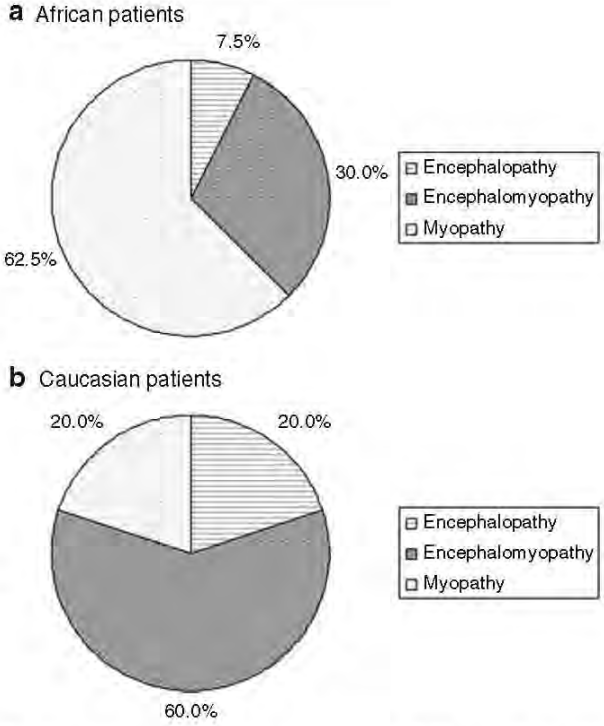

Fig. 3 **a** African patients. **b** Caucasian patients. Differences in clinical manifestation of South African patients with mitochondria-related neuromuscular disorders

Thirteen African patients (32.5%) had single enzyme deficiencies, with four CI, one CII, six CIII, one CIV, and one PDHc. Complex I as a single deficiency was only found in four African patients (10.0%); 27 (68.0%) had combined deficiencies, with CI+III or CII+III involved in 21 of these (77.8%). Two African patients (5.0%) had PDHc combined with other RC deficiencies, and four (10.0%) had nonspecific combined RC deficiencies (Figs. 4

Table 2 Encephalopathic versus myopathic manifestations in different population groups

Predominant manifestations	Number of African patients		Number of Caucasians and patients of other ethnicity		*P* value
Encephalopathy	3	7.5%	5	21.7%	0.1030
Encephalomyopathy	12	30.0%	13	56.6%	0.0385
Myopathy	25	62.5%	5	21.7%	0.0018
Total	40		23		

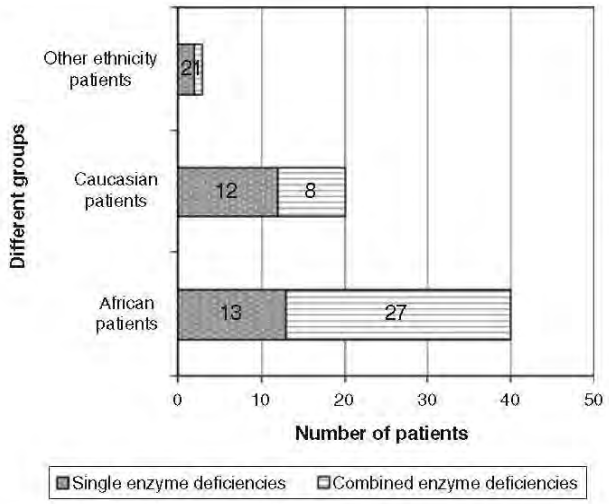

Fig. 4 Combined versus single enzyme deficiencies in various South African patients

and 5a). However, CIII as a single deficiency or combined with other defects were found in 29 African patients (72.5%), of whom 19 (47.5%) had a CII+III (succinate-cytochrome *c* reductase activity) deficiency. Twelve Caucasian patients and those of other ethnicities (60.0%) had single enzyme deficiencies, with four CI, six CIV, one CIII, and two PDHc. Complex I as a single deficiency was found in four of these 23 patients (17.4%), and CIII as single or in combination with other defects was found in nine (39.1%; Fig. 5b). In this group, there were only four patients (17.4%) with CI+III or CII+III deficiencies and five (21.7%) with combined single enzyme RC deficiencies (Figs. 4 and 5b). The z-test for the differences between two proportions yielded a p value of 0.0061 for the difference between the proportion of Africans and Caucasians and those of other ethnicities with CI+III or CII+III deficiencies, indicating that significantly more African patients had these deficiencies than Caucasians and other ethnicities.

The Jaccard cluster analysis (Fig. 6) illustrated clearly that African patients tend to cluster together in terms of predominant myopathic involvement. It is also evident that a CII+III deficiency is mainly found in this group. It was also found in three Caucasian patients with exercise intolerance. However, no Caucasian patient with predominant CNS involvement was found in this group of deficiencies.

Discussion

To our knowledge, this study was the first in South Africa to characterize mitochondrial disorders clinically and biochemically in a group of patients over a period of 10 years. Screening for point mutations was not done routinely owing to the low yield of positive results and relative high cost. Although this study had many limitations, including the lack of genetic data, important logistic and procedural challenges were overcome, in addition to refinement of analyses and reference values. This resulted in an increased yield of positive results from 21.2% initially to 72.1%.

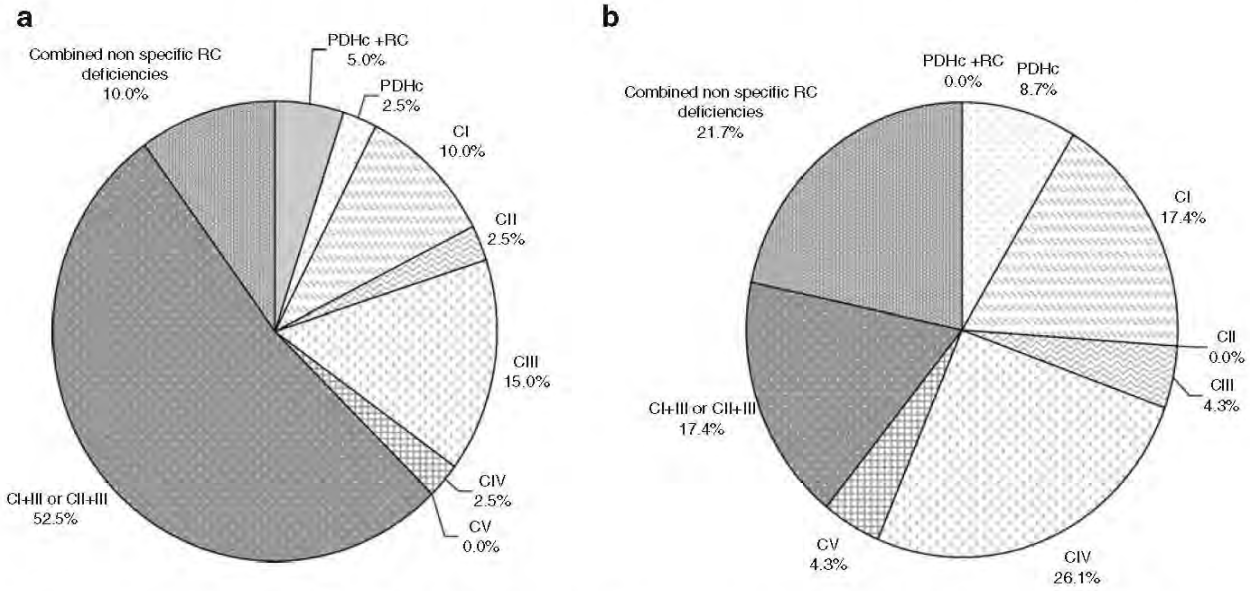

Fig. 5 **a** African patients. **b** Caucasian, mixed ancestry, and Indian patients. Combined nonspecific respiratory chain (RC), pyruvate dehydrogenase complex (PDHc), and specific RC deficiencies in the South African population

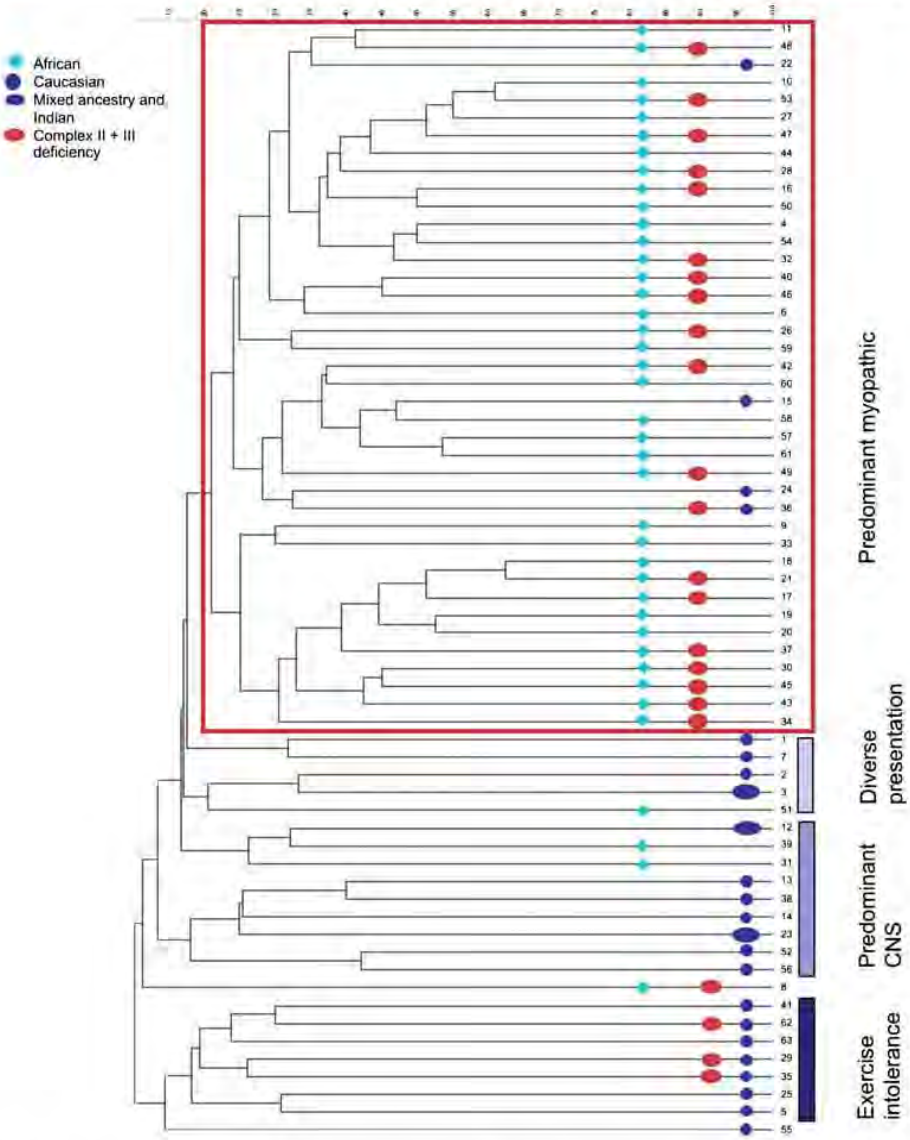

Fig. 6 Jaccard cluster analysis of South African patients with confirmed mitochondrial disorders

Using existing data and observing the age-matched population of the three provinces that form the source of this study, a prevalence of 1 per 100,000 children can be calculated. This value, however, is most likely an underestimation considering the strict inclusion criteria followed and the misdiagnosis or nondiagnosis of patients who were thus given no referral for further investigation. Although the number of patients with biochemically confirmed mitochondrial disorders reported in this study was limited to 63, and many aspects were in line with previous descriptions (Munnich et al. 1996; Munnich and Rustin 2001; Nissenkorn et al. 1999; Sciacco et al. 2001), important differences were observed. Of three recent studies with comparable data (Skladal et al. 2003a; Scaglia et al. 2004; Debray et al. 2007) our study, included the largest number of African patients (63.0%), with 31.7% Caucasian patients (Table 3).

The overall South African male:female ratio was comparable with other studies: 1.2:1 (Skladal et al. 2003a; Scaglia et al. 2004; Debray et al. 2007), but it is interesting to observe that the male:female ratio for African patients was 1:1.2, with females more affected. In the Austrian cohort, 41.3% of patients had early neonatal onset in contrast to only 33.0% of South African patients. African patients presented earlier than Caucasian patients. Population differences were noted in other contexts as well; Skladal et al. (2003b) documented that the age of presentation in Lebanese patients was significantly lower than in non-Lebanese patients and the minimum birth prevalence of respiratory chain disorders was 12-fold higher in this group.

Table 3 Summary of selected findings in four different studies describing mitochondrial disorders in population groups

Patients	Austria and Czech Republic (Skladal et al. 2003a) $n=75$	USA (Scaglia et al. 2004) $n=113$	Canada (Debray et al. 2007) $n=73$	South African study (2009) $n=63$
Ethnicity				
Caucasian	*	46.0%	93.0%	31.7%
Hispanic	*	33.0%	*	*
African	*	9.0%	*	63.5%
Southeast Asian	*	6.0%	*	*
Arabian Peninsula	*	4.0%	*	*
Native American	*	2.0%	*	*
Other	*	*	7.0%[a]	3.8%[b]
M:F	1.8:1	1.4:1	1.4:1	1.2:1
Onset				
Neonatal	41.3%		9.6%	33.3%
First year	57.0%[c]		*	33.3%
Mean/median age of onset (months)	*	Cardiac group: 33 Other: 44	7	*
Clinical presentations				
Intermittent neurological symptoms	*	*	5.5%	*
Mitochondrial syndromes[d]	53.3%	21.2%	23.2%	*
Encephalopathy	45.3%	39.0%	31.5%	C: 20.0% A: 7.5%
Encephalomyopathy	*	*	19.2%	C: 60.0% A: 30.0%
Myopathy	*	*	*	C: 20.0% A: 62.5%
Visceral	*	*	11.0%	*
Cardiomyopathy and myopathy	1.3%	39.8%	*	*
Involved organ systems				
Skeletal muscle	88.0%	79.0%[e]	*	All: 87.3% A: 92.5% C: 20.0%
CNS	73.3%	68.0%[f]	90.4%	52.4%
Eye	53.3%	32.0%	42.0%	44.4%
GIT	48.0%	*	8.2%	7.9%
Heart	42.7%	*	17.8%	6.3%
Bone marrow	33.3%	*	6.8%	*
Liver	18.7%	*	16.4%	17.5%
Kidney	10.7%	*	11.0%	20.6%
Developmental delay	60.0%[g]	68.0%[h]	79.0%	65.1%
Failure to thrive	26.7%	*	52.1%	22.2%
Exercise intolerance	27.0%[e]	*	*	15.9%
Hearing loss	9.3%	21.0%	26.0%	8.0%
RC and PDHc analyses				
CI	20.0%	32.0%	25.0%	12.7%
CII	*	7.0%	*	1.6%
CII/III	7.7%	*	*	*
CIII	*	*	*	11.1%
CIV	29.2%	19.0%	27.0%	11.1%
CV	*	*	*	1.6%

Table 3 (continued)

Patients	Austria and Czech Republic (Skladal et al. 2003a) *n*=75	USA (Scaglia et al. 2004) *n*=113	Canada (Debray et al. 2007) *n*=73	South African study (2009) *n*=63
PDHc	10.8%	*	25.0%	4.8%
CI+III or II+III	*	16.0%	5.0%	39.7%
Combined I, III & IV	*	26.0%	*	*
Combined non-specific	15.4%	*	13.0%	14.3%
PDHc+RC	10.8%	*	*	3.2%
PC	*	*	5.0%	*

A African, *C* Caucasian, *CNS* central nervous system; *CI – CIV* respiratory chain (RC) enzyme complexes I – IV and combinations, *CV* ATP synthase, *F* female, *GIT* gastrointestinal tract, *M* male, *PDHc* pyruvate dehydrogenase complex

Blocks containing asterisks (*) indicate information not specified.

[a] Includes one patient of each of the following: Haitian Black, Indian, Pakistani, Moroccan, Turkish (n=5)

[b] Includes two Indian patients and one patient of mixed ancestry

[c] Late onset implies onset after the first month of life

[d] Includes CPEO, chronic progressive external ophthalmoplegia; KSS, Kearns Sayre; LS, Leigh syndrome; LIMD, lethal infantile mitochondrial disease; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; Pearson syndrome; Leigh syndrome; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; NARP, neuropathy, ataxia and retinitis pigmentosa and Pearson syndrome

[e] Was specified as hypotonia only

[f] Symptoms were specified as seizures (51.0%), movement disorders (12.0%) and ataxia (6.0%)

[g] Present in the late onset group

[h] Developmental delay was present in all the patients with no cardiac involvement

In the South African study, African patients predominantly (62.5%) had muscular involvement, with a distinct phenotype not observed in Caucasian patients; 30.0% presented with encephalomyopathy; and only 7.5% presented primarily with CNS involvement (Fig. 3a, b). In addition, involvement of a CII+III deficiency was evident and statistically significant, and although it was not measured in the tissue, an underlying coenzyme Q deficiency may be a possibility. The contribution of mitochondrial DNA haplogroups in the expression of mitochondria-related disorders is often described (Brown et al. 2002; Khusnutdinova et al. 2008; Herrnstadt and Howell 2004), and thus, along with other genetic factors, diet or lifestyle may also contributed to the differences in phenotypic expression among these diverse patients. Caucasian patients presented with an encephalomyopathic phenotype in 60.0% of cases, 20.0% had pure CNS involvement, and in the older patient group exercise intolerance with unexplained myalgia was prominent (Fig. 3b). Patients were recruited from the pediatric neurology clinic, which explained the bias toward the neuromuscular presentation of mitochondrial disorders and the limited number of older patients. Patients might also have been missed owing to strict inclusion criteria for a muscle biopsy. The calculated MDC score might have resulted in underscoring, as limited histology was available. Furthermore, criteria to define a deficiency were relatively strict, as a deficiency expressed against two markers was required.

The frequency of hearing loss was comparable in the Austrian and South African studies, with 9.3% and 8.0%, respectively (Skladal et al. 2003a). All South African patients with hearing impairment were Caucasian. The presence of altered muscle tone, including hypotonicity and/or hypertonicity, was present in 77.0% of Austrian patients (Skladal et al. 2003a) and in 71.0% of the South African group.

Single enzyme deficiencies in the Caucasian group (60.0%) were more common than combined deficiencies, but combined deficiencies were found in 67.5% of African patients (Table 1; Fig. 4). It is interesting to note that of the five patients identified as having a PDHc deficiency, only one was male. The Jaccard cluster analyses (Fig. 6) clearly illustrates that African patients tend to cluster together in terms of predominant myopathic involvement, and CII+III is mainly found in this group.

Mitochondrial syndromes were identified in 53.0% of cases in the Austrian study, which is considerably higher than in the American and Canadian studies, which reported this as 21.2% and 23.2%, respectively (Skladal et al. 2003a; Scaglia et al. 2004; Debray et al. 2007). Owing to a lack of mutation analyses, the South African study cannot be compared in this regard.

Conclusion

Phenotypes of mitochondrial disorders in the South African population have been recognized by physicians in the past. Within a health system facing significant and wide-ranging challenges, as recently extensively reviewed by Lawn and Kinney (2009), these disorders are mostly underdiagnosed. The calculated prevalence for this study, which served three provinces, is comparatively low (five to 20 times) considering the existing epidemiological data for developed countries and exposes the limitations of the existing capacity to diagnose these disorders in the South African population.

The different groups of patients with mitochondria-related neuromuscular disorders in South Africa have different phenotypes. African patients present predominantly with myopathy associated with CII+III deficiency, and Caucasian patients present predominantly with encephalopathy or encephalomyopathies. The confirmation of mitochondrial disorders in a developing country remains a challenge. A useful and simplified approach to enhancing awareness of mitochondrial disorders and prompt referral of patients in the South African scenario would be to consider a mitochondrial disorder in cases in which two or more seemingly unexplained and unrelated symptoms are observed (Munnich and Rustin 2001; Nissenkorn et al. 1999). In addition, biochemical and molecular genetic analyses for diagnosing these inherited metabolic disorders should be well coordinated in a country in which resources are limited and the burden of other, more pressing, health issues is increasing.

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We embarked on a metabolomics approach in order to identify markers that could be used as a screening tool to refine the selection of patients for a confirmatory muscle biopsy. For the purpose of the metabolomics study, a group of 101 patients was selected. The inclusion criteria were that the muscle biopsy should have been done after 2006 and urine specimens taken at the time of the biopsy should have been available for the metabolomics study. The detailed methodology is explained in the article in Section 3.3 where a group of 24 metabolites was identified as useful. Furthermore, a global metabolomics profile of carbohydrate, fatty acid and amino acid catabolism associated with respiratory chain disorders (RCDs) was constructed, and is also discussed in the same article in Section 3.3.

3.3 METABOLOMICS OF URINARY ORGANIC ACIDS IN RESPIRATORY CHAIN DEFICIENCIES IN CHILDREN

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ORIGINAL ARTICLE

Metabolomics of urinary organic acids in respiratory chain deficiencies in children

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Abstract Metabolomic analysis of the urinary organic acids from 39 selected children with defined respiratory chain deficiencies (RCDs) was performed using untargeted gas chromatography–mass spectrometry, revealing the presence of 255 endogenous and 46 exogenous substances. Variable reduction identified 92 variables from the endogenous substances, which could be analysed by univariate and multivariate statistical methods. Using these methods, no characteristic organic acid biomarker profile could be defined of practical value for diagnostic purposes for complex I (CI), complex III (CIII) and multiple complex (CM) deficiencies. The statistical procedures used did, however, disclose 24 metabolites that were practical highly ($d > 0.75$) and statistically ($P < 0.05$) significant for the combined and clinically closely related group of RCDs. Several of these metabolites occur in single enzyme inherited metabolic diseases, but most were not previously reported to be linked to the metabolic perturbations that are due to RCDs. Ultimately, we constructed a global metabolic profile of carbohydrate, amino acid and fatty acid catabolism, illuminating the diverse and complex biochemical consequences of these disorders. This metabolomics investigation disclosed a metabolite profile that has the potential to define an extended and characteristic biosignature for RCDs and the development of a non-invasive screening procedure for these disorders.

Keywords Metabolomics · Respiratory chain deficiencies · Urinary organic acids · Data reduction · Global metabolite profile

1 Introduction

Impaired energy metabolism, due to genetically based dysfunction of one or more components of the mitochondrial oxidative phosphorylation (OXPHOS) system, underlies the pathology of what are generally referred to as mitochondrial disorders (MD) in humans. The OXPHOS system consists of several multimeric and heterologous enzyme complexes, designated complex I to IV (respiratory chain, RC) and complex V (F-type ATP synthase, ATPase), which are encoded by nuclear as well as mitochondrial DNA (reviewed by Anderson et al. 1981). Although the RC primarily supports the transport of electrons from reducing cofactors (e.g. $NADH_2$ and $FADH_2$,), metabolic consequences of RC deficiencies (RCDs) include increased concentrations of organic acids (Munnich et al. 1992; Esteitie et al. 2005) and perturbed amino acid profiles (Rabier et al. 1998) in blood and cerebrospinal fluid. These result in a diverse profile of organic acids and amino acids excreted in the urine. Unlike many other inherited metabolic disorders due to single enzyme deficiencies, however, there are no known specific diagnostic biochemical biomarkers for disorders involving RC deficiencies (Suomalainen 2010; Haas et al. 2008; Mancuso et al. 2009). As with all inherited MDs there is no cure for these

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deficiencies, and the variety of treatments proposed over time for different RCDs may or may not be effective (Kurlemann et al. 1995). Several scoring systems, which include some of the RCD-associated biochemical markers in body fluids as minor criteria, have been developed to assist in the diagnosis of these disorders (Bernier et al. 2002; Wolf and Smeitink 2002; Morava et al. 2006). However, the diagnostic process is still complicated and tedious. The organic acid profile from suspected cases for a MD provides only information for a tentative indication of RCDs. A complete and reliable diagnosis of RCDs requires costly and invasive procedures and relies almost exclusively on enzyme analyses on muscle biopsy material and/or molecular genetics analyses. The initial diagnosis of patients with suspected MDs would thus greatly benefit from the identification of biochemical biomarkers in an easily obtainable specimen as a useful decision-guiding instrument.

Barshop (2004) attempted to refine the metabolic markers for MDs by means of a correlation study of a retrospective analysis of 3646 randomly selected urine samples referred to a laboratory for diagnostic purposes. The metabolomic profile from this investigation confirmed that abnormalities in the organic acid profiles are a feature of most MDs and provided further quantitative and qualitative insights into these disorders. Although somewhat limited for diagnostic purposes, elevated fumaric and malic acids were found to be the most useful in distinguishing patients with a mitochondrial deficiency from those with the organic acidaemias. Barshop, however, noted that further refinements were required to develop a characteristic metabolomic profile and may improve the use of quantitative organic acid analysis in MDs. More recently, Mancuso et al. (2009) remarked that the ideal biomarker for mitochondrial disorders should improve on the timing and accuracy of the diagnosis. Furthermore, it should contribute to limiting the number of invasive diagnostic procedures and ultimately be useful for monitoring the effect of drug treatment and disease progression (Mancuso et al. 2009). In this regard, the use of urine samples provides minimal invasiveness and several practical advantages, aspects which are of great importance where improved screening using metabolic data in the diagnostic process is an imperative. The availability of urine samples from a relatively large group of clinically and biochemically well-defined South African patients with an enzymatically confirmed respiratory chain disorder (Smuts et al. 2010) presented a unique opportunity to address these issues through a metabolomics investigation.

For this investigation we asked the question whether a metabolomics approach, using an organic acid profile of urine, could expand the existing information on the metabolites elevated due to an RCD, contribute to the better understanding of the perturbations due to the RCD, and provide a critical first step towards the future development of biomarkers for RCDs. Urine was selected as the source for data generation as it is a primary option in clinical metabolic investigations and because variations in several urinary organic acids in RCDs have been reported. A significant aspect of the cohort of RCD patients we investigated was the availability of enzyme activity data of the RC complexes I–IV, measured in muscle biopsy material. This opened up the possibility of determining if the origin of these elevated metabolites could be explained in light of the well-defined, but wide-ranging, biochemical disturbances associated with RCDs. To explore these questions, the organic acid-containing section of the metabolome from RCD patients and controls was investigated, and the organic acid profile was determined by untargeted gas chromatographic–mass spectrometric (GC–MS) analysis. Metabolic profiling depends on a clear understanding of the metabolic pathways of the subsection of the metabolome in question and the analytical methods and technologies used for the analyses (Dunn and Ellis 2005). As reported here, with the metabolomics approach followed, we succeeded in producing the first comprehensive list of statistically and practically significant elevated levels of several metabolites that have been previously described for RCDs, as well as increased levels of several metabolites not previously associated with these disorders. Our procedure also enabled the construction of a model of biochemical pathways indicating the putative origin of this complex array of metabolites from various, but related, catabolic pathways. This also paved the way for the development of possible biosignature(s) for RCDs.

2 Materials and methods

2.1 Sample selection

Ethical approval for the study was obtained from the relevant Ethics Committees of the University of Pretoria (No. 91/98 and amendments) and North-West University (No. 02M02). Informed consent was obtained from the parents of patients and controls for the use of the urine samples and biopsy material (where applicable) of their children for research purposes.

The urinary samples were obtained from patients referred to the Paediatric Neurology Unit at the Steve Biko Academic Hospital, Pretoria, South Africa. The original experimental group consisted of 101 clinically selected patients, including the cohort of South African patients as described by Smuts et al. (2010). The mean age of the original patient group was 5 years, ranging from 1 to 25 years of age. To improve a case–control comparison,

age as a first criterion for exclusion was used. Only data for patients of 13 years or younger, i.e. by definition a paediatric group, were initially selected for this investigation. Of these patients, 48 were identified with an RC enzyme deficiency (see Sect. 2.3). These patients constituted the group used for the metabolomics investigation (see Fig. 1) and were not further sub-divided on clinical criteria, like hypotonia or central nervous system presentations. The 92 controls were selected from a large group of 139 children referred to the clinic, but for whom no prevailing disorder could be detected. This known complexity of obtaining comparative control groups in investigations of inherited metabolic disorders is generally recognized (Barshop 2004). No match on race and gender could be achieved between the controls and patient groups, but there are no reports that indicate race or gender as confounding factors for rather comparable age groups as selected by us. The urinary organic acid profile does seem to vary with age, although little is known about age-specific changes or circadian rhythms of urinary organic acids reflecting metabolic changes and/or maturation of tubular function and how this might influence diagnosis of disorders of organic acids (Hoffman and Feyh 2005). The reference values given by Hoffman and Feyh (2005), never the less classify them according to four age groups: (1) Term newborns (>36 weeks), (2) children (<5 years), (3) children (>5 years) and (4) adults. A common practice followed in the Human Metabolomic Data Base is to classify urinary organic acids according to (1) newborns, (2) children (1–13 years) and (3) adults. From our experience with the present and other studies, the age difference between the control (mean = 4.6; s.d. = 2.7) and patient groups (mean = 8.9; s.d. = 2.9) as used here, does not produce a confounding factor.

2.2 Enzyme analyses and sample collection in the patient group

The enzyme analyses were performed on muscle biopsies from the *vastus lateralis* muscle of all patients complying with the Mitochondrial Disease Criteria (Wolf and Smeitink 2002). The activity of the following enzymes was determined to identify possible RC deficiencies: complex I to IV (EC 1.6.5.3, EC 1.3.5.1, EC 1.9.2.2 and EC 1.9.3.1, respectively) and citrate synthase (CS, EC 2.3.3.1), as a marker for mitochondrial content, using standard operating procedures based on existing methods (Shepherd and Garland 1969; Rahman et al. 1996; Janssen et al. 2007). Pyruvate dehydrogenase complex (PDHc, EC 1.2.4.1) was measured using the pyruvate dehydrogenase (PDH) Enzyme Activity Dipstick Assay Kit (MitoSciences, Eugene, OR). PDHc activity was measured to determine if any of the selected cases had this related deficiency. The criteria used for identification of an enzyme deficiency in this case cohort were somewhat more stringent than originally described by Smuts et al. (2010). Here two criteria were invoked to decide if a respiratory chain enzyme deficiency was present: the one was that the activity value should be lower than the lowest control value when expressed on CS; the other was that the activity also had to be less than at least the 5th percentile of the control group when expressed against either CII or CIV, if not deficient (if both were deficient only the first criterion was applied). As summarized in Table 1, using these criteria, 39 of the 48 paediatric patients complied with a muscle deficiency of complex I (CI), complex III (CIII) or a multiple deficiency of more than one RC enzyme (CM).

The urine specimen for metabolomics analysis was obtained at the time when the muscle biopsy was performed; the patients did not receive any specific treatment or supplements often given to patients with MD. Use of anti-convulsants was not stopped.

2.3 Acquisition of metabolite data

The organic substances were isolated from the urine, and consisted mostly of organic acids, derivatized and separated by gas chromatography according to a standardized technique described below, which we have refined since our first description of a South African organic aciduria case (Erasmus et al. 1985). This is also the procedure that we use in our participation in the quality assurance programme of the European research network for evaluation and improvement of screening, diagnosis and treatment of inherited disorders of metabolism (ERNDIM, www.erndim.unibas.ch). The semi-quantitative identification of the organic acids was conducted according to Chen et al. (2009). All organic acids identified above the detection limit of the equipment used were expressed as mmol per mol creatinine.

2.3.1 Extraction and derivatization of organic acids

Urine samples of approximately 20 ml were obtained from patients and controls. The samples were frozen at −80°C upon delivery and transported to the laboratory on dry ice. The samples were thawed at room temperature and an aliquot was prepared for creatinine and organic acid analysis, respectively. The volume of urine used for organic acid analysis was based on urinary creatinine values, according to the following guidelines:

- Creatinine values higher than 8.8 mmol/l: 0.5 ml of urine.
- Creatinine values lower than 8.8 mmol/l and higher than 0.44 mmol/l: 1 ml of urine.

