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Mitochondrial DNA (mtDNA) mutations in patients with suspected myoclonic epilepsy and ragged red muscle fibres (MERRF), Leigh syndrome (LS), and mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS)

BY

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To my parents and Craig

ABSTRACT

Mitochondrial disorders are considered to be the most common cause of metabolic abnormalities in the paediatric neurology population (Zeviani *et al.*, 1996). These authors reported that the phenotypes observed in 25-30% of the paediatric patients in their neurology clinics were due to a mitochondrial aetiology. The genetic aetiology in an equivalently affected paediatric population in South Africa is currently unknown. This study investigated the possibility that reported mutations could account for the mitochondrial phenotypes observed in the South African population. It focussed on the most frequent paediatric mitochondrial disorders namely: Leigh Syndrome (LS), mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), and myoclonic epilepsy and ragged red muscle fibres (MERRF).

A clinically well characterised group of 25 patients with mitochondrial disorders was included in this study. The molecular analysis of the mitochondrial genome was initially based on a restriction fragment length polymorphism (RFLP) screening strategy for the ten most common mitochondrial DNA (mtDNA) mutations associated with the above-mentioned three disorders. However, during the study the mutation analysis strategy was modified to a sequencing strategy as this provided more information than the RFLP approach. The modified sequencing strategy extended the study to incorporate fifteen additional mtDNA mutations, associated with other mitochondrial disorders, and individuals included in the study were thus investigated for the presence of 25 mtDNA mutations. Moreover, the modified strategy provided additional information of the regions encompassing the reported mutations.

A single patient was observed to harbour the reported A3243G MELAS mutation. This mutation was noted to be heteroplasmic in the proband and two of her maternal relatives. None of the other 24 reported mutations were observed in this patient population. One novel mtDNA alteration in the $tRNA^{Leu(UUR)}$ gene was observed in a single patient, although the pathogenicity of this mutation remains to be investigated. Novel and reported polymorphisms, some of which are associated with specific haplogroups, were also observed when comparing sequencing data against the Cambridge reference sequence.

The data generated during this study contributed towards the understanding of the uniqueness of the South African population in the global context. This was apparent from the fact that only one of the reported mutations was observed in our patient population who were clinically well characterised and displayed phenotypes similar to those reported internationally. Results form this study underlined the complexity of mitochondrial disorders and argues in favour of whole mitochondrial genome sequence information to be used for diagnostic purposes. Moreover, the results confer with the hypothesis that novel mitochondrial mutations may account for the majority of mitochondrial phenotypes observed in the South African population.

OPSOMMING

Mitochondriale toestande word beskou as die algemeenste oorsaak van metaboliese afwykings in die pediatriese neurologie populasie (Zeviani *et al.* 1996). Volgens hierdie outeurs is die fenotipes wat waargeneem word in 25-30% van die pediatriese pasiënte in hul neurologie kliniek te wyte aan 'n mitochondriale etologie. Die genetiese basis van 'n ekwivalente pediatriese populasie in Suid-Afrika is tans onbekend. Hierdie studie het die moontlikheid ondersoek dat bekende mutasies die fenotipes wat waargeneem word in die Suid-Afrikaanse populasie, kon veroorsaak. Dit het gefokus op die algemeenste pediatriese mitochondriale toestande, naamlik: Leigh Sindroom (LS), mitochondriale enkefalomiopatie, laktaatasidose en beroerte-agtige episodes (MELAS) en miokloniese epilepsie met gerafelde spiervesels (MERRF).

'n Groep van 25 pasiënte met mitochondriale toestande was in hierdie studie ingesluit. Molekulêre analise van die mitochondriale genoom was aanvanklik gebaseer op 'n restriksie fragment lengte polimorfisme (RFLP) sifting strategie. Hierdie strategie is aangepas tot 'n volgorde bepaling strategie ten einde meer inligting te versamel. Dit het daartoe gelei dat vyftien addisionele mtDNA mutasies, wat met ander mitochondriale toestande geassosiëerd is, ingesluit kon word. Individue was dus vir 'n totaal van 25 mtDNA mutasies ondersoek. Die gewysigde strategie het ook addisionele inligting ingewin oor die gebiede waarin die bekende mutasies geleë is.

Die bekende A3243G MELAS mutasie is waargeneem in slegs een pasiënt. Hierdie mutasie was heteroplasmies in die indekspasiënt en twee van haar maternale familielede. Geen van die ander 24 bekende mutasies is waargeneem in die res van hierdie pasiënt populasie nie. Een nuwe mtDNA verandering in die $tRNA^{Leu(UUR)}$ geen is waargeneem in 'n enkele pasiënt en die patogenisiteit van hierdie mutasie moet verder ondersoek word. Data is vergelyk met die Cambridge verwysingsvolgorde en bekende sowel as nuwe polimorfismes, waarvan sommiges geassosiëerd is met spesifieke haplogroepe, is aangetoon.

Slegs een van die bekende mutasies is in hierdie pasiënt populasie, wat fenotipies ooreenstem met internasionale beskryfde gevalle, gevind. Dit mag aanduidend wees

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van 'n beduidende uniekheid in die Suid-Afrikaanse populasie. Resultate van hierdie studie het die kompleksiteit van mitochondriale toestande bevestig en steun die gebruik van volledige mitochondriale genoom volgorde inligting vir diagnoses. Verder ondersteun hierdie resultate die hipotese dat nuwe mitochondriale mutasies waarskynlik verantwoordelik is vir die meerderheid van mitochondriale fenotipes wat in die Suid-Afrikaanse populasie waargeneem word.

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LIST OF ABBREVIATIONS

Abbreviations are listed in alphabetical order.

12 S-rRNA	12 S ribosomal RNA
16 S-rRNA	16 S ribosomal RNA
I	respiratory chain complex I
II	respiratory chain complex II
III	respiratory chain complex III
IV	respiratory chain complex IV
V	oxidative phosphorylation system complex V
α	alpha
α^{32}P-dATP	dATP labelled in the α position with ^{32}P isotope
α^{35}S-dATP	dATP labelled in the α position with ^{35}S isotope
A	alanine (in amino acid sequence)
A and a	adenine (in DNA sequence)
A.A.	amino acid
A.C.	anti-codon
A_{260}/A_{280}	ratio of absorbency measured at 260 nm and 280 nm
ACRS	amplification-created restriction site
AD	autosomal dominant
ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
APS	ammonium persulphate: $(NH_4)_2S_2O_8$
AR	autosomal recessive
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
ATP 6	ATP synthetase subunit 6
ATP 8	ATP synthetase subunit 8
ATPase	adenosine triphosphatase
ATPase 6	adenosine triphosphatase 6
ATV	active trypsin versene
Ava I	restriction endonuclease isolated from an *E.coli* strain that carries the cloned *Ava* I gene from *Anabaena variabilis*, with recognition site 5'-C↓PyCGPuG-3'
β	beta
Ban II	restriction endonuclease isolated from *Bacillus aneurinolyticus*, with recognition site 5'-G(A/G)GC(T/C)↓C-3'
BER	base excision repair
boric acid	boracic acid: H_3BO_3
bp	base pair
BPB	bromophenol blue: $C_{14}H_{10}Br_4O_5S$
BSA	bovine serum albumin
%C	percentage crosslinking monomer

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°C	degrees centigrade
C	cysteine (in amino acid sequence)
c and c	cytosine (in DNA sequence)
C. elegans	*Caenorhabditis elegans*
ca.	circa: approximately
Ca^{2+}	calcium ion
cDNA	complementary DNA
Ci	curie: quantity of any radioactive nuclide in which there are 3.7×10^{10} disintegrations per second
cM	centimorgan
cm	centimetre: 10^{-2} metre
CNS	central nervous system
CO I	cytochrome *c* oxidase subunit I
CO II	cytochrome *c* oxidase subunit II
CO III	cytochrome *c* oxidase subunit III
CO_2	carbon dioxide
CoA	coenzyme A
CoQ	coenzyme Q
COX	cytochrome c oxidase
COX III	subunit III gene of the cytochrome *c* oxidase complex
CPEO	chronic progressive external ophthalmoplegia
CPT-I	carnitine palmitoyltransferase
CPT-II	carnitine-acylcarnitine translocase
CRS	Cambridge reference sequence
CsCl	cesium chloride
CSF	cerebrospinal fluid
CT scan	computerised tomography scan
Cys	cysteine
Cyt	cytochrome
cyt b	cytochrome b
cyt c	cytochrome c
δ	delta
ΔTnt	deletion of T nucleotide
D	aspartic acid
D. yakuba	*Drosophila yakuba*
D. melanogaster	*Drosophila melanogaster*
Da	dalton
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
ddATP	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-dideoxycytidine-5'-triphosphate
Dde I	restriction endonuclease isolated from *Desulfovibrio desulfuricans*, with recognition site 5'-C↓TNAG-3'
ddGTP	2',3'-dideoxyguanosine-5'-triphosphate
ddH_2O	double distilled water
ddNTP	2',3'-dideoxynucleotide triphosphate
ddNTPs	2',3'-dideoxynucleotide triphosphates
del	deletion
dGTP	2'-deoxyguanosine-5'-triphosphate
DHU	dihydro-uridine
D-loop	displacement loop

DLD	dihydrolipoamide dehydrogenase
DMSO	dimethyl sulfoxide: C_2H_6SO
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol: threo-1,4-dimercapto-2,3-butanediol: $C_4H_{10}O_2S_2$
dTTP	2'-deoxythymidine-5'-triphosphate
ε	epsilon
E	glutamic acid
E	energy
E1	pyruvate decarboxylase
E1α	pyruvate decarboxylase alpha
E2	dihydrolipoamide acetyltransferase
E3	dihydrolipoamide dehydrogenase
EDTA	ethylenediamine tetraacetic acid: $C_{10}H_{16}N_2O_8$
EtBr	ethidium bromide: $C_{21}H_{20}BrN_3$
EtOH	ethanol: CH_3CH_2OH
Exo I	exonoclease I
F	phenylalanine
F_0	transmembrane proton channel of complex V
F_1	hydrophobic component of complex V
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
Fe^{2+}	iron - ferrous oxidation state
Fe^{3+}	iron – ferric oxidation state
Fe-S	iron sulphur
FMN	flavin mononucleotide
Formamide	carbamaldehyde: CH_3NO
F_P	flavoprotein
γ	gamma
g	gram
G	glycine (in amino acid sequence)
G and g	guanine (in DNA sequence)
gDNA	genomic DNA
Genbank	GenBank® [1] : United States repository of DNA sequence information
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GPI	glucose phosphate isomerase
H	histidine
H^+	hydrogen
H_2O	water
Hae III	restriction endonuclease isolated from *Haemophilus aegyptius*, with recognition site 5'-GG↓CC-3'
HCl	hydrochloric acid
HETPP	α-hydroxyethyl thiamin pyrophosphate
Hinf I	restriction endonuclease isolated from an *E.coli* strain that carries the cloned *Hinf* I gene from *Haemophilus influenzae Rf*, with recognition site 5'-G↓ANTC-3'
His	histidine

[1] GenBank® is a registered trademark of the National Institutes of Health, U.S.A.