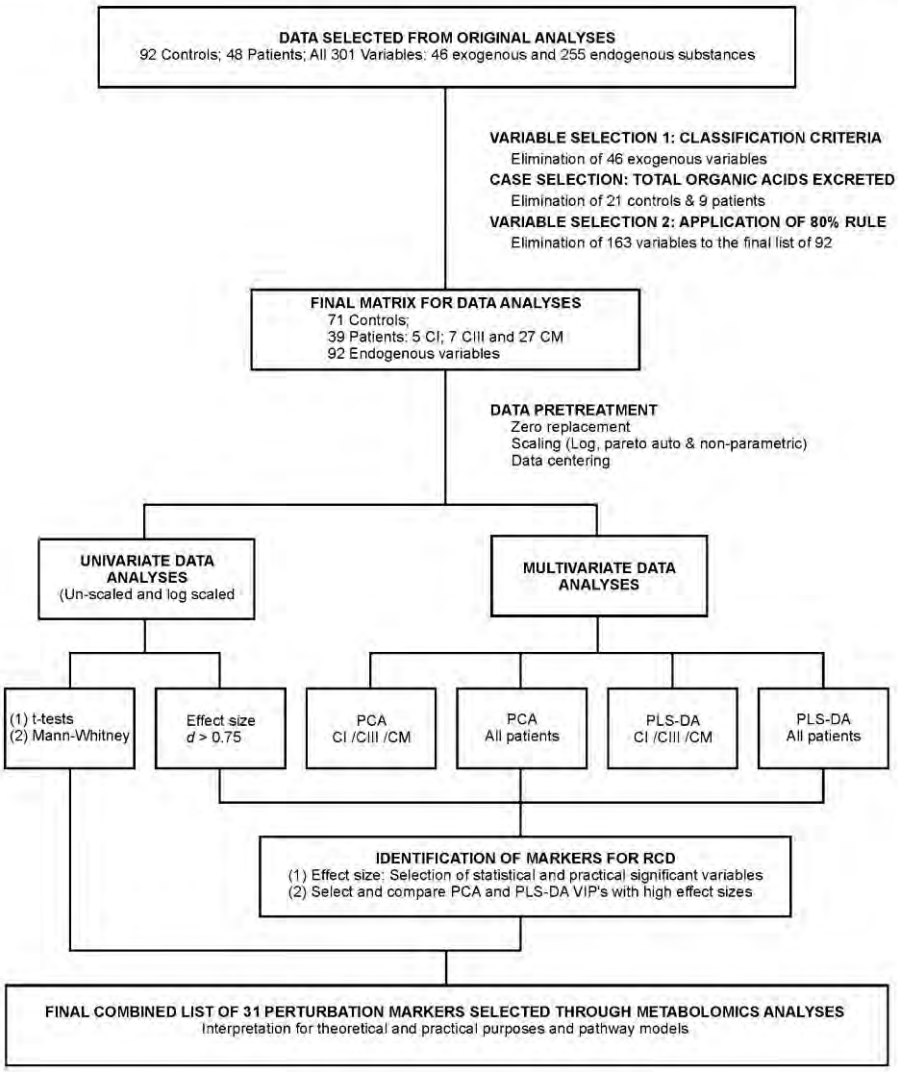


Fig. 1 Schematic representation of the work flow following data generation, through statistical preprocessing and data analysis towards identification of perturbation markers

- Creatinine values lower than 0.44 mmol/l and higher than 0.18 mmol/l: 2 ml of urine.
- Creatinine values lower than 0.18 mmol/l: 3 ml of urine.

Urine samples were transferred to silanized glass tubes (Kimax) and the internal standard (3-phenylbutyric acid, Sigma Chemical Company) was added to a final concentration of 180 mmol/mol creatinine. This ensured a fairly constant ratio between the urinary organic acids and internal standard, which contributes to more constant extraction efficiency of the organic acids, including the internal standard. 3-Phenylbutyric acid was used as internal standard due to its absence in normal urine and in known pathological conditions and because it elutes almost in the middle of the organic acid profile and theoretically co-elutes with very few, if any, other organic acids.

Urine samples were acidified with 5 N HCl (approximately 5 drops) and the pH was monitored to ensure it was less than 2. Six ml of ethylacetate (Merck Chemicals) was added to each sample and the mixture shaken on a rotary wheel for 20 min. The mixture was centrifuged for 2 min at $1300 \times g$ and the upper ethylacetate phase transferred to a clean glass tube. Three ml of diethylether (Merck Chemicals) was added to the water phase and shaken for a further 10 min. After centrifugation ($1300 \times g$, 10 min), the upper phase was removed and added to the ethylacetate. A small amount of sodium sulphate (BDH) was added to the ethylacetate/diethylether mixture to remove any residual water. After a subsequent centrifugation step, the organic phase was transferred to a clean glass tube. The organic solvents were evaporated to dryness under nitrogen at 37°C.

Table 1 Meta data on the final cohort of 39 cases selected for biomarker identification

Enzyme deficiency(ies)[a]	Case	Total organic acids[c]	CI[d]	CII	CIII	CII + III	CIV	PDHc	Ethnicity		Gender		Age
			(%)	(%)	(%)	(%)	(%)	(%)	B	C	M	F	(years)
CI	P10	1559	**95**	146	134	149	185	778	x		x		4
CI	P27	2972	**94**	237	173	119	204	306		x		x	2
CI	P30	1061	**76**	230	145	126	147	754		x	x		3
CI	P47	3319	**66**	129	139	137	357	368	x			x	1
CI	P73	2088	**72**	120	117	144	121	624		x	x		4
CIII	P36	1457	199	200	**90**	103	166	446	x			x	3
CIII	P59	1262	138	108	**71**	108	139	849	x			x	1
CIII	P60	1373	150	340	**95**	211	150	593	x		x		8
CIII	P63	1095	154	124	**64**	142	105	396	x			x	1
CIII	P84	1634	114	171	**97**	168	134	688	x			x	2
CIII	P97	832	105	130	**93**	141	142	339	x		x		6
CIII	P100	1457	145	121	**63**	107	129	220	x			x	7
CM (CI & CII + III)	P01	971	**0**	135	132	**0**	410	146	x			x	5
CM (CII & CII + III)	P07	2206	120	**80**	113	**64**	193	683	x		x		3
CM (CI & CIII)	P11	1163	**79**	148	**78**	148	163	1061		x	x		3
CM (CI & CII + III)	P14	2103	**77**	118	110	**89**	117	611	x			x	4
CM (CIII & CII + III)	P21	3380	104	102	**98**	**92**	111	301	x			x	4
CM (CI & CII + III)	P32	877	**63**	212	113	**90**	137	1704	x		x		4
CM (CII & CIII & CII + III)[b]	P39	2007	**91**	**93**	**81**	**83**	188	734	x		x		2
CM (CIII & CII + III & CIV)[b]	P41	1603	**89**	109	**97**	**85**	**81**	523		x		x	2
CM (CII + III & CIV)[b]	P43	1360	**99**	**98**	**94**	**70**	**91**	143	x			x	4
CM (CI & CIII)	P55	1489	**86**	185	**71**	142	137	548		x		x	2
CM (CIII & CIV)	P62	954	101	141	**87**	140	**80**	640	x			x	8
CM (CIII & CIV)	P67	1005	130	115	**65**	112	**92**	Nd		x	x		6
CM (CII + III & CIV)[b]	P69	918	117	112	**94**	**46**	**43**	125	x		x		8
CM (CIII)[b]	P70	741	**84**	120	**82**	130	109	750	x		x		8
CM (CIII & CIV)	P71	1372	109	171	**99**	123	**71**	778		x		x	9
CM (CII + III)	P72	2562	245	136	244	**55**	ND	525	x		x		10
CM (CIII & CIV)[b]	P75	896	**90**	121	**97**	143	**37**	786	x			x	7
CM (CI & CIII)	P76	931	**37**	191	**33**	159	102	426	x			x	3
CM (CI & CIII & CIV)	P78	1188	**28**	229	**38**	101	**54**	336		x		x	9
CM (CI & CIII)	P80	7666	**88**	140	**83**	120	108	601	x		x		6
CM (CI & CIII & CIV)	P82	3475	**72**	155	**70**	109	**65**	532	x		x		3
CM (CIII & CIV)	P83	2463	114	164	**65**	123	**93**	736	x		x		1

Table 1 continued

Enzyme deficiency(ies)[a]	Case	Total organic acids[c]	CI[d] (%)	CII (%)	CIII (%)	CII + III (%)	CIV (%)	PDHc (%)	Ethnicity B	Ethnicity C	Gender M	Gender F	Age (years)
CM (CII + III)	P85	775	126	147	129	**95**	130	786		x		x	10
CM (CI & CIII & CII + III & CIV)	P86	1958	**11**	174	**65**	**76**	**39**	607	x		x		5
CM (CII + III)	P87	775	127	144	100	**86**	152	633		x		x	5
CM (CII + III)	P94	1317	139	118	124	**97**	135	412	x		x		3
CM (CIII & CII + III)	P101	4795	146	103	**93**	**82**	149	508	x			x	2
Patients ($n = 39$)		1822[e]							72[f]	28[f]	46[f]	54[f]	4.6[e]
Controls ($n = 71$)		459[e]	Nd	Nd	Nd	Nd	Nd	Nd	55[f]	45[f]	67[f]	33[f]	8.9[e]

Nd not determined

[a] An enzyme deficiency of the respiratory chain was identified using the following two criteria: firstly, the enzyme activity expressed against citrate synthase had to be lower than the lowest control value (columns 4–9 indicate the activity as a percentage of the lowest control value). This criterion was also used to identify PDHc deficiencies. Any patient with a PDHc deficiency was excluded from the group of patients investigated with the metabolomics technology. Secondly, the activity had to be lower than the 5th percentile of the control group when expressed against either CII or CIV, if not deficient (if both were deficient only the first criterion was applied)

[b] Note that this second criterion determines that certain enzymes were not expressed as deficient in selected cases, although expressed on CS they were lower than the lowest control value (compare Smuts et al. 2010)

[c] Total organic acids are expressed in mmol/mol creatinine

[d] The values expressed for CI to PDHc (columns 4–9) gives the final values for each patient for these six parameters, and the numerical value of 100 and above was designated as "normal". All values below 100 are printed in bold, indicating the enzyme deficiency. Columns 10–12 indicate the ethnicity, gender and age for each patient. For this purpose the following notation was used: *B* black, *C* Caucasian, *M* male; *F* female

[e] Average of group

[f] Percentage within group

O-bis(trimethylsilyl)trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS): piridine (5:1:1, and volume added based on the creatinine values) was used for derivatization. The volume of urine used gave a creatinine concentration equivalent to 21 μmol/ml derivatization reagent. The samples were derivatized at 85°C for 45 min in a sand bath. The derivatized mixture was transferred to a 1.5 ml vial for GC–MS analysis. This approach ensured a fairly constant concentration of organic acids in the derivatization mixture, which improved the repeatability of the analysis.

2.3.2 *GC–MS analysis*

The Agilent GC–MS system used in this study consisted of a model 7890A gas chromatograph, a model 5975C mass selective detector, an HP 5970C MS and Agilent Chemstation (Revision E.02.00). A fused-silica capillary column (DB-1MS UI, 30 m, 2.50 μm i.d., 0.25 μm film thickness) was used for the fractionation. The initial GC temperature was 60°C, kept for 2 min. It was then increased to 120°C at a rate of 5°C/min, to 295°C at a rate of 7°C/min, and held at a final temperature of 295°C for 2 min. Helium (1 ml/min) was used as carrier gas at a constant flow rate. The mass spectra of all GC peaks were generated by a mass spectrometer operated at 70 eV in the electron impact mode with SCAN (50–600 amu) positive ion monitoring. The MS source and quadrupole temperatures were 230 and 150°C respectively.

2.3.3 *Deconvolution, peak identification and quantification*

Deconvolution and data analyses were conducted using AMDIS software (Version 2.66) linked to NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.0F, built Oct. 8, 2008). An AMDIS library file (more than 800 mass spectra) was generated by transferring organic acid (TMS derivatives) mass spectra from the NIST/EPA/NIH Mass Spectral Library, as well as from the Wiley Registry™ of Mass Spectral Data (8th Edition). In addition to the mass spectra of commercial compounds and those synthesized in our laboratory, we obtained mass spectra in the 1980s from Prof. S.K. Wadman of the Wilhelmina Kinderziekenhuis, University of Utrecht, The Netherlands (Erasmus et al. 1985).

Since January 2001, more than 30 000 organic acid analyses have been performed in our laboratory for diagnostic purposes. Mass spectra of unknown compounds present in relatively high concentrations were added to the target file, using the retention times as part of the identification. The identity of some of these spectra was resolved using the software ACD/MS Fragmenter (Release 10.00). Where possible all mass spectra were confirmed by analysing authentic standards. During this period, the mass spectra of compounds that were never detected were deleted from the library, leaving the spectra of more than 800 compounds in the reference version of the library. Where authentic standards were available, their respective responds factors were used, and for those compounds where no authentic standards were available, a response factor of 1 was assumed (See the note in this regard in the legend to Table 2). The analytical setting of the AMDIS software was as follows: minimum factor = 60% and type of analysis = "Use of an internal standard for RI". The deconvolution settings were: component width = 20; adjacent peak subtraction = 1; resolution = low; sensitivity = very low; and shape requirements = low.

The first hit of identified compounds and integrated area of the peaks were exported to Microsoft Excel©. Some peaks with uncertain identification were indicated by the AMDIS software with a question-mark against the name of the most likely organic acid in the library. These peaks were manually inspected and compared to mass spectra and retention times of the pure compound for identification purposes. Some compounds may produce multiple peaks, so that the SUMIF function of Excel© was used to calculate the sums of the areas of these peaks. A relative concentration, expressed as mmol/mol creatinine, was calculated using the formula: Concentration = Area (compound)/Area (IS) × concentration (IS) (Chen et al. 2009). A data matrix was created by aligning all the metabolites against the samples using MATLAB software and the statistical R-program.

2.4 Statistical analysis

The data pretreatment used in this investigation consisted of zero replacement, data transformation and centering. The zero values (representing the detection limit of the analytical equipment) were replaced by a random sample of values from a Beta (0.1, 1) distribution. After comparison with pareto-, auto- and non-parametric-scaling methods, a shifted log transformation was selected and applied to these data, so that the scales of the various metabolite concentrations were more comparable. The transformed data were then centred prior to multivariate analysis.

The data analysis used in this investigation consisted of principal component analysis (PCA) as an unsupervised pattern recognition method (Johnson and Wichern 1998), and a partial least squares discriminant analysis (PLS-DA) as a supervised method (Baker and Rayens 2003). From these multivariate analyses, the coefficients for all variables important in projection (VIP) were calculated, and ranked from high to low.

Table 2 Summary of elevated metabolites identified as being associated with RC deficiency in this metabolomics study

Class	Metabolite	Effect size		Controls[a]		Patients[a]		*t*-test	Literature[b]	Upper limit of reference value[a]
		Log	Value	Mean	S.D.	Mean	S.D.	*P*		
Carbohydrate metabolism										
1	Lactic acid	1.85	0.52	6.01	6.85	78.4	138	0.002	Mochel et al. (2005)	131
Krebs cycle										
(2)	2-Hydroxyglutaric acid	2.29	0.89	1.97	1.56	18.8	18.9	0.0001		13.9
2	Fumaric acid	1.38	0.54	0.09	0.21	12.3	22.8	0.002	Barshop (2004)	3.7
2	Succinic acid	1.29	0.76	11.8	9.80	101	118	0.0001	Hoffman and Feyh (2005)[c]	81.3
2	Malic acid	0.89	0.38	0.13	0.34	12.0	31.1	0.022	Barshop (2004)	5.5
Fatty acid metabolism										
3	3-Hydroxy-3-methylglutaric acid	1.71	0.78	2.82	5.87	22.4	25.2	0.0001		28
3	Adipic acid	1.67	0.56	1.02	2.20	36.1	62.4	0.001	Enns et al. (2000) and Barshop et al. (2000)[d]	5.3
3	Suberic acid	1.32	0.53	0.64	1.50	28.2	51.7	0.002	Enns et al. (2000) and Barshop et al. (2000)[d]	8.8
(3)	Glycerol	1.06	0.64	0.16	0.53	5.49	8.37	0.0001		n.d.
3	Methylsuccinic acid	0.99	0.70	0.32	0.68	4.63	6.13	0.0001	Lalani et al. (2005)	4.4
3	2-Keto-octanoic acid[e]	0.83	0.57	0.63	1.95	12.0	20.1	0.001		n.r.
3/7	3-Methyladipic acid[e]	0.77	0.45	0.42	0.90	5.40	11.0	0.008		n.d.
3	3-Hydroxyadipic acid	0.76	0.43	0.03	0.18	14.2	32.8	0.01	Enns et al. (2000)	13.3
3	3-Hydroxysebacic acid	0.76	0.37	0.29	1.14	19.5	52.6	0.028	Enns et al. (2000)	2
Amino acid metabolism										
4	3-Hydroxyisovaleric acid	1.84	0.71	5.23	3.69	49.2	61.8	0.0001		50.2
4	3-Hydroxyisobutyric acid	1.72	1.09	3.60	3.82	27.7	22.1	0.0001	Bennett et al. (1993)	137
4/6	4-Hydroxyphenyllactic acid	1.13	0.55	0.38	0.60	5.78	9.79	0.001	Enns et al. (2000)	3.6
4	3-Methylglutaconic acid	1.13	0.52	0.62	1.22	7.77	13.6	0.002	Bennett et al. (1993), Wortmann et al. (2005) and Sperl et al. (2006)	11.4
4	2-Methyl-3-hydroxybutyric acid	1.01	0.39	0.43	0.74	7.43	17.8	0.019		23.3
4/3	Glutaric acid	0.91	0.63	0.29	0.66	10.6	16.3	0.0001	Christensen et al. (1993) and Barshop et al. (2000)[d]	3.8
4	2-Ethylhydracrylic acid[e]	0.10	0.45	0.05	0.27	5.70	12.6	0.008	Kumps et al. (2002)[c]	n.r.
4/6	Phenylacetylglutamine[e]	0.84	0.56	2.99	7.23	25.6	40.2	0.001		n.r.

Table 2 continued

Class	Metabolite	Effect size		Controls[a]		Patients[a]		*t*-test	Literature[b]	Upper limit of reference value[a]
		Log	Value	Mean	S.D.	Mean	S.D.	*P*		
Neurological/stress										
5	3-Methoxy-4-hydroxyphenylacetic acid	2.13	0.78	3.90	2.90	27.7	30.5	0.0001		10.3
5/4	Pyroglutamic acid	1.33	0.60	2.93	1.65	18.4	25.8	0.001		n.d.
Other										
7	Citramalic acid	1.10	0.79	0.70	0.98	6.72	7.60	0.0001		n.a.
7	Uracil	0.88	0.29	0.27	0.49	9.53	32.3	0.082		64.5
7/8	Stearic acid	0.83	0.53	1.72	5.49	10.5	16.3	0.002		n.a.
Diet/bacterial										
6	4-Hydroxybenzoic acid	1.79	0.35	2.60	2.65	35.8	96.3	0.038		n.a.
Unclassified										
7/1	2,3,4-Trihydroxybutyric acid[e]	1.19	0.74	0.15	0.41	0.60	2.14	0.0001		n.a.
7/1	2,3-Dihydroxybutanoic acid[e]	1.12	0.71	3.73	2.40	15.4	16.6	0.0001		n.a.
7/1	3,4-Dihydroxybutanoic acid[e]	1.12	0.85	0.97	2.26	6.88	6.91	0.0001		n.a.
Additionally reported in the literature										
2	Aconitinic acid	1.13	0.94	29.3	12.7	103	78.3	0.0001	Kumps et al. (2002)[c] and HMDB (2010)[c]	135
(1)	2-Hydroxybutyric acid	0.69	0.22	0.01	0.07	8.59	40.2	0.180	Kumps et al. (2002)[c], HMDB (2010)[c] and Hoffman and Feyh (2005)[c]	7.3
4	3-Methylglutaric acid	0.44	0.34	0.06	0.22	0.72	2.02	0.043	Wortmann et al. (2005)	n.d.
7	Uric acid	0.56	0.32	1.33	2.40	12.9	36.4	0.56	Kuwertz-Bröking et al. (2000)	n.r.
3	3-Hydroxybutyric acid	80% rule							Bennett et al. (1993)	7.6
4	Isovalerylglycine	80% rule							Christensen et al. (1993)	n.r.
3	Ethylmalonic acid	80% rule							Christensen et al. (1993)	8.4
4	4-Hydroxyphenylpyruvic acid	80% rule							Enns et al. (2000)	0.3
4	Tiglylglycine	80% rule							Bennet et al. (1993, 1994)	n.d.
4	Isobutyrylglycine	80% rule							Christensen et al. (1993)	n.r.
4	2-Ketoadipic acid	80% rule							Barshop et al. (2000)[d]	n.d.
1	Pyuvic acid	80% rule							Hoffman and Feyh (2005)[c], Kumps et al. (2002)[c], and HMDB (2010)[c]	17.3
2	2-Ketoglutaric acid	80% rule							Hoffman and Feyh (2005)[c], Kumps et al. (2002)[c] and HMDB (2010)[c]	94.8

Table 2 continued

Class	Metabolite	Effect size		Controls[a]		Patients[a]		*t*-test *P*	Literature[b]	Upper limit of reference value[a]
		Log	Value	Mean	S.D.	Mean	S.D.			
3	Sebacic acid	80% rule							Wolf and Smeitink (2002)[c]	1.5
n.d.	Glutaconic acid	n.d.							Shah et al. (2002)[d]	n.d.

The class shown in the first column was selected according to the criteria applied to the initial data set of 301 variables. Some metabolites may originate from more than one catabolic pathway, in which case the classes are shown in brackets or indicated as alternative classes. The information on each variable for each row include the *d*-value for the effect size, determined for the log-scaled and un-scaled data, the mean value and standard deviation of the variable for the control and patient groups, followed by the *P*-value of the *t*-tests. The next three items in the row indicate a literature reference if the variable has been reported in a publication on a deficiency in any one or more of complexes I, II, III or IV of the RC, followed by the upper limit of the normal value listed by Hoffman and Feyh (2005). Within the column of variables, the members in each category are ranked according to the *d*-value for the effect size calculated from the log-scaled data

n.d. Not detected; *n.a.* not applicable; *n.r.* not reported

[a] Values are in mmol/mol creatinine with the reference value as described for children >5 years (Hoffman and Feyh 2005)

[b] Literature references included here were cited where an RC enzyme deficiency was reported except where [c] the report was made only in a review publication, or [d] the report was made where only mtDNA mutation/deletion was reported without RC enzyme activity data

[e] For these substances no authentic standards were available, and response factors of 1.0 was assumed in determining their relative concentrations

Effect size (Ellis and Steyn 2003) was used as a univariate analysis to ascertain the importance of a variable. The effect size d is defined as $d = |\bar{X}_1 - \bar{X}_2|/S_{\max}$, where $\bar{X}_1$ and $\bar{X}_2$ are the group means and $S_{\max}$ is the maximum standard deviation of the two groups. An effect size of $d > 0.5$ can be considered as being of medium practical importance, whereas an effect size of $d > 0.75$ can be considered as highly practically significant. In addition, standard *t*-tests and the non-parametric Mann–Whitney tests were used as univariate analyses to test the equality of group means and group medians, respectively.

3 Results and discussion

3.1 Data reduction

Metabolic fingerprinting of GC–MS data requires pre-processing to generate a data matrix of variables and cases of an operational size, to be followed by multivariate analysis to identify only the relevant analytical information. The work-flow that we followed is shown schematically in Fig. 1. Our selected experimental group consisted of 92 controls and 48 patients having an experimentally detected deficiency in one or more complexes of the RC. The total number of organic acids identified by GC–MS analysis of urine samples from this experimental group consisted of 301 components. From information in the Human Metabolomics Database, other published material on the origin of organic acids, and from knowledge of organic acids acquired over decades in our Laboratory for Metabolic Disorders, we could classify these 301 components into two major groups. Group 1 consisted of 255 endogenous substances, categorized as Class 1–7, as shown in Table 2. These substances originated from the metabolism of carbohydrates (Class 1, 9%); the Krebs cycle (Class 2, 3%); fatty acid metabolism (Class 3, 19%); amino acid metabolism (Class 4, 20%); neurological, stress or impaired organ function (Class 5, 11%); or from the diet or gut flora (Class 6, 15%). This group also contained substances of known metabolic pathways (e.g. purine and pyrimidine metabolism), as well as metabolites which could not be classified unambiguously (Class 7, 23%). Group 2 originated from medication or from other xenobiotic origin (Class 8). All substances from Group 2 were eliminated from the data matrix.

A scatterplot with drop-lines of the 255 organic acids for the 92 controls and the 48 patients which all complied to the two criteria based on the enzyme analyses, is shown in Fig. 2a. The organic acids are ranked from low to high retention times, as actually observed in the GC separation. The controls and patients were ranked by group, from those

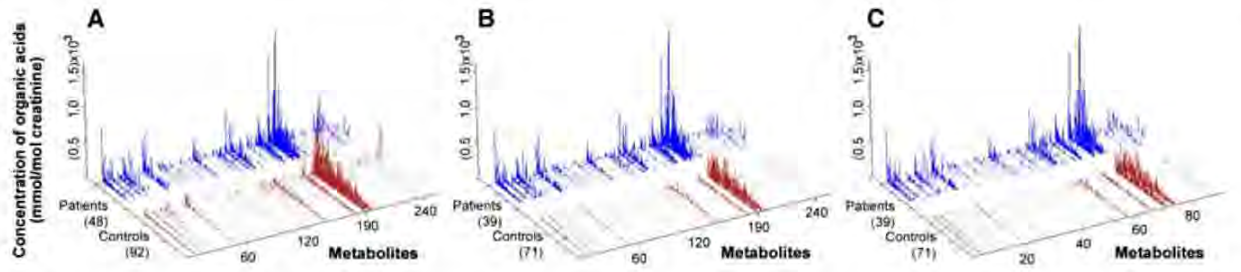


Fig. 2 Drop-line representation of variables present in the control and patient groups. **a** Data for all initial 140 cases and 255 selected variables of endogenous origin. **b** Data for the reduced cases (110, including 71 controls and 39 patients) and the 255 variables. **c** Representation of the information included in the final data matrix, consisting of the 110 cases as in **b** and the 92 variables, selected after applying the 80% rule

with the lowest total excretion of the urinary organic acids to those with the highest values. A visual inspection of this representation clearly indicates (1) a greater abundance of organic acids in the patient group than in the controls, (2) an extensive number of substances with very low or zero values, and (3) an overlap between patients with low and controls with a high excretion of urinary organic acids. The low excretion of total organic acids in some patients could be related to subjects in a near-normal clinical state when the urine sample was taken. The high values in the control group may be due to the clinical condition of individuals, which could not be discounted as a mitochondrial disorder or another metabolic condition. These observations required further selection of cases as the next step in the process of data reduction. For this, the total organic acids excreted were first calculated. Controls with total organic acid values greater than the corresponding lower quartile for patients were eliminated from the group. Patients with total organic acid concentrations less than the upper quartile of the corresponding acids excreted by the controls were similarly eliminated. From this, the final experimental group of 71 controls and 39 patients were selected, for whom the outcome is shown in Fig. 2b. Relevant meta data of these 39 patients are summarized in Table 1.

The next data reduction step involved the variables. All variables which had 80% or more zeros in both the patient and the control groups were then eliminated (compare also the approach by Bijlsma et al. 2006). This yielded a final list of 92 variables, the outcome of which is shown in Fig. 2c. A comparison of the various results shown in Fig. 2 indicates that the essential characteristics of the data set were still retained after reduction of the disease cases and variables. The selected data consisted of (1) a reduced but still comprehensive set of endogenous variables, with the mean concentration of total organic acids excreted by the controls and the patients amounting to 459 (s.d. = 161) and 1822 (s.d. = 1323) mmol per mol creatinine, respectively, but (2) with the elimination of the overlap between the controls and the patients.

3.2 Selection of important variables

The subsequent data pre-treatment focused only on the variables selected after data reduction as discussed in Sect. 3.1 and shown in Fig. 2c. Mean values, as well as the standard deviation of all variables for the controls and patients, were determined on the un-scaled data, followed by *t*-test and Mann–Whitney analyses. The effect size analyses were subsequently conducted on the un-scaled as well as on the scaled data. The most powerful discrimination between the RCD patients and the controls was obtained from the effect size analysis of the data. For the 92 variables, $d > 0.5$ for 45 and 59 for the un-scaled and log-scaled data, respectively. Variables from the log-scaled data, for which $d > 0.75$ (practical highly significant) and were statistically significant, were selected for further analysis of the consequences of RCD on the cellular metabolism. This selection produced 44 variables, which were subsequently compared with the 44 VIPs identified by multivariate analyses. It should be noted that this list include metabolites that are not organic acids per definition, but which are co-extracted by the procedures used. They are glycerol, uric acid (a purine) and uracil (a pyrimidine).

Two traditional methods of multivariate analysis (PCA and PLS-DA) proved to be valuable for variable selection and were subsequently applied to four sets of data: the three groups (CI-, CIII- and CM-deficiencies) of RCD taken separately, and also as one combined group. All 71 controls were included in every analysis. The outcome of the PCA, shown in three-dimensional score plots (PC1, PC2 and PC3) for the four data sets, is shown in Fig. 3. For all three sub-groups of RCD (Fig. 3a–c), the patient groups were distinguished from the controls, indicating that the metabolic profiles of these three groups were altered by the perturbation induced by the respective CI, CIII or CM deficiencies. These observations hold the potential to identify biomarkers that could distinguish these three RCDs. However, this was excluded by the results obtained

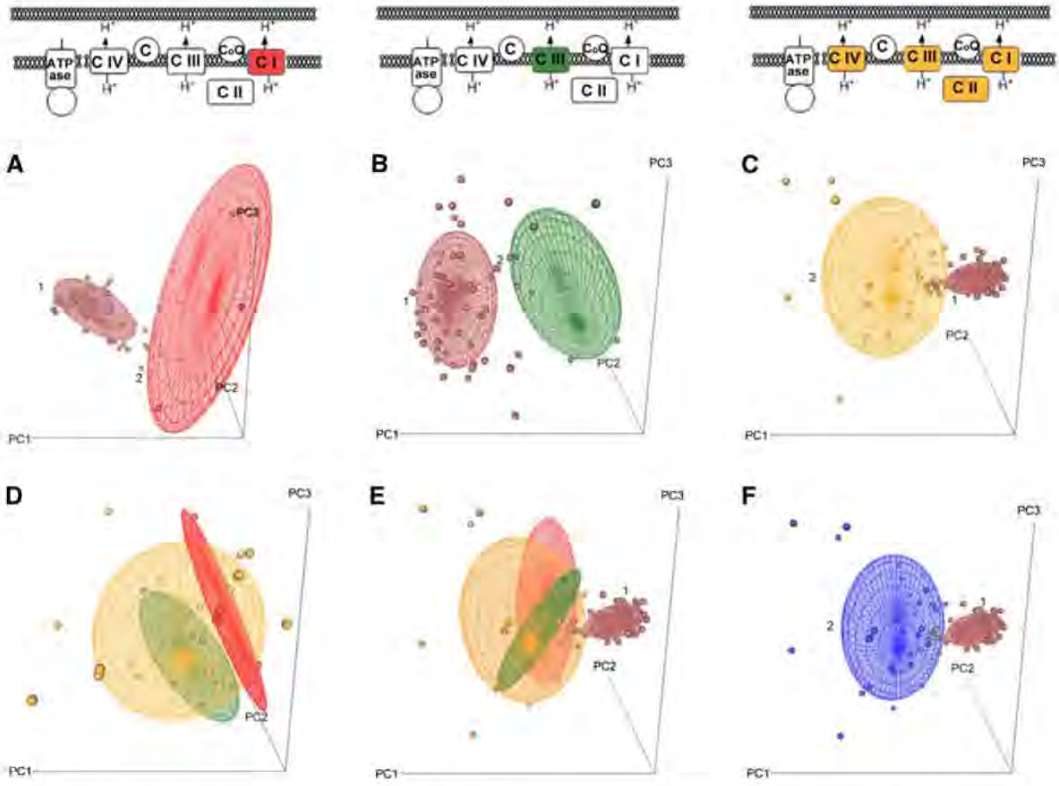


Fig. 3 Score plots of PCA shown in 3-dimensional plots of identical rotation in all six subsections of patients. The upper section of the figure depicts a model of the association of complexes CI, CII, CIII, CIV and the ATPase with the inner mitochondrial membrane. Complexes CI, CIII and CI, CII, CIII and CIV (designated together as CM) are coloured red, green and yellow, respectively. This indicates the sites of the RCD for the PCA analysis conducted for each of the three patient subgroups (CI, CIII and CM). The respective PCA analysis is shown directly below each presentation (**a**–**c**). The same controls were used for the PCAs shown in **a**–**c**, **e** and **f**, and are indicated in brown as in Fig. 2. The patient groups used in these six PCA analyses were CI (**a**), CIII (**b**), CM (**c**), and CI, CIII and CM together in **d**–**f**. The cumulative proportions of PC1, PC2 and PC3 for the data shown in **f** (blue as in Fig. 2) were 40.0, 46.6 and 51.0%. The lists of variables important in projection (VIPs) were generated for the data shown in **a**–**c** (Fig. 4a) and for **f** (Fig. 4b; Table 2)

from the PCA of the three combined groups (Fig. 3d–f). In a score plot of the PCA of the three groups taken together, but excluding the controls, they could not be distinguished from one another (Fig. 3d). The same holds true for a PCA in the presence of the controls, although the three groups could still be distinguished from the control group (Fig. 3e, f). Following these results, the search for possible biomarkers was continued with the individual (CI, CIII or CM) as well as the combined groups of patients.

In this investigation the important variables for each of the three sub-groups were identified by using three statistical methods of analysis. The three methods were distinctly different, being a univariate approach (effect size), unsupervised multivariate analysis (PCA), and a supervised multivariate analysis (PLS-DA). All variables with $d > 0.75$ were first identified from the VIP lists of the PCA and PLS-DA analyses for the three sub-groups. From these lists, variables originating from the diet, gut flora or of unknown source were eliminated, and were found to be four for the CI group, one in the CIII group, and zero in the CM group. The remaining important metabolites for which $d > 0.75$ were found to be 12 for CI, 24 for CIII and 25 for CM.

In order to compare the three lists of metabolites, we developed a *Venn diagram* shown in Fig. 4a, indicating the number of variables which define the three groups, as well as those which are common to all of them. Eleven of the 12 metabolites of the CI group were shared with the CIII and CM groups. These were adipic acid, fumaric acid, lactic acid, suberic acid, succinic acid, 2-hydroxyglutaric acid, 3-hydroxy-3-methylglutaric acid, 3-hydroxyisobutyric acid, homovanillic acid, 3-methylglutaric acid and 4-hydroxyphenyllactic acid. The remaining metabolite, not present among the important variables of the CIII and CM groups, was vanillylmandelic acid. Eleven of the remaining metabolites were also common to the CIII and CM groups. They were ethylhydracrylic acid, glutaric acid, glycerol, malic acid, methylsuccinic acid, phenylacetylglutamine,

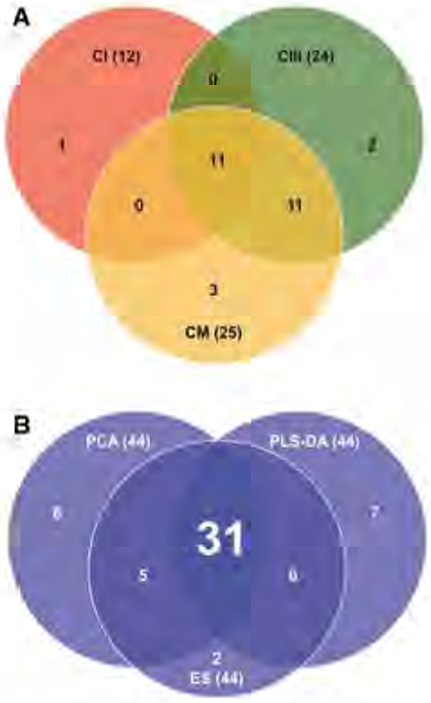


Fig. 4 *Venn diagrams* highlighting the common variables observed in the four patient groups. **a** Diagram for subgroups CI, CIII and CM. **b** Diagram for the variables identified through the effect size (ES), PCA and PLS-DA for the combined patient group. In all cases all controls were used and the effect size of the log-scaled values has been used as the basic benchmark for the selection of variables. The colour scheme used in **a** and **b** is the same as in Fig. 3

uracil, 2-hydroxysebacic acid, 2-methyl-3-hydroxybutyric acid, 3-hydroxyisovaleric acid and 3-methyladipic acid. The remaining metabolites not shared by any of the other groups were glyceric acid and 3-hydroxysuberic acid for the CIII, and pyroglutamic acid, 2-keto-octanoic acid and 3-hydroxyadipic acid for the CM groups, respectively. This comparison clearly indicates great overlap of the important metabolites that define the three sub-groups and substantiates the observation that the CI, CIII and CM patient groups were not distinguishable, as indicated by the PCAs shown in Fig. 3d, e. We therefore concluded that no characteristic organic acid biomarker profile could be defined for the CI, CIII and CM RCDs.

Secondly, the variables from the effect size, PCA and PLS-DA lists were used to construct a *Venn diagram* to identify the important metabolites in the combined patient group. The outcome is shown Fig. 4b. In the case of the PCA, 8 variables, and for the PLS-DA 7 variables from their respective lists of the first 44 VIPs had $d < 0.75$, and were discarded, not being practically significant. In the effect size list, two of the variables with $d > 0.75$ were not among the 44 variables in either the PCA or the PLS-DA list. Of the remaining 11 variables with $d > 0.75$, five and six, respectively, were not common to either multivariate analysis. This comparison thus produced 31 variables (33.7% from the 92 variables in the final matrix, and 12.1% of the original endogenous substances) which were common to all three statistical methods of analysis. All 31 variables were subsequently evaluated to explore which metabolic pathways were affected in patients with RCD, as compared to the control group. This comparison is summarized in Table 2, and can be considered to be the most important outcome of our metabolomics investigation of RCDs.

3.3 Comparative analysis of the important metabolites

Table 2 presents the information on the 31 variables, classified according to the criteria which we applied to the initial data set of 301 variables, followed by their grouping in terms of their most important functions in intermediate metabolism. Twenty-three of the 31 variables are clearly primary or secondary metabolites associated with the metabolism of carbohydrates, fatty acids, amino acids or the Krebs cycle. Two further variables could be related to neurological or stress conditions that are known to prevail in RCD, while the remaining six variables probably originate from sources not directly related to RCD. For these 25 metabolites, the *d*-value for the effect size of the log-scaled data was greater than 0.75, indicating that all are highly significant from a practical point of view. Apart from one (uracil), all 25 had a *P*-value smaller than 0.05 in the *t*-tests. The Mann–Whitney test showed that the group medians differed significantly for all 25 variables at the 5% level. Fifteen of the 25 metabolites were previously reported for deficiencies in one or more complexes of the RC. Of the remaining 10 metabolites not previously mentioned in investigations on RC, most are known to be normal metabolites, or metabolites that become elevated due to an inherited metabolic defect other than RCD. The presence of 2-keto-octanoic acid was rather unexpected. It might originate from fatty acid degradation in the peroxisomes, but then 2-hydroxyoctanoic acid would be the more likely metabolite (Foulon et al. 2005). This aspect should thus need further investigations. The biological significance and potential diagnostic value of the other 24 metabolites will be discussed in sect. 3.4 below.

Table 2 also lists all other metabolites (15) that were described in other studies on RCDs. With the exception of glutaconic acid, all were included among the 92 variables in our final data matrix. Of these, acotinic acid is noteworthy for its high effect sizes ($d = 1.13$ and 0.94 for the log-scaled and un-scaled data, respectively) and its statistically significant ($P = 0.0001$) higher value for the patients than the controls. The mean value for the patient group was, however, lower than its reported normal

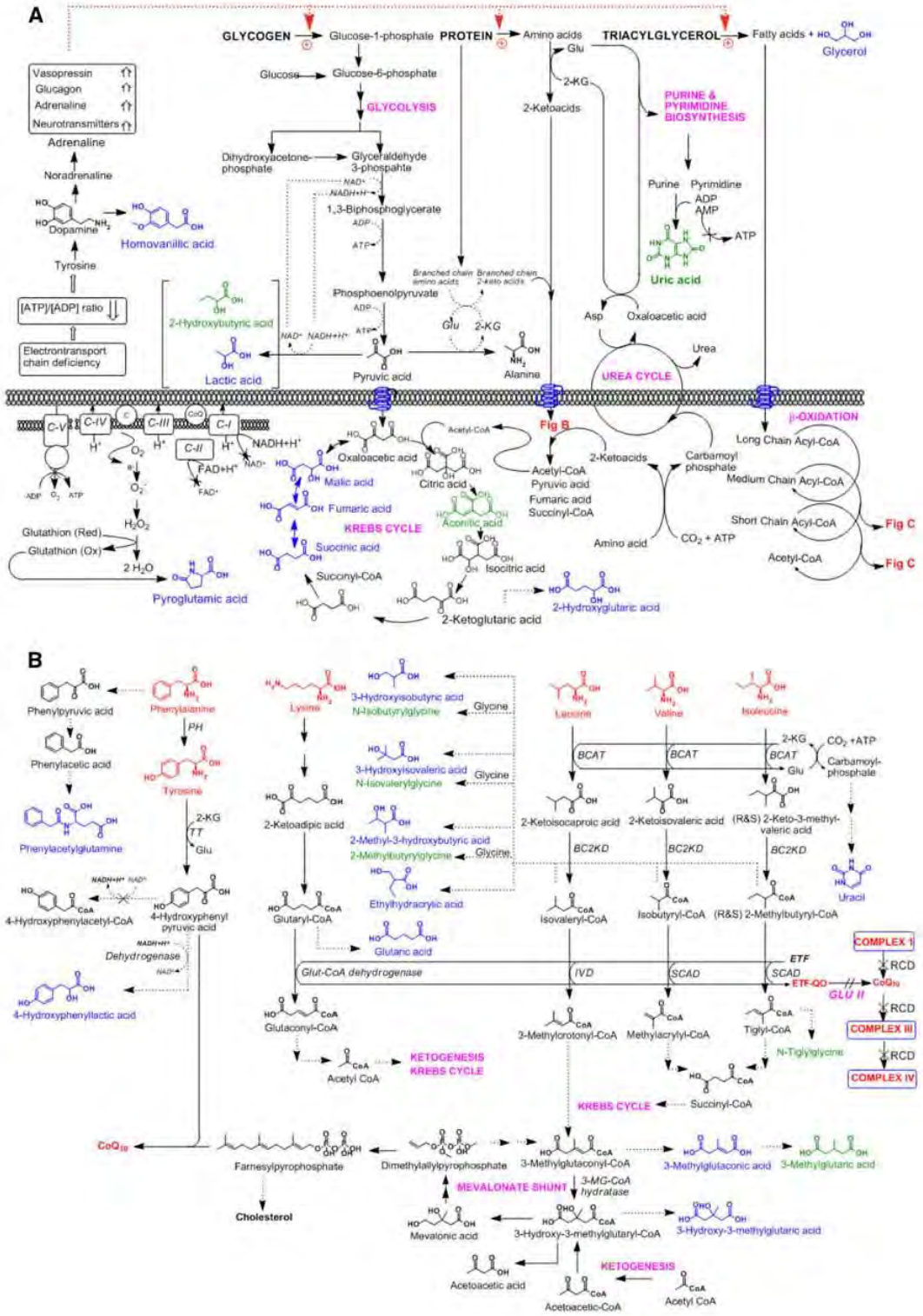
maximum value. Three of the remaining metabolites had low d values and the other 10 were excluded by the 80% test, indicating that they were present in only a very few cases. It, therefore, appears that these 15 metabolites are not common to patients with RCDs in general, but observed in individual cases with such a deficiency.

3.4 A global metabolite profile associated with organic aciduria due to RCD

The metabolite profile as discussed here refers to changes in the cellular profile of the organic acids and a few other metabolites in cases of RCD, as derived from the information disclosed by metabolomics analysis of urine samples. We interpret the variation in this profile, compared to controls, as a result of the immediate and downstream consequences of RCDs, which include allosteric regulation of metabolic processes, as well as transcriptional/translational responses to the deficiencies. As RC is essentially involved in the cellular reduction/oxidation (redox) status and energy (ATP) production, deficiencies inevitably affect a plethora of metabolic and other processes (Reinecke et al. 2009), of which only a selective group of metabolites is unravelled here. We derived a comprehensive set of metabolic pathways shown in Fig. 5, to illustrate the complex metabolic perturbations as revealed by this investigation. Although we investigated three subsets of RCD (CI, CIII and CM), the proposed metabolic pathways affected by RCDs are based on the metabolites of the combined patient group, implicating a mutual relationship between the individual components of the RC, assuming that all complexes affected redox status and ATP production as key final consequences of the disease. Such a mutual relationship supports the prevailing view on the structure, functioning and regulation of the OXPHOS system (Boekema and Braun 2007; Dudkina et al. 2008; Reder 1988; Rossignol et al. 2003), implying that the control of mitochondrial respiration is shared among all of the OXPHOS complexes. Other investigations demonstrated the existence of a biochemical threshold effect for the expression of a specific RCD and mitochondrial energy production (Mazat et al. 2001), which accounts for the severity of the pathogenic mutation and for extrinsic factors, such as the tissue affected. Finally, kinetic studies indicated the modulation of the activity of RC complexes by variations in the concentrations of intermediate metabolites, designated as network attenuation (Heinrich and Rapoport 1974; Rossignol et al. 2003). These concepts thus support the notion of a global, perturbed metabolite profile in RCD, as presented here, rather than a linear relationship between one or a few metabolites as biomarkers for a specific complex deficiency, as seen in other inherited metabolic mitochondrial disorders like isovaleric acidaemia and propionic acidaemia.

Fig. 5 A representation of global metabolic perturbations associated with organic aciduria due to RCD. The 25 metabolites indicated in Table 2, as being statistically and practically significant, are indicated in blue. Others frequently observed in individual cases of RCD, also included in Table 2, are shown in green. Names or structural formulas of metabolites printed in red are normal intermediates in the respective metabolic pathways. The six amino acids, from which the secondary metabolites and markers of RCD originate, are shown in red. **a** The extramitochondrial consequences of RCD, and its link to the intramitochondrial metabolism. Stage 1: Decreased ATP production and the decreased [ATP]/[ADP] ratio is compensated for by the action of various neurotransmitters, e.g. dopamine, and/or hormones like vasopressin, glucagon and adrenaline. Stage 2: Increased catabolism of glycogen, proteins, triacylglycerols and purines and pyrimidines. Stage 3: Intramitochondrial disarray of the Krebs cycle and production of secondary metabolites due to impaired catabolism of fatty acids and some amino acids. Production of pyroglutamic acid is probably a consequence of oxidative stress (alternatively: a degradation product of glutamine). **b** Proposed origin of secondary metabolites observed in RCD and formed from amino acid catabolism. The consequences for phenylalanine and tyrosine are shown on the left of the figure. Phenylacetylglutamine may be an endogenous product, as indicated, or, alternatively a bacterial metabolite from the gut. Degradation of lysine and the branched-chain amino acids resemble aspects of glutaric aciduria Type II (GLU II). Formation of 3-methylglutaric acid in RCD might be due to the mevalonate shunt; the link to the Krebs cycle and the clinical characteristic of ketosis in RCD is indicated where applicable. **c** Proposed origin of secondary metabolites observed in RCD and formed from fatty acid catabolism due to impaired β-oxidation, resembling glutaric aciduria Type II (GLU II)

Figure 5a summarizes the regulation of carbohydrate, protein and triacylglycerol metabolism in the extra-mitochondrial compartment and its link to RCD metabolic alterations, with a focus on the Krebs cycle, amino acid metabolism (Fig. 5b) and fatty acid metabolism (Fig. 5c). Figure 5a shows how the catabolic degradation products of carbohydrates, fatty acids and amino acids assemble in the final stages of cellular oxidation, in which the energy generated by the RC process drives the synthesis of ATP, generally providing the bulk of cellular ATP. In RCD's a decrease in cellular ATP occurs, corresponding to a smaller ATP/ADP ratio, which is normally very high, as the ATP-ADP system is then virtually fully phosphorylated. Reduced mitochondrial production of ATP can be directly compensated for by an equivalent increase in glycolysis (Hofhaus et al. 1996), as the [ATP][ADP]/[Pi] ratio functions as the primary cytosolic sensor of altered energy production, initially via glycolysis (Pfeiffer et al. 2001). With these regulatory processes in mind, a theoretical model has been proposed (Korzeniewski 2001), indicating how compensation of OXPHOS defects could occur at the cellular level through the stimulation of ATP production by the action of various neurotransmitters, such as dopamine and/or hormones like vasopressin, glucagon and adrenaline. This is


A
GLYCOGEN
Glucose-1-phosphate
PROTEIN
Amino acids
TRIACYLGLYCEROL
Fatty acids + Glycerol
Glucose
Glucose-6-phosphate
GLYCOLYSIS
2-Ketoacids
PURINE & PYRIMIDINE BIOSYNTHESIS
Vasopressin
Glucagon
Adrenaline
Neurotransmitters
Adrenaline
Noradrenaline
Dopamine
Homovanillic acid
Tyrosine
Dihydroxyacetone-phosphate
Glyceraldehyde 3-phospahte
1,3-Biphosphoglycerate
Purine
Pyrimidine
ADP
AMP
ATP
Uric acid
[ATP]/[ADP] ratio
Electrontransport chain deficiency
2-Hydroxybutyric acid
Lactic acid
Phosphoenolpyruvate
Pyruvic acid
Alanine
Asp
Oxaloacetic acid
Urea
UREA CYCLE
Fig B
β-OXIDATION
Long Chain Acyl-CoA
Medium Chain Acyl-CoA
Short Chain Acyl-CoA
Acetyl-CoA
Fig C
Oxaloacetic acid
Malic acid
Citric acid
Fumaric acid
KREBS CYCLE
Aconitic acid
Isocitric acid
Succinic acid
Succinyl-CoA
2-Ketoglutaric acid
2-Hydroxyglutaric acid
Glutathion (Red)
Glutathion (Ox)
Pyroglutamic acid
Pyruvic acid
Fumaric acid
Succinyl-CoA
Amino acid
Carbamoyl phosphate
CO2 + ATP
B
Phenylpyruvic acid
Phenylalanine
Phenylacetic acid
Tyrosine
Phenylacetylglutamine
4-Hydroxyphenylacetyl-CoA
4-Hydroxyphenyl pyruvic acid
Dehydrogenase
4-Hydroxyphenyllactic acid
Lysine
3-Hydroxyisobutyric acid
N-Isobutyrylglycine
3-Hydroxyisovaleric acid
N-Isovalerylglycine
2-Keloadipic acid
2-Methyl-3-hydroxybutyric acid
2-Methylbutyrylglycine
Ethylhydracrylic acid
Glutaryl-CoA
Glutaric acid
Glutaconyl-CoA
Acetyl CoA
KETOGENESIS
KREBS CYCLE
Glycine
Leucine
Valine
Isoleucine
BCAT
2-KG
Glu
CO2 +ATP
Carbamoyl-phosphate
2-Ketoisocaproic acid
2-Ketoisovaleric acid
(R&S) 2-Keto-3-methyl-valeric acid
BC2KD
Uracil
Isovaleryl-CoA
Isobutyryl-CoA
(R&S) 2-Methylbutyryl-CoA
ETF
Glut-CoA dehydrogenase
IVD
SCAD
ETF-QO
CoQ10
GLU II
COMPLEX I
COMPLEX III
COMPLEX IV
RCD
3-Methylcrotonyl-CoA
Methylacrylyl-CoA
Tiglyl-CoA
N-Tiglylglycine
Succinyl-CoA
CoQ10
Farnesylpyrophosphate
Dimethylallylpyrophosphate
3-Methylglutaconyl-CoA
3-Methylglutaconic acid
3-Methylglutaric acid
MEVALONATE SHUNT
3-MG-CoA hydratase
Cholesterol
Mevalonic acid
3-Hydroxy-3-methylglutaryl-CoA
3-Hydroxy-3-methylglutaric acid
KETOGENESIS
Acetoacetic acid
Acetoacetic-CoA
Acetyl CoA

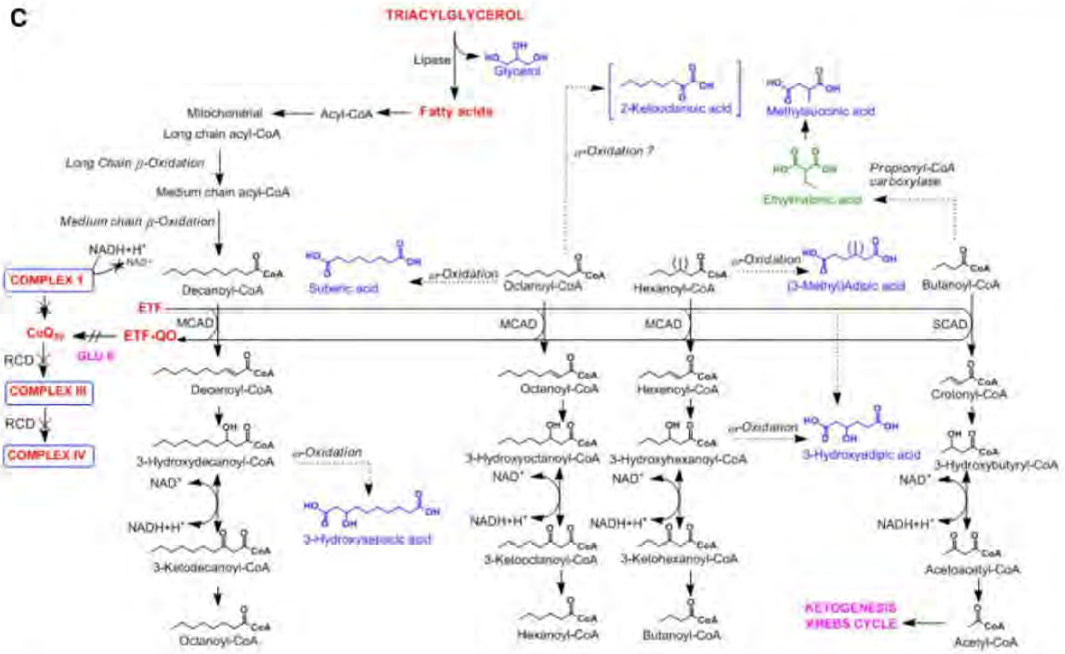


Fig. 5 continued

supported by the increased levels of homovanillic acid (HVA, 3-methoxy-4-hydroxyphenylacetic acid), which we found in all RCD groups, and by vanillylmandelic acid (VMA, 4-hydroxy-3-methoxyphenyl-glycolic acid) in the CI subgroup. These metabolites have not been described before for RCDs, although increased urinary HVA concentrations are known to arise from neurological stress conditions (Frankenhaeuser et al. 1985; Rauste-von Wright and Frankenhaeuser 1989). The elevated neurotransmitter–hormone model and catabolism of carbohydrates (glycogen → glycolysis) similarly apply to catabolism of proteins (→amino acid catabolism) and triacylglycerols (→fatty acid oxidation and the production of glycerol).

As indicated before, a key consequence of RCD is increased production of NADH (NADH/NAD^+ ratio) and $FADH_2$ due to the dysfunctional RC at any site within the chain. Although several enzymes are regulated by this change in redox state, extramitochondrially it can result in commonly observed (as in this study) elevated lactic acid, an increased lactate/pyruvate ratio, elevated alanine (not reported here due to our metabolome selection), as well as raised uric acid (Kuwertz-Bröking et al. 2000). Moreover, it may result in elevated TCA intermediates of which several were observed in this study, and which are included as one of the disease criteria for MD (Morava et al. 2006). Barshop (2004) highlighted increased urinary fumaric acid and malic acid but in our study the order of significance of elevated TCA metabolites was succinate >fumarate >malate. We anticipate that this order may be a result of irreversible conversion of 2-ketoglutaric acid to succinic acid, and a consequent reverse TCA cycle towards succinic acid and possibly an impaired malate–aspartate shuttle which is known to be physically closely linked to consecutive enzymes of the Krebs cycle (Beeckman and Kanarek 1981).

Accumulation of succinic acid contributes to a decreased conversion of 2-ketoglutaric acid to succinic acid, resulting in the statistically significant increase in a-conitinic acid in the first half of the TCA cycle (Table 2). Although 2-ketoglutaric acid is present in the list of PCA VIPs, the expected increase in its concentration was not observed above the level of the normal maximum in any of the RC deficiencies studied here. However, 2-hydroxyglutaric acid, which has not been described in terms of RC deficiencies before, was statistically significantly increased in all three of our subgroups. Although some methane-producing prokaryotes utilize 2-hydroxyglutaric acid in a metabolic pathway (Van Beelen et al. 1984), it is not an intermediate in any known metabolic pathway in man or other eukaryotes. A first case of L-2-hydroxyglutaric aciduria was nonetheless identified in 1980 (Duran et al. 1980) and later confirmed by mutational analysis (Topçu et al. 2004; Rzem et al. 2004). Our proposal for the accumulation of 2-hydroxyglutaric acid seen in our patient groups favours the NADH-dependent conversion of 2-ketoglutaric acid to 2-hydroxyglutaric acid by mitochondrial L-malate

dehydrogenase. This proposal is based on (1) the known redox potential for the 2-hydroxuglutaric acid/2-ketoglutaric acid couple (Buckel and Miller 1987), (2) the anticipated high 2-ketoglutaric content in the mitochondria due to the reversible operation of the latter part of the TCA cycle, and (3) the relative specificity of mitochondrial L-malate dehydrogenase (Banaszak and Bradshaw 1975). This mechanism was recently proposed as operating in L-2-hydroxyglutaric aciduria (Van Schaftingen et al. 2009).

The interrelated involvement of the NAD redox state (increased $NADH_2$), formation of reactive oxygen species (ROS) and the glutathione redox state in RCDs can explain the observed elevated urinary pyroglutamic acid as also reported by Topaloğlu et al. (2008). We interpret the increase in pyroglutamic acid (Fig. 5a) as an increase in the flux through glutathione rather than a change in the thiol redox state, as the mitochondrial and cytosolic thiol redox states are not inevitably affected, as shown in studies using complex I-deficient fibroblasts (Verkaart et al. 2007).

The consequences of RCD on amino acid degradation are shown in Fig. 5b. Similarities with known inherited single enzyme deficiencies of lysine, leucine, valine and isoleucine catabolism are again conspicuous. Glutaric acid, 3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 2-methyl-3-hydroxybutyric acid, 2-ethylhydracrylic acid (Table 2) and the glycine conjugates of isobutyric acid, isovaleric acid, and 2-methylbutyric acid are all secondary metabolites seen in glutaric aciduria type II [electrotransfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase deficiency, and ETF-dehydrogenase deficiency, reviewed by Gordon (2006)]. This implies that the defective flow of electrons in RCDs impairs the functionality of the ETF-dependent enzymes, followed by an increase in their respective substrates from which the secondary metabolites, disclosed by the metabolomics analysis of urine samples from RCD patients, arises. The origin of 3-methylglutaconic acid and 3-hydroxy-3-methylglutaric acid, which we also observed in our RCD cohort (Table 2), is controversial (Walsh et al. 1999; Wortmann et al. 2005). To account for their presence in RCDs, the proposed origin of these catabolites from leucine catabolism (Duran et al. 1982) and the mevalonate shunt (Kelley and Kratz 1995) is indicated in Fig. 5b. Comprehensive genetic mapping will be required, however, to establish unequivocally the aetiology of the heterogeneous profile associated with 3-methylglutaconic acid. Finally, increased tyrosine and phenylalanine catabolism, which occurs exclusively in the liver, can account for 4-hydroxyphenyllactic acid and phenylacetylglutamine, respectively. This relates to the perturbed $NADH/NAD^+$ ratio, and may also be an indication of liver damage in RCDs.

Finally, the consequences of RCDs for fatty acid degradation are shown in Fig. 5c. Decreased electron transfer flavoprotein (ETF) and increased electron transfer flavoprotein: ubiquinone oxidoreductase (ETF-QO) activity due to a deficient flow of electrons past CoQ_{10} may give rise to adipic, 3-methyladipic acid and 3-hydroxyadipic acid, suberic and 3-hydroxysuberic acid, as well as 3-hydroxysebacic acid. These are formed by ω-oxidation from their respective short- and medium-chain fatty acid-CoA precursors, comparable with the observation of impaired mitochondrial fatty acid oxidation (reviewed by Duran 2005) of short-chain (SCAD), medium-chain (MCAD) and very long-chain acyl-CoA dehydrogenase deficiencies (VLCAD). The elevated levels of two of these ω-oxidation products, 3-hydroxysebacic acid and 3-hydroxyadipic acid, again illustrate the involvement of NAD redox state. Furthermore, elevated butanoyl-CoA due to reduced SCAD activity is proposed to give rise through an acetyl-CoA- and propionyl-CoA-carboxylase catalysed production of ethylhydracrylic and methylsuccinic acid, respectively (Sweetman and Williams 2001).

3.5 Potential clinical applications

The clinical and genetic heterogeneity of RCDs are the most important aspects complicating diagnosis in these disorders. The number of known mutations is rapidly growing; around 743 different mutations had been reported on MITOMAP (http://www.mitomap.org) by October 2010. The final confirmation of a diagnosis is further complicated by the logistic, invasive and expensive nature of the current biochemical approach including enzyme analyses on muscle biopsies. Using a metabolomic technique to simplify this complex diagnostic challenge can be anticipated a priori to be a multi-faceted process, because large and complex data sets are created, requiring special skills to derive meaningful information from them.

In a metabolomics experiment the biological sample provides a profile of the metabolome at that time. In clinical studies on the diagnosis of RCDs, the samples most commonly used for biochemical analysis come from urine collected in clinics from more or less critically ill patients (Smeitink et al. 2006). Rigorous requirements for sample collection to comply with the minimum standards advocated for metabolomics investigations (Dunn and Ellis 2005; Goodacre et al. 2007) are thus not fully feasible in a metabolomics study of RCDs and many other inherited metabolic diseases. We could overcome this handicap, however, with samples from a large cohort of clinically well-described patients (Smuts et al. 2010) and from an even larger number of controls, and by specifically focusing on the organic aciduria profiles of the patients. The comparative approach of using the effect size, PCA and PLS-DA unequivocally established that no single organic acid biomarker, or combination of such biomarkers, can be defined for sub-classes

of RC complex deficiencies. However, the insight into the perturbations caused by RCDs gained by the metabolomics approach has significance for possible clinical applications, and strengthens expectations for a new frontier in pediatrics research (Carraro et al. 2009).