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Hpa II	restriction endonuclease isolated from *Haemophilus parainfluenzae*, with recognition site 5'-C↓CGG-3'
HSP	heavy strand promoter
H-strand	heavy strand
I	isoleucine
IAA	isoamyl alcohol
Ile	isoleucine
ins	insertion
K	lysine
K^+	potassium ion
kb	kilo (10^3) base pair
KCl	potassium chloride
kDa	kilo dalton
kJ	kilo joule
L	leucine
L^{CUN}	leucine amino acid with anticodon CUN
L^{UUR}	leucine amino acid with anticodon UUR
Leu	leucine
LHON	Leber's hereditary optic neuropathy
loading buffer	0.25% xylene cyanol FF; 0.25% bromophenol blue; 30% glycerol
LS	Leigh Syndrome
LSP	light strand promoter
L-strand	light strand
Lys	lysine
µ	micro
µCi	micro Curie
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
M	molar: moles per litre
m	milli: 10^{-3}
M	methionine
Mae II	restriction endonuclease isolated from *Methanococcus aeolicus* PL-5/H with recognition site 5'-A↓CGT-3'
Mae III	restriction endonuclease isolated from *Methanococcus* aeo*licus* PL-5/H with recognition site 5'-↓GTNAC-3'
Mbo I	restriction endonuclease isolated from *Moraxella bovis* ATCC 10900, with recognition site 5'-↓GATC-3'
MCAD	medium chain acyl-CoA dehydrogenase
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonus epilepsy and ragged red muscle fibres
Met	methionine
mg	milligram
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
MILS	maternally inherited Leigh Syndrome
MIMyCa	mitochondrial myopathy and cardiomyopathy
min	minutes
ml	millilitre
mM	millimolar

mm	millimetre
Mn	manganese
MNGIE	myoneurogastrointestinal disorder and encephalopathy
MRI	magnetic resonance imaging
mRNA	messenger RNA
Msp I	restriction endonuclease isolated from an *E. Coli* strain that carries the cloned *Msp* I gene from *Moraxella* species, with recognition site 5'-C↓CGG-3'
mtDNA	mitochondrial DNA
mTERF	mitochondrial transcription termination factor
mtRNA polymerase	mitochondrial RNA polymerase
mtTFA	mitochondrial transcription factor A
n	nano: 10^{-9}
N	asparagine
Na	sodium
N/A	not available
Na_2EDTA	disodium EDTA: $C_{10}H_{14}N_2Na_2O_8.2H_2O$
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NARP	neurogenic atrophy, ataxia, retinitis pigmentosa
ND1	NADH dehydrogenase subunit 1
ND2	NADH dehydrogenase subunit 2
ND3	NADH dehydrogenase subunit 3
ND4	NADH dehydrogenase subunit 4
ND4L	NADH dehydrogenase subunit 4L
ND5	NADH dehydrogenase subunit 5
ND6	NADH dehydrogenase subunit 6
nDNA	nuclear DNA
NDUFS7	NADH-ubiquinone oxidoreductase Fe-S protein 7
NDUFS8	NADH-ubiquinone oxidoreductase Fe-S protein 8
NER	nucleotide excision repair
ng	nanogram
NIH	National Institutes of Health, U.S.A.
nm	nanometre: 10^{-9} meter
nM	nanomolar
O_2	oxygen
OAA	oxaloacetate
OD	optical density
O_H	heavy strand origin of replication
O_L	light strand origin of replication
OSCP	oligomycin sensitive conferring protein
OXPHOS	oxidative phosphorylation
ϕX174/*Hinf* I	bacteriophage ϕX174 am3cs70 DNA, cut with restriction endonuclease *Hinf* I
%	percent
p	pico: 10^{-12}
P	proline
^{32}P	phosphor isotope
PA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
pBR322	bacteriophage pBR322

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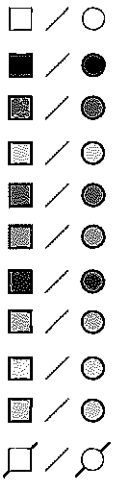
pBR322/*Hae* III	bacteriophage pBR322, cut with restriction endonuclease *Hae* III
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
PDHA1	pyruvate dehydrogenase alpha 1
PDHC	pyruvate dehydrogenase complex
PEO	progressive external ophthalmoplegia
pH	indicates acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
P_H	heavy strand promoter
Phe	phenylalanine
P_i	inorganic phosphate
P_L	light strand promoter
PL	*Paracentrotus lividus*
pmol	pico mole
Pro	proline
prot K	proteinase K: endopeptidase: *EC 3.4.21.14*
Q	glutamine
R	arginine
RC	respiratory chain
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RNase MRP	RNase maturation of RNA priming
RNase P	ribonuclease P
RP	retinitis pigmentosa
rpm	revolutions per minute
RRF	ragged red fibres
rRNA	ribosomal RNA
S	serine
S.U.	sea urchin
SA	South African
S^{AGY}	serine amino acid with anticodon AGY
S^{UCN}	serine amino acid with anticodon UCN
SAP	shrimp alkaline phosphatase
SCAD	short chain acyl-CoA dehydrogenase
SDH	succinate dehydrogenase
SDHA	succinate dehydrogenase 2, flavoprotein subunit
SDS	sodium dodecyl sulphate: $C_{12}H_{25}NaSO_4$
SDS-EDTA	0.5% SDS; 50 mM EDTA
sec	seconds
Sequenase	Sequenase® [1] Version 2.0 T7 DNA Polymerase
Sequenase buffer	200 mM Tris-HCl (pH 7.5); 100 mM $MgCl_2$; 250 mM NaCl
Ser	serine
SHY1	Surf Homolog of Yeast
SP	*Strongylocentrotus purpuratus*
SSCP	single stranded conformational polymorphism
SURF-1	surfeit locus
T	threonine (in amino acid sequence)
T and t	thymine (in DNA sequence)

[1] Sequenase ® is a registered trademark of United States Biochemical Corporation, Cleveland, Ohio, U.S.A.

T_a	optimal annealing temperature
Taq polymerase	DNA deoxynucleotidyltransferase from *Thermus aquaticus*: *EC 2.7.7.7*
TBE	89.15 mM Tris (pH 8.0), 88.95 mM boric acid, 2.498 mM Na_2EDTA
TCA	tricarboxylic acid
TE	10 mM Tris-HCl (pH 7.5), 1 mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine: $C_6H_{16}N_2$
temp	temperature
Thr	threonine
T-loop	TψC loop
T_m	calculated annealing temperature
TPP	thiamine pyrophosphate
Tris	Tris®: tris(hydroxymethyl)aminomethan: 2-Amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride: $C_4H_{11}NO_3.H_2O$
Triton X-100	Triton X-100®: octylphenolpoly(ethylene-glycolether)$_n$: $C_{34}H_{62}O_{11}$, for n = 10
tRNA	transfer RNA
$tRNA^{Cys}$	transfer RNA cysteine
$tRNA^{Leu(UUR)}$	transfer RNA leucine with anticodon UUR
$tRNA^{Lys}$	transfer RNA lysine
$tRNA^{Phe}$	transfer RNA phenylalanine
$tRNA^{Val}$	transfer RNA valine
Trp	tryptophan
Tyr	tyrosine
U	units
UK	United Kingdom
UQ	ubiquinone
UQH_2	dihydro-ubiquinone
Urea	H_2NCONH_2
UV	ultra violet
V	volt
V	valine
V-loop	variable loop
Val	valine
VLCAD	very long chain acyl-CoA dehydrogenase
W	tryptophan
W	watt
Xba I	restriction endonuclease isolated from *Xanthomonas badrii*, with recognition site 5'-T↓CTAG-3'
XC	xylene cyanole FF: $C_{25}H_{27}N_2O_6S_2Na$
xg	gravitational acceleration
Y	tyrosine

LIST OF SYMBOLS



male/female: unaffected
male/female: with MELAS clinical phenotype
male/female: with Leigh Syndrome clinical phenotype
male/female: with MERRF clinical phenotype
male/female: positive for the MELAS A3243G mitochondrial mutation
male/female: with a suspected MERRF clinical phenotype
male/female: with cardiac and renal failure
male/female: with migraines
male/female: with encephalitis
male/female: with hydrocephalus
male/female: deceased
spontaneous abortion/ still birth
gender unknown
proband
distant relation

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CHAPTER ONE

INTRODUCTION

In the 1940's it was postulated that the mitochondrion was the primary site for energy production. The structures themselves had been observed since the 1890's when Benda introduced the term mitochondria from the Greek words of 'mitos' meaning thread and 'chondros' for granule (Benda, 1898). Since that time the metabolic paths which are functional within the mitochondria have been under investigation. The majority of the proteins and enzymes constituting this metabolic process have been singularly characterised. Their function and interaction with surrounding pathways are however still under investigation (Dahl and Thornburn, 2001).

The presence of DNA within the mitochondria was discovered in 1963 by Nass and Nass, and in 1981 its entire sequence was determined (Anderson *et al.*, 1981). The sequencing of this genome paved the way for the identification of numerous mutations within the mitochondrial DNA (mtDNA) which are responsible for various clinical phenotypes. To date more than one hundred point mutations, and more than one hundred mtDNA rearrangements, have been associated with mitochondrial disease (Vogel, 2001).

The first clinical disorder to be associated with an alteration in the mtDNA was Leber's hereditary optic neuropathy (LHON) described by Wallace *et al.* (1988a). Since that time numerous clinical symptoms were reported to be associated with mitochondrial disease (Wallace *et al.*, 1992). Mitochondrial disorders usually present with amongst others, blindness, deafness, hypotonia, and cardiac failure. These are, however, non-specific symptoms and only offer an indication towards a particular disorder when presented as a group. Multisystem involvement is therefore the clinical sign most indicative of a mitochondrial disorder (Schapira and DiMauro, 1994). The clinical evaluation of distinct mitochondrial myopathies is therefore complex. Many disorders are termed mitochondrial myopathies when there is any indication of deficient energy metabolism and skeletal muscle involvement, together with evidence of ragged red fibres (RRF). In contrast, mitochondrial encephalomyopathies display central nervous system (CNS) involvement as well (Schapira and DiMauro, 1994).

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The disorders presented in this study were first described on a clinical level before their genetic aetiology was elucidated. The most frequent mitochondrial disorders observed in paediatric patients are NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa) and a more severe form which is referred to as Leigh Syndrome (LS), MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy and ragged red muscle fibres). According to a study by Zeviani *et al.* (1996) 25 - 30% of the children treated in their paediatric neurology clinic were found to harbour a mitochondrial aetiology. These authors stated that "mitochondrial disorders should be considered as the most frequent cause of metabolic abnormality in child neurology" (Zeviani *et al.*, 1996).

Since 1988 when mutations within the mitochondrial genome were first associated with spontaneous mitochondrial encephalomyopathies very few prevalence studies have been conducted. It is only during the past five years that the prevalence or incidence of the mitochondrial mutations within specific populations has been estimated.

In 1996 Rahman *et al.* studied the incidence of LS in the Australian population and concluded that the incidence of LS was 1 in 77,000 compared to an incidence of 1 in 40,000 when taking LS and Leigh-like disorders into account. This incidence rate has been substantiated by a more recent study in western Sweden (Darin *et al.*, 2001). These authors estimated the incidence of LS within pre-school children to be 1 in 32,000. This same study was extended to evaluate the prevalence of mitochondrial encephalomyopathies within the group of pre-school children, and determined to be 1 in 11,000. The prevalence of mitochondrial encephalomyopathies within children under the age of 16 was noted to be 1 in 21,000 (Darin *et al.*, 2001).

The above investigations regarding the incidence of the mitochondrial myopathies utilised the most common mitochondrial mutations, and the prevalence was calculated for all the mutations as a group. However, only the MELAS A3243G mutation has been studied individually by Majamaa *et al.* (1998). These authors calculated the prevalence of the A3243G mutation in an adult population and revealed a prevalence of 16.3 per 100,000 (1 in 6,000).

In 2000 the North East England population was investigated for the prevalence of mtDNA point mutations within the adult population (Chinnery *et al.*, 2000). From this

data it was concluded that mitochondrial myopathies had a prevalence of 1 in 15,000 adults. This was comparable to the prevalence of amyotrophic lateral sclerosis and Huntington's disease. However, the mitochondrial myopathies were observed to be more prevalent in this population than other neuromuscular disorders, for instance Duchenne dystrophy with a prevalence of 1 in 31,000 and myotonic dystrophy with a prevalence of 1 in 20,000 (Chinnery *et al.*, 2000).