The criteria defined for new novel biomarkers for cardiovascular disorders (Morrow and de Lemos 2007) are equally well applicable in a search for biomarkers for inherited metabolic disorders. For the present, our hypothesis is that a biosignature for RCDs, derived from a metabolomics investigation of different parts of the metabolome, rather than a search for a few highly specific biomarkers, has the potential to define a practical diagnostic instrument to detect the presence of an RCD. Ultimately, enzyme analysis of the complexes of the RC in skeletal muscle biopsy material will probably remain the golden diagnostic standard when an RCD is suspected (Smeitink et al. 2006). However, a global metabolic effect as shown here can be exploited in screening for an RCD by analysis of biofluids, such as urine, obtainable by non-invasive procedures. From our experience, the adoption of such an approach for diagnosis and in the follow-up of disease progression or treatment of patients is in the offing in the health-care systems of a developing country such as South Africa, where this investigation was conducted. The general inclusion of a biosignature for RCDs in routine clinical practice will require, however, assessment of larger, prospective and validated studies, as was recently emphasized by Mamas et al. (2011). Meanwhile, our recommended preference in clinical practice on RCDs would be to shift the emphasis from an approach "to scrape the barrel" (DiMauro and Andreu 2000; Smeitink 2003), in search of a definitive answer to a highly variable and evasive disease, to a focus on caring, affordability and personalized medicine.

4 Concluding remarks

This study demonstrates that pattern recognition of urinary organic acids in metabolomics analysis can inform on the complex differences that can be observed in the global metabolite profiles of controls and RCD patients. It rules out the possibility of identifying one unique and distinctive organic acid biomarker. Significantly, however, the global metabolite profile has the potential to define an extended biosignature and the development of a non-invasive screening procedure for RCDs. We suggest that such a procedure for diagnosis and monitoring of disease progression or treatment will add considerable value to the current clinical practice followed for RCDs.

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CHAPTER 4

APPLICATIONS

4.1 INTRODUCTION

The first application was a case study, based on some of the expertise used in the experimental Section and additional investigations were done to elucidate the findings. A very interesting young male adult patient with Kearns–Sayre Syndrome (KSS) was identified, and biochemical analyses revealed deficiencies of CI, II+II and IV. Blue native polyacrylamide gel electrophoresis was performed and the presence of two catalytically active complexes was revealed. Molecular genetic characterisation was performed and a novel deletion of 3,431 bp was found between the nucleotide positions 7,115 and 10,546 in 80.0% of muscle mitochondrial DNA (mtDNA). It was further established that the absence of the *ATPase6* and *ATPase8* genes resulted in the aberrant synthesis of adenosine triphosphate (ATP) synthase. This published case study is shown in Section 4.2.

The second application described a proposed biosignature for respiratory chain disorders (RCDs) based on the extension of the global metabolite profile identified in the urinary organic acid analysis of the metabolome, as described in Section 3.3. Although it was possible to use the organic acids to distinguish between healthy controls and patients with RCDs, no single biomarker for RCDs was found. It was then decided to include additional components of the metabolome. Amino acid as well as carnitine analyses and an untargeted approach using nuclear magnetic resonance (NMR) analyses were added. The final application was the development of a biosignature that, if validated, can be utilised to improve the selection of patients for a muscle biopsy to confirm an RCD. The outcome of this study is described in Section 4.3.

The third application was focussed to increase the awareness of mitochondrial disorders (MDs) among the South African medical community and inform them about the availability of facilities in South Africa, although such availability was still in the form of a research project. The publication that resulted from this is given in Section 4.4.

4.2 CASE STUDY

Van der Westhuizen FH, Smet J, Levanets O, Meissner-Roloff, Louw R, Van Coster R, Smuts I (2010) Aberrant synthesis of ATP synthase resulting from a novel deletion in mitochondrial DNA in an African patient with progressive external ophthalmoplegia. J Inherit Metab Dis DOI10.1007/s10545-009-9020-y.

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CASE REPORT

Aberrant synthesis of ATP synthase resulting from a novel deletion in mitochondrial DNA in an African patient with progressive external ophthalmoplegia

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Abstract A young, adult, African male patient presented with progressive proximal muscle weakness, external ophthalmoplegia and ptosis, as well as cardiac conduction abnormalities resembling Kearns–Sayre syndrome (KSS). Magnetic resonance imaging (MRI) of the brain revealed normal basal ganglia but bilateral well-circumscribed lesions in the cerebellar peduncles. Enzyme deficiencies in oxidative phosphorylation (OXPHOS) complexes I, IV and V was measured in muscle tissue. Blue native polyacrylamide gel electrophoresis (BN-PAGE) confirmed decreased protein content and activity of these complexes and revealed the presence of two catalytically active complex V sub-complexes. Upon investigation by molecular genetics, the mitochondrial DNA (mtDNA) copy number was found to be elevated and a novel deletion of 3431 bp was found in 80% of muscle mtDNA between positions 7115 and 10546, flanked by a 5 bp direct repeat sequence. In addition, it could also be concluded that the absence of mtDNA-encoded *ATPase6* and *ATPase8* genes in this patient clearly resulted in aberrant synthesis of ATP synthase.

Abbreviations

BN-PAGE	blue native polyacrylamide gel electrophoresis
ECG	electrocardiogram
EMG	electromyogram
MRI	magnetic resonance imaging
FLAIR	fluid-attenuated inversion-recovery
DWI	diffusion-weighted imaging

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References to electronic databases: Progressive external ophthalmoplegia: OMIM 157640, 609286, 258450, 610131, 609283, 258400. Retinitis pigmentosa: OMIM 600105, 608133, 500004, 612572, 312612, 268025, 268000, 312600, 300389, 180104. Kearns–Sayre syndrome: OMIM 530000. Pearson marrow–pancreas syndrome: OMIM 557000. ATP:creatine *N*-phosphotransferase (creatine kinase): EC 2.7.3.2. NADH:ubiquinone oxidoreductase (complex I): EC 1.6.5.3. Succinate:ubiquinone oxidoreductase (complex II): EC 1.3.5.1. Ubiquinol:ferricytochrome-*c* oxidoreductase (complex III): EC 1.10.2.2. Ferrocytochrome-*c*:oxygen oxidoreductase (complex IV): EC 1.9.3.1. ATP phosphohydrolase (complex V): EC 3.6.1.3. Pyruvate:[dihydrolipoyllysine-residue acetyltransferase]-lipoyllysine 2-oxidoreductase (decarboxylating, acceptor-acetylating; pyruvate dehydrogenase complex): EC 1.2.4.1. Acetyl-CoA:oxaloacetate *C*-acetyltransferase [thioester-hydrolysing, (*pro-S*)-carboxymethyl forming] (citrate synthase): EC 2.3.3.1. Deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (DNA-directed; DNA polymerase gamma): EC 2.7.7.7.

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HbA_{1C}	glycohaemoglobin
mtDNA	mitochondrial DNA
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species

Introduction

Mitochondrial disorders are caused by any one of a great number of mutations in nuclear DNA and mitochondrial DNA (mtDNA). Amongst these, mtDNA deletions are often associated with chronic progressive ophthalmoplegia (OMIM 157640, 609286, 258450, 610131, 609283, 258400), Kearns–Sayre syndrome (OMIM 530000), and Pearson marrow–pancreas syndrome (OMIM 557000; Yamashita et al. 2008; Mancuso et al. 2007). These deletions often occur in an area of the mitochondrial genome that encodes the two mitochondrial genes for ATP synthase, *ATPase6* and *ATPase8* (Mita et al. 1990; Samuels et al. 2004). In our study, clinical, biochemical and molecular genetic findings in an adult African patient with a novel mtDNA deletion in this area of the mitochondrial genome are reported. In addition, the effect of this deletion on the native structure and function of oxidative phosphorylation (OXPHOS) complexes is demonstrated.

Patient and methods

Patient

A 22-year-old male patient with progressive proximal muscle weakness, external ophthalmoplegia and ptosis was referred for assessment. The presenting symptom had been ptosis when he was aged 17 years. He had been healthy previously, and there was no family history of any mitochondrial disorder. His initial school performance was satisfactory, and he completed grade 10. However, at the time of the assessment, the results of the Beery–Buktenica developmental test of visual motor integration correlated with that of an 8-year-old child.

He had asymmetrical but bilateral ptosis, with the left side more affected. He experienced progressive external ophthalmoplegia, and complete ophthalmoplegia was present within 15 months after the initial presentation. Retinitis pigmentosa (OMIM 600105, 608133, 500004, 612572, 312612, 268025, 268000, 312600, 300389, 180104) was diagnosed. He had proximal muscle weakness and complained of exercise intolerance. The deep tendon reflexes were diminished. He had dysarthria, a slight tremor and past-pointing, but no ataxia was present initially. Although he did not have ataxia initially, the dysarthria, slight tremor and past pointing were indicative of cerebellar involvement and therefore fulfilled the criteria for Kearns–Sayre syndrome (KSS) (OMIM 530000) as proposed by Moraes et al. (1989).

Gynaecomastia was noted. He had an irregular pulse, but no cardiomegaly or cardiac failure.

Methods

Ethical aspects

For the specialised investigations described in this report, ethics approval was obtained from the University of Pretoria (number 91/98 and amendments), the North-West University (02M02). Informed consent and assent were obtained from the patient.

Enzyme assays

A muscle biopsy was taken from the vastus lateralis muscle for further biochemical and molecular genetics analyses. Mitochondrial respiratory chain enzymes complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3), complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1), complex III (ubiquinol:ferricytochrome-*c* oxidoreductase, EC 1.10.2.2), complex IV (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1), combined activity for complex II and III (succinate-cytochrome c reductase) and pyruvate dehydrogenase complex (PDHc, EC 1.2.4.1) were measured in the muscle, essentially as has been described previously (Rahman et al. 1996; Janssen et al. 2007; Chretien et al. 1995). As frozen muscle tissue was used to prepare 600 *g* supernatants for assay of respiratory chain enzymes, an assay of complex V (EC 3.6.1.3), which requires freshly isolated mitochondria, was not performed. Citrate synthase (CS; EC 2.3.3.1) activity was determined by the method of Shepherd and Garland (1969), and enzyme activities were expressed as a ratio to CS (as mitochondrial marker enzyme) to compensate for mitochondrial enrichment in the sample (Janssen et al. 2007). Values were compared to reference values obtained from muscle samples from 18 healthy controls undergoing orthopaedic surgery.

Blue native polyacrylamide gel electrophoresis followed by in-gel activity staining

Blue native polyacrylamide gel electrophoresis (BN-PAGE) and in-gel activity staining were performed as has been described previously (Van Coster et al. 2001). Briefly, mitochondria were prepared from the patient together with a control muscle specimen. The mitochondria were solubilised, and 50 μg of protein were loaded in duplicate.

The first set of lanes was used for activity staining of complexes I, III (Meulemans et al. 2007) and IV, and the second set was used for activity staining of complexes II and V.

Western blot analysis of mitochondrial proteins resolved by two-dimensional BN-PAGE

After the first dimension (BN-PAGE), a second denaturing electrophoresis [tricine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)] was performed, for which the BN-PAGE gel was turned 90° and run in a perpendicular second dimension. In this way, the protein subunits within each OXPHOS complex were separated (Devreese et al. 2002). The two-dimensional (2D) gels were electro-blotted onto a nitrocellulose membrane using a tank blotting apparatus from Bio-Rad, as has been described previously (De Vriese et al. 2006). The relative levels of the five OXPHOS complexes were evaluated by immunodetection, using the MS601 MitoProfile® human total OXPHOS complexes detection kit from MitoSciences in a 1/1000 dilution. Detection was done with the enhanced chemiluminescence kit, ECL Plus™ from GE Healthcare. The signals were captured with a ChemiDoc charge coupled device (CCD) camera and processed with Quantity One® software, both from Bio-Rad.

Molecular genetic analyses

DNA was extracted from muscle with the NucleoSpin® kit from Macherey–Nagel. In order to investigate the integrity of mtDNA, we performed Southern blot analysis (Selden 1989). For this, 0.1 μg to 0.5 μg of DNA were digested with *Pvu*II before electrophoresis and membrane transfer. A probe encompassing the complete mtDNA sequence was prepared by polymerase chain reaction (PCR) (Long PCR Enzyme Mix, Fermentas), using an ND1 gene primer set (forward 5′-GTCTCAGGCTT CAACATCG-3′; reverse 5′-GCATTAGGAATGC CATTGCG-3′). The probe was labelled with alpha 32-phosphorus deoxycytidine triphosphate (α-[P^{32}]dCTP; Izotop) using random prime labelling. After autoradiography, the percentage of deleted DNA was determined by densitometry (GeneTools software, Syngene). In addition to Southern blot analysis, PCR was used to identify and verify the general position of the deletion. The mtDNA primer sets described by Taylor et al. (2001) were used for this purpose.

The total DNA extracted from the patient's muscle and the muscle control DNA samples (n=18) were used to determine the relative mtDNA content. Relative mtDNA content was measured by real-time PCR and calculated using nuclear DNA as a normaliser. The primers and probe for the nuclear reference β-globin gene were 5′-GTGCACCTGACTCCTGAGGAGA-3′ (forward), 5′-CTTGATACCAACCTGCCCAG-3′ (reverse) and 5′-FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3′ (probe), synthesised by Metabion International. For mtDNA amplification, an MT-ND2 TaqMan® gene expression assay from Applied Biosystems was used (assay Hs02596874_g1). The PCR was performed with Applied Biosystems's ABI 7300 real-time PCR system in a 25 μl volume. Each reaction mixture contained 12.5 μl TaqMan® universal PCR master mix, No AmpErase® UNG (2×; Applied Biosystems), 0.5 μM forward and reverse primers, 0.2 μM probe for β-globin gene or 1 × dilution of primers/probe mixture for the MT-ND2 gene, and 10 ng of DNA. The PCR conditions were 10 min at 95°C, followed by 40 cycles of denaturation at 95°C and annealing/extension at 60°C for 1 min, with fluorescence measurement during this step. C_T values were calculated with 7300 System Sequence Detection software (version 1.4; Applied Biosystems). All reactions were performed in triplicate. Each assay also included a no-template control, three serial dilution points (in steps of five-fold) of a DNA mixture, and each of the test DNAs (patient and controls). In order to calculate the relative mtDNA content in the patient's DNA, C_T we exported the values obtained from real-time PCR analyses of the patient and control samples to REST© software (Relative Expression Software Tool; Pfaffl et al. 2002). PCR efficiency for each primer set was calculated by serial dilution using the REST software tool. The specific mtDNA fragment between positions 6113 and 11727 that contained the deletion was sequenced with an AB1 3130XL genetic analyser (Applied Biosystems) at Inqaba Biotechnical Industries in Pretoria, South Africa. DNA polymerase gamma (EC 2.7.7.7) subunit 1 gene (*POLG*) was sequenced, essentially as described by Nguyen et al. (2006).

Results

The results of the special clinical investigations revealed a right bundle branch block on the electrocardiogram (ECG). The findings of an ultrasound examination of the heart were within normal limits. The findings of an electromyogram (EMG) and nerve conduction studies were within normal limits. The MRI of the brain revealed normal basal ganglia, but bilateral well-circumscribed lesions in the cerebellar peduncles were demonstrated. Low signal intensity on T1-weighted images and high signal intensity on T2-weighted images were observed (Fig. 1). The intensity was mixed on the fluid-attenuated inversion-recovery (FLAIR) images, and restricted diffusion was found on diffusion-weighted imaging (DWI).

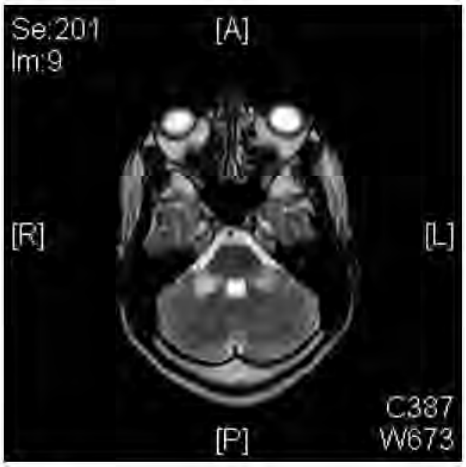

Fig. 1 T2-weighted magnetic resonance image demonstrating the high signal intensity, well-circumscribed lesions in the cerebellar peduncles

Urine analyses revealed the presence of elevated levels of lactic acid, elevated levels of Krebs cycle metabolites, a mild generalised amino-aciduria and glucosuria, but the glycohaemoglobin (HbA_{1C}) and serum glucose levels were within normal limits. Serum lactate was 4.2 mM and the lactate-to-pyruvate ratio was 31.3. Blood creatine kinase (EC 2.7.3.2) activity was initially 278 U/l, but it increased over a period of 18 months to 2,083 U/l. Human immunodeficiency virus (HIV) infection was excluded. The patient did not have any proven endocrinological abnormalities.

Muscle histology revealed ragged red fibres and mild lymphocyte infiltration (results not shown). Biochemical analyses of the muscle biopsy confirmed the suggestion of a mitochondrial disorder and were informative as to the deficiency that existed. From the data from respiratory chain and PDHc enzyme activity, which are summarised in Table 1, it was concluded that there was a combined deficiency of complexes I, II+III, and IV. BN-PAGE analysis of muscle provided additional information on the deficiency (Fig. 2). The OXPHOS complexes were already visible in the gel without additional staining, owing to the coomassie dye, which induced a charge shift of the proteins and which thus revealed the protein content of the complexes (Smet et al. 2005). By comparing the intensities of the OXPHOS protein bands between the lane loaded with the patient's muscle mitochondria and a control lane, we noticed decreased protein content of complexes I and IV and the V holo-complex (Fig. 2, left side of panel A). Consequently, following in-gel activity staining, decreased catalytic activity of those complexes could be demonstrated. The enzyme activities of complexes II and III seemed comparable. Two catalytically active complex V sub-complexes in the patient's sample were observed (Fig. 2, right side of panel A).

Using western blotting following 2D BN/SDS-PAGE, we observed a reduced signal for the complex I subunit (NDUFB8-20 kDa) and the complex IV subunit (COX II, 26 kDa) in the patient. Three different bands originating from the complex V alpha subunit were also detected. The signal intensities of the complex II subunit (Ip, 30 kDa) and the complex III subunit (core 2, 47 kDa) were, however, comparable between patient and control samples (Fig. 2, panel B).

Initial analysis of mtDNA structure (Fig. 3a, b) clearly revealed a deletion of ~3.4 kbp, which was present in 80% of the mtDNA copies of the patient's muscle biopsy according to the densitometry of various DNA dilutions. Further investigations revealed that the deletion occurred in an area between nucleotide positions ~6000 and ~12000 (Fig. 3c, d). It was also observed that the mtDNA copy number was notably higher (219%) than the average of that of controls (n=8). In addition, no pathogenic mutations were detected in the *POLG* gene. From sequencing data, the position of the deletion on mtDNA was determined to be present between positions 7115 and 10546, as shown in Fig. 4. This 3,431 bp deletion included five transfer RNA (tRNA) genes, two genes of complex I (*ND4L* and *ND3*), all three genes of complex IV (*CO I-III*) and both mitochondrially encoded genes of complex V (*ATPase6* and *ATPase8*). The deletion occurred immediately after, and on the 3′ end also included, the sequence ACACC.

Table 1 Respiratory chain and PDHc enzyme activities in the patient's muscle. Activities are expressed against several reference enzymes as shown: mU, nmol/min; UCS, μmol/min citrate synthase activity; PDHc, pyruvate dehydrogenase complex

Sample	Complex I			Complex II		Complex III			Complex II+III			Complex IV		PDHc	
	mU/UCS	CI/CII	CI/CIV	mU/UCS	CII/CIV	mU/UCS	CIII/CII	CIII/CIV	mU/UCS	CII+III/CII	CII+III/CIV	mU/UCS	CIV/CII	mU/mg	mU/UCS
Patient	150[a]	1.9[a]	12.0	79.2	6.3	392	4.95	31.2	75[a]	0.94[a]	5.94	13[a]	0.2[a]	42.6	101
Control range (n=18)	219 372	1.9 4.5	2.1 7.1	72.8 149	0.78 2.1	327 787	2.97 9.32	5.04 14.5	117 234	1.14 2.2	1.28 3.67	43.1 124	0.5 1.3	4.0 96.5	7.8 104

[a] Indicates patient's values equal to or lower than the lowest control value

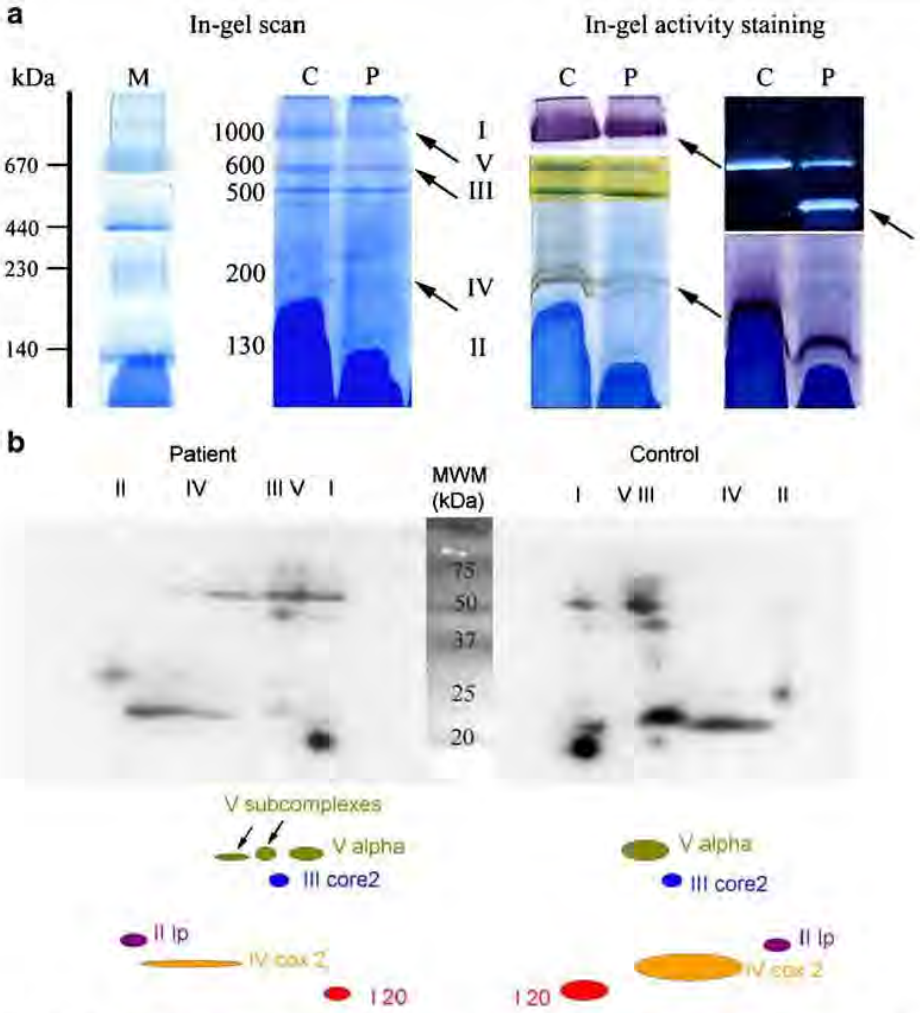

Fig. 2 BN-PAGE and 2D BN-PAGE western blotting. Panel A. Legend: (M) native molecular weight marker, (C) control muscle, (P) patient muscle. Left side: scan of the gel following BN-PAGE; arrows mark decreased amounts of OXPHOS complex proteins (I, IV and V) in the patient compared to those in the control sample. Right side: in-gel activity of the patient's muscle, indicating decreased activity of complexes I and IV, whilst the activities of complexes III and II are comparable to those of the control sample. Note the presence of catalytically active complex V sub-complexes, which are suggestive of disturbed intra-mitochondrial protein synthesis. Panel B. Immunostaining of OXPHOS proteins from patient and control samples following 2D BN-PAGE, using specific antibodies against subunits of the five OXPHOS complexes. MWM: molecular weight marker. The signals from the complex I subunit (NDUFB8, 20 kDa) and the COX II subunit are reduced in the patient in comparison with those of the control. The signals of the other OXPHOS subunits, complex III core 2 and complex II Ip, are comparable to those of the control. Notice the presence of two additional signals from the complex V alpha subunit, indicating the presence of two sub-complexes of complex V

Discussion

A patient of African origin with a novel deletion in his mtDNA has been described. The patient presented with external ophthalmoplegia, ptosis and retinitis pigmentosa resembling KSS. The MRI findings comprising normal basal ganglia and bilateral well-circumscribed lesions in the cerebellar peduncles (Fig. 1) might not comply entirely with the classical description of MRI findings in KSS, but there is limited correlation between the MRI findings and the neurological deficits in KSS (Chu et al. 1999; Lerman-Sagie et al. 2005). Bilateral lesions of high signal intensity on T2-weighted images of the globus pallidus and subcortical cerebral white matter are characteristic of KSS (Lerman-Sagie et al. 2005). Other common MRI findings include cerebral and cerebellar atrophy, bilateral lesions of high signal intensity in the thalamus, substantia nigra and brain stem (Leutner et al. 1994; Wray et al. 1995; Saneto et al. 2008). On the other hand, according to Barragan-Campos et al. (2005), basal ganglia involvement might not always be

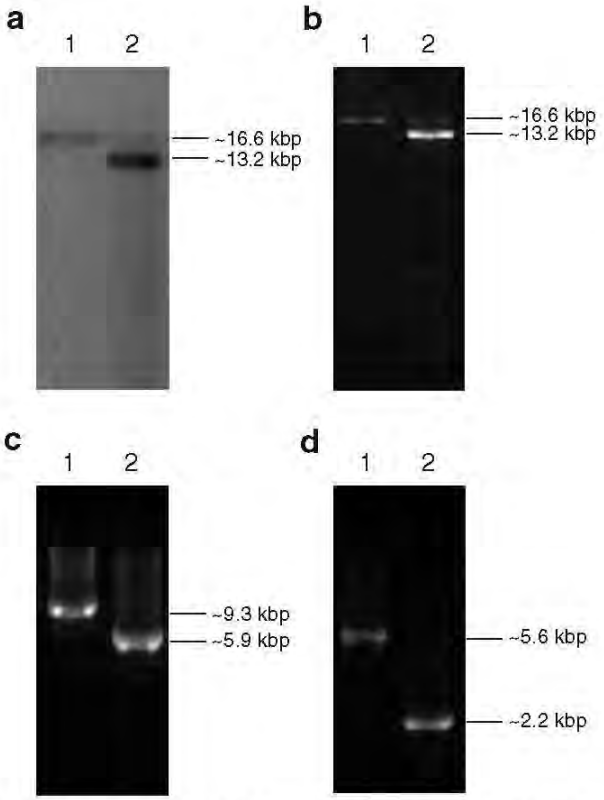

Fig. 3 Southern blot and PCR analysis of mtDNA. DNA of a healthy control (1) and the patient (2) was pre-treated with *Pvu*II and subjected to Southern blot analysis (a). PCR and ethidium bromide-containing gel electrophoresis using several primers were used to identify the region of deletion (b–d). The primers used were complementary to the following positions on the mtDNA genome: 37–58 (forward) and 16537–16558 (reverse), which amplified the complete mtDNA genome (b); 6113–6133 (forward) and 15431–15409 (reverse) in (c); 6113–6133 (forward) and 11748–11727 (reverse) in (d)

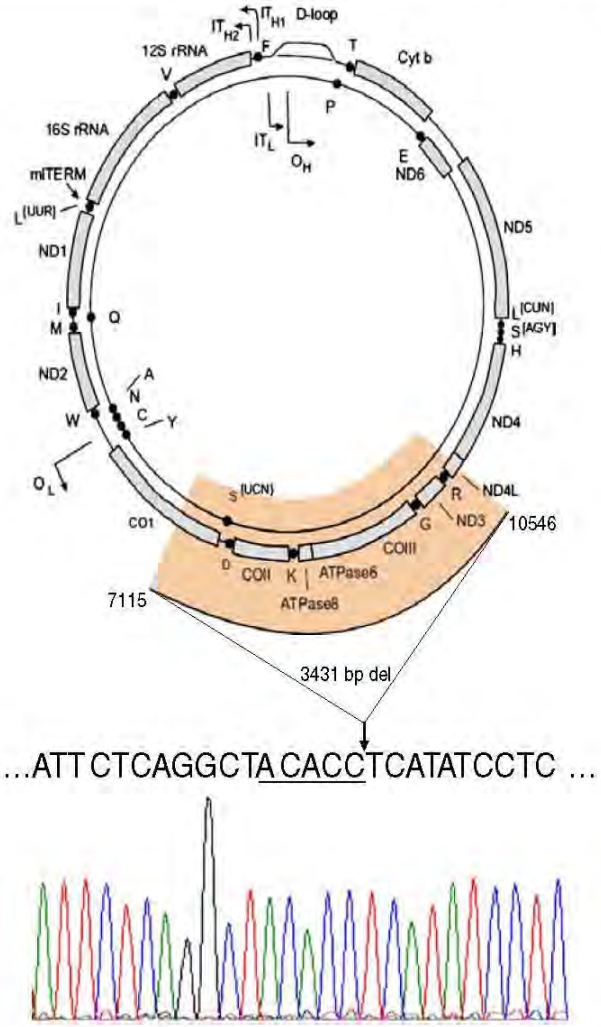

Fig. 4 Position of deletion in the patient's mtDNA. An electropherogram on the bottom indicates the position of the 3431 bp deletion, with the flanking repeat sequence underlined (the repeat sequence occurs at the 3′ end of the deletion as well). The position of the deletion on mtDNA and the genes involved are highlighted in the top part of the figure (adapted from Taanman 1999). Nucleotide positions are according to the Revised Cambridge Reference Sequence (GenBank J01415.2)

present. The white matter lesions can affect both the deep cerebral and cerebellar white matter (Lerman-Sagie et al. 2005). Heidenreich et al. (2006) described involvement of the cerebellar peduncles in their study of patients with chronic progressive external ophthalmoplegia (CPEO), including a patient with KSS. Bianchi et al. (2007) classify the MRI changes found in KSS as the syndromic type II pattern. The rest of the spectrum constitutes the type I pattern, which includes the more non-specific changes, and type III, the more leukodystrophy-like pattern.

A combined deficiency of OXPHOS complexes I, II+III, and IV was identified from the initial enzyme analyses. From our initial diagnostic analyses, which included a very simple but valuable PCR analysis to screen for mtDNA macro-structural changes, an mtDNA deletion was clearly present in this case, which required further investigation. The additional results obtained following BN-PAGE revealed reduced protein content and activities of complex I, IV and V. These findings were in agreement with the genes deleted from mtDNA in this patient. Both the protein content and activity of complex III, which, apart from absent tRNA genes, is not affected by the deletion, as well as nuclear-encoded complex II, were comparable to those of the control sample. Additionally, notable aspects of complex V synthesis and activity were demonstrated in the patient's sample. It was observed that the activity of complex V was reduced in the patient but that a catalytically active sub-complex of complex V existed. Some activity remained in the holo-complex, which might be attributed to the 20% wild-type mtDNA present in the patient. This finding points to disturbed intra-mitochondrial

protein synthesis, which can be attributed to the absence of the two mtDNA genes for complex V, *ATPase6 and ATPase8* (Carrozzo et al. 2006; Smet et al. 2009).

Several characteristics of the deletion should be noted. The position of this relatively small deletion (3,431 bp), present in 80% of mtDNA copies, has not previously been reported in the literature. The deletion break points are flanked precisely by direct repeats, which identify it as a class I deletion according to the classification by Mita et al. (1990). Deletions of this class are the most common and occur in ~60% of all known cases (Samuels et al. 2004). The reported deletion is located in the mtDNA region, where a high deletion frequency occurs. The 5′ end of the deletion (7115) falls within the 5′-end distribution range (5835 to 12112) that is common for most deletions, as reported by Mita et al. (1990). It also falls within the distribution range of around 8 kbp to 9 kbp (7832 bp to 8653 bp median values) for 5′ ends of mtDNA deletions, as reported by Samuels et al. (2004). However, the 3′ end of the reported deletion (10546) is not situated within the common 3′-end distribution range, which is mostly found in the region between 12661 and 15945. Samuels et al. (2004) reported that the distribution of 3′-end deletion has two peaks, which is different from the unimodular distribution for 5′ ends, consisting of a broad peak, centred on the median values of 13958 to 14643, and a sharp peak, at position 16000 to 16100.

The associated increased mtDNA replication observed is known to occur in patients with mtDNA deletions (Bai and Wong 2005). Although increased oxidative stress or oxidative damage was not measured in the tissue, mtDNA overexpression may occur through the reactive oxygen species (ROS)-mediated induction of mtDNA transcription and replication factors (Miranda et al. 1999; Lee and Wei 2005).

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4.3 BIOSIGNATURE

Smuts I, van der Westhuizen FH, Louw R, Mienie LJ, Engelke UFH, Wevers RA, Mason S, Engelke UFH, Koekemoer G, Reinecke CJ. The search for non-invasive biomarker for patients with respiratory chain deficiency biomarker: a metabolomic contribution. Manuscript submitted for publication in the J Inherit Metab Dis.

DISCLOSURE OF A PUTATIVE BIOSIGNATURE FOR RESPIRATORY CHAIN DISORDERS THROUGH A METABOLOMICS APPROACH

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Abbreviated title:

A biosignature for respiratory chain disorders

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Keywords:

Metabolomics, respiratory chain disorders, urinary organic acids, urinary amino acids, data reduction, biosignature

Abstract

The diagnosis of respiratory chain deficiencies (RCDs) is complicated and the need for a diagnostic biomarker or biosignature has been widely expressed. In this study, the metabolic profile of a selected group of 29 RCD patients with a predominantly muscle disease phenotype and 22 controls were investigated using targeted and untargeted analyses of three sub-sections of the human metabolome, including organic acids and amino acids (measured by gas chromatography–mass spectrometry (GC-MS)), as well as acylcarnitines (measured by electrospray ionization tandem MS TMS)). Although MS technologies are highly sensitive and selective, they are restrictive by being applied only to sub-sections of the metabolome; an untargeted nuclear magnetic resonance (NMR) spectroscopy approach was therefore also included. After data reduction and pre-treatment, a biosignature, comprising of six organic acids (lactic, succinic, 2-hydroxyglutaric, 3-hydroxyisobutyric, 3-hydroxyisovaleric and 3-hydroxy-3-methylglutaric acids), six amino acids (alanine, glycine, glutamic acid, serine, tyrosine and α-aminoadipic acid) and creatine, was constructed from uni- and multivariate statistical analyses and verified by cross-validation. The results presented here provide proof-of-concept that the metabolomics approach is capable of defining a biosignature for RCDs, based on an extended metabolite profile of these diseases, offering an improved ability to assign individual patients to a group with defined RCD characteristics. The biosignature could be useful to reduce the need for invasive biopsy procedures, especially in infants and children, and could eventually influence or even change clinical practice with regard to RCD diagnosis and monitoring of treatment.

Introduction

Several reviews on mitochondrial medicine appeared in the April 2011 edition of the Journal for Inherited Metabolic Diseases and were placed in perspective by Koene and Smeitink (2011). Among the mitochondrial disorders (MDs), the respiratory chain disorders (RCDs) are a group of more than 100 different conditions related to defects in many of the proteins that constitute the four complexes (CI to CIV) of the respiratory chain and ATP synthase (complex V, CV), the major site of ATP regeneration in the cell. These proteins are encoded by nuclear- and mitochondrial DNA (nDNA and mtDNA, respectively), which implies that autosomal recessive and dominant, X-linked and maternal modes of inheritance, as well as incidental *de novo* mutations, are involved in the genetic basis for these disorders, which can present from the neonatal period to adulthood (Thornburn 2006). Ultrastructure research firmly established that mitochondrial structure and function varies across cell and tissue types, and has now been well substantiated by comprehensive investigations of the human mitochondrial proteome (reviewed by Calva and Mootha 2010). All these characteristics, along with the extensive adaptive responses that occur during RCDs (Reinecke et al 2009; Elstner and Turnbull 2011), contribute to the clinical heterogeneity of these disease, so that a wide range of medical specialists, including paediatricians, cardiologists, gastroenterologists, neurologists and ophthalmologists, may first encounter these patients (Wong et al 2010). Accordingly, various criteria, apart from genomic analyses, have been developed from both clinical and biochemical points of view to direct the diagnosis of the RCDs.

Skeletal muscle provides the key material for histological and biochemical analysis of mitochondrial function and RCD diagnosis. Given the invasive procedure of a muscle biopsy under general or local anaesthesia in children or adults, respectively, distinct clinical and biochemical information is preferably required as indication for a biopsy. Elevated transaminases and creatine phosphokinase are generally accepted as non-specific enzymatic indicators of MD (Wong et al 2010). Recently Suomalainen et al (2011) recently proposed fibroblast growth factor (FGF-21) as a biomarker for muscle-manifesting

mitochondrial respiratory chain deficiencies, which need to be confirmed by a prospective study, including appropriate patient groups (Turnbull 2011). An analysis of urinary metabolites including lactate, alanine, other amino acids, Krebs cycle intermediates and other organic acids provides the least invasive indicators of RCD, but still lacks specificity as well as selectivity, as pointed out by Koene and Smeitink (2011). From the use of metabolic profiling of data generated by mass spectrometry (MS), plasma creatine was recently proposed as a specific and sensitive indicator of RCDs (Shaham et al2010). A subsequent preliminary study confirmed that plasma creatine is elevated in RCDs, suggesting that it could be used in combination with other biomarkers for the diagnosis of MD (Boenzi et al 2011). In this regard, it has been suggested that ~~-o~~mics" approaches, such as metabolite profiling, might expand the global view of metabolism due to RCD pathology directly or indirectly (Soumalainen 2011), and so support the more efficient identification of improved biomarkers for RCDs. This concurs with the findings from a metabolomics investigation which disclosed the presence of 24 organic acid metabolites that were practically and statistically highly significant for a well-defined group of RCD patients (Reinecke et al 2011).

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological conditions or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). A profile of combined biomarkers is called a biosignature. Measuring single markers seems insufficient in dealing with complex diseases, like RCDs, as outlined above. It has been argued that for complex infectious diseases, such as tuberculosis, a combination of molecular profiles is likely to have more value than single biomarkers (Jacobsen et al 2008), and that global approaches would be the analytical route to reveal such markers. Metabolic profiling through a global approach thus proved valuable in the search for biomarkers of complex conditions using an experimental model for an infectious condition (Wikoff et al 2008) as well as for the inherited RCDs (Shaham et al 2010). We recently proposed that a global approach might disclose a metabolite profile with the potential to define an extended

and characteristic biosignature that can be used as a non-invasive screening instrument for RCDs (Reinecke et al 2011).

In the study reported here we have further investigated the metabolite profile in RCDs through analysis of three sub-sections of the human metabolome, included in the evaluation by the Mitochondrial Medicine Society‘s Committee on Disease as different laboratory modalities that can contribute to the establishment of RCDs (Haas et al 2008). The three sub-sections are the organic acids and amino acids (measured by gas chromatography–mass spectrometry (GC-MS)) and acylcarnitines (measured by electrospray ionization tandem mass spectrometry (TMS). MS technologies are highly sensitive and selective, but also restrictive by being applied only to sub-sections of the metabolome. We therefore also included the untargeted NMR spectroscopy in this investigation. Although less sensitive than MS analysis, NMR spectroscopy proved to be highly successful as a complementary technique in studies of inherited metabolic diseases (Engelke et al 2007). The results presented here provide proof-of-concept that the metabolomics approach is capable of defining a biosignature for RCDs, based on an extended metabolite profile of these diseases, and offers an improved ability to assign individual patients to a group with defined RCD characteristics. This knowledge paves the way for a prospective study on a biosignature for RCDs which could be used to reduce the need for invasive biopsy procedures, especially in infants and children. Availability of such a biosignature could eventually also influence or even change clinical practice with regard to RCD diagnosis and monitoring of treatment, especially in sophisticated clinical settings.

Materials and methods

Reagents

Reagents and standards for the extraction of the organic acids were purchased from Merck Chemical Co. (Darmstadt, Germany) and ethylacetate, diethylether and sodium sulphate and 3phenylbutyric acid from Sigma-Aldrich (St Louis, USA). All the reagents for the amino acid analysis, including the standards (200 µM each), GC column (10 m×0.25 mm ZB-AAA) and liner were provided in the EZ:faast^{TM} amino acid analysis sample testing kit by Phenomenex, Inc. (Torrance, CA). For the carnitine analysis, acetonitrile, formic acid, and methanol were purchased from Merck Chemical Co. Butanolic HCl (3N) was purchased from Sigma-Aldrich Co. The following standards were obtained from Dr H J ten Brink, Free University Medical Center (VUMC), Amsterdam, The Netherlands: L-carnitine·HCl, acetyl-L-carnitine·HCl, propionyl-L-carnitine·HCl, isovaleryl-L-carnitine·HCl, octanoyl-L-carnitine·HCl, hexadecanoyl-L-carnitine·HCl, [methyl-d_3]-L-carnitine·HCl, [d_3]-acetyl-L-carnitine·HCl, [3,3,3-d_3]-propionyl-L-carnitine·HCl, [d_9]-isovaleryl-L-carnitine·HCl, [8,8,8-d_3]-octanoyl-L-carnitine·HCl, and [16,16,16-d_3]-hexadecanoyl-L-carnitine·HCl.

Subjects and the selection of samples for the metabolomics analysis

Ethical approval for the study was obtained from the relevant Ethics Committees of the University of Pretoria (No. 91/98 and amendments) and North-West University (No. 02M02). Informed consent was obtained from the parents of patients and controls for the use of the urine samples and biopsy material (where applicable) of their children for research purposes.

The original RCD experimental group consisted of 101 clinically selected patients, including the cohort of South African patients described by Smuts et al (2010). Urine samples were obtained at the Paediatric Neurology Unit at the Steve Biko Academic Hospital, Pretoria, South Africa, at the time when the muscle biopsy was performed; the patients did not receive any specific treatment or supplements often given to patients with MDs. Use of anti-convulsants was not stopped. The controls were selected from among children referred to

the clinic, but for whom no prevailing disorder was detected. Aliquots of all samples were stored at minus 80^{o}C prior to metabolomics analyses. This cohort provided the basis for the selection of samples from patients and controls for the metabolomics analyses.

Metabolomic investigations are most successfully conducted with control and patients groups which are well-distinguished from one another. As sample selection is one of the most important aspects of any metabolomics analysis, special care was taken to include clinical as well as biochemical parameters that would ensure the clear distinction between controls and patients, while retaining the intrinsic heterogeneity of the selected RCD group. Three inclusion criteria were thus defined: (1) clinical criteria characteristic of RCDs (including the intrinsic property of having a predominantly myopathic phenotype as described in Smuts et al (2010)); (2) a proven deficiency in one or more complexes of the RC; and (3) a complete separation between controls and the patients, based on the numerical value of the sum of all organic acids excreted in a urinary sample by these cases. These samples were selected for metabolomics analysis. Samples from 51 cases (29 patients and 22 controls, designated as Group 1) satisfied these criteria and were available for the MS analyses of the organic acids, amino acids and acylcarnitines, but sufficient urine from only 34 of these cases (20 patients and 14 controls, designated as Group 2) were available for the NMR analyses. The characteristics of Groups 1 and 2 are shown in Tables 1 and 2.

Biopsy material and enzyme analyses from the patient group

Enzyme analyses were performed on muscle biopsies from the *vastus lateralis* muscle of all patients complying with the Mitochondrial Disease Criteria as defined by Wolf and Smeitink 2002, and performed according to the procedures fully described previously, including the two criteria used for identification of an enzyme deficiency in this patient group (Reinecke et al 2011). As summarized in Table 1, the 29 patients selected thus had a muscle deficiency of either complex I (CI; five cases), complex III (CIII; four cases) or a number of different deficiencies of more than one RC enzyme (CM; 20 cases).

Acquisition of metabolite data

Untargeted metabolic analysis using nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (1H-NMR) spectroscopy was included in this investigation for its high selectivity, provision of unambiguous information about a metabolite and the direct analysis of samples that did not require any prior sub-fractionation for metabolite selection. This work was conducted at the Laboratory for Genetic, Endocrine and Metabolic Diseases, Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre according to standard procedures used there (Engelke et al 2007). Urine samples from 34 cases of Group 2 were used in the 1H-NMR study. These urine samples were analyzed using one-dimensional (1D) 1H NMR spectroscopy. One millilitre of urine was centrifuged at 3000 rpm for 10 min and 700 µL supernatant transferred into a clean test tube. To this, 70 µL of 20.2 mM standard trimethylsilys-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP) in $^{2}H_2O$ was added, the pH adjusted to 2.5 ± 0.05 with concentrated HCl, and 650,µL was transferred to a 5-mm NMR tube. Each sample was analyzed in a 500 MHz Bruker DRX spectrometer at 256 scans with a pulse of 7 µsec and a delay of 4 sec. The resulting free induction decay (FID) was converted into frequency domain by Fourier transformation, thereby yielding an 1H-NMR spectrum. The instrument was equipped with a sample changer and each urine 1H-NMR spectrum was analyzed individually. The dominant metabolites typically present in urine were detected in all samples. Six notable metabolites were identified based upon their chemical shift resonances at pH 2.5, namely, alanine (1.51 ppm (doublet)), betaine (3.26 ppm (singlet)), creatinine (3.13 ppm (singlet)), creatine (3.05 ppm (singlet)), lactic acid (1.41 ppm (doublet)) and succinic acid (2.66 ppm (singlet)). Using a software program (Bruker Amix), each of the above peaks was manually selected and the area under the peak was calculated. Each selected metabolite was quantified relative to creatinine, using the integral and the number of protons with respect to each peak. Interference from medication made selection, and thus quantification, of certain selected metabolites (particularly alanine) not possible for some urine samples. The creatine and

betaine values obtained for 14 controls and 20 patients are included in Table 2 where applicable.

Analyses of organic acids using gas chromatography-mass spectrometry

MS-technology was likewise selected as it affords sensitive, accurate and reproducible measurements covering a wide dynamic range, indispensable in urine analyses. Untargeted gas chromatography-mass spectrometry (GC-MS) analysis of the urinary organic acids was performed as described previously (Reinecke et al 2011).

Analyses of amino acids using gas chromatography-mass spectrometry

GC-MS analysis of the amino acids was conducted on an Agilent Technologies (Chemetrix, Midrand, South Africa) 6890 series GC system with an Agilent Technologies 5973 Mass Selective Detector and a 7683 series dual tower and autosampler, all controlled by the MSD ChemStation E.02.00 (Palo Alto, CA, USA). The amino acid standards and urine were prepared as prescribed by the suppliers of the EZ:faast^{TM} amino acid analysis sample testing kit. One hundred µL of internal standard (norvaline at 200 µM) and amino acid standards (10, 25, 50, and 100µL each standard at 200 µM) or 100 microlitres urine were combined in a glass vial and further procedures were conducte according to the method supplied with the testing kit. Two microlitres of the extracts prepared according to the prescribed method was injected into the GC-MS for analysis and also analyzed according to the prescribed method. The standard range analysis was used to calibrate the identification and quantification of the amino acids, using the MSD ChemStation E.02.00 software with a linear regression curve fit.

Analyses of carnitines using tandem mass spectrometry

The electrospray ionisation a TMS method was used to quantify serum acylcarnitines. Ten microlitres of urine was added to a 1.5 ml centrifuge tube before 400 microlitres of the deuterated acylcarnitines (internal standard solution) with the following concentrations were added: [methyl-d_3]-L-carnitine·HCl (30.45 µmol/l), [d_3]acetyl-L-carnitine·HCl (20.83 µmol/l), [3,3,3-d_3]propionyl-L-carnitine·HCl (19.69 µmol/l), [d_9]isovaleryl-L-carnitine·HCl (17.73 µmol/l), [8,8,8-d_3]octanoyl-L-carnitine·HCl (15.43 µmol/l) and [16,16,16-d_3]hexadecanoyl-L-carnitine·HCl· (11.47 µmol/l). After the samples were evaporated to

dryness under a gentle stream of nitrogen (55°C), the remaining procedures were followed as described by Mels et al (2011). Acylcarnitines were quantified by comparing the signal intensity of carnitine and acylcarnitines against that of the corresponding deuterated analogues. The concentrations of carnitine and acylcarnitines analyzed were expressed as mmol/mol creatinine.

Statistical analysis

Variables with no variation (e.g. the internal standards) were removed from the original data sets for the organic acids, amino acids and carnitines and each of these data sets was initially analyzed separately to identify their role as potential biomarkers. In addition, a data filter, based on the approach of Bijlsma et al (2006), was applied to each variable to eliminate those that contained more than 40% zero values (~~"6~~0% rule") for the control and patient groups. Standard univariate analyses, including t-tests and the Mann-Whitney U-test, were applied to all these resulting variables. The subsequent data pretreatment, in the first instance, consisted of zero replacement, where the zero values represented the detection limit of the analytical equipment. The zeros were replaced by a random sample of values from a Beta (0.1;1) distribution bounded between zero and the detection limit. Thereafter, a shifted logarithmic transformation with a shift parameter set at one was performed, ensuring that the scales of the various metabolite concentrations were more comparable, after which the transformed data were centred prior to further statistical analyses.

The effect size of each individual variable was measured to ascertain the importance of the single variables (Ellis and Steyn 2003). An effect size of $d > 0.5$ can be considered as being of medium practical importance, whereas an effect size of $d > 0.8$ can be considered as highly practically significant. Descriptive statistics, such as minimum and maximum values, means and standard deviations were included as applicable.

Multivariate analyses used for the identification of important variables were principal component analysis (PCA), as an unsupervised pattern recognition method (Johnson and

Wichern 1998), and a partial least squares discriminant analysis (PLS-DA) as a supervised method (Baker and Rayens 2003). Variables listed by the PCA with a modelling power greater than 0.5 were regarded as potential biomarkers (Brereton 2003); and for variables important in projection (VIPs) from PLS-DA, the ‚greater than one rule' was used as the criterion for variable selection (Chong and Jun 2005). The primary criterion for selection of important metabolites was those identified by PLS-DA, based on a VIP > 1.0 for each variable in the three data sets. The specificity and sensitivity estimates of the outcomes of the PLS-DA approach were evaluated by cross-validation as described below. Fit statistics of the PCA and PLS-DA models were reported as the percentage variance explained for the metabolites (R^2X), the percentage variance for the group membership of the patients and controls (R^2Y), and the predictive R^2Y values (Q^2).

A putative biosignature was derived from a consolidated data set, consisting of the important metabolites identified by the PLS-DA of the three MS-based analyses and the two important variables from the 1H-NMR analysis. Because the scales of the four data sets were not comparable, the data of the consolidated data set was first scaled by using $Y = \log[X/\sqrt{\{1/(n - 1)\Sigma X^2\}} + 1]$. The transformed data were then centred prior to PLS-DA analysis, and the important variables identified and validated as described below.

A generic description of the cross-validation, which was constructed on the outcomes of the applicable PLS-DA models, includes the following aspects:

A data set was constructed which included only the important metabolites that were identified. Next, a PLS-DA model was constructed for this data set and an appropriate cutoff point was determined by calculating the Youdin index (Fluss et al. 2005). Then, we let $\mathbf{P_{CON}}$ and $\mathbf{P_{PAT}}$ be the observed occurrence probabilities of a control and a patient and let α be the fraction of cases to be removed in the cross-validation. Then, 10 000 unique stratified samples of size $n_{CV} = [\alpha \cdot n]$, with n = total number of control and patients, were selected from the data, stratified according to the observed occurrence probabilities, that is, $n_{CV} = n_C + n_P$, where n_C and n_P are the sample sizes from the controls and patients, respectively. For each

of the 10 000 samples the n_{CV} cases were withheld, a PLS-DA model was built using the remaining cases and the group membership of the withheld cases were predicted. For this, the sensitivity and specificity as well as the percentage of misclassified cases were recorded. Lastly, the standard deviation and the average of the recorded information were calculated over the 10 000 samples and reported as cross-validated estimates of sensitivity, specificity and percentage of misclassifications, as well as the respective values for α and the cut point.

Results and discussion

Profile of the control and patient groups

Table 1 summarizes the inclusion criteria and selected metabolomics data of the 29 RCD patients investigated. With regard to criterion 1 (5 aspects), assessments were based on a detailed history and clinical examination of all patients (Smuts et al 2010), indicating that an intrinsic property of the selected patients was their predominant myopathic phenotype. Original baseline investigations included lactate (L), pyruvate (P), creatine kinase (CK), and ammonia (NH_3) determinations. Lactic acidosis was present in five (17%) and a raised pyruvate and lactate:pyruvate ratio (>18) in nine (31%) cases of the selected group. All patients had a deficiency in one or more complexes of the RC (criterion 2), established by enzyme assays of biopsy material; and no patients with a deficiency in the pyruvate dehydrogenase complex (PDH) were included in the group. The total excretion of organic acids (criterion 3) of the patients was statistically significantly ($p < 0.0001$) increased relative to the controls, and no patients (minimum = 579 mmol/mol creatinine) or controls (maximum = 565 mmol/mol creatinine) were included in the group with an overlap in the total organic acid content, as shown in Table 2. With regard to the 7 sets of metabolomics data shown, statistically significant differences between the patients and controls were also found for the total amino acid excretion ($p < 0.0001$), total acylcarnitines ($p < 0.001$), free carnitine ($p < 0.001$), creatine ($p < 0.0001$) and betaine ($p < 0.047$). Although the mean values for the

ratios of octanoyl-carnitine:octenoyl-carnitine and decanoyl-carnitine:decenoyl-carnitine were respectively three times and five times higher than the controls, these differences were not statistically significant ($p > 0.05$), due mainly to the presence of outliers among these metabolites. Such high values, however, coincides with the view that the ratio of certain acylcarnitine esters may be useful in supporting specific diagnosis (Haas et al 2008).

A distinct difference between the biochemical profile of the patients and the controls, as shown in Table 2, is an important point of departure for metabolomics investigations. This was substantiated by a PCA conducted on the 29 patients and 22 controls for all the original 291 variables measured in the organic acid, amino acid and acylcarnitine (including free carnitine) analysis (Fig. 1). The outcome of the PCA, shown as a two-dimensional (PC1 and PC2) score plot for all the cases, shows that the patient group was distinguished from the controls. This indicates that the metabolic profiles of the two groups were distinctly different due to the perturbation induced by the respective CI, CIII or CM deficiencies. Moreover, the heterogeneity, which is characteristic of RCD, was retained in the patient group, as shown by the spread of these cases in the PCA. These observations already held the potential to identify biomarkers that could distinguish RCDs, but required data reduction for this identification.

Identification of important metabolites

The work-flow followed to identify important metabolites is shown schematically in Fig. 2. NMR-based metabolic profiling enables the simultaneous examination of a complex mixture of metabolites in a biological sample and requires only a limited knowledge of sample composition prior to analysis. NMR metabolomics may thus be regarded as an untargeted mode of analysis. By contrast, MS-based analyses are mostly semi-targeted as they distinguish a specific sub-section of the metabolome, extracted from a biofluid by an appropriate analytical procedure. Thus, as shown in Fig. 2, we included both these analytical

approaches in our metabolomics investigation to optimize the detection of possible biomarkers for RCDs.

As indicated, urine samples from only 20 patients and 14 controls, which included information on the urinary organic acids, amino acids and acylcarnitines, were available for the 1H-NMR analysis. Although these cases were fewer than the 51 cases of the total group, the clinical profile of the 34 cases strongly resembled that of the group of 51 patients. Alanine, lactic acid, succinic acid, creatine and betaine were found to be the important variables that distinguished the patient and control groups. As the first three of these are included in the MS-based analysis, only the values obtained for creatine and betaine were considered for the final consolidation of all important variables identified by the different analytical approaches.

Identification of important metabolites from GC–MS and TMS data required pre-processing to generate a data matrix of variables and cases of an operational size, to be followed by multivariate analyses to identify only the relevant analytical information. The total number of features in the original data set of the 51 cases, generated by an untargeted analysis in each of the three metabolite groups, yielded 291 substances that could be annotated as metabolites, namely, 189 organic acids, 51 amino acids, 50 acylcarnitines and free carnitine. Using the data filter, these were reduced to 120 metabolites: 39 organic acids, 36 amino acids, 44 acylcarnitines and free carnitine. With regard to the long-chain acylcarnitines, it should be noted that they are strongly protein-bound in the plasma and thus escape from excretion into the urine, similar to the free fatty acids. Their presence in the urine in appreciable amounts may thus be due to renal malfunctioning or damage resulting in proteinuria and the rather elevated amino acid excretion found in the RCD patients.

The subsequent data pre-treatment first included zero replacement and logarithmic scaling. Mean values, as well as the standard deviation of all variables for the controls and patients, were determined on the unscaled data, followed by t-test and Mann-Whitney analyses. The

two traditional methods of multivariate analysis chosen (PCA and PLS-DA), proved to be valuable for variable selection and were subsequently applied to all three data sets, followed by effect size analyses. All variables with a VIP > 1.0 and/or a power value > 0.5 and an effect size > 0.8 were designated as important metabolites due to the RCDs in the patient group. A total of 26 metabolites were identified through this selection method, and included 11 organic acids (adipic, fumaric, homovanillic, lactic, suberic, succinic, vanilmandelic, 2-hydroxyglutaric, 3-hydroxyisobutyric, 3-hydroxyisovaleric, and 3-hydroxy-3-methylglutaric acids), 13 amino acids (alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, lysine, proline, serine, threonine, tyrosine, α-amino adipic acid and β-alanine), acetyl carnitine and free carnitine. Thus from the 1H-NMR-based and the MS-based analyses a total of 2 + 26 = 28 metabolites were identified as important indicators of RCD, from which a final list of biomarkers was selected and validated according to the cross-validation procedure described in the statistical methods section.

The outcome of the cross-validation for the two experimental groups (51 or 34 cases, respectively) is shown in Table 3. The cut-off points for the metabolite groups were determined for each group separately; the differences relate to the numerical characteristics of the data sets for these variables. The sensitivity refers to the percentage of patients in the experimental group who were correctly classified as such by using the important metabolites identified from the organic acids (11), amino acids (13), carnitine (2) and creatine plus betaine, respectively. The specificity relates to the ability of the selected metabolites to identify the controls. The percentage misclassification includes the results obtained for the patients and controls taken together. The values of 100 obtained for the selectivity and specificity for the organic acids clearly relates to the selection of the control and patient groups on the basis of a complete separation of the total urinary organic acids excreted by the groups (criterion 3). From the misclassification outcome it is clear that the ranking of importance of the metabolite groups are organic acids ≈ amino acids > creatine and betaine > carnitines, with the respective percentage of misclassifications being 0%, 3.08%, 16.58%

and 26.64% respectively. A comparable ranking is obtained for the outcome of the sensitivity and specificity measures. The final conclusion from these cross-validations is that all 26 important metabolites from the three MS-based analysis should be included in a consolidated matrix with the two metabolites identified by 1H-NMR analysis. From this matrix a biosignature for the group of RCD patients could then be constructed.

Identification of a biosignature for the RCD patient group

First, the consolidated data set of 28 metabolites was formed, followed by data pre-treatment as described above. Subsequently, a PLS-DA model was constructed for this data set to identify the metabolites that could qualify for a biosignature for the group of RCD patients. Sixteen metabolites with a VIP > 1.0 were identified as possible components of a biosignature and are summarized in Table 4. Eight of the organic acids could be related directly to a consequence of RCDs and were included in the biosignature. Vanilmandelic acid (VMA) and homovanillic acid (HVA) were excluded from the biosignature because of their properties as indicators of neurological stress conditions (Frankenhaeuser et al 1985; Rauste-von Wright and Frankenhaeuser 1989), rather than being specifically related to RCDs.

The remaining 14 components of the biosignature can be related to RCDs in the following way. The RC is essentially involved in cellular reduction/oxidation (redox) status and energy (ATP) production; deficiencies in any component of this supramolecular complex inevitably affect a wide array of metabolic and other processes (Reinecke et al 2009; Elstner and Turnbull 2011). An important consequence of RCDs is a relative increase in levels of NADH ($NADH/NAD^+$ ratio) and $FADH_2$, as well as decreased ATP production, which may result from a defect at any site within the RC and result in the well-established elevations in lactic acid and the lactate/pyruvate ratio. Increased succinic acid, 3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 3-hydroxy-3-methylglutaric acid and 2-hydroxyglutaric acid were all described in a metabolomics investigation on global changes in organic acid metabolism (Reinecke et al 2011). Re-absorption of the amino acids as well as carnitine in the kidneys

may be affected by reduced energy production due to a defect of the electron transport chain, causing a Fanconi-Bickel excretion pattern of these metabolites (Odièvre et al 2002). However, some amino acids may also increase as a response to other primary and secondary abnormalities due to RCDs. These amino acids include alanine, due to an increase in pyruvate and its consequent transamination, as well as glutamic acid resulting from an increase in amino acid catabolism (indicating also the possible hyperammonemia in RCD disorders) and tyrosine due to underlying liver damage (Levine and Conn 1967). Among the amino acids, α-aminoadipic acid has not been described for RCDs before, and clearly reflects a deficiency in lysine catabolism due to high FADH concentrations. Elevated creatine in plasma was recently described for RCDs as a consequence of a low energy state of tissues using the phosphocreatine shuttle (Shaham et al 2011). Furthermore, several of the metabolites that can be attributed to increased catabolism of fatty acids and amino acids share a bioenergetics-sensing (hormone modulated) induction pathway with FGF-21, which is also associated with a muscle disease phenotype response (Soumalainen et al 2011).

From the metabolomics and statistical analyses, as well as the biochemical considerations discussed here, the proposed biosignature for our experimental group consisted of 6 organic acids, 6 amino acids and creatine, as shown in Table 4

Specifications for a biosignature

It has been proposed that the specification for a single metabolite (a biomarker) or a combination of metabolites (a biosignature) would be the consistent assignment of an individual to a unique group with defined characteristics (Jacobsen et al 2008). The evaluation of a biosignature thus requires the use of a data set to validate the capacity of a putative biosignature to correctly classify individual samples. The data set from which a biosignature was defined may also be used for the validation, but preferably an independent data set should be used for this purpose. For inherited metabolic disease, the latter can only be generated over a period of time or by the creation of a data set through information

gathered by participants from several medical centres, as was recently reported for FGF-21 as a potential biomarker for an RCD (Soumalainen et al 2011). In our investigation the original data sets had to be used to validate the RCD biosignature, as an independent data set was not available for this purpose.

The cross-validation procedure described in the statistical section was used for the validation of the biosignature, and the outcome is summarized in Table 3. This validation was conducted for the data set consisting of the 13 metabolites (Table 4) and the 20 patients and 14 controls used in the 1H-NMR analysis. The cut-off point for the cross-validation of the biosignature shown in Table 3 was determined for the consolidated set of variables. The cross-validation of the biosignature, in comparison with the individual metabolites, indicates the advantage of a biosignature as an indicator of an RCD compared with the use of a limited number of markers. In addition, the results presented here give proof-of-concept that metabolomics investigations should include inherited metabolic diseases in their field of investigation.