To date no extensive study has been conducted within the South African population regarding the presence of mitochondrial mutations responsible for the phenotypes associated with mitochondrial myopathies. Internationally, mitochondrial myopathies were regarded to be rare. Prior to this investigation it was also thought that mitochondrial myopathies were rare in South Africa. However, since 1996 it was suspected that a mitochondrial aetiology in patients with metabolic disorders is not as rare as originally believed (Zeviani *et al.*, 1996). Subsequent to this report several authors have confirmed that mitochondrial myopathies have a high prevalence.

It was envisaged that this investigation would characterise the genetic aetiology of the LS, MERRF and MELAS phenotypes observed within specific patients from the South African paediatric population. It would thus represent the first extensive molecular characterisation of individuals with mitochondrial disorders in South Africa. A definitive diagnosis for the particular disorders will enhance the patient care and genetic counselling of the families and affected individuals. Knowledge regarding the prevalence of these mitochondrial mutations within the South African paediatric population will ultimately contribute towards the current understanding of the genetic basis of mitochondrial disorders.

CHAPTER TWO

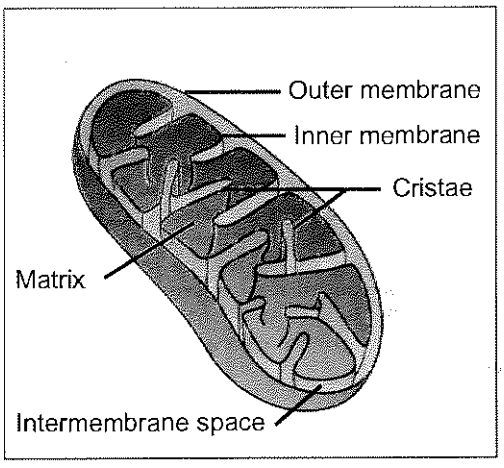
BIOCHEMICAL AND GENETIC ASPECTS OF MITOCHONDRIA

Mitochondria are the biochemical powerhouses of cells. They are responsible for the oxidative degeneration of foods using the energy-yielding substrates to produce the majority of the energy required by the cell. Cells of different organs contain different numbers of mitochondria, with the functional demand of the organ determining the number of mitochondria within the cell. For this reason organs, with high-energy demands, such as the central nervous system (CNS), eye, skeletal muscle, heart and kidneys have a much higher mitochondrion content than organs with lower energy demands i.e. hormone-producing tissues (Wallace, 1997).

2.1 THE STRUCTURE OF THE MITOCHONDRION

The mitochondria are organelles, which are typically the size of bacteria, approximately (ca.) 2 micrometers (µm), consisting of two membranes, a smooth outer membrane and a folded inner membrane. As illustrated in Figure 2.1 the folds, or cristae, of the inner membrane serve to increase the surface area available for adenosine triphosphate (ATP) production within the mitochondria. The inner membrane encloses the mitochondrial matrix, where the majority of the biochemical pathways are executed. An intermembrane space is formed between the outer membrane and the inner membrane (DiMauro and Wallace, 1993).

Figure 2.1: Structure of the mitochondrion



Adapted from Koolman and Röhm (1996).

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The membranes of the mitochondria contain integral membrane proteins but both have specific functions according to their location. The outer membrane consists mainly of porin proteins which ensure permeability of the membrane to molecules smaller than 10 kilo daltons (kDa). Porins are generally trimeric proteins with water-filled pores in the centre, allowing free diffusion of molecules across this membrane (Garrett and Grisham, 1995). The inner mitochondrial membrane is impermeable to most molecules and consists of cardiolipin, a phospholipid that is unique to the mitochondrial inner membrane. The proteins located within the inner membrane include specific transport proteins, so-called carriers, which facilitate the controlled exchange of metabolites from the cytoplasm (Garrett and Grisham, 1995). Enzymes and components of the respiratory chain (RC) and the ATP synthase complex (complex V) are also located within the inner membrane.

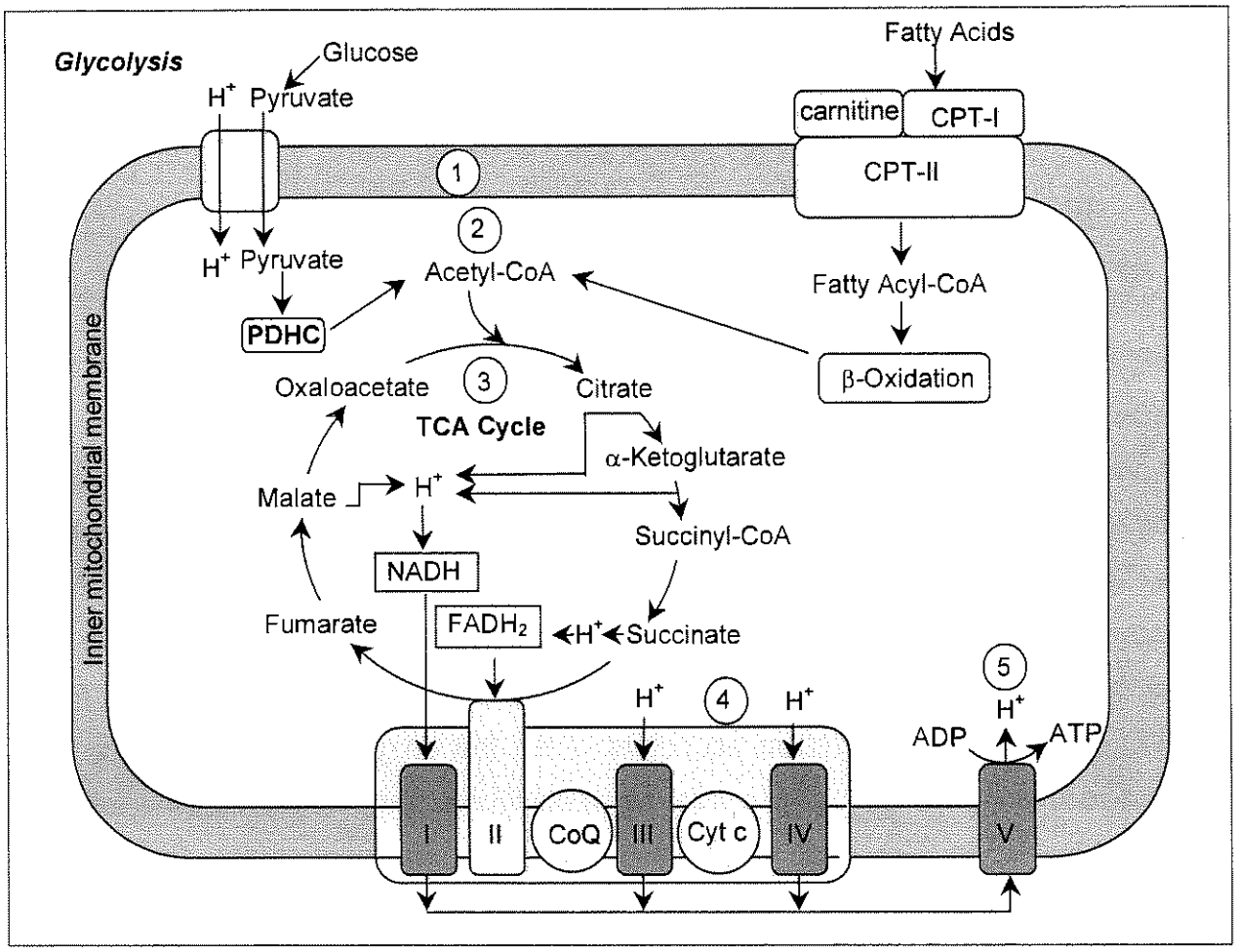
The mitochondrial matrix is filled with proteins that are involved in the tricarboxylic acid (TCA) cycle. This cycle is discussed in paragraph 2.2.2. Another biochemical pathway, which is functional within the mitochondria, is the pyruvate dehydrogenase complex (PDHC), discussed in paragraph 2.2.1. This complex is responsible for the conversion of pyruvate to acetyl-coenzyme A (acetyl-CoA). The TCA cycle is initiated to catalyse acetyl-CoA, which enters the RC, coupled to the synthesis of ATP, as discussed in section 2.2.3. The final stages of haem synthesis and the degradation of fatty acids by β-oxidation and components of the urea cycle all form part of the metabolism processes found within the mitochondria.

2.2 BIOCHEMISTRY WITHIN THE MITOCHONDRIA

Within the mitochondria the metabolism of glucose to energy and oxygen takes place via a number of inter-related pathways as illustrated in Figure 2.2. These include the transportation of the substrates from the cytoplasm across the outer mitochondrial membrane into the mitochondria. Once pyruvate has entered the mitochondria, substrate utilisation takes place via the conversion of pyruvate to acetyl-CoA through the PDHC. The resulting substrate, acetyl-CoA, then enters the TCA cycle. During this cyclic pathway the electron acceptors nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) are required for the flow of electrons down the RC. As depicted in Figure 2.2 the RC is composed of four complexes along which electrons are

transported creating an electron potential gradient across the inter-membrane space of the mitochondria. The final complex of the oxidative phosphorylation (OXPHOS) system, the ATP synthase complex (complex V), utilises this electron gradient for the production of ATP with the release of water (Campbell, 1991; Berg, 1996).

Figure 2.2: Inter-related metabolic pathways within the mitochondria



1 = Transport of substrates; 2 = Substrate utilisation; 3 = Tricarboxylic acid (TCA) cycle; 4 = Respiratory chain (RC); 5 = Oxidative phosphorylation (OXPHOS); PDHC = Pyruvate dehydrogenase complex; NADH = reduced nicotinamide adenine dinucleotide; $FADH_2$ = reduced flavin adenine dinucleotide; CPT-I = carnitine palmitoyltransferase I; CPT-II = carnitine-acylcarnitine translocase; CoA = Coenzyme A; I = complex I; II = complex II; CoQ = coenzyme Q; III = complex III; Cyt *c* = cytochrome *c*; IV = complex IV; V = complex V; ADP = adenosine diphosphate; ATP = adenosine triphosphate. Adapted from Berg (1996).

2.2.1 The Pyruvate Dehydrogenase Complex

The PDHC is responsible for the conversion of pyruvate to acetyl-CoA in the mitochondrial matrix (Wieland, 1983). Only once pyruvate has been converted to acetyl-CoA can this substrate enter the TCA cycle. For this reason the PDHC is considered to be one of the most important enzyme complexes in controlling aerobic energy metabolism (Maragos *et al.*, 1989).