From metabolomics to the clinic

According to Mancuso et al (2009), the requirements for an ideal biomarker for a metabolic disorder such as RCD should improve the timing and accuracy of diagnosis, minimize the invasiveness for the final diagnosis and be useful to monitor disease progression and efficacy of treatment. They concluded, however, *–that to date, no one can bet on this, but we are all looking forward to find it"*. The requirements to develop such a biosignature to become a practical and useful instrument in clinical settings are clear, but complex (Turnbull, 2011). Another proposal regarded metabolomics as the new frontier in paediatric research (Carraro et al 2009) but, as has recently been emphasized, omics-based biomarkers and biosignatures can potentially be useful. However, the translation of research findings into clinical practice is not straightforward (Hu2011). Notwithstanding these reservations, the results presented in this paper clearly indicate that a metabolomics analysis of RCDs

disclosed a biosignature with distinct potential as a screening instrument for clinicians specializing in these diseases, and may eventually diminish the need for the invasive diagnostic biopsy procedure. Although this biosignature can be considered as only a putative instrument at this stage we already benefit from this new knowledge in dealing with children in our paediatric clinic as well as in our diagnostic laboratory.

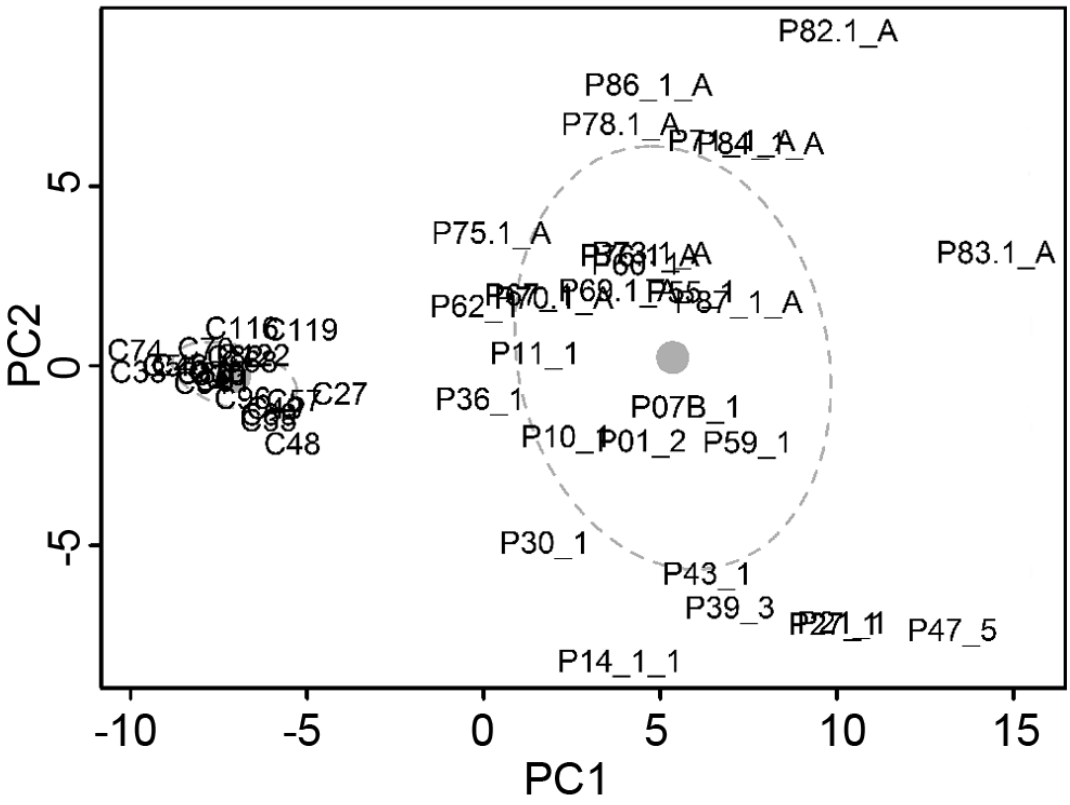

Figure 1 Two-dimensional principal component analysis of the controls (indicated by a C and the case number) and patients (indicated by a P and the case number). This analysis was based on all 291 variables present before data reduction. The circles were drawn to indicate a 50% probability level and the average of the group scores are indicated by the solid dots. Due to the density of the controls, the dot and most of the circle remained obscured in their case.

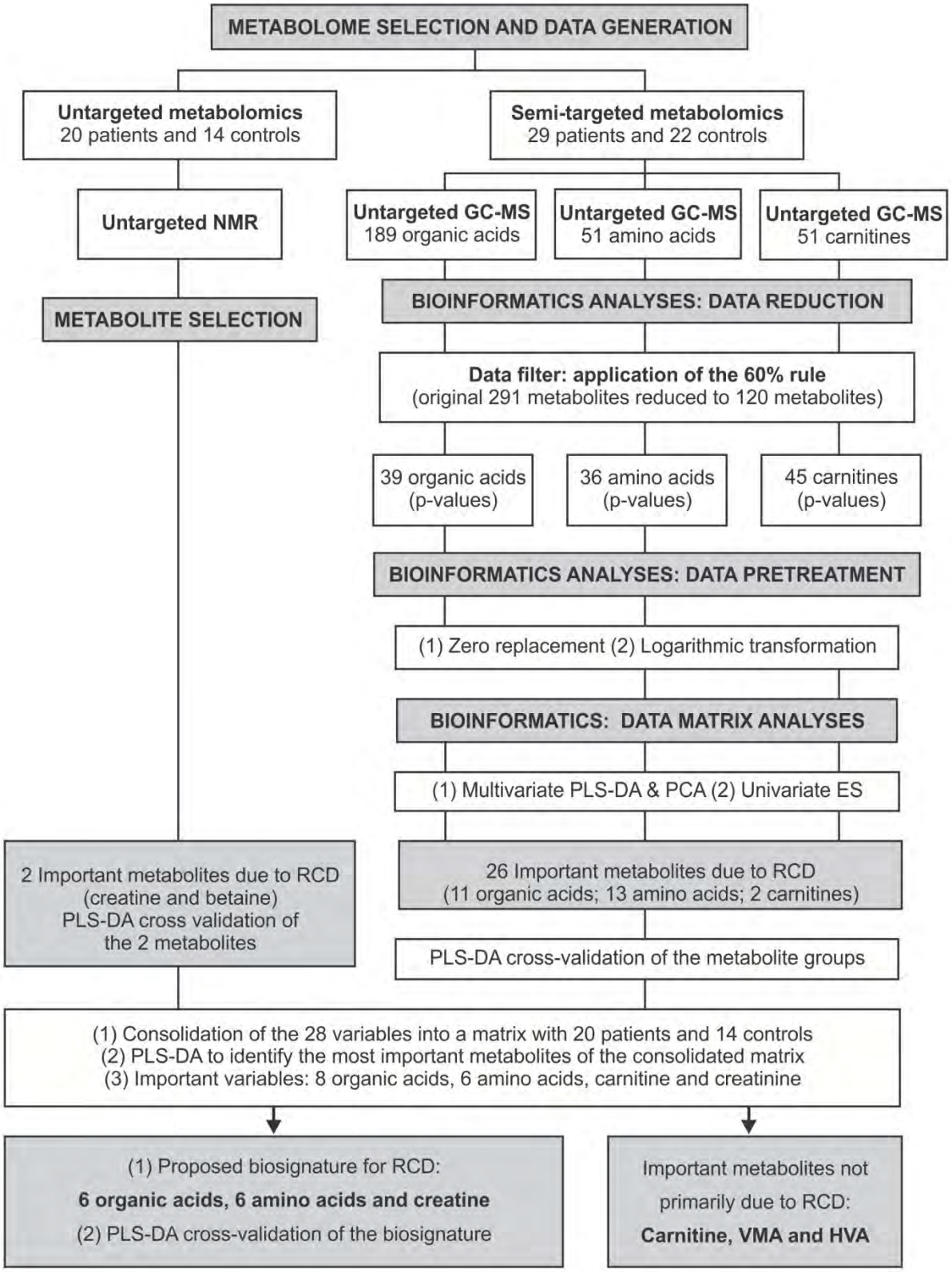

Figure 2 Metabolomics work-flow and cross validation of metabolite groups and thebiosignature

Table 1 Summary of the three inclusion criteria and selected metabolomics data of patients used in this study

Patients	Criterion 1	Criterion 1	Criterion 1	Criterion 1	Criterion 1	Criterion 2	Criterion 3	Data 1	Data 2	Data 3	Data 4	Data 5	Data 6	Data 7
Number	Clinical profile[1]	MDC score[2]	Lactate (mmol/l)	Pyruvate (mmol/l)	L/P	RC enzyme defect: Percentage of the lowest control value	Total OA (mmol/mol Cr)	Total AA (mmol/mol Cr)	Acetyl-Car (mmol/mol Cr)	Carnitine (mmol/mol Cr)	DC372/370	OC344/342	Creatine (mmol/mol Cr)	Betaine (mmol/mol Cr)
P10_1	M, CNS, DD, DYS	6	2.50	0.10	25.00	CI: 95	1159	1485	2.10	3.39	0.12	0.86	0.84	0.05
P27_1	M, CNS, Eye, ENT, DD, DYS	7	6.00	0.47	12.80	CI: 94	2695	4765	15.27	35.80	0.10	0.15	nd	nd
P30_1	CNS	6	3.53	0.11	32.18	CI: 76	619	1874	8.45	13.30	0.24	1.81	nd	nd
P47_5	M, Eye, R, DD, PNS	7	1.60	0.07	22.85	CI: 66	3049	9719	17.96	44.24	0.42	0.08	3.3	0.38
P73.1_A	M, CNS, Eye, L, DD, PNS	8	0.80	0.08	10.00	CI: 72	1514	901	25.48	22.88	0.41	2.83	0.46	0.04
P36_1	M, CNS, DD	3	1.60	0.13	12.31	CIII: 90	1397	1034	12.85	7.43	0.10	0.11	0.77	0.04
P59_1	M, CNS, Eye, L, DR	7	2.40	0.12	20.70	CIII: 71	1137	3284	14.71	35.02	0.10	0.08	0.66	0.19
P60_1	M, AID	4	1.07	0.13	8.20	CIII: 95	1223	1399	4.57	3.82	0.41	0.24	nd	nd
P84_1_A	M, CNS, Eye, G, R, DD	8	nd	nd	nd	CIII: 97	1297	1119	5.06	6.56	0.14	0.07	0.56	0.03
P01_2	M, Eye, PNS, DD	7	2.80	0.20	14.00	CI, CII+III: 0, 0	873	2877	4.12	2.29	0.41	0.67	nd	nd
P07B_1	M, Eye, DD	3	1.90	0.14	13.57	CII, CII+III: 80, 64	2041	2068	6.18	12.35	0.21	0.25	3.23	0.06
P11_1	M, CNS, Eye, R, DD	8	2.50	0.35	7.10	CI, CIII: 79, 78	845	1058	2.12	1.32	0.58	2.76	0.26	0.01
P14_1_1	M, CNS, Eye, DD, DR	6	1.10	0.10	11.00	CCI, CII+III: 77, 89	1985	2492	4.94	12.86	0.39	12.51	2.03	0.1
P21_1	M, Eye, PNS, End	8	2.80	0.32	8.80	CIII, CII+III: 98, 92	3218	4781	10.07	15.36	0.17	0.09	0.52	1.44
P39_3	M, R, DD	4	1.30	0.20	6.50	CI, CII, CIII, CII+III: 91, 93, 81, 83	1841	6544	29.42	74.93	0.40	0.13	nd	nd

Table 1 Continue...

Patients	Criterion 1	Criterion 1	Criterion 1	Criterion 1	Criterion 1	Criterion 2	Criterion 3	Data 1	Data 2	Data 3	Data 4	Data 5	Data 6	Data 7
Number	Clinical profile[1]	MDC score[2]	Lactate (mmol/l)	Pyruvate (mmol/l)	L/P	RC enzyme defect: Percentage of the lowest control value	Total OA (mmol/mol Cr)	Total AA (mmol/mol Cr)	Acetyl-Car (mmol/mol Cr)	Carnitine (mmol/mol Cr)	DC372/370	OC344/342	Creatine (mmol/mol Cr)	Betaine (mmol/mol Cr)
P43_1	M, Eye, DD	7	2.00	nd	nd	CII, CIII, CII+III, CIV: 98, 94, 70, 91	1208	3961	9.71	27.13	0.28	0.35	2.56	0.07
P55_1	M, End, ENT, G, DD	10	nd	nd	nd	CI, CIII: 86, 71	849	1784	5.33	7.55	0.25	0.13	2.24	0.13
P62_1	M, CNS, End, S, DD	8	3.30	0.17	19.41	CIII, CIV: 87, 80	864	904	1.80	0.54	0.07	0.19	0.92	0.04
P67_1	M, CNS, L, DD	6	1.01	0.08	12.60	CIII, CIV: 65, 92	941	850	3.94	7.83	0.29	0.55	0.88	0.12
P69.1_A	M, End, DD, DYS	5	1.10	0.10	11.00	CCII+III, CIV: 09, 97	779	1901	9.44	2.07	0.06	1.21	nd	nd
P70.1_A	BE, CNS & PNS	4	1.90	0.16	11.90	CCI, CIII: 84, 82	586	1862	6.44	12.06	0.08	0.17	0.85	0.06
P71_1_A	M, G, Car, DD	4	2.20	0.12	18.30	CIII, CIV: 99, 71	1077	1745	5.56	4.78	0.38	0.18	0.93	0.03
P75.1_A	CNS, Eye, S, DD, DR, BE	6	3.00	0.14	22.40	CI, CIII, CIV: 90, 97, 37	820	695	9.85	6.54	0.20	0.26	0.13	0.01
P76.1_A	M, CNS, Eye, ENT, S, DD	5	nd	nd	nd	CI, CIII: 37, 33	814	1622	6.47	1.82	0.31	0.19	nd	nd
P78.1_A	M, CNS, L, S, DD	5	1.30	0.08	16.25	CI, CIII, CIV: 28, 38, 54	746	1054	6.39	1.25	1.25	11.29	nd	nd
P82.1_A	M, CNS, Eye, G, ENT, DD	8	3.60	0.15	24.00	CI, CIII, CIV: 72,70, 65	2297	1739	31.15	8.41	0.14	0.33	nd	nd
P83.1_A	M, CNS, End, G, DD, DYS	6	2.30	0.14	16.40	CIII, CIV: 65, 93	1762	4222	3.29	5.94	10.22	0.67	0.14	0.12
P86_1_A	M, CNS, G, End, BE, DD, DR	8	3.70	0.20	18.50	CI, CIII, CII+III, CIV: 11, 65, 76, 39	1416	980	12.30	9.05	0.12	0.16	0.21	0.01
P87_1_A	M, CNS, Eye, Skin, DD, DR	8	1.90	0.17	11.20	CII+III: 86	580	3186	10.01	30.93	0.11	0.18	0.02	0.15

[1]Clinical profile includes: M, muscle involvement; CNS, central nervous system involvement; Eye, vision involvement; DD, developmental delay; DR, developmental regression; Dys, dysmorphism (minor and major); BE, behaviour and emotional abnormalities; ENT, sensori-neural deafness; PNS, peripheral neuropathy; G, gastro-intestinal tract involvement; R, renal involvement; Car, cardiac involvement; End, endocrine abnormalities; AID, auto-immune disorder; L, liver involvement; S, skeletal involvement. [2]MDC score: Mitochondrial Disease Score (Wolf and Smeitink, 2002). AA, amino acids; AcCar, acylcarnitines; CI-IV; complexes I-IV; CAR, carnitines; Crea, creatine; L/P, lactate:pyruvate ratio; nd, not done; OA, organic acids; RC, respiratory chain; SD, standard deviation.

Table 2 Summary of the urinary parameters for the respective controls (22/12) and patients (29/22)

Patients	OA	AA	AcCar	CAR	DC372/370	OC344/342	Creatine	Betaine
Minimum	579	694	1.80	0.5	0.06	0.07	0.02	0.01
Mean	1366	2479	9.83	24.2	0.62	1.33	1.08	0.15
Maximum	3217	9719	31.15	74.9	10.22	12.51	3.3	1.44
SD	724	2006	7.73	22.4	1.86	3.02	1.01	0.31
Controls								
Minimum	164	246	0.59	0.26	0.05	0.08	0.02	0.01
Mean	348	494	3.96	6.5	0.19	0.26	0.12	0.02
Maximum	565	882	20.54	10.6	0.41	0.72	0.5	0.03
SD	123	164	4.05	5.8	0.09	0.17	0.15	0.01
***p*-value**	**> 0.0001**	**> 0.0001**	**> 0.001**	**> 0.001**	0.232	0.070	**> 0.0001**	0.047

AA, amino acids; AcCar, acylcarnitines; CI-IV, complexes I-IV; CAR, carnitines; Crea, creatine; L/P, lactate:pyruvate ratio; nd, not done; OA, organic acids; RC, respiratory chain; SD, standard deviation

Table 3 Cross-validation of individual metabolite groups and of the biosignature

Number of cases (validation size)	Metabolite class (number)	Cut-off points	Sensitivity mean (SD)	Specificity mean (SD)	% Mis-Classification (SD)
Cross-validation of individual groups of metabolites					
51 (n_C = 7, n_P = 9)	Organic acids (11)	0.11	100 (0)	100 (0)	0 (0)
51 (n_C = 7, n_P = 9)	Amino acids (13)	0.17	96.19 (6.67)	97.83 (5.67)	3.09 (4.02)
51 (n_C = 7, n_P = 9)	Carnitines (2)	0.06	73.78 (14.19)	72.79 (16.30)	26.64 (9.25)
34 (n_C = 6, n_P = 8)	Creatine and betaine	-0.01	72.95 (16.11)	97.39 (6.26)	16.58 (9.01)
Cross-validation of the biosignature					
34 (n_C = 6, n_P = 8)	Organic acids (6), amino acids (6) and creatine	0.23	98.12 (4.88)	97.96 (6.40)	1.95 (3.67)

n_c, number of controls; n_p, number of patients

Table 4 The proposed biosignature

Metabolite	VIP	ES	C[Mean]	S.D.	P[Mean]	S.D.	P/C	t-value	p-value
lactic acid	1.15	2.2	3.2	2.7	65.1	95.2	20	+3.51	< 0.001
succinic acid	1.29	2.1	6.0	5.9	97.5	108.6	16	+4.53	< 0.001
2-OH-glutaric acid	1.26	2.6	1.5	1.3	15.3	13.1	10	+5.65	< 0.001
3-OH-isobutyric acid	1.39	2.3	2.8	2.9	26.8	17.5	10	+7.27	< 0.001
3-OH-valeric acid	1.35	2.5	3.7	2.8	42.1	51.1	11	+4.03	< 0.001
3-OH-3-me-glutaric acid	1.18	1.8	1.4	2.4	17.8	17.4	13	+5.02	< 0.001
alanine	1.05	1.9	22.9	11.3	196.9	213.4	9	+4.38	< 0.001
glycine	1.06	1.9	93.6	45.6	638.8	622.1	7	+4.70	< 0.001
glutamic acid	1.01	1.8	4.7	1.9	38.7	39	8	+4.69	< 0.001
serine	1.00	2	35.9	11	216	188.1	6	+5.14	< 0.001
tyrosine	1.04	2	14.5	5.6	64.8	45.6	4	+5.89	< 0.001
α-aminoadipic	1.04	1.7	2.5	1.5	34.6	42.7	14	+4.04	< 0.001
creatine	1.11	0.94	0.12	0.15	1.08	1.02	9	+4.11	< 0.001

P/C : P[Mean] / C[Mean] per metabolite; All p-values of the Mann-Whitney analyses for the metabolites of the biosignature were below 0.001 and are not included in the table.

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4.4 AWARENESS OF MITOCHONDRIAL DISORDERS IN SOUTH AFRICA

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MITOCHONDRIAL DISORDERS

DIAGNOSTIC APPROACHES AND THEIR APPLICATION IN THE SOUTH AFRICAN CONTEXT

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Primary mitochondrial disorders result from deficiencies of the oxidative phosphorylation system and other mitochondrial proteins or enzymes such as the pyruvate dehydrogenase complex (PDHc), tricarboxylic acid (TCA) cycle enzymes or mitochondrial carrier proteins. It has a minimum prevalence of 1 in 5000 and can present at any age with single or combinations of symptoms in any system. The diagnosis is extremely complicated and requires an integrated approach including clinical, biochemical, histological and molecular genetics data.

INTRODUCTION

The first patient with a mitochondrial disorder (MD) was described by Luft and colleagues in 1962.[1] Since then, and especially after the discovery in the late 1980's that mitochondrial DNA (mtDNA) mutations cause human disease,[2,3,4,5] the number of reported cases has escalated. MD's are now regarded as the group of inherited metabolic disorders that occurs most frequently with a minimum prevalence of 1 in 5000, which is higher than Duchene's muscular dystrophy with a prevalence of 1 in 6000.[6,7] Accurate data on the prevalence of MD's in South Africa are still lacking.

Mitochondrial dysfunction has until now been described as a deficiency of the mitochondrial oxidative phosphorylation (OXPHOS) system. However, a primary mitochondriopathy may also result from deficiencies of other mitochondrial proteins or enzymes such as the pyruvate dehydrogenase complex (PDHc), tricarboxylic acid (TCA) cycle enzymes or mitochondrial carrier proteins.

Since the first disorders were described, significant progress has been made to understand mitochondrial bioenergetics and pathology. On the other hand, it has also become clear that diagnosis of MD's ideally requires a demanding multidisciplinary approach that involves clinical, biochemical and molecular investigations.[8,9,10,11] These diagnostic approaches involve a merging of routine and rapid diagnostic analyses and applied research investigations that present practical dilemmas, especially when the spectrum of molecular genetic analyses is considered.

AETIOLOGY OF MITOCHONDRIAL DISORDERS

The OXPHOS system in the mitochondrion is the major source of cellular energy production in the form of adenosine triphosphate (ATP). The system consists of five multi-subunit enzyme complexes (complex I - V) located in the inner mitochondrial membrane. With the exception of complex II, of which all subunits are encoded by the nuclear genome, the other enzyme complexes are encoded by both the nuclear and mitochondrial genomes. The mitochondrial genome (mtDNA) is relatively small (only 16.6 kbp) and encodes 13 structural subunits of the OXPHOS enzymes, 22 transfer-RNAs and two ribosomal-RNAs used in mitochondrial transcription and translation.[12] MtDNA is maternally inherited and multiple copies are present in each mitochondrion, with every nucleated cell having numerous mitochondria. These copies may all have the same sequence (homoplasmy) or some may contain nucleotide changes proportionally (heteroplasmy) which may segregate differentially in dividing cells (or tissues) during embryogenesis to produce different levels of heteroplasmy.[13,14] The remainder of the estimated 1000-2000 proteins that constitute the mitochondrial proteome are nuclear encoded and include several import, folding and assembly factors of the OXPHOS system, mtDNA replication, transcription, translation and maintenance machinery.[15,16] Thus, the genetic defect of patients with MD's may originate from either the nuclear or mitochondrial genome and inheritance can be maternal, autosomal recessive, autosomal dominant or X-linked.

Since the first pathogenic mutations of mtDNA were described in 1988 more than 350 pathogenic mtDNA mutations have been reported in more than 30 mtDNA genes.[2,4,17] MtDNA point mutations or rearrangements account for more than 40% of adult MD's, but much less (~10 - 25%) in paediatric cases.[18,19,20,21] The occurrence may even be lower with Bernier *et al* reporting mtDNA involvement of any type in only 5% of children with MD's.[22]

Factors that may affect the pathophysiology of mtDNA mutations include the type of mutation, the affected gene, the level of heteroplasmy and its tissue distribution, the energy requirements of the tissue, environmental influences and the genetic background including the mitochondrial haplogroup.[10,23,24] The role of mtDNA and nuclear mutations in MD's has been reviewed extensively in the literature[10,11,13] and an updated overview of these mutations, as well as nuclear DNA mutations, can be viewed on the MITOMAP mitochondrial genome database.[17]

Nuclear DNA mutations are responsible for the majority of MD's especially in children where they account for up to 90% of mutations.[21,25] Spinazolla and Zeviani[26] suggested clinical-genetic classification in 2009 of MDS due to abnormalities of nuclear genes in the following scenarios:

1. mutations affecting OXPHOS subunits;
2. mutations affecting ancillary proteins;
3. impaired intergenomic communication that may affect the integrity, stability, amount and expression of mtDNA;
4. defects in biosynthetic enzymes for lipids and cofactors;
5. mutations affecting proteins implicated in mitochondrial biogenesis.[26]

It has also become apparent recently that mutations in the nuclear encoded mitochondrial DNA polymerase γ (POLG), which is responsible for mtDNA replication and proof-reading, may account for as much as 25% of all mutations that result in MD's.[27]

DIAGNOSTIC CRITERIA

There are no uniform guidelines for the diagnosis of MD's, or more accurately, laboratories worldwide have developed strategies and methods that differ. Especially problematic is the fact that methods for biochemical laboratory analyses are not always comparable and reference values are developed, defined and interpreted differently.

Nevertheless, the general diagnostic approach has common features that include essentially three levels of investigations if a mitochondrial disorder is suspected: clinical, biochemical/histochemical and molecular genetic investigations (*Figure 1*).

Clinical criteria

MD's can present at any age and are characterised by highly variable phenotypes. They usually present with multi-systemic involvement including skeletal muscle, central nervous system, and various degrees of visceral involvement.[28,29,30] MD's should always be considered in a single or multi-system disorder with a chronic, progressive or intermittent course.[8] Munnich and Rustin (2001) suggested in 2001 that the diagnosis of an MD is simpler if two unrelated symptoms are present.[18]

A number of well established phenotypes are classified as classical MD's and include such as Leber's hereditary optic neuropathy (LHON), mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres MERRF, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), neuropathy, ataxia and retinitis pigmentosa (NARP), chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre, Pearson or Leigh syndromes, and the phenotypes are well described.[14]

The problem arises when the patient does not fulfil the criteria for one of these syndromes, and in children this is the case in the majority of patients. The mitochondrial disease criteria (MDC) are a useful guideline.[31] Symptoms associated with MD's are summarised in *Table 1* and various eloquent reviews discussing the different aspects are available.[29,32,33,34]

According to Munnich *et al*, 44% of children with MD's present with neuromuscular signs.[35] Although MRI findings for many MD's are non-specific, Bianchi *et al* classified the changes in three main categories:

Type I is the non-specific findings including mild to moderate cerebral and cerebellar atrophy and high signal intensities in sub-cortical or periventricular white matter. The predominant myopathic and non-syndromic encephalomyopathic phenotypes are included in this group.

Type II includes the syndromic phenotypes and they may have more specific MRI changes, including cortical and subcortical grey matter involvement with the basal ganglia, cerebellar dentate, brainstem grey matter and colliculi more often affected than the thalami. KSS is included in this group.

Type III involves primarily the white matter. Small cysts may appear in the white matter and signal changes in the brainstem and cerebellar white matter. The basal ganglia and nuclei in the brain-stem are seldom involved.[36]

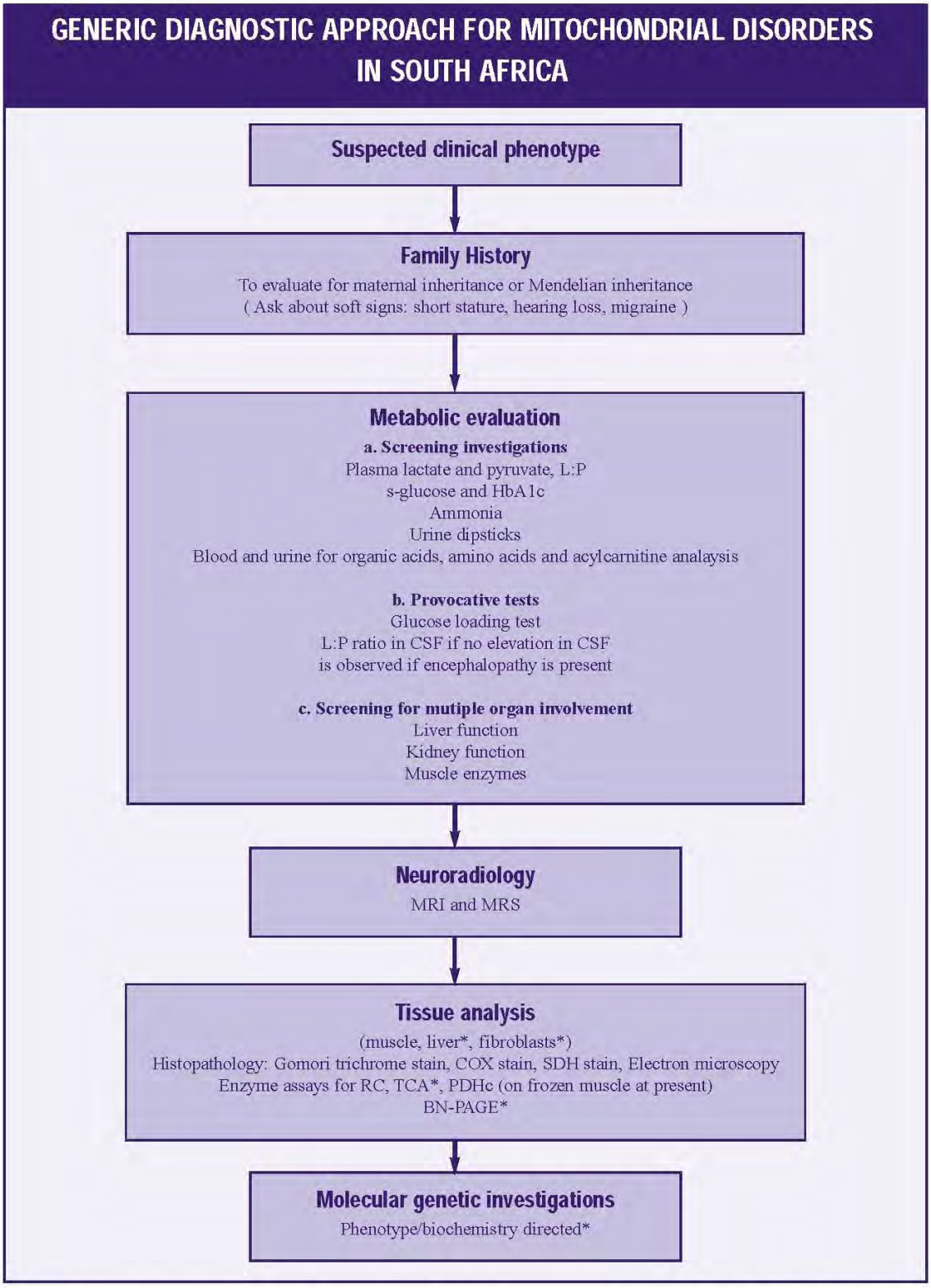

Figure 1: Analyses marked with an asterisk are specialised analyses not performed on a routine basis, or performed at specific institutions

SUMMARY OF CLINICAL FINDINGS IN MITOCHONDRIAL DISORDERS

NEUROMUSCULAR INVOLVEMENT

Central and peripheral nervous system	**Muscle**	**Eye**	**General**	**Skin**
Epilepsy	Weakness	Visual impairment	Exacerbation of symptoms	Hypertrichosis
Myoclonic epilepsy	Hypotonia ± hypertonia	Ptosis	with minor illness	Pili torti
Developmental delay	Myalgia	External ophthalmoplegia	Failure to thrive	Subcutaneous lipomas
Developmental regression	Exercise intolerance	Strabismus		
Ataxia	Rhabdomyolysis	Retinitis pigmentosa		
Dystonia	Myopathy	Cataracts		
Tremor and chorea		Optic atrophy		
Peripheral neuropathy				
Stroke-like episodes				
Migraine				
Brainstem involvement				
Apnoea				
Hypertension				
Strabismus				
Autonomic disturbances				
Swallow difficulties				
Sensori-neural deafness				

VISCERAL INVOLVEMENT

Gastro intestinal tract	**Heart**	**Kidney**	**Endocrine system**	**Blood**
Hepatomegaly	Arrhythmia	Proximal tubular	Delayed puberty	Anaemia
Liver failure	Cardiomyopathy	dysfunction	Short stature	Thrombocytopaenia
Steatosis	Wolf Parkinson White	Focal-segmental sclerosis	Diabetes mellitus	Neutropaenia
Dysmotility			Diabetes insipidus	
			Exocrine pancreatic dysfunction	
			Hypoparathyroidism	

Table 1

Histopathology and histochemistry

The role of histopathology and histochemistry in the diagnosis of MD's differs among diagnostic centres and depends on the logistics and expertise available.