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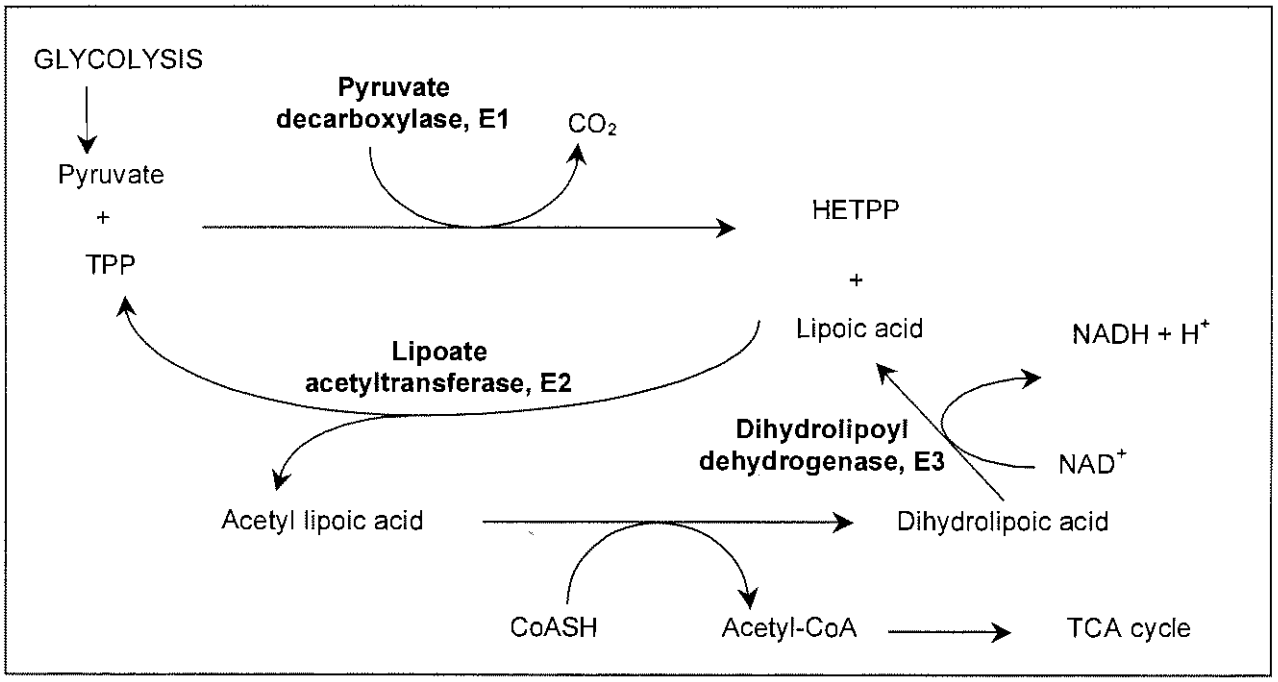
The entire complex has a molecular weight of ca. $7x10^6$ Da and consists of multiple copies of the three enzyme components (Dahl *et al.*, 1987). The three catalytic enzymes were described by Wieland in 1983 and are pyruvate decarboxylase (E1; EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2; EC 2.3.1.12), and dihydrolipoamide dehydrogenase (E3; EC 1.6.4.3). The E1 enzyme consists of two alpha (α) and two beta (β) subunits (Wieland, 1983; Dahl *et al.*, 1987) and acts as the regulatory site for the complex (Dahl *et al.*, 1987; Brown *et al.*, 1989). Each of the different components of the complex are encoded for by various genes located on different chromosomes as discussed below (Scherer *et al.*, 1991; Thekkumkara *et al.*, 1988).

As depicted in Figure 2.3, the enzyme E1 catalyses a thiamine pyrophosphate (TPP) dependent decarboxylation of pyruvate. The phosphorylation sites on the E1α subunit are responsible for the pyrophosphate binding while the E1β subunits bind the thiazolium ring of TPP. Following the decarboxylation of pyruvate, the acetyl group is transferred to a lipoic acid molecule attached to enzyme E2, a 59 kDa subunit, which has a lipoyl-bearing domain (Thekkumkara *et al.*, 1988). Acylation of CoA is subsequently performed via enzyme E3, a 50 kDa protein which is responsible for the transfer of electrons from reduced lipoyl groups (lipoic acid) to NAD within the PDH complex. Two active site electron acceptors within the enzyme, namely FAD and a redox-active disulphide, achieve the transfer of electrons, thereby allowing the release of oxidised lipoate to participate in reductive acylation (Dahl *et al.*, 1987; Otulakowski and Robinson, 1987; Scherer *et al.*, 1991).

The regulation of the PDHC is achieved by phosphorylation and dephosphorylation of the E1α subunit. The phosphorylation sites on enzyme E1α are responsible for the regulation of the entire complex's activity, modulated by the availability of particular metabolites within the mitochondria, namely adenosine diphosphate (ADP), pyruvate, NAD and CoA (Dahl *et al.*, 1987; Chun *et al.*, 1995)

Figure 2.3: Schematic representation of the pyruvate dehydrogenase complex



TPP = thiamin pyrophosphate; HETPP = hydroxyethyl thiamin pyrophosphate; TCA = tricarboxylic acid; CoA = coenzyme A; NADH = reduced nicotinamide adenine dinucleotide; NAD = nicotinamide adenine dinucleotide. Adapted from Zubay (1988).

2.2.1.1 The pyruvate decarboxylase (E1) subunit

In 1987 Dahl *et al.* isolated the complementary DNA (cDNA) of the E1α subunit. In this study the authors described the presence of the leader peptide in the immature protein. This sequence shared features with other mitochondrial targeting sequences. The sequence contained no acidic amino acids and the predicted secondary structure had amphiphilic properties with an α-helical structure. The leader peptide is cleaved within the mitochondrial matrix to form a mature E1α protein of 40 534 Da (Dahl *et al.*, 1987).

Although the cDNA had been isolated by Dahl *et al.* (1987), the gene was only mapped two years later. In 1989 Brown *et al.* localised the functional E1α subunit to the Xp22.1-22.2 region. The mapping of the E1α gene allowed for clarification of the clinical and biochemical heterogeneity of PDH deficiency. It is unusual for an X-linked disorder to demonstrate equal numbers of affected male and female patients. However, the biochemical properties of the PDHC permit this occurrence with regard to PDH deficient patients, as will be discussed in section 3.3.3.3.4 (Brown *et al.*, 1989).

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The E1α gene is 17 kilo bases (kb) in length and contains 11 exons (Maragos *et al.*, 1989; Koike *et al.*, 1990). Most of the mutations associated with the LS phenotype occur at the 3' end of the gene, in exons 10 and 11. These two exons are either mutational hotspots, or are the sites at which mutations are compatible with life. Appendix B refers to the various mutations which have been observed to be scattered throughout the gene, generally localised to functionally important regions such as the TPP-binding site and the phosphorylation sites (Chen *et al.*, 1995).

2.2.1.2 The dihydrolipoamide acetyltransferase (E2) subunit

In 1988 Thekkumkara *et al.* isolated the cDNA for the dihydrolipoamide acetyltransferase (E2) component of the PDHC. The cDNA was observed to have an open reading frame of 1,848 bp. The first 54 amino acids were determined to be the leader peptide required for transport into the mitochondrion. Once the leader sequence had been cleaved within the mitochondrion the mature E2 protein consisted of 561 amino acids and had a molecular weight of 59,551 Da. The authors were able to identify the lipoyl-binding sites within the complex to be at residues 35-72 and 162-199. These two regions consist of 38 highly conserved amino acids each (Thekkumkara *et al.*, 1988).

2.2.1.3 The dihydrolipoamide dehydrogenase (E3) subunit

The cDNA for the 51 kDa subunit of the PDHC was isolated in 1987 by Otulakowski and Robinson. The authors initially isolated the cDNA of the E3 subunit from a porcine library and subsequently utilised regions of the porcine sequence to screen human libraries. The human E3 subunit was observed to consist of 474 amino acids with a leader sequence of 35 amino acids (Otulakowski and Robinson, 1987). The sequence of the cDNA was confirmed by Pons *et al.* in 1988. The cDNA of the E3 subunit of the PDHC has an open reading frame of 1,527 bp which encode for a precursor protein of 509 amino acids with the leader sequence being ascribed to the first 35 amino acid residues (Pons *et al.*, 1988).

In 1991 the gene for the E3 subunit of the PDHC was named dihydrolipoamide dehydrogenase gene (DLD) and localised to chromosome 7q31-q32 by Scherer *et al.*

Two years later the first mutations within this gene were identified in a patient deficient for E3. The patient was noted to be a compound heterozygote for two mutations as described by Liu *et al.* (1993). The first mutation altered the highly conserved Lys37 for a glutamine residue. Lys37 was indicated to be located within the FAD-binding region of the E3 subunit and the altered charge was predicted to modify the charge distribution at this active site. The second mutation altered the highly conserved Pro453 for a leucine residue. This change occurs in the region of the subunit which is responsible for the acceptance and donation of protons within the subunit. The changes within the subunit are predicted to alter the activity and binding capacity of the enzyme (Liu *et al.*, 1993).

2.2.2 The Tricarboxylic Acid (TCA) cycle

In this cyclic metabolic pathway acetyl-CoA generated by the pyruvate dehydrogenase complex is reduced via oxidation to release electrons which are donated to the coenzymes NAD and FAD as illustrated in Figure 2.4. The net outcome of the TCA cycle is the oxidation of the acetyl group to two molecules of carbon dioxide. The energy released during this oxidation process enables the subsequent formation of one molecule of energy (ATP). The high-energy electrons, liberated by the oxidative process of the TCA cycle, are carried by coenzymes NAD and FAD to the respiratory chain. Here these electrons are sequentially passed along the electron transport pathway to their final acceptor, oxygen. This transference of electrons is coupled to the formation of a proton gradient across the mitochondrial inner membrane. The gradient drives the production of ATP through the ATPase complex. Hence the TCA cycle serves to convert carbon in the form of acetyl-CoA to carbon dioxide (CO_2) and release metabolic energy in the form of ATP, NADH and $FADH_2$ (Garrett and Grisham, 1995). Through the OXPHOS process a pair of electrons from NADH generates three molecules of ATP and a pair of electrons donated from $FADH_2$ results in the formation of two ATP molecules. Therefore one mole of acetyl-CoA results in the formation of 12 moles of ATP (Zubay, 1988).

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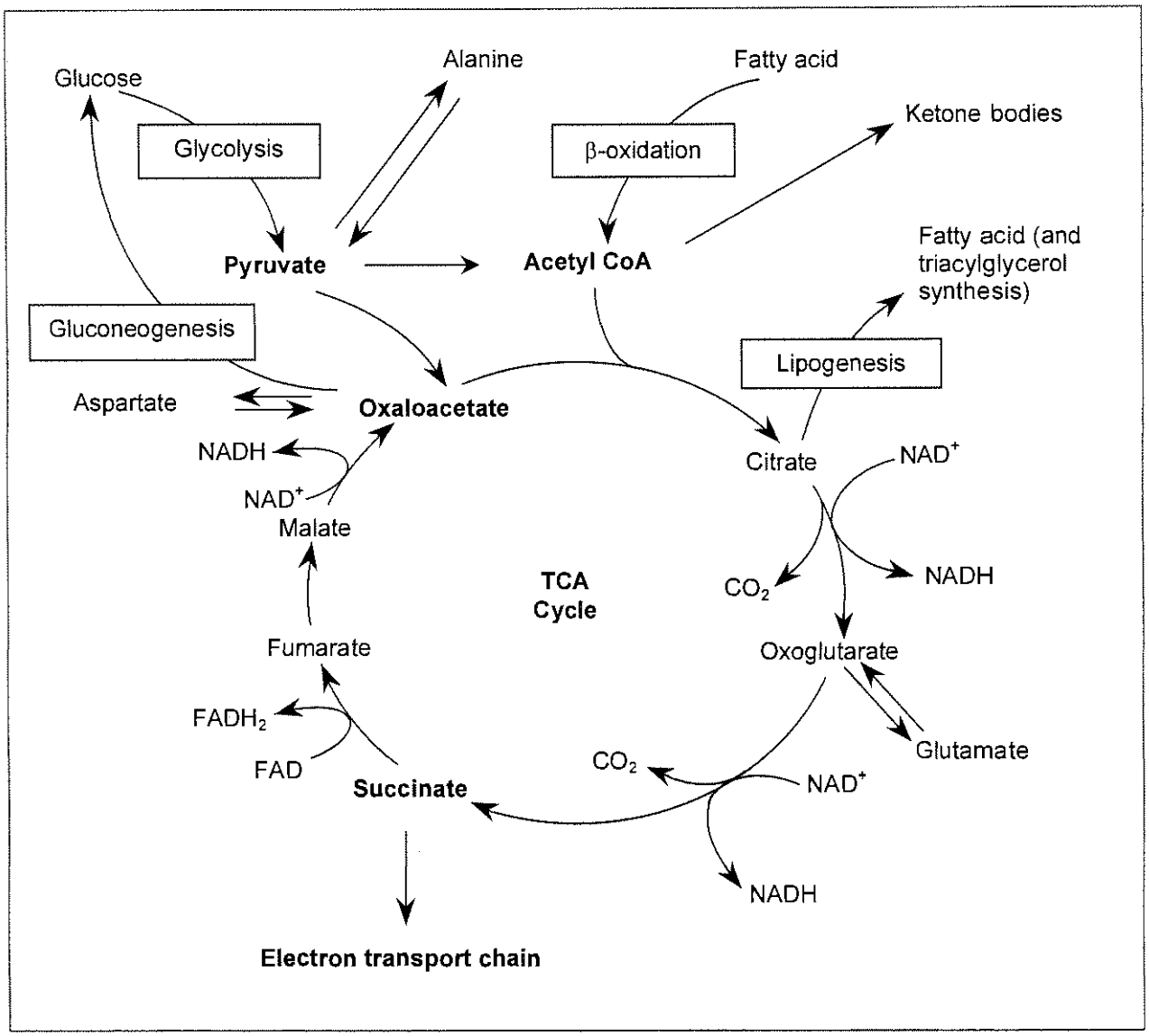
Figure 2.4: Schematic representation of the TCA cycle and associated metabolic pathways

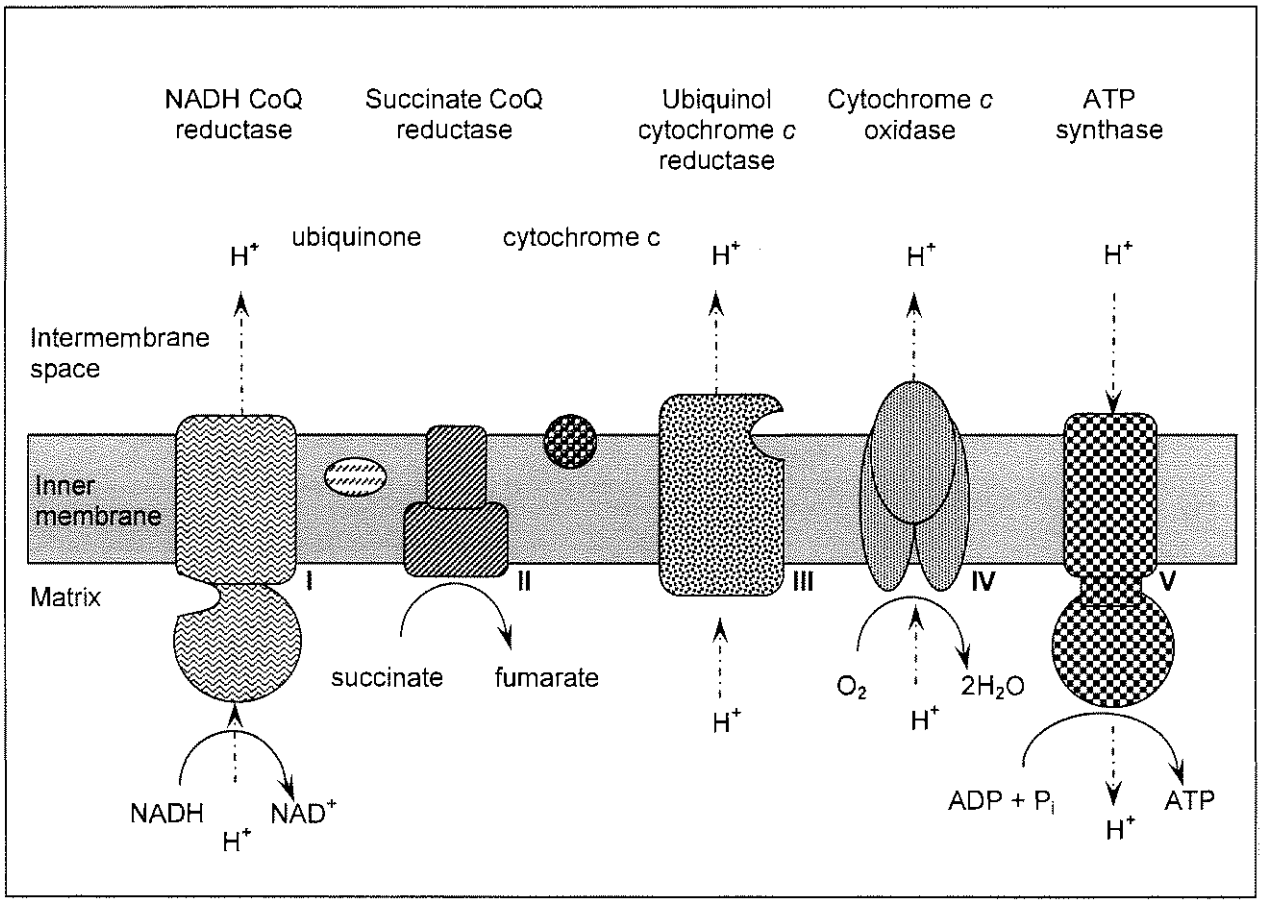


CoA = coenzyme A; TCA = tricarboxylic acid; NADH = reduced nicotinamide adenine dinucleotide; NAD = nicotinamide adenine dinucleotide; $FADH_2$ = reduced flavin adenine dinucleotide; FAD = flavin adenine dinucleotide. Adapted from Campbell and Smith (1994).

2.2.3 The mitochondrial oxidative phosphorylation pathway

The mitochondrial OXPHOS pathway is made up of the respiratory chain together with the ATP synthase complex (complex V), as illustrated in Figure 2.5. The RC is responsible for the formation of the proton gradient across the inner membrane of the mitochondria. This gradient then serves as the driving force of the ATP synthase complex for the production of ATP.

Figure 2.5: Complexes of the mitochondrial oxidative phosphorylation system



CoQ = coenzyme Q; NADH = reduced nicotinamide adenine dinucleotide; NAD = nicotinamide adenine dinucleotide; ADP = adenosine diphosphate; ATP = adenosine triphosphate.

The OXPHOS pathway is made of five complexes, namely complex I – V, with the first four complexes comprising the respiratory chain. These four multi-polypeptide complexes are listed in Table 2.1, and function in conjunction with two mobile electron carriers: ubiquinone and cytochrome *c*.

Table 2.1: Complexes of the mitochondrial respiratory chain

Complex Number	Complex name
Complex I	NADH-ubiquinone reductase
Complex II	succinate-ubiquinone reductase
Complex III	ubiquinol-cytochrome *c* reductase
Complex IV	cytochrome *c* oxidase (COX)

Adapted from Adams and Turnbull (1996).

The complexes of the RC are comprised of different molecular species as listed in Table 2.2. All the intermediates of the RC are membrane bound to the mitochondrial inner membrane, except for the mobile electron carriers, which are located within the intermembrane space (Garrett and Grisham, 1995).

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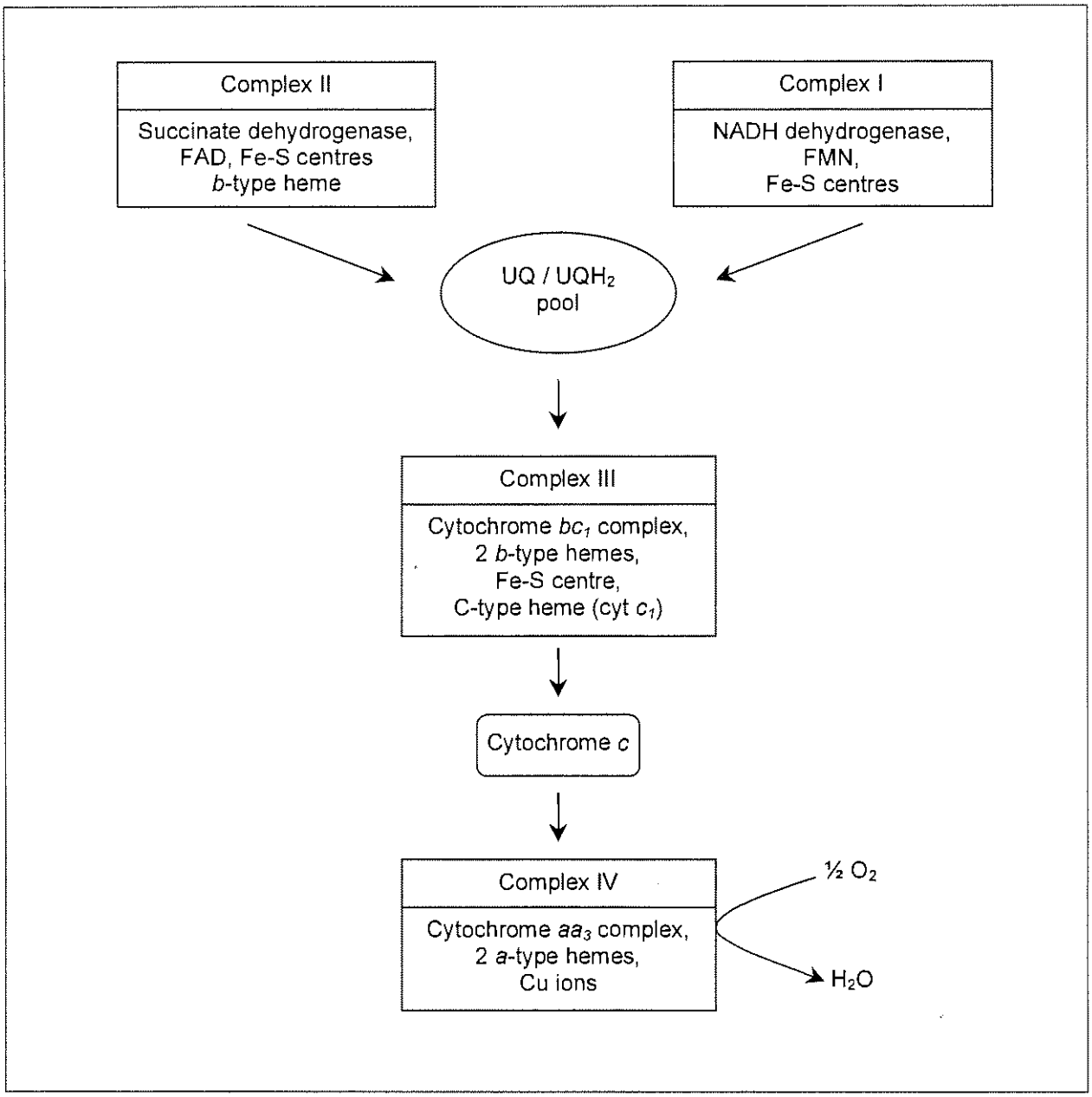
Table 2.2: The various molecular species which constitute the complexes of the respiratory chain

Complex	Molecular species	Description	Function
I, II	Flavoproteins	contain tightly bound FMN or FAD as prosthetic groups	One- or two-electron transfer events
Co Q	Coenzyme Q (ubiquinone)	a mobile electron carrier	One- or two-electron transfer reactions
III, IV	Cytochromes (Cytochromes *b*, *c*, c_1, *a* and a_3	proteins containing heme prosthetic groups to carry or transfer electrons	One-electron transfer agents where heme is converted from Fe^{2+} to Fe^{3+} and back
I, II, III	Iron-sulphur proteins	proteins containing iron-sulphur clusters to carry or transfer electrons	One-electron transfers involving iron (Fe) states
IV	Copper (protein-bound)	participate in electron transfer	One-electron transfer site, converts between Cu^{+} and Cu^{2+}

I = complex I; II = complex II; III = complex III; IV = complex IV; Co Q = coenzyme Q; FMN = flavin mono-nucleotide; FAD = flavin adenine dinucleotide. Garrett and Grisham (1995).