Histopathological or histochemical investigations using several types of stains can be very informative as changes in the central and peripheral nervous system as well as other affected tissues can be severe.[37,38] From a practical point of view, when OXHOS disorders are clinically suspected, a muscle biopsy is usually the most informative tool, even if muscle symptoms are not apparent. For muscle histopathology and in particular the detection of subsarcolemmal collection of mitochondria (ragged-red fibres), Gomori trichrome staining of frozen and sectioned muscle samples can be done. More informative are histochemical stains for succinate dehydrogenase (SDH, complex II) and cytochrome c oxidase (COX, complex IV). Staining of SDH, which is exclusively nuclear encoded and very seldom deficient, can demonstrate mitochondrial distribution and their subsarcolemmal proliferation. Staining of COX, which contains subunits encoded by both mitochondrial and nuclear genomes, can distinguish between type I (oxidative) and type II (glycolytic) fibres in normal tissues. Mosaic patterns after COX staining are highly suggestive of a heteroplasmic mtDNA disorder and most ragged-red fibres are COX negative.[39] A uniform lack of COX staining can indicate a nuclear mutation of structural subunits or assembly or import factors of COX. With no stains currently available for the complex I, III and V, histochemistry has obvious limitations. Furthermore, false negative results from histochemistry and histophathology on muscle tissues are common and even electron microscopy add little to the diagnostic yield.[37]

Analysis of organic acids in urine and acylcarnitines in urine and plasma is informative; not only to identify possible associated metabolites, but also to exclude other related inherited metabolic defects.

Biochemical metabolic investigations

On a cellular level, a deficiency of OXPHOS results in several consequences that can be measured in biological fluids or specific tissues. One important consequence is the increased redox state, reflected by the NADH/NAD^+ ratio, which have a regulatory effect on several enzymes, including PDHc and lactate dehydrogenase (LDH).

The resulting effect, which is most often key metabolites used to indicate possible MD's is the increase in lactate, (>2.1 mM in blood) or an increase in lactate/pyruvate ratio (>20 in blood) and alanine (>450 μM in plasma).[11,31] The measurement of these and other metabolites needs consideration of the type of body fluid, collection, sample handling and analytical method, appropriate reference values (e.g. age-dependent) and careful interpretation as none of these metabolites or ratios are specific markers for MD's.[11] Blood lactate may be normal in patients even after glucose loading. One centre reports that ~15% of all patients with a proven complex I deficiency do not have increased blood lactate.[40] On the other hand, increased lactate in blood and cerebrospinal fluid (CSF) may occur in other conditions or because of poor collection or handling techniques.[34] Levels of alanine in plasma are increased in most patients with OXPHOS disorders[31,34] and isolated cases of increased plasma glycine, proline, and sarcosine have been reported.[42] Generalised aminoaciduria can also be indicative of MD's, especially when associated with Fanconi syndrome.[43]

Analysis of organic acids in urine and acylcarnitines in urine and plasma is informative; not only to identify possible associated metabolites, but also to exclude other related inherited metabolic defects. Commonly found organic acids in MD's are elevated TCA cycle intermediates as well as ethylmalonic acid and 3-methyl glutaconic acid.[44,45] Quantification of total and free carnitine, as well as acylcarnitine analysis in plasma can identify secondary fatty acid oxidation defects and carnitine deficiency that can be associated with primary OXPHOS disorders.[11]

Biochemical analyses on tissue

The mainstay of diagnosing MD's is biochemical analyses on tissues using enzyme and functional assays.[46] Ideally, biochemical analyses should be performed in affected tissue. For primary diagnosis, most centres use muscle tissue which is most commonly affected and fibroblasts. The type of assays performed depends on the availability of fresh or frozen tissues. Fresh tissues allow functional analysis on intact mitochondria, which includes ATP production, polarography and substrate oxidation assays in addition to enzyme assays.

Substrate oxidations using radiochemically labelled compounds in combination with ATP + creatine phosphate production, are particularly useful as it eloquently tests the PDHc/TCA cycle/OXPHOS system in an environment closer to *in vivo* conditions.[47,48] This approach seems particularly sensitive with Janssen *et al.* reporting that ~25% of patients show reduced oxidation rates without defects of OXPHOS or PDHc enzymes activities detected.[46] This approach requires adequate material, experience and a competent setup. Alternatively, polarography, which measures oxygen consumption, is also often used. In our experience, as well as in that of others[11] however, polarography did not show the same sensitivity and correlated poorly in cases where enzyme deficiencies were detected (unpublished data). When frozen tissues are available, only enzyme assays (excluding complex V) can be done spectrophotometrically. With all the variation in diagnostic approaches, enzyme assays remain the golden standard for diagnosis of MD's. The good stability of respiratory chain enzymes allows the use of frozen samples for biochemical analysis where logistical constraints exist.

Notwithstanding the diagnostic value of biochemical assays, many aspects of biochemical analyses are problematic. These have been documented extensively before.[11,18,49,50] The most crucial of these are the variations in tissue specificity and assay protocols, lack of universal standards/quality control and the variations in the developing and defining of reference ranges.

Thorburn *et al.* highlight the major problem of distinguishing between a primary and a secondary enzyme deficiency, which is compounded by the way enzyme activities are defined.[9] The issue at hand is which markers to use to normalise enzyme activities with. In spite of efforts to solve many of them through standardization, several aspects will probably remain problematic for some time due to the biological variability of these disorders.

Molecular genetic and complementary investigations

Due to the complex genotype-phenotype variation in MD's, the approach to molecular genetic investigations is complicated. The number of disease-causing mutations is escalating but not surprising considering the great number of genes that are involved. Moreover, conditions where secondary OXPHOS deficiencies occur necessitate the availability of molecular genetic analysis to differentiate it from primary MD's.

Centres that perform systematic molecular investigations use clinical, biochemical and histopathological data to direct the course for molecular investigations.[8,9,11,39,51] Several approaches are used to limit the number of candidate genes to sequence.

Cell lines that express deficiencies can be manipulated by transfection/infection with candidate genes or by creation of cybrids to investigate mtDNA/nuclear gene involvement.[52] Other, less intensive, protein-based techniques such as Blue Native polyacrylamide gel electrophoresis (BN-PAGE) immunoblotting and determination of mtDNA copy number and integrity, could further limit the number of candidate genes. Even though the number of candidate genes can be limited for each specific patient, molecular genetic analysis on a routine/frequent basis demands the capacity to analyse a great number of genes.

The availability of comprehensive genetic diagnostic facilities is crucial for genetic counselling and prenatal diagnosis. However, Smeitink repeats Di Mauro's question: "should we scrape the bottom of the barrel with every patient", referring mainly to the complex, labour intensive and expensive molecular analysis of MD's which is currently only performed by a few experienced centres in developed countries.[8,53] It is especially on this aspect that the (multi-disciplinary) interplay between diagnostics and research is clearly evident. In cases where capacities are limited, however, it is crucial that the resources, expertise and communication between local medical and scientific entities are maximised and that networks with experienced centres are developed.

TREATMENT

An extensive Cochrane review of six randomised trials on treatments of MD's concluded that there is currently no established treatment for MD's.[54] However, therapeutic approaches for MD's includes oral treatments with supplements such as co-enzyme Q10 (ubiquinone) or other quinone derivatives, vitamins and metabolic supplements as well as other pharmacologic agents that manages treatable symptoms of the disorder. In adult patients, exercise therapy has also been tested.[55,56] All of these treatments demonstrated only varied improvement in isolated cases. Treatment of patients with MD's is problematic, but numerous creative novel approaches including alternative gene expression, enzyme replacement and modulation of cell signalling are currently being investigated.[57]

CURRENT AND FUTURE DIAGNOSTIC APPROACH IN SOUTH AFRICA

In recent years a basic, but structured strategy for research and diagnosis of MD's in mainly paediatric patients has been developed between two academic institutions in South Africa, University of Pretoria and North-West University, as well as *ad hoc* collaboration with other centres. This approach in its current form is illustrated in *Figure 1*, but includes limited molecular genetic evaluation. Diagnoses of possible disorders relied mainly on RC and PDHc enzyme analyses in muscle tissue.

The approach for the investigations on muscle has changed from using fresh to frozen tissue for practical and logistical reasons. Muscle tissue from age matched controls was obtained from patients, without possible MD's, who underwent routine orthopaedic surgery to develop reference values for enzyme assays. The diagnostic yield improved from an initial 21% to 72% over a period of 10 years. Histology did not contribute to finalise a diagnosis of MD's in any patients additionally to the enzyme assays.

The limitations of this strategy have been the same as those expressed by other centres, such as how to distinguish between primary and secondary mitochondrial deficiencies in the absence of a complete molecular genetic screening of all genes that may possibly be associated with MD's. An in-depth diagnostic approach requires a multi-disciplinary, but highly specialised, approach that has a diagnostic as well as a research character. This places a huge burden on the capacity of any single centre at any South African academic/medical diagnostic facilities.

Recent advances in the understanding of mitochondrial function and mitochondria-nuclear communication, in addition to technological developments to investigate these processes, may provide novel approaches to identify primary mitochondrial involvement in diseases.

The inclusion of additional analyses, such as BN-PAGE and proteomics, on tissue material has already proven to be of significant value in order to identify structural and assembly processes of mitochondrial proteins and aberrations of those processes in MD's.[58] Gene expression profiles in cell lines with various OXPHOS deficiencies using DNA micro-arrays have been reported in recent years.[59,60,61] These studies have shown that a systems biology approach to investigate MD's may become an important additional tool to unravel the complexities of this group of disorders.[41] Thus, to include nucleic acid, protein or metabolite profiles is a promising possibility for future diagnostic strategies.

CONCLUSION

MD's can present at any age with any combination of systems involved, and require a holistic approach to patients, something that might be compromised by sub-specialising. A high index of suspicion and thorough clinical assessment remains the cornerstone in the complicated diagnostic process to select the appropriate further special investigations for confirmation.[34] Moreover, considering current priorities on health care, it appears that the most viable strategy is to extend the network of inter-institutional collaboration, if a more extended diagnostic approach is sought.

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MITOCHONDRIAL DISORDERS

Which of the following statements is/are true?

1. **Diagnostic features for mitochondrial disorders differ from centre to centre making diagnosis difficult.**
2. **The OXPHOS system in the mitochondrion is the major source of cellular energy production in the form of adenosine triphosphate (ATP).**
3. **MD's can present at any age and are characterised by a limited number of phenotypes.**
4. **There is currently no established treatment for MD's.**
5. **Nuclear DNA mutations are responsible for the majority of MD's especially in children where they account for up to 90% of mutations.**

CHAPTER 5

DISCUSSION AND CONCLUSIONS

The mitochondrial investigation described in this thesis was part of a long-term a multi-disciplinary project, which has been undertaken since 1998. It resulted directly from the absence of a comprehensive diagnostic facility for mitochondrial disorders (MDs) in South Africa. As described in previous sections, the diagnosis of MDs is challenging under the best of circumstances. Consequently the lack of a comprehensive service and complexity of the diagnostic procedure posed an opportunity to obtain novel data and to develop imaginative strategies in a developing country such as South Africa with other major health-related issues.

The actual research presented in this thesis was initiated in 2008 and important contributions resulted from this investigation. The South African patient population with MDs was characterized and the findings were documented for the first time. It was found that the population had unique characteristics. The African patients predominantly presented with muscular involvement (62.5%), 30.0% presented with encephalomyopathy and only 7.5% had primary central nervous system (CNS) involvement. In addition, involvement of a CII+III deficiency was evident in this group of patients, which was found to be statistically significant. On the contrary, Caucasian patients predominantly presented with encephalomyopathy (60.0%), 20.0% had pure CNS involvement, and the older patients had exercise intolerance and myalgia as their primary symptoms. The Caucasian group tended to have more single enzyme deficiencies (60.0%) and the African patients had combined deficiencies in 67.5% of cases (Smuts et al 2010). Equivalent biochemical differences between the population groups, as observed in the clinical study, were not observed in the metabolomics studies, but the statistical processes in variable and case selection aimed to

achieve complete separation between controls and patients. This resulted in a more homogenous patient group, but only a limited number of Caucacian patients was finally included in the two different metabolomics studies, 11/39 (28.2%) and 5/20 (25.0%) respectively. Except for one Caucasian patient with a pure encephalopathy, all the others had muscle involvement as well. The clinical differences observed between African and Caucasian patients therefore remain to be investigated on a biochemical level in a follow up study.

The clinical study helped to raise awareness about MDs among clinicians and a network for the referral of patients was developed (Smuts and van der Westhuizen 2010).

During the study, interesting case presentations were identified, including a young adult male patient who presented with Kearns–Sayre Syndrome (KSS). In order to identify primary MDs, it was realised that additional investigations, besides those which are routinely performed, should be undertaken, including blue native polyacrylamide gel electrophoresis (BN-PAGE) and molecular genetics studies. A novel deletion of 3,431 bp was found in 80% of muscle mitochondrial DNA (mtDNA) between positions 7,115 and 10,545 flanked by a five bp direct repeat sequence. In addition to describing the first African case with mtDNA deletion in KSS, this clearly demonstrated that the absence of the mtDNA-encoded *ATPase6* and *ATPase8* genes resulted in the aberrant synthesis of adenosine triphosphate (ATP) (van der Westhuizen et al 2010).

A metabolomics approach was then adopted. It was possible to compile the first comprehensive list of 24 metabolites associated with RCDs, which were, both statistically and practically, significantly elevated. It was obtained through the analysis of the organic acid-containing section of the metabolome by untargeted GC-MS analysis of urine obtained from patients with respiratory chain disorders (RCDs) (Reinecke et al 2011). A total set of 301 components was identified initially, which consisted of 255 endogenous substances. These metabolites were categorized further into seven subclasses, according to their

role/position in the metabolome, and a global metabolic profile was constructed. The global metabolic profile that was constructed from these results indicated, firstly, the involvement of increased carbohydrate, amino acid and fatty acid catabolism. It was concluded that the RCD-induced, decreased ATP production and consequent reduced ATP/ADP ratio may lead to the elevation of neurotransmitters, e.g. dopamine and other hormones like vasopressin, glucagon and adrenaline responsible for the increased catabolism of glycogen, protein, triacylglycerols, purines and pyrimidines. This observation was consistent with the induction pathway of fibroblast growth factor 21 (FGF-21), which was recently identified as a possible biomarker in plasma of RCD patients with a predominantly myopathic phenotype (Suomalainen et al 2011). The global metabolic profile, secondly, also revealed elevation of several metabolites that can be associated with a change in nicotinamide redox state and resulting modulation of several oxidoreductases. The Krebs cycle is consequently influenced and secondary metabolites arise from impaired fatty acid and amino acid metabolism. This profile was discussed in detail in Section 3.3.

This initial metabolomic study clearly illuminated the diversity and complexity of the complex biochemical consequences in RCDs. Moreover, it also indicated that there was no single characteristic organic acid biomarker to distinguish between the CI, CIII and CM RCDs (Reinecke et al 2011).

Twenty percent of the 24 metabolites originated from amino acid metabolism. Although the initial study was designed to focus on the organic acids, it was realised that the amino acids should be included in the metabolomic study. Amino acid and carnitine analyses were thus included in the urinary metabolic profile of the patient cohort to further assist in the development of a possible biosignature. As described in detail in Chapter four, an explorative biosignature comprising of six organic acids, six amino acids and creatine. It included succinic acid, lactic acid, 3-OH-isobutyric acid, 3-OH-valeric acid, 3-OH-3-Me-glutaric acid, 2-OH-glutaric acid, α-aminoadipic acid, glutamic acid, alanine, glycine, serine,

tyrosine, and creatine. Extensive cross validation was done. From the misclassification outcome it is clear that the ranking of importance of the metabolite groups are organic acids ≈ amino acids > creatine and betaine > carnitines, with the respective percentage of misclassifications being 0%, 3.08%, 16.58% and 26.64% respectively.

The limitations of the study should be acknowledged. The prevalence of MDs in South Africa is still unknown. The calculated prevalence for this group, with the available data, was 1 per 100,000 children, which was about 20 times less than the reported prevalence of 1 per 5,000 (Schaefer et al 2004). The low prevalence could be explained by a number of reasons. Although the diagnostic criteria proposed by the Nijmegen group of infants and children (the Mitochondrial Disease Criteria, MDC) by Wolf and Smeitink (2002), as was used in this study, was found to be a valuable diagnostic aid, it still remained complicated to apply these criteria directly to the South African population, as one-third of the diagnostic criteria was based on muscle biopsy findings. Patients were underscored with the MDC, as we did not always have complete sets of data available and muscle biopsies were only performed if the MDC was six or more, or if a very distinct and characteristic MD phenotype was present. Another reason for biopsies not being performed was logistic issues, e.g. theatre lists that were cancelled on short notice, resulting in patients‘ details being lost, which prevented follow-up studies. The study was performed in a clinic mainly handling paediatric patients and adults were definitely missed. There was also a lack of knowledge about MDs and patients were not referred to the clinic for further management. Unfortunately, not all interesting cases can be offered the comprehensive work-up that includes genetic analyses. This needs to be addressed in future.

A comprehensive validation of these results, based on a large cohort of cases, would be a key for future implementation of such an explorative metabolic biosignature. Moreover, the biosignature has the potential to be a valuable diagnostic tool to better select patients before a confirmatory biopsy is performed. It is foreseen that a biopsy will only be performed after determination of the biosignature, in order to increase the yield of positive biopsies and to

limit the number of inconclusive results. As mentioned, it is also important that the genetic testing is explored further to identify primary MDs, and that the contribution of the mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in the genetic diversity of the South African population is investigated. This would be essential in the development of a genetic counselling service. The recent advances in next-generation DNA sequencing technology and exome sequencing provide exciting new prospects to address these issues.

Considering the current expertise and technical- and financial capacities that exist in South Africa (excluding exome sequencing or similar explorative molecular approaches or specific research approaches), Figure 5.1 illustrates a possible diagnostic approach that can be followed in the near future.

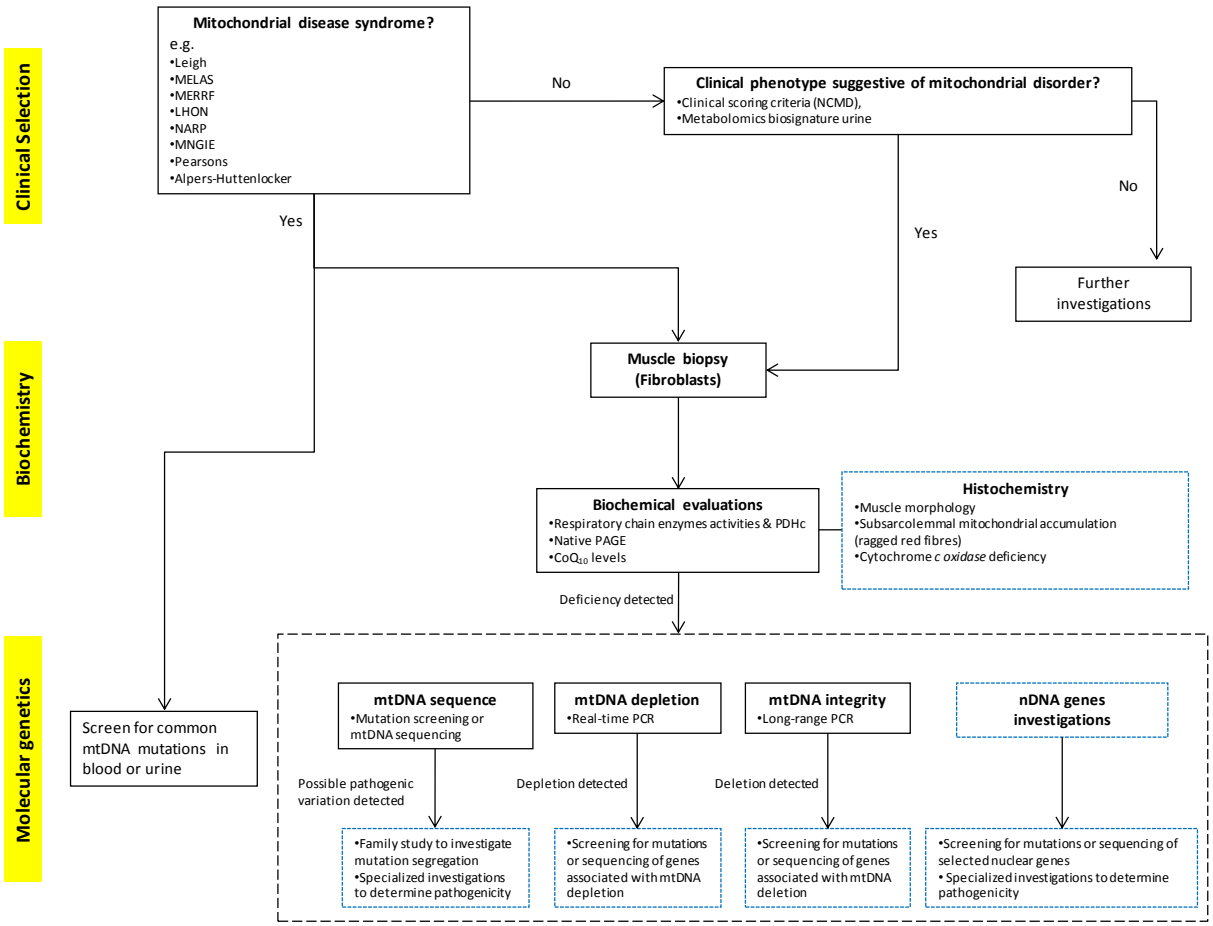

Figure 5.1. Broad strategy for the diagnosis of mitochondrial disorders that can be applied in the South African context.

Solid line squares represent the part of the strategy that is already in place and which represents the short term objective (mtDNA investigation on an expanded cohort). The dashed line squares represent the additions to the strategy than need to be developed. Adapted from McFarland et al 2010.

In conclusion, this study was a long-term project that has, from the perspective of a relevant and urgent health problem statement, resulted in new scientific knowledge on RCDs in South Africa and on RCDs in general. This new scientific knowledge includes both clinical and biochemical descriptive data, using current as well as novel diagnostic approaches and investigations. From a clinical point of view, the study has described the phenotypic differences that presented in an African population group. It has, furthermore, applied new advances in the field of natural sciences (i.e. in metabolomics) to identify an explorative metabolic biosignature in the urine of patients with RCDs, which have the potential to be of specific value to the diagnostic process of these patients in the future.

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CHAPTER 6

SUPPLEMENTARY MATERIAL

This section includes supplementary information including copies of:

6.1 APPENDIX A: CONSENT FORM

6.2 APPENDIX B: ASSENT FORM

6.3 APPENDIX C: MITOCHONDRIAL DISEASE CRITERIA SHEET

6.4 APPENDIX D: LIST OF SCIENTIFIC CONTRIBUTIONS OF THE MITOCHONDRIAL PROJECT IN SOUTH AFRICA

6.5 APPENDIX E: COPYRIGHT LICENCES

6.6 APPENDIX F: INSTRUCTIONS TO AUTHORS

6.6 APPENDIX G: PERMISSION OF CO-AUTHORS

6.1 APPENDIX A: CONSENT FORM

THE INVESTIGATION OF THE BIOCHEMICAL PROFILES OF PATIENTS WITH POSSIBLE MITOCHONDRIAL DISORDERS

AIM OF THE STUDY

Mitochondrial disorders are a group of disorders that may affect the brain, muscle and several other organs, because a defect exists in the energy production of the cell. It is caused by a genetic defect. The aim of the project is to measure respiratory function in your child‘s muscle in conjunction with the enzyme activity of the energy producing system as your child is identified as having a possible mitochondrial disorder. The urine will also be examined for chemical compounds that may be excreted in the urine if such a disorder is present. The long-term goal of this project is to establish a diagnostic service in which mitochondrial disorders could be diagnosed properly in South Africa.

EXPECTED DURATION OF THE PROJECT

This is a long term project.

PROCEDURES FOLLOWED DURING THIS PROJECT

The study will be explained to you and you will be allowed time to ask questions and decide whether you would prefer to give consent for your child to take part.

A family history will be taken and a complete physical examination will be performed.

In the case of a patient with a suspected disorder appropriate special investigations, including urine will be performed to confirm the diagnosis of a mitochondrial disorder, to determine involvement of other organ systems and to monitor the course of the disease.

Not more than 15 ml of blood will be taken, depending on the age of the child. This blood will be used for appropriate biochemical and haematological investigations and a small volume will be stored away to do genetic studies when an appropriate test comes available. If it would be applicable in future, you will be contacted again for a follow up blood sample to establish cell lines to ensure a continuous supply of DNA for further analyses.

In some instances where the nervous system symptoms are unexplained it will be necessary to do a lumbar puncture. It implies that fluid surrounding the brain will be obtained through a thin needle in the back. A maximum of 5 ml cerebrospinal fluid will be needed.

Nerve conduction studies and electromyography in which the electrical functioning of the nerves and the muscle will only be done if it has not been done before and it seems to be affected.

A muscle biopsy will be done in some cases if the blood tests were inconclusive. The muscle biopsy will be taken under general anaesthetics by a qualified paediatric surgeon. A 3-4 cm incision will be made on the thigh and a small piece of muscle about 1 cubic cm will be removed for the biochemical analyses and an additional smaller piece of muscle will be taken for histology. The wound will be sutured. The muscle biopsy taken for biochemical analyses will be processed and the excess solution will be frozen for possible future use if better biochemical or genetic tests come available.

A specialised type of X-Ray, e.g. computerised tomography (CT) scan or magnetic resonance imaging (MRI) of the brain will be done to obtain a photographic picture only if it has not been done before and your doctor is of opinion it might be helpful to clarify some of the symptoms and signs.

A formal hearing and/or vision test will be performed if deafness or visual impairment is suspected.

RISKS AND POSSIBLE DISCOMFORT FOR THE PATIENT / INDIVIDUAL

Blood sampling is causing mild pain, but the risks are minimal.

A lumbar puncture will cause moderate pain and discoloration at the puncture site. The patient may suffer from a headache afterwards.

The EMG and nerve conduction study may cause pain, but the greatest care will be taken to assure the comfort of the patient as far as possible.

The muscle biopsy has the following risk factors: possible bleeding, discomfort including pain, possible scarring, and a remote risk of infection.

DECLARATION OF CONFIDENTIALITY

All the Information provided for the purpose of this study will be treated as highly confidential. Only individuals of the research group and officials, who are entitled to it by law, will have access to information. Data published in a medical journal will include no information that could identify a patient or his/her family.

WITHDRAWAL CLAUSE

I understand that I am entitled to withdraw from the project at any time without penalty or loss of benefits. The participation of my child, in this project is therefore on a voluntary basis until I request withdrawal in writing.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING ADDITIONAL INFORMATION POINTED OUT TO ME

- That the entire urine, blood and muscle biopsy sample is used for research purposes and no compensation for this will be provided.

- That I will be kept informed of significant developments in the project.
- That the withdrawal clause was explained to me and that I understand its implications.
- That I will receive a copy of this informed consent form.

ENQUIRIES ABOUT THIS PROJECT SHOULD BE DIRECTED TO:

- General enquiries should be directed to: Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria Private Bag X323, Arcadia, 0007
- Enquiries regarding ethical aspects of the project can be addressed to: The Chairperson of the Ethics Committee, Steve Biko Academic Hospital.

DECLARATION OF CONSENT

I, _____

(Print full name and surname)

Hereby consent to the participation of my child,

_____ in the above-mentioned project.

(Print full name and surname or stick a sticker)

I confirm that I am fully aware of the content of this form and that by signing it I give the necessary consent.

Signed at: _____ on _____

(Place) (Date)

_____

(Signature of a parent/guardian)

Witness 1: _____W**itness 2:** _____

(Signature) (Signature)

_____

(Signature of responsible who explained the project and assent to the participant)

6.2 APPENDIX B: ASSENT FORM

THE INVESTIGATION OF BIOCHEMICAL PROFILES OF PATIENTS WITH POSSIBLE MITOCHONDRIAL DISORDERS

We are going to explain the study to you. If you understand and want to take part, we ask you to sign the form.

WHAT DO WE WANT TO DO IN THE SUDY?

Mitochondrial disorders are diseases that may cause a problem in many parts of the body, because too little energy is made. You were born with the problem. The aim of the project is to measure if your body makes enough energy. We hope to be able to have a centre one day in South Africa where patients with these problems could be helped.

HOW LONG WILL THE STUDY BE?

This project is a long-term project.

WHAT ARE WE GOING TO DO?

We will explain to you what we are going to do and give you time to ask your questions.

We are going to ask your parents about other people in your family and whether they are ill. We will examine your body.

If we think that you have a this possible problem, we will do some test to see if there are other parts of your body that is also ill and to see if the problem gets worse. It can be sore if we do some of the test, but then we will give you medicine. It will help you then to sleep and lie very still. It will also help to take away the pain.

Less than 15 ml blood will be taken. It may be necessary to take some of the fluid from your back. Afterwards you may have a sore head, and then you will have to lie in bed for a while. If your muscle or nerves are weak we are going to test it with a machine. It is sore for a short while. If we really do not get an answer from those tests it may be necessary to do a small operation to get a small piece of muscle. The little operation may cause bleeding, pain and leave a small mark on your e.g. Your urine will also sent to be tested in a big machine, but that is not sore.

Sometimes we need a picture of your brain. That is not painful at all; you just have to lie very quiet in the machine that we call a scanner. It is like taking photos of your brain.

If you cannot see or hear well, we may also test your eyes and or ears. It is not sore.

WILL ANYBODY KNOW ABOUT YOU?

Only the doctors that know you and the people in the laboratory that work with your blood, urine or muscle see the answers and your hospital number and name. If we find some answers we would like to write about it in a medical journal, but nobody that will read the article will be able to know that you took part, only the answers will be given.

WHAT HAPPENS IF YOU DO NOT WANT TO TAKE PART?

You or your parent may at any time tell us if you do not want to take part any further at any time, but your parent must please write us a letter. We will not be cross with you and we shall still carry on to treat you as good as possible.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING EXTRA POINTS WERE TOLD TO ME.

The blood, urine and muscle are given for research and that I will not be paid for it.

You will let us know if you find the answers.

I may decide not to take part any further in the study

WHO SHOULD WE CONTACT IF WE HAVE QUESTIONS?

Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria, Private bag 323, Arcadia, 0007.

Enquiries regarding ethical aspects of the project can be addressed to the: Chairperson of the Ethics Committee, Steve Biko Academic Hospital, Private Bag X169, Pretoria, 0001.

That I will receive a copy of assent form.

DECLARATION OF ASSENT

I, _____

(Print full name and surname)

Hereby assent to take part in the project.

I understand that I take part in the research project as a **patient.**

I confirm that I understand everything.

Signed at: _____on _____.

(Place) (Date)

_____

(Signature of a minor)

Witness 1: _____W**itness 2:** _____

(Signature) (Signature)

_____

(Signature of responsible who explained the project and assent to the participant.)