An overview of the interaction between the complexes of the RC is presented in Figure 2.6. A detailed description of complexes I-V and their function is discussed in sections 2.2.3.1 – 2.2.3.5. In short, the RC functions in the following sequential manner. Complex I accepts electrons from NADH which serves as a link between the RC and the TCA cycle as well as glycolysis and fatty acid oxidation. Simultaneously complex II, which contains succinate dehydrogenase, links the TCA cycle directly to the RC. Complexes I and II both yield the same product, that being reduced coenzyme Q (UQH_2), which then serves as the substrate for complex III. The reduced coenzyme Q is then oxidised by complex III while reducing cytochrome *c*. Reduced cytochrome *c* is utilised as the substrate for complex IV which is responsible for reducing molecular oxygen to form water as the by-product.

Figure 2.6: Interaction between complexes of the respiratory chain



FAD = flavin adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide; FMN = flavin mono-nucleotide; UQ = ubiquinone; UQH_2 = dihydro-ubiquinone. Adapted from Garrett and Grisham (1995).

The oxidative phosphorylation system consists of the respiratory chain together with a fifth complex (complex V), which is also referred to as ATP synthase. The five complexes are localised to the inner mitochondrial membrane and are essential for oxidative metabolism and the formation of ATP. Complexes I, III, IV and V are under dual genetic control, while nuclear DNA (nDNA) alone encodes for all the components of complex II. (Scholte, 1988; Hanna and Nelson, 1999). Table 2.3 summarises the current terminology by which the five complexes are distinguished. From this point forward the RC complexes will be referred by their complex number.

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Table 2.3: Terminology regarding the respective complexes of the oxidative phosphorylation system

Complex	Other names
Complex I	NADH ubiquinone oxidoreductase[1] (EC 1.6.5.3)[4]; NADH CoQ reductase[2]; NADH dehydrogenase (EC 1.6.99.3)[3]
Complex II	Succinate ubiquinone oxidoreductase[1] (EC 1.3.5.1)[4]; Succinate CoQ reductase[2]; Succinate dehydrogenase (EC 1.3.99.1)[3]
Complex III	Ubiquinol cytochrome *c* oxidoreductase[1]; Ubiquinol cytochrome *c* reductase[2] (EC 1.10.2.2)[3]
Complex IV	Cytochrome *c* oxidase[1, 2] (EC 1.9.3.1)[3]
Complex V	F_0-F_1-ATP synthase[1]; ATP synthase[2] (EC 3.6.1.34)[4]

1 = Adams and Turnbull (1996); 2 = Zubay (1988); 3 = MITOP (2001), Scharfe *et al.* (1999), Scharfe *et al.* (2000); 4 = Scriver *et al.* (1995).

2.2.3.1 Complex I

Complex I is the largest complex located within the mitochondrial inner membrane. It consists of 26 different polypeptides with a molecular weight of 10^6 daltons (Da) (Walker, 1995). This complex has the ability to pump protons acquired from the negatively charged matrix across to the positively charged intermembrane space of the mitochondria. Coupled to this proton translocator, electrons are transferred from NADH to ubiquinone (Adams and Turnbull, 1996).

Of the 26 subunits of complex I, seven are encoded for by the mtDNA. The structure of complex I was observed to be L-shaped with one arm embedded into the inner membrane and the other protruding into the matrix (Adams and Turnbull, 1995). The prosthetic group flavin mono-nucleotide (FMN), which removes electrons from NADH, is contained within the peripheral arm of the complex and is responsible for NADH dehydrogenase. In addition to the FMN group, the peripheral arm also contains approximately four different iron-sulphur centres (Zubay, 1988; Adams and Turnbull, 1996). The arm embedded in the membrane is composed of all seven mitochondrial encoded subunits, one or two iron-sulphur centres and makes up the ubiquinone hydrogenase component of the complex (Adams and Turnbull, 1996).

Ubiquinone is proposed to be reduced in the following manner: NADH reacts with the FMN, reducing it to $FMNH_2$, the reduced flavin then transfers the electrons to the iron-sulphur centres. These electrons are passed along from the iron-sulphur centres to their final destination of ubiquinone, where the formation of dihydro-ubiquinone (UQH_2)

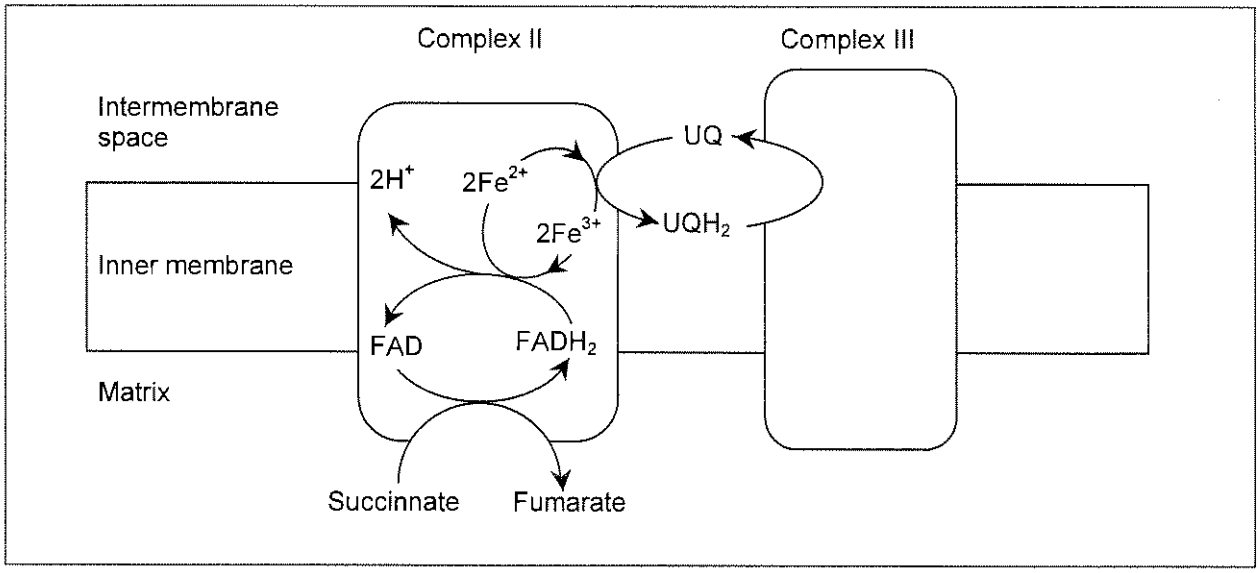
takes place. UQH_2 diffuses to complex III by way of a central, hydrophobic region of the inner mitochondrial membrane's phospholipid bilayer, catalysing the reduction of ubiquinone by NADH (Zubay, 1988).

2.2.3.2 Complex II

Complex II is the only enzyme involved in the TCA cycle that is completely embedded within the mitochondrial inner membrane with a molecular mass of ca. 140 kDa. This complex is also the only complex of the RC which is entirely encoded for by nuclear genes. It is responsible for the oxidation of succinate to fumarate and transfers electrons across to the ubiquinone pool (Adams and Turnbull, 1996).

The complex is composed of two domains, one for succinate dehydrogenase (SDH) and the other with a membrane anchoring function (Adams and Turnbull, 1996). The SDH component consists of a flavoprotein subunit (70 kDa) and an iron-sulphur protein (30 kDa). The larger flavoprotein contains a succinate binding site and a FAD molecule, which is bound to the protein via a histidine residue. The membrane-anchoring fraction is composed of two subunits of 15 kDa and 13 kDa respectively. These polypeptides each contain a heme group (cytochrome b_{558}) which is essential for ubiquinone binding (Adams and Turnbull, 1996). Complex II is responsible for the reduction of ubiquinone via succinate to form UQH_2, which diffuses to complex III, as illustrated in Figure 2.7 (Zubay, 1988; Garrett and Grisham, 1995).

Figure 2.7: Schematic representation of electron flow from complex II to complex III



UQH_2 = dihydro-ubiquinone; UQ = ubiquinone; FAD = flavin adenine dinucleotide; $FADH_2$ = reduced flavin adenine dinucleotide; Fe^{2+} = iron - ferrous oxidation state; Fe^{3+} = iron - ferric oxidation state. Adapted from Garrett and Grisham (1995).

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2.2.3.3 Complex III

Complex III is composed of eleven subunits and has a molecular weight of approximately 400 kDa (Koolman and Röhm, 1996). The cytochrome *b* subunit is the only subunit encoded and synthesised by the mitochondrial genome, the ten remaining subunits are nuclear encoded. The five largest subunits all contain four redox centres which are responsible for the translocation of protons from the mitochondrial matrix across the inner mitochondrial membrane, coupled to the electrons which are transferred from ubiquinol to cytochrome *c* (Zubay, 1988; Adams and Turnbull, 1996).

The subunits of complex III which do not contain redox centres and are therefore not involved in the transport of electrons between complexes, have a structural function. It has been proposed that these subunits play an important role in the maintenance and assembly of the complex (Adams and Turnbull, 1996).

Cytochrome *c* is located between complexes III and IV and serves as an intermediate carrier of electrons between these two complexes. It is a heme protein of approximately 12.5 kDa and is localised within the intermembrane space. As a carrier it is loosely associated with the cytosolic surface of the inner mitochondrial membrane and has specific binding sites for complexes III and IV as depicted in Figure 2.8. Cytochrome *c* binds to subunit IV of complex III and subunit II of complex IV (Adams and Turnbull, 1996).

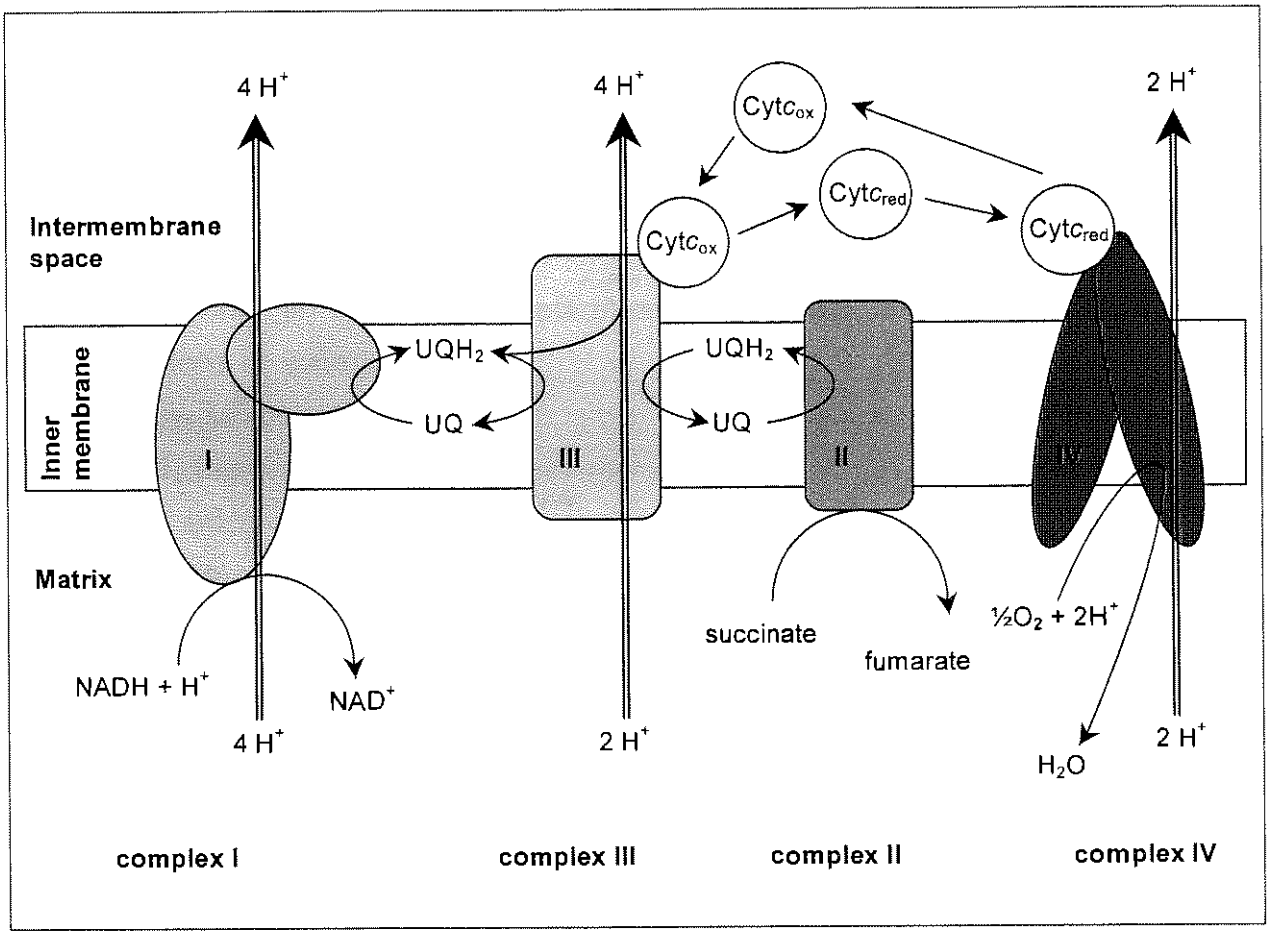
2.2.3.4 Complex IV

Complex IV is the terminal complex of the respiratory chain system and is responsible for the transfer of electrons from the cytochrome *c* pool to form oxygen and water. The complex is composed of 13 subunits and has a molecular weight of ca. 200 kDa (Koolman and Röhm, 1996).

The three mitochondrial-encoded subunits are the largest and form the catalytic core of the enzyme. These subunits harbour the redox centres (heme a, heme a_3, Cu_A and Cu_B) of complex IV. The heme and copper atoms are located within two of the larger subunits. The ten subunits encoded by the nuclear genome either have a modulating function or are responsible for the stability of the structure of the holoenzyme (Poyton,

1998). The complex spans the phospholipid bilayer of the inner membrane, with a large domain protruding into the intermembrane space. Electron transfer is initiated by the binding of cytochrome *c* to subunit II of complex IV on the matrix side of the membrane. The electrons are carried by two copper atoms and heme groups of cytochrome *a* and a_3, which are located in subunit II of this complex (Zubay, 1988; Adams and Turnbull, 1996). A summary of the electron flow through the four complexes of the respiratory chain is illustrated in Figure 2.8.

Figure 2.8: Proposed model for electron flow through the respiratory chain



NADH = reduced nicotinamide adenine dinucleotide; NAD = nicotinamide adenine dinucleotide; UQ = ubiquinone; UQH_2 = dihydro-ubiquinone; H^+ = hydrogen; O_2 = oxygen; $Cytc_{ox}$ = cytochrome *c* oxidation; $Cytc_{red}$ = cytochrome *c* reduction. Adapted from Garrett and Grisham (1995).

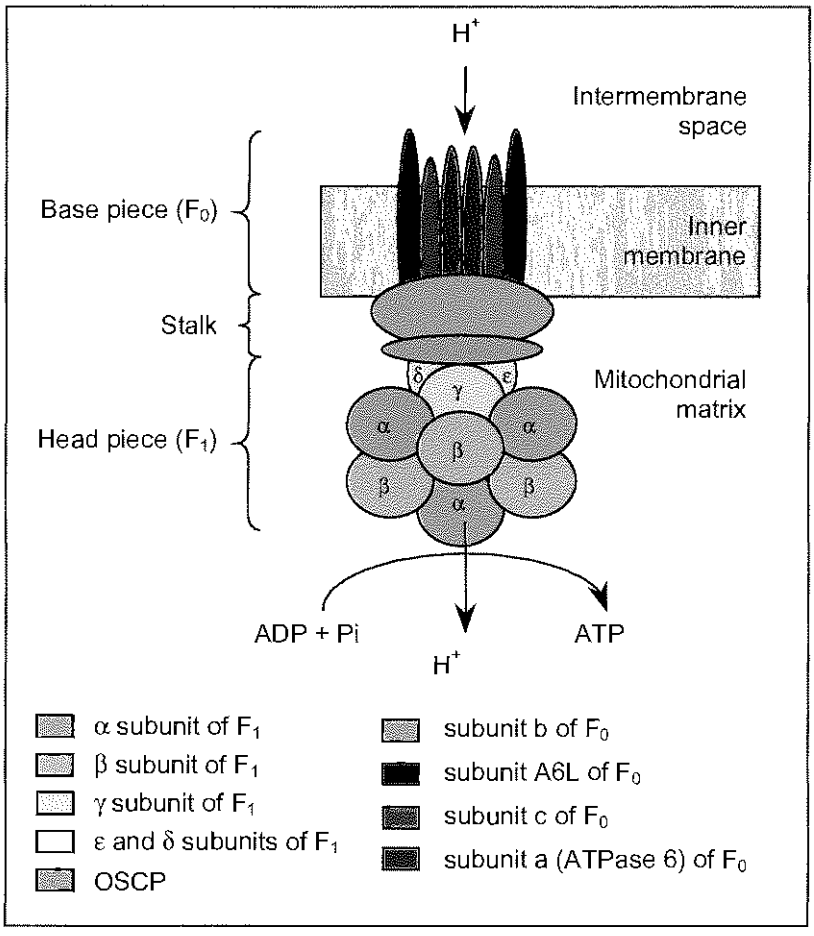
2.2.3.5 Complex V

Complex V is the terminal complex of the OXPHOS system and is also termed the ATP synthase (ATPase) complex. It is responsible for the catalytic formation of ATP from ADP and inorganic phosphate (Pi) via the proton gradient formed during the transfer of electrons down the RC (Mitchell, 1961). The complex is composed of 16 different

polypeptides, two of which are encoded for by the mtDNA, namely the subunit a and A6L. These two subunits are encoded by the ATPase 6 and ATPase 8 genes respectively of the mitochondrial genome (Anderson *et al.*, 1981; Adams and Turnbull, 1996). The entire complex has a molecular weight of ca. 400 kDa (Koolman and Röhm, 1996).

The structure of the ATPase complex is described as a "lollipop" configuration as depicted in Figure 2.9. The two principle domains, F_1 and F_0, are connected together within the inner membrane by the stalk. The stalk consists of subunits of the F_1 and F_0 domains. The F_0 domain is a hydrophobic component situated within the inner mitochondrial membrane and facilitates proton translocation. The F_1 domain protrudes into the mitochondrial matrix and contains the catalytic centre and the water-soluble portion of the ATPase complex (Schapira *et al.*, 1994)

Figure 2.9: A diagrammatic representation of complex V



OSCP = oligomycin sensitive conferring protein. Adapted from Schapira and DiMauro (1994).

ATP is the universal currency of energy within the biological world. The first phosphate bond, as in adenosine mono-phosphate (AMP), is a low energy-bond, while the second, ADP and the third phosphate (ATP) bonds are high-energy bonds. The high-energy bonds release 30.5 kilo joules (kJ) of energy while the low-energy bonds only release 9.2 kJ upon cleavage. Release of the third bond yields three substrates: an inorganic phosphate group (P_i), ADP and energy (E). ADP and P_i are used in the

resynthesis of ATP and the released energy is utilised in anabolism (Meyer *et al.*, 1994).

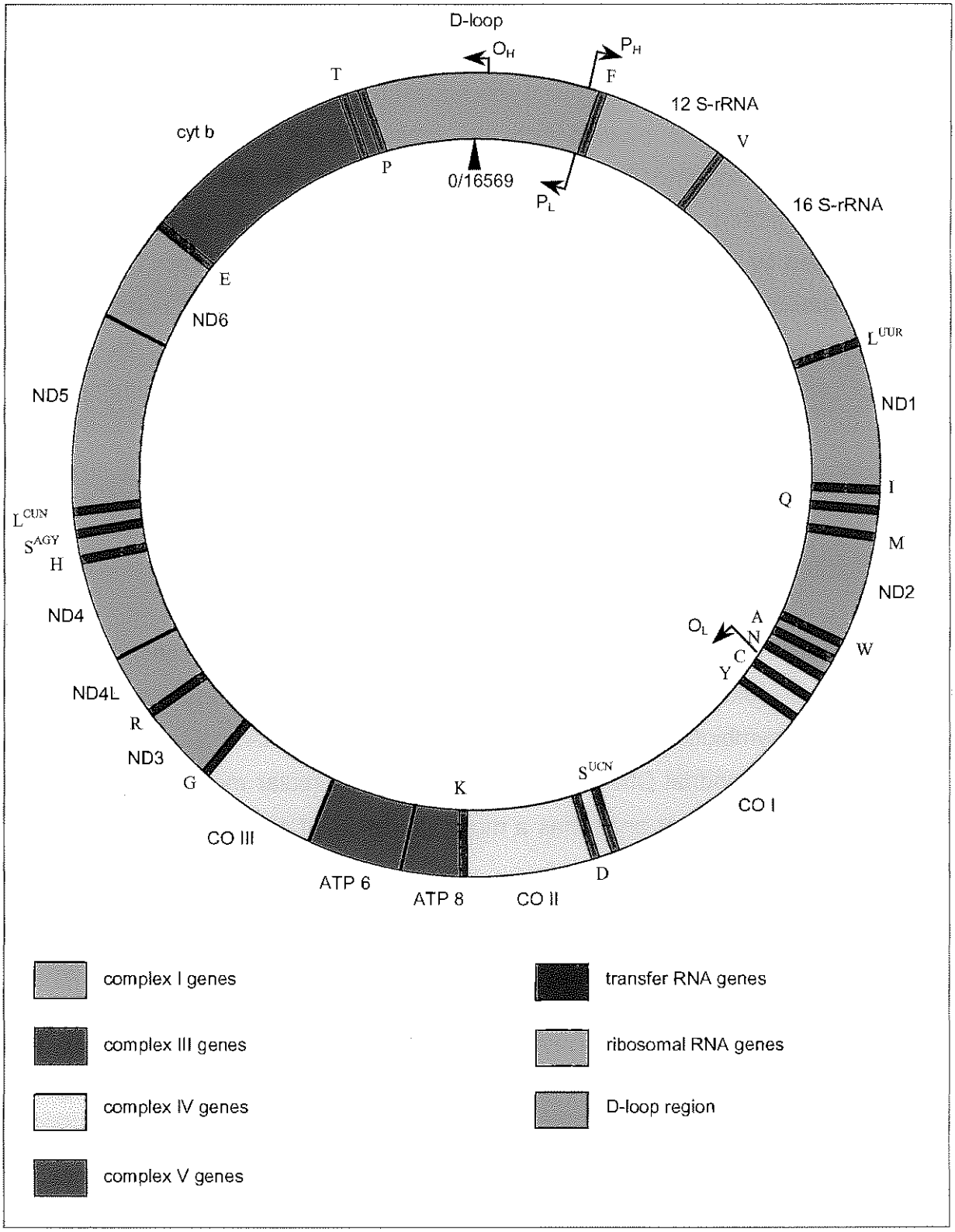
As the high-energy electrons flow down the sequential carriers in the RC, the energy released is used to pump protons from the matrix across the inner mitochondrial membrane to the intermembrane space. This results in the formation of an electrochemical proton gradient which is utilised by complex V to generate ATP. Protons released by the RC flow down the electrochemical gradient, back to the matrix side of the inner mitochondrial membrane, through the transmembrane proton channel (F_0). The energy released during this backflow of protons is captured by the ATPase complex to synthesise ATP from ADP and inorganic phosphate (Mitchell, 1961; Berg, 1996).