6.3 APPENDIX C: MITOCHONDRIAL DISEASE CRITERIA SHEET

1.1	**Muscular presentation** plus CNS plus multisystem	max 2 pt max 1 pt max 2 pt **max 4 pt**		**Evaluation** 1 point: Unlikely 2 to 4 points: Possible 5 to 7 points: Probable 8 to 12 points: Definite
	PEO	2		
	Ptosis, myopathic face	1		
	Exercise intolerance	1		Premature fatigue, weakness, cramps, myalgia with normal ADL
	Reduced muscle power	1		Including Gower sign, poor head control, DD motor delay if cognitive fair, slipping through, frog like position in babies < 6 months
	Rhabdomyolysis/ abnormal EMG	1		Reduced amplitude and duration of motor unit potentials, increased polyphasic potentials
	OR			
1.2	**CNS signs and symptoms** plus muscle plus multisystem	max 2 pt max 1 pt max 2 pt **max 4 pt**		
	DD/IQ<70	1		Significant delay in two or more domains
	Loss of acquired skills	1		
	Stroke like episodes			Transient hemianopia, hemiplegia
	Migraine			
	Epilepsy or abnormal EEG	1		Slowing of background, generalised epileptiform seizures, focal slow wave, seizure activity
	Myoclonus/myoclonic epilepsy	1		
	Cortical blindness	1		Loss of vision, optokinetic nystagmus with rest of eye exam normal and intact pupillary light response
	Pyramidal tract involvement	1		Increased tone, opisthotonus, tendon reflexes, extensor plantar responses, etc.
	Extrapyramidal involvement	1		Athetosis, dystonia, involuntary movements
	Brainstem involvement	1		Autonomic disturbances, central apnoea, hypoventilation, sinus bradycardia or tachycardia, swallowing difficulties, nystagmus, strabismus, abnormal or absent waves III to V in ABR
	Cerebellar involvement	1		
	OR			

1.3	**Multisystem involvement** plus muscle or CNS	max 3 pt max 1 pt **max 4 pt**		
	Haematology	1		
	Sideroblastic anaemia			
	Pancytopaenia			
	Gastrointestinal tract	1		
	Abnormal LFT acute or chronic			Elevated LFT, decreased synthesis of liver proteins, decreased bilirubin excretion, hypoglycaemia
	FTT			No adequate weight gain, weight < 3rd percentile/-2SD of percentiles
	Exocrine pancreatic dysfunction			> 7% excretion of fat
	Intestinal pseudo-obstruction			Constipation, colicky pain, vomiting without organic obstruction
	Unexplained chronic diarrhoea			> 3 weeks
	Endocrine	1		
	Short stature			< 3rd percentile/-2SD of percentiles
	delayed puberty			
	DM I or II			
	Hypoparathyroidism			
	DI			
	Heart	1		
	Cardiomyopathy			Hypertrophic or dilated
	Conduction block			AV block I-III, bundle branch block, pre-excitation syndromes
	Kidney	1		
	Proximal tubular dysfunction			Partial or complete Fanconi
	FSG			
	Eyes	1		
	Cataracts			
	Retinopathy			Impairment or loss or retinal function confirmed with ERG
	Optic atrophy			
	Ears	1		
	Sensorineural deafness			
	Nerve	1		
	Peripheral neuropathy			
	General	1		
	Exacerbation of symptoms with minor illness			
	SIDS in family			

2	**Metabolic or other investigations**	**max 4 pt**		
	Elevated lactate > 2 mmol/l	2		Confirmed at 3 occasions
	L:P > 18 if lactate is elevated	1		Only if the lactate is elevated
	Elevated alanine > 450 μmol/l	2		
	CSF lactate > 1.8 mmol/l Score only if blood lactate is normal	2		
	Elevated CSF protein	1		
	Elevated CSF alanine	2		
	Urine: Lactate or TCA intermediates	2		Citrate, α-Ketoglutaric acid, succinyl-CoA, succinate, fumarate, malate, oxaloacetate
	Ethylmalonic acid, 3-methylglutaconic acid or dicarbonic acids	1		adipic, suberic and sebacic acid
	Other			
	^{31}P-MRS reduced phosphocreatine/P_i	2		
	Leigh syndrome	2		T2 hyperintense lesions in globus pallidus, putamen, caudate nuclei
	Strokelike picture, leukodystrophy, cerebral/cerebellar atrophy	1		
	^{1}H-MRS: clear lactate peak	1		
3	**Morphology**	**max 4 pt**		
	RRF	2 to 4		For any in a child score 2, if > 2% score 4
	COX negative	2 to 4		For any in a child score 2, if > 2% score 4
	Strongly reduced overall COX-staining	4		
	Reduced SDH-staining	1		
	Strongly SDH reactive blood vessels	2		
	EM: Abnormal mitochondria	2		# Subsarcolemmal or intermyofibrillar aggregates 1 point # Increased cristae with irregular orientation, honeycomb, paucity of cristae 2 points # Enlarged or elongated mitochondria 2 points # Abnormal mitochondrial inclusions 2 points #Lipid droplets 1 point

ADL, activities of daily living; CNS, central nervous system; CSF, cerebrospinal fluid; COX, cytochrome oxidase; DD, developmental delay; DI, diabetes insipidus, DM, diabetes mellitus; EEG, electroencephalogram; EM, electron microscopy; EMG, electromyography, FSG, focal segmental glomerulosclerosis; FTT, failure to thrive; IQ, intellectual quotient; LFT, liver function tests; max, maximum, PEO, progressive external ophthalmoplegia; pt, points; RRF, ragged red fibres, SD, standard deviations; SDH, succinate dehydrogenase; SIDS, sudden infant death syndrome (Adapted from Wolf and Smeitink 2002)

6.4 APPENDIX D: LIST OF SCIENTIFIC CONTRIBUTIONS OF THE MITOCHONDRIAL PROJECT IN SOUTH AFRICA

6.4.1 Publications in peer-reviewed or refereed journals

Reinecke CJ, Koekemoer G, van der Westhuizen FH, Louw R, Lindeque JZ, Mienie LJ, Smuts I (2011) Metabolomics of urinary organic acids in respiratory chain deficiencies in children. Metabolomics DOI:10.1007/s11306-011-0309-0

Smuts I, Louw R, Du Toit H, Klopper B, Mienie LJ, van der Westhuizen FH (2010) An overview of a cohort of South African patients with mitochondrial disorders. J Inherit Metab Dis DOI:10.1007/s10545-009-9031-8

Smuts I, van der Westhuizen (2010) Mitochondrial disorders-diagnostic approaches and their application in the South African context. SA Paed Rev 7(2):6-15

Van der Walt E, Smuts I, Taylor R, Elson J, Louw R, van der Westhuizen FH. Characterisation of mtDNA variation in a cohort of South African paediatric patients with mitochondrial disease. (Submitted for publication to Eur J Hum Gen)

Van der Westhuizen FH, Smet J, Levanets O, Meissner-Roloff, Louw R, Van Coster R, Smuts I (2010) Aberrant ATP synthesis resulting from a novel deletion in mitochondrial DNA in an African patient with progressive external ophthalmoplegia. J Inherit Metab Dis DOI:10.1007/s10545-009-9020-y

6.4.2 Posters and presentations at international meetings

Cawood D, Hosseini SH, Smuts I, Brown MD, Wallace DC, Olckers A (2002) Mitochondrial genome screening of five South African mitochondrial myopathy paediatric patients: Novel changes. 5th World Muscle Society Congress, Skukuza, South Africa

Cawood D, Smuts I, Engelbrecht S, Wallace DC, Olckers A (1999) Mitochondrial encephalopathies: molecular investigation of causative mutations within the South

African population. 4th European meeting on Mitochondrial Pathology (EUROMIT), Queens College, Cambridge, UK

Koekemoer G, van der Westhuizen, Smuts I, Reinecke C (2011) Description of concurrent class analysis and its application in defining a biosignature for mitochondrial repsiratory chain deficiencies. 7th International Conference of the Metabolomics Society, Cairns, Australia

Olckers A, Prosser D, Maree FF, Brown MD, Wallace DC, Smuts I (2002) Mitochondrial myopathies in the South African population: implications for diagnosis – lessons from the first four years. 52nd Annual Meeting of the American Society of Human Genetics, Baltimore, USA

Olckers A, Prosser D, Wallace DC, Brown MD, Smuts I (2001) Mitochondrial Myopathies: a South African perspective. 51st Annual meeting of the American Society of Human Genetics, San Diego, USA

Reinecke C, Koekemoer, Mienie J, Louw R, van der Westhuizen FH, Smuts I (2010) Metabolomics define a biosignature for respiratory chain deficiencies. 6th International Conference of the Metabolomics Society, Amsterdam, The Netherlands

Reinecke CJ, Koekemoer G, van der Westhuizen FH, Mienie LJ, Smuts I (2011) The search for biomarkers for respiratory chain deficiencies: A metabolomics approach. Annual symposium of the Society for the study of inborn errors of metabolism (SSIEM), Geneva, Switzerland, Oral presentation

Smuts I, van Brummelen AC, Wallace DC, Olckers A (2003) Evaluation of the mitochondrial disease criteria scoring system in the South African population. 53rd Annual Meeting of the American Society of Human Genetics, Los Angeles, USA

Smuts I, van der Westhuizen FH (2008) Mitochondrial Disorders in South African paediatric patients: recent advances, diagnostic strategy and future Prospects. 7th European meeting on Mitochondrial disorders (Euromit), Stockholm, Sweden

Van Brummelen AC, Smuts I, Wallace DC, Olckers A. Molecular screening of patients with mitochondrial disorders in the South African population. 53rd Annual Meeting of the American Society of Human Genetics, Los Angeles, USA

Van der Walt EM, Smuts I, Louw R, Taylor RW, Elson JL, Turnbull DM, van der Westhuizen FH (2010) The molecular genetic characterisation of mitochondrial DNA in a cohort of South African patients with mitochondrial disorders using next-generation DNA sequencing. 7th Annual Meeting of ASMRM and 10th J-mit, Fukuoka, Japan

Van der Walt EM, Smuts I, Louw R, Taylor RW, Elson JL, Turnbull DM, van der Westhuizen FH (2011) Molecular genetic characterisation of mitochondrial DNA in a cohort of South African patients with mitochondrial disorders using next-generation DNA sequencing. Joint Conference of the African and Southern African Societies of Human Genetics, Cape Town, South Africa

Van der Westhuizen, van der Walt EM, Elson JL, McFarland R, Turnbull DM, Taylor RW, Louw R, Smuts I (2011) mtDNA variation in muscle of 42 African paediatric patients with respiratory chain deficiencies. European meeting on Mitochondrial disorders (Euromit), Zaragoza, Spain

Wortmann SB, Kluijtmans LAJ, Rodenburg RJ, Sass JO, Nouws J, van Kaauwen EP, Kleefstra T, De Vries MC, Oshanni P, Smuts I, van der Westhuizen FH, Reinecke C, Thorburn D, Smeitink JAM, Morvava E, Wevers RA (2011) 3-Methylglutaconicaciduria-Lessons form nearly 50 genes and more than 900 patients. Annual symposium of the Society for the study of inborn errors of metabolism (SSIEM), Geneva, Switzerland

6.4.3 Poster and presentations at national meetings

6.4.3.1. Invited speaker

Smuts I (2002) Mitochondrial disorders. Panda meeting. Mount Aux Source, South Africa

Smuts I (2003) Genetic analysis of mitochondrial disease in South Africa. Mitochondrial Workshop, PUCHE, Potchefstroom, South Africa

Smuts I (2003) Clinical Phenotype of mitochondrial disorders in South Africa. GSK Neurology meeting. Mount Aux Source, South Africa

Smuts I (2005) New challenges in the diagnosis of mitochondrial disorders. Neurology Association of South Africa. Annual Conference, Clarens, South Africa

Smuts I (2009) Experience with mitochondrial disorders in South Africa. 1st South African Metabolic Disease Symposium, Cape Town, South Africa

6.4.3.2 Posters or Presentations

Cawood D, Smuts I, Engelbrecht S, Wallace DC, Olckers A (2000) Molecular investigation of Mitochondrial encephalopathies within the South African Population. Biennial Congress ~~M~~illipaed 2000". Sun City Pilanesberg, South Africa

Cawood D, Smuts I, Engelbrecht S, Wallace DC, Olckers A (2000) Molecular investigation of the mitochondrial genome in South African mitochondrial myopathy paediatric patients. University of Pretoria, Faculty day, South Africa

Olckers A, Prosser D, Smuts I, Wallace DC, Brown MD. Mitochondrial Myopathies in the South African Paediatric Population. 9th South African Society of Human Genetics Congress, Kruger National Park South Africa, South Africa

Prosser D, Smuts I, Brown M, Wallace DC, Olckers A (2002) Molecular screening of South African mitochondrial myopathy patients lends credence to a unique genetic aetiology. Annual Congress of the Neurological Association of South Africa, Johannesburg, South Africa

Prosser D, Smuts I, Wallace DC, Olckers A (2001) Molecular Analysis of Mitochondrial Myopathies within South Africa. Annual Congress of the Neurological Association of South Africa, Wild Coast Sun, Kwa-Zulu Natal, South Africa

Smuts I, Du Toit H, van der Westhuizen FH (2008) Respiration analyses in muscle of patients with possible mitochondrial disorders. The Sky's the Limit; Combined

Conference of SAPA, ALLSA, Ipokrates, PANDA, SACCSG, SAAPS and UNAPSA, Sun City, South Africa and Faculty day University of Pretoria, South Africa

Smuts I, Van Brummelen AC, Wallace DC, Olckers A (2004) Dilemmas in the application of mitochondrial disease criteria (MDC) in the Southern African Population. Kidz n All, Combined conference of SAPA, ALLSA and PANDA SA, Cape Town, South Africa

Smuts I, van der Westhuizen FH (2007) The contribution of OXPHOS and PDHc analysis to the diagnostic yield in the paediatric patients with neuromuscular disorder at the Pretoria Academic Hospital. University of Pretoria Faculty day, South Africa

Smuts I, van der Westhuizen FH (2008) The contribution of OXPHOS and PDH_c analyses to the diagnostic yield in paediatric patients with neuromuscular disorders at the Pretoria Academic Hospital. The Sky's the Limit; Combined Conference of SAPA, ALLSA, Ipokrates, PANDA, SACCSG, SAAPS and UNAPSA, Sun City, South Africa

Van der Walt EM, van der Westhuizen FH, Smuts I (2010) The molecular genetic characterisation of mitochondrial DNA in a selection of South African patients with mitochondrial disorders: Preliminary results. Faculty day University of Pretoria, South Africa

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6.6 APPENDIX F: INSTRUCTIONS TO AUTHORS

6.6.1 Journal of Metabolic Disease (JIMD)

JIMD – Journal of Inherited Metabolic Disease

Aims and Scope

The JIMD is the official journal of the Society for the Study of Inborn Errors of Metabolism, SSIEM. By enhancing communication between workers in the field throughout the world, the JIMD aims to improve the management and understanding of inherited metabolic disorders. It publishes results of original research and new or important observations pertaining to any aspect of inherited metabolic disease in humans and higher animals. This includes clinical (medical, dental and veterinary), biochemical, genetic (including cytogenetic, molecular and population genetic), experimental (including cell biological), methodological, theoretical, epidemiological, ethical and counselling aspects. The JIMD also reviews important new developments or controversial issues relating to metabolic disorders and publishes reviews and short reports arising from the Society's annual symposia. A distinction is made between peer-reviewed scientific material that is selected because of its significance for other professionals in the field, and non-peer-reviewed material that aims to be important, controversial, interesting or entertaining ("Extras").

The Journal of Inherited Metabolic Disease exists as two sister publications which are served by a single Editorial Team and a single manuscript submission and review process: the traditional print and online journal "JIMD" and the online-only "JIMD Reports". The latter publishes scientifically sound research findings or clinical observations that warrant communication in the peer-reviewed literature but are of more limited interest to the readers. In addition to full electronic publication as "JIMD Reports", the abstracts of these articles are also printed in the non-online section of the "JIMD" to reach the widest possible readership. All other types of articles are published electronically and in print in the JIMD.

Scientific contributions

Full Articles

The JIMD welcomes scientific contributions for publication as printed full articles in the following categories:

- **Original Articles:** Important manuscripts that may be expected to influence or change clinical or research practice with regard to inherited metabolic disorders. Original articles may include comprehensive studies on disease features in groups of patients, important novel information on a disease or relevant research findings. Exceptional case reports that are judged to be of general interest to the readers may also be accepted as original articles. The editors may reject submitted manuscripts as original articles but invite revision or resubmission for publication as Reports in "JIMD Reports". Anecdotal observations may also be submitted as "Extras".
- **Rapid Communications:** Highly competitive and timely manuscripts; please discuss this with the editors: editor@jimd.org.
- **Reviews:** Concise summaries of metabolic pathways, specific disorders, methods, treatment options etc.

- **Metabolic Dissertations:** The JIMD invites all researchers who have completed a Ph.D. or M.D. thesis in the field of inborn errors of metabolism to submit a comprehensive review of the topic of their thesis. The article should not focus solely on the research findings but should cover all relevant information in the respective field. Such reviews preferably (but not necessarily) have a single author (other contributors should be acknowledged) and will be published with a photograph of the investigator.

All authors are invited to provide a colour picture that may be used for the front cover of the issue in which the article appears.

Images in Metabolic Medicine

The Editors will consider clear and interesting clinical pictures or other types of images (e.g. laboratory results or observations) submitted with a descriptive paragraph of up to 250 words. Prints, slides, or electronic copy are all acceptable. Authors must obtain informed consent for publication of patient-related materials. Case reports or additional information may be added as supplementary material. Images will be fully printed; title and author(s) will be listed in bibliographical databases such as Medline.

Editorials

The JIMD invites communicating editors and reviewers of articles that have been accepted for publication in the JIMD to provide an editorial that places the article in a broader context. Editorials have no abstract, may be comprised of up to 500 words and should contain no more than two (if any) references. Additional material can be added as supplementary material online. Editorials will be fully printed; title and author(s) will be listed in bibliographical databases such as Medline.

Letters

The JIMD invites comments on previously published articles in the journal which should reach the editorial office within 4 weeks of publication of the original item. Correspondence may be subjected to peer-review and counter-replies are usually invited from the authors of the original publication. The concise form of a letter may also be used to report exceptionally important clinical or research information unrelated to a previous JIMD publication.

Letters should have no more than five authors. They have no abstract, are limited to a maximum of 500 words and should contain no more than two (if any) references. Additional material can be added as supplementary material online. Letters will be fully printed; title and author(s) will be listed in bibliographical databases such as Medline.

Reports (Online Articles)

Some manuscripts present scientifically sound research findings or clinical observations that are worth communicating but are of more limited interest to the readers of JIMD and may be sufficiently summarised in an abstract of 250 words. In order to facilitate publication of these types of manuscripts, **"JIMD Reports"** has been introduced as a sister of the traditional "JIMD". It is positioned as an independent periodical with its own ISSN number. All manuscripts submitted as Reports to the JIMD website will be considered for "JIMD Reports" rather than for the traditional journal. They will undergo the same review process as Original Articles (and in exceptional cases may be reassigned for publication in the traditional "JIMD"). In addition, the Editorial Team (based on the advice of reviewers and

Communicating Editors) may reject Original Articles for publication in the traditional "JIMD" but offer publication in "JIMD Reports". After acceptance, articles in "JIMD Reports" will be professionally typeset in the same manner as articles in "JIMD". Reports will be available online and fully referenced in bibliographical databases. They will be submitted to relevant international abstracting and indexing services with an embargo of no more than 12 months and thus (in contrast to traditional "JIMD" articles) will be available free of charge after a certain time period. In addition, titles and abstracts of Reports are printed in the print-only "Extras" section of the traditional "JIMD". It is recommended to use of the full allowance of 250 words for the abstract of Reports to convey the message of the article to the widest possible readership.

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Extras in the JIMD

The Editors of the JIMD invite submission of short items that are interesting, stimulating, important or entertaining to professionals working in the field of inborn errors of metabolism. These items will not usually be reviewed outside the editorial board and usually will not be referenced in bibliographic databases. All items of this type should be submitted by Email to the editorial office (editor@jimd.org); please provide full personal details for all authors of each contribution.

Observations and opinions

The JIMD wishes to provide a forum for relevant or stimulating opinions, ideas, experiences or personal views that merit communication without fulfilling the requirements for scientific articles or short reports. Items in this section may include anecdotal experiences that are deemed important to others in the field, unusual clincal observations, puzzling complications or novel side effects, or summaries of contributions e.g. to the metabolic Email list metab-l.

Observations should consist of one to two short paragraphs (maximum 500 words) and should contain no more than two (if any) references. No more than five authors may be included.

Fillers

Small texts that are used to fill gaps, e.g. at the end of original articles, have been a long and cherished tradition in some journals. They usually have the added advantage of entertaining readers and stimulating thought. The Editors invite interesting stories or personal experiences of up to a few hundred words on topics such as:

- A patient / paper / experience that changed my practice
- A memorable patient / experience

- An error that proved educational or informative for lab operation or clinical care
- How I embarked on this career path, and lessons learned along the way
- Any other story conveying instruction, pathos, or humour

If the filler refers to an identifiable person, written consent for publication from that person or an appropriate relative is required.

Book Reviews

Instructive reviews of up to 400 words are invited on new books published in the field of inborn errors of metabolism, or closely affiliated areas.

Obituaries

The Editors of the JIMD strongly encourage submission of obituary notices for all recently deceased SSIEM members or other persons in the field of inborn errors of metabolism. Obituary notices should be mailed to the editorial office. Please give your name and contact details, including a phone number and email address. Obituaries will be considered by the editorial board and may be shortened; they will be published (without proofs) with the name of the person(s) who submitted the notice.

Please provide:

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2. A (black and white) photograph
3. A summary of **Important data:**
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    b. *Date and place of birth*
    c. *Primary degree with university and year when obtained*
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    e. *Date of death, Cause of death*
4. The **main text** summarising important contributions and personal characteristics of the deceased. The last sentence should state the remaining relatives such as spouse and/or the number of children and grandchildren.

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All scientific contributions for publication in the JIMD (including JIMD Reports) must be submitted by the web-enabled online manuscript submission and review system. As the review process is also fully web-based, this system allows editors to keep review times as short as possible and offers authors the option to track progress of the review of their manuscripts. The online manuscript submission and review system for the Journal of

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- their substantial contribution to the work (all authors should have been involved in (a) conception and design, or analysis and interpretation of data, and (b) drafting the article or revising it critically for important intellectual content)
- their agreement to submission.

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6.6.2 Metabolomics

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Journal no. 11306

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Instructions for Authors

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  Keywords
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Online document

Abou-Allaban, Y., Dell, M. L., Greenberg, W., Lomax, J., Peteet, J., Torres, M., & Cowell, V. (2006). Religious/spiritual commitments and psychiatric practice. Resource document. American Psychiatric Association. http://www.psych.org/edu/other_res/lib_archives/archives/200604.pdf. Accessed 25 June 2007.

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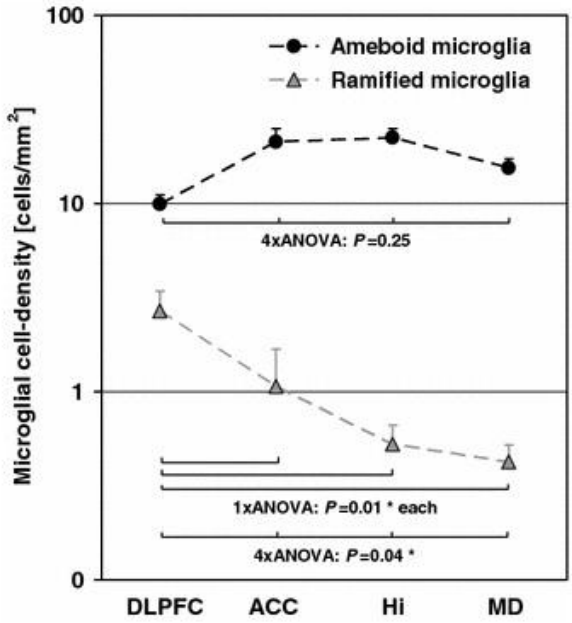

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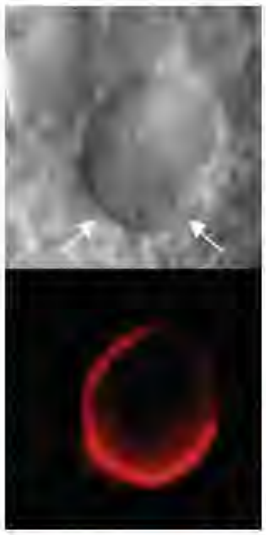

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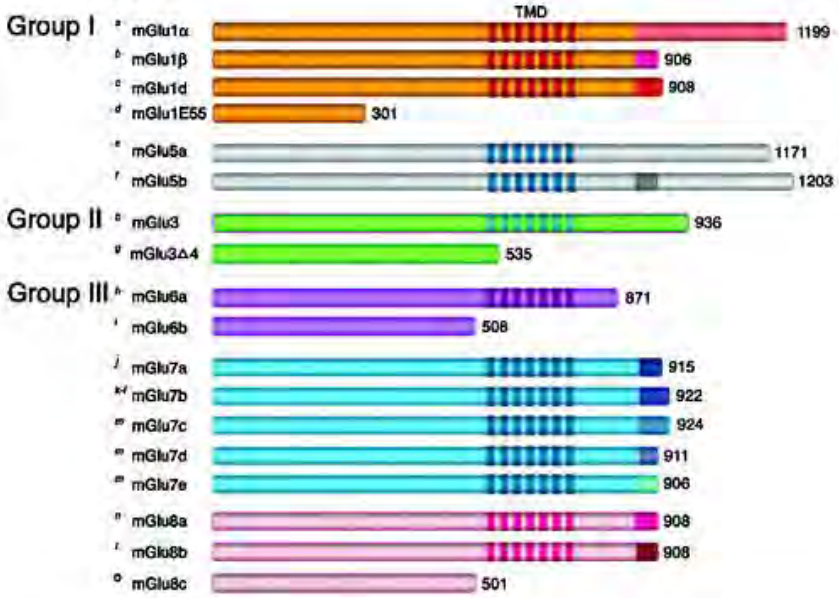

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6.7 APPENDIX G: PERMISSION OF CO-AUTHORS

6.7.1 Permission from co-authors: JIMD 1

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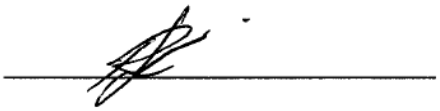
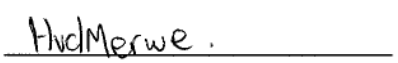
The contributions of the co-authors for this publication were as follows: Dr Izelle Smuts was the clinician primarily responsible for the patient care, scientific planning, data interpretation and preparation of the manuscript; Dr Roan Louw and Ms Hanli Du Toit conducted the enzyme assays for complexes I-IV; Prof Japie Mienie and Dr Brenda Louw were involved in the biochemical analyses of urine specimens; Prof Francois van der Westhuizen was responsible scientific supervision and preparation of the manuscript.





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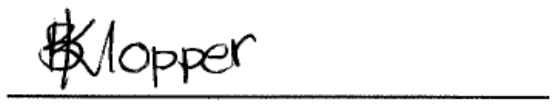

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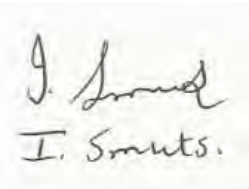

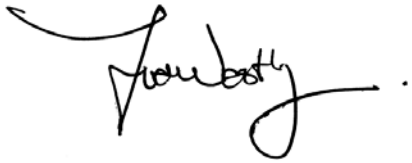

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6.7.2 Permission from co-authors: Metabolomics

We, the co-authors of the paper titled — **Metabolomics of urinary organic acids in respiratory chain deficiencies in children** hereby give permission that the data originating from the experiments in the metabolomics study, **as presented in Chapter Three**, **Section 3.3** may be submitted as part of the thesis submitted for the degree *Philosophiae Doctor.*

The contributions of the co-authors for this publication were as follows: Prof Carolus J Reinecke was involved in the data analyses and data interpretation. Dr Gerhard Koekemoer was responsible for the statistical analyses; Prof Francois H van der Westhuizen provided scientific supervision; Dr. Roan Louw and Mr Jeremie Z Lindeque were responsible for assistance in the laboratory; Prof Lodewyk J Mienie was involved in the data interpretation and construction of the metabolic profile; Dr Izelle Smuts was the clinician responsible for the management of the patients, involved in the scientific planning and interpretation of results. All the authors participated in the writing of the manuscript.

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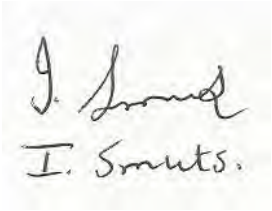

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6.7.3 Permission from co-authors: JIMD 2

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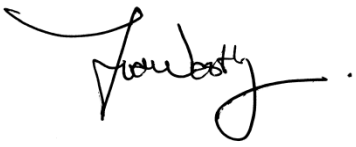
The contributions of the co-authors for this publication were as follows: Prof Francois H van der Westhuizen conducted the enzyme assays for complexes I-IV and molecular genetic analyses. He provided scientific supervision and contributed in the preparation of the manuscript; Prof Joél Smet and Prof Rudy van Coster were responsible for the BN-PAGE blotting and in-gel activity assays. Dr Oksana Levanets, Dr Roan Louw and Mrs Madelein Meissner-Roloff were responsible for assistance in the laboratory; Dr Izelle Smuts treated the patient, was involved in the scientific planning, interpretation of results and responsible for the preparation of the manuscript.



_____ _____

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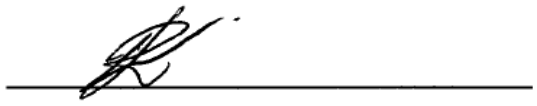

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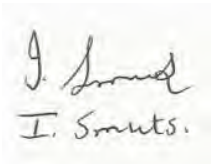

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6.7.4 Permission from co-authors: JIMD 3

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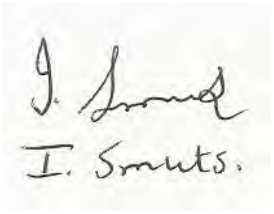
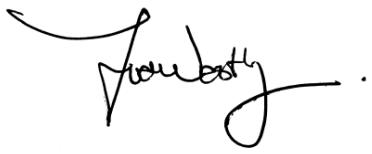
The contributions of the co-authors for this publication were as follows: Dr Izelle Smuts was the clinician responsible for the management of the patients, involved in the scientific planning and interpretation of results; Prof Francois H van der Westhuizen provided scientific supervision; Dr Roan Louw and Mr Shayne Mason were responsible for the GC-MS and NMR analyses respectively; Prof Lodewyk J Mienie was involved in the data interpretation; Dr Udo FH Engelke and Prof Ron A Wevers directed on the nuclear magnetic resonance analyses and data interpretation; Dr Gerhard Koekemoer was responsible for the statistical analyses and Prof Carolus J Reinecke was involved in the data analyses and data interpretation. All the authors participated in the writing of the manuscript.





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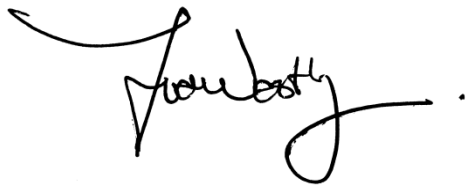
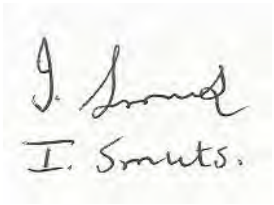
We, the co-authors of the paper titled —**Mitochondrial disorders: diagnostic approaches and their application in the South African context** hereby give permission that the data originating from the experiments conducted on the patients, **as presented in Chapter Four, Section 4.4** may be submitted as part of the thesis submitted for the degree *Philosophiae Doctor.*

The contributions of the co-authors for this publication were as follows: Prof Francois H van der Westhuizen conducted the enzyme assays for complexes I-IV, provided scientific supervision and contributed in the preparation of the manuscript; Dr Izelle Smuts was the paediatrician primarily responsible for the patient care, scientific planning, data interpretation and she was responsible for the preparation of the manuscript.





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