2.3 THE MITOCHONDRIAL GENOME

The mitochondrial genome is believed to have evolved from the symbiotic relationship between aerobic prokaryotic bacteria and anaerobic host cells (DiMauro and Wallace, 1993). This endosymbiotic theory explains the presence of DNA molecules within the mitochondria. Mitochondrial DNA is a covalently closed circular molecule, its physical length is approximately 5μm and it has a molecular mass of 10^7 Da (Bogenhagen and Clayton, 1974; Borst, 1977).

The 16,569 bp circular double-stranded human mtDNA molecule, as depicted in Figure 2.10, is present as multiple copies within each mitochondrion, with single cells harbouring 1,000 – 10,000 mtDNA molecules (Bogenhagen and Clayton, 1974). The two strands of the DNA molecule are referred to as the G-rich, or heavy (H) strand, and the C-rich, or light (L) strand, respectively. Both strands contain genetic information and encode various proteins or RNA molecules. The human mtDNA contains 37 genes, as listed in Table 2.4. Even though the copy number of the mtDNA is high, approximately 10^3 to 10^4 molecules per cell, it comprises less than one percent of the total genomic DNA (Larsson and Clayton, 1995; Berg, 1996).

Figure 2.10: Schematic representation of the mitochondrial genome



The heavy strand encodes all genes indicated on the outside of the genome, the light strand of the mitochondrial genome encodes genes indicated on the inside of the genome. All tRNA genes are indicated by the single letter amino acid abbreviation. A = alanine; C = cysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; 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; L^{UUR} = leucine with anticodon UUR; L^{CUN} = leucine with anticodon CUN; S^{UCN} = serine with anticodon UCN; S^{AGY} = serine with anticodon AGY; cyt b = cytochrome b; D-loop = displacement loop; ND1-6 = NADH dehydrogenase 1-6; CO I-III = cytochrome *c* oxidase I-III; ATP 6 = ATP synthetase subunit 6; ATP 8 = ATP synthetase subunit 8; O_H = heavy strand origin of replication; O_L = light strand origin of replication; P_H = heavy strand promoter; P_L = light strand promoter. Adapted from MITOMAP (2001).

The mitochondrial genetic code differs from the universal nuclear genetic code as indicated in Table 2.5 (Barrell *et al.*, 1979). The differences between the mtDNA code and the nDNA code ensure the incompatibility of the mtDNA genes within the nucleus environment. Therefore, any DNA transfer from the mtDNA to the nucleus would result in non-functional genes (Scriver *et al.* 1995).

Table 2.4: Molecules encoded by the mitochondrial genome

13 structural polypeptide-coding genes
seven subunits of complex I
one subunit of complex III
three subunits of complex IV
two subunits of complex V
22 transfer RNA's (tRNA's) genes
2 ribosomal RNA's (rRNA's) genes

Adapted from Anderson *et al.* (1981).

Table 2.5: Differences between the nuclear genetic code and the mitochondrial genetic code

	Amino acid coded for in different genomes	
Codon	**nDNA**	**mtDNA**
AGA	Arginine	STOP
AGG	Arginine	STOP
AUA	Isoleucine	Methionine
UGA	STOP	Tryptophan

Adapted from Barrel *et al.* (1979) and Anderson *et al.* (1981).

The entire mitochondrial genome was sequenced in 1981 by Anderson *et al.* and this sequence is now known as the Cambridge reference sequence (CRS) and is used as the reference sequence to analyse all mtDNA sequence data generated. However the sequence was reanalysed in 1999 and variations were noted, as listed in Table 2.6 (Andrews *et al.*, 1999). Although there are differences between the CRS and the revised mitochondrial genome sequence it was decided that the substitutions should be corrected, the rare polymorphisms should be retained and that the nucleotide numbering should remain the same (Andrews *et al.*, 1999). The revised CRS belongs to European haplogroup H and would therefore differ significantly when compared to mitochondrial sequence data from an African individual.

Table 2.6: Revision of the Cambridge reference sequence

Nucleotide number	Original sequence	Revised sequence	Remarks
263	A	A	rare polymorphism
311-315	CCCCC	CCCCC	rare polymorphism
750	A	A	rare polymorphism
1438	A	A	rare polymorphism
3106 – 3107	CC	C	error
3423	G	T	error

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Table 2.6: continued ...

Nucleotide number	Original sequence	Revised sequence	Remarks
4769	A	A	rare polymorphism
4985	G	A	error
8860	A	A	rare polymorphism
9559	G	C	error
11335	T	C	error
13702	G	C	error
14199	G	T	error
14272	G	C	error (bovine)
14365	G	C	error (bovine)
14368	G	C	error
14766	T	C	error (HeLa)
15326	A	A	rare polymorphism

Adapted from Andrews *et al.* (1999).

2.3.1 Dual genetic control

Nuclear genes, as well as mitochondrial genes encode for the proteins involved in energy production within the mitochondria. The majority of the subunits of these proteins are encoded for by the nDNA. However, mitochondrial DNA genes generally code for the catalytic subunits, which are functionally important (Schapira and DiMauro, 1994).

The proteins, which are encoded by the nDNA, are translated into precursor proteins, which in turn are transported to the mitochondria where they function together with proteins encoded for by the mitochondrial genome. The precursor proteins are cleaved to form mature proteins upon entrance into the mitochondria. This mechanism allows for mutations in nuclear genes to result in defective mitochondrial metabolism. Due to dual genetic control it is thus possible that DNA mutations in genes segregating in any inheritance pattern - maternal, autosomal, sex-linked or sporadic, can compromise the energy production of the cell (von Kleist-Retzow *et al.*, 1998).

2.3.2 Mitochondrial genome replication, transcription and translation

Transcription and replication of the mtDNA is under control of the displacement-loop (D-loop). This region has been described to often occur as a short three-stranded structure when involved in transcription or replication. The D-loop contains the promoter sequences for both the heavy and light strands of the mitochondrial genome as well as the origin of replication for the heavy strand. During replication the nascent D-loop strand results in the displacement of the parental H-strand (Clayton, 1982; Larsson and Clayton, 1995).

2.3.2.1 Replication of the mitochondrial genome

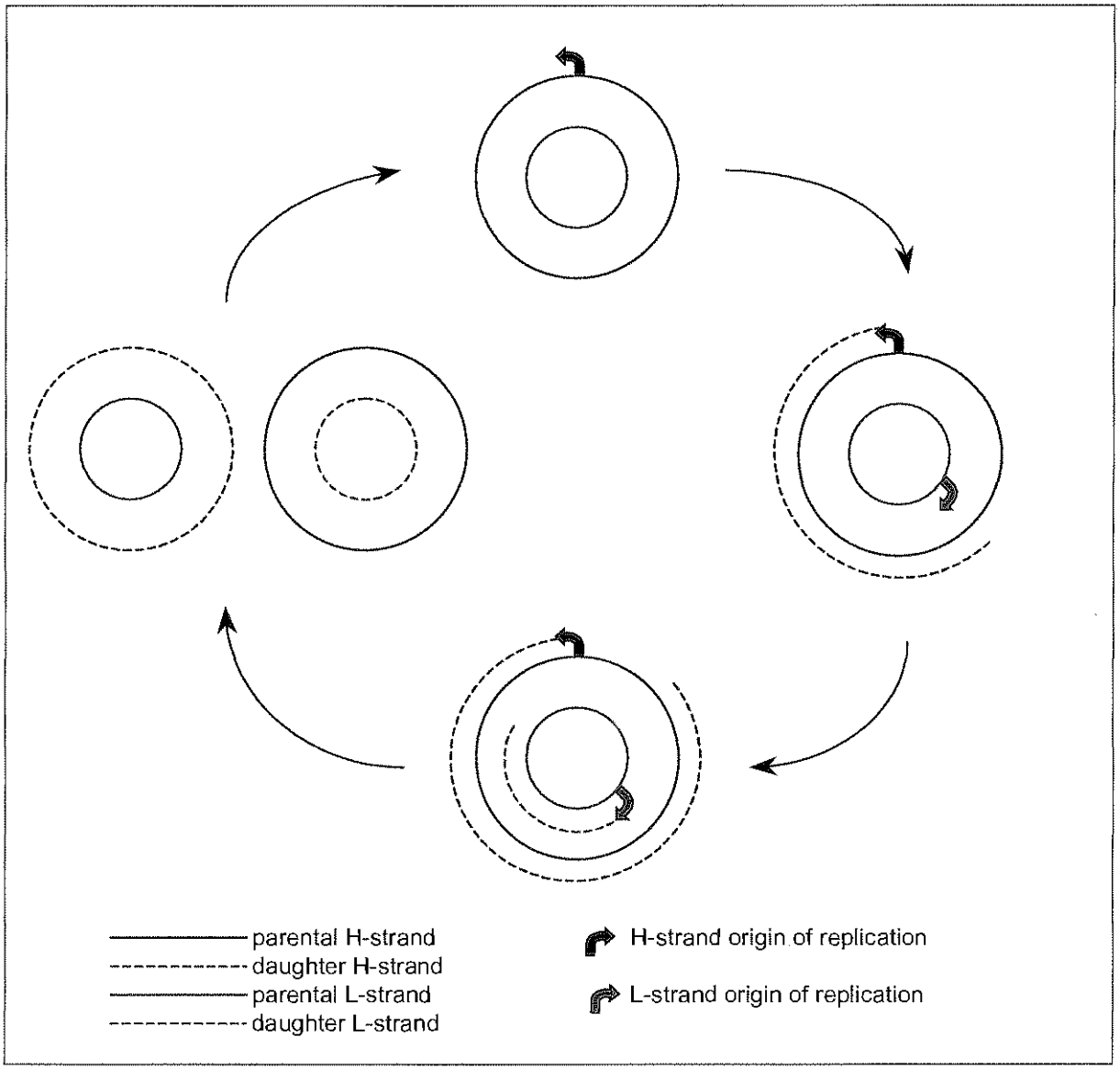
The replication of the mitochondrial genomes within the cells is performed under relaxed control. There is no evidence that each molecule is replicated only once per cell cycle. mtDNA replication is also not restricted to a specific cell cycle phase (Clayton, 1982; Larsson and Clayton, 1995).

The mitochondrial genome has two separate origins of replication, one for the H-strand (O_H) situated within the D-loop and the other for the L-strand (O_L) which is located within a cluster of five tRNA genes (Figure 2.10). In the mitochondria the replication and transcription of the genomes are linked. As mentioned above, transcription of the L-strand initiates replication of the H-strand. This is accomplished by utilising the 5'-end of the L-strand transcript to prime replication (Clayton, 1982). When the L-strand transcript is cleaved the required 3'-OH primer is created for DNA synthesis. A ribonuclear protein enzyme termed RNase maturation of RNA priming (RNase MRP) facilitates this process (Larsson and Clayton, 1995).

Once the priming of DNA synthesis has taken place a DNA gamma polymerase proceeds to synthesise the daughter H-strand across the D-loop initiating replication at the O_H and proceeding along the genome in a unidirectional process. As depicted in Figure 2.11 synthesis of the leading strand continues until two thirds of the genome is complete before exposing the O_L, and initiating replication of the lagging strand (Clayton, 1982; Larsson and Clayton, 1995).

The replication of the H-strand is completed first, followed by the completion of L-strand replication. DNA ligase proceeds to form the closed circular structures of the newly synthesised mtDNA's. At the same time DNA gyrase introduces supercoils into the mtDNA helix (Clayton, 1982; Shoffner and Wallace, 1995a).

Figure 2.11: Schematic representation of replication of the mitochondrial genome



H strand = heavy strand; L strand = light strand. Adapted from Clayton (1982).

2.3.2.2 Transcription of the mitochondrial genome

Transcription of the mtDNA is initiated from the dedicated promoters on each strand, H-strand promoter (HSP) and L-strand promoter (LSP), as illustrated in Figure 2.10 (Clayton, 1984). Both promoters consist of a transcriptional start site of approximately

15 bp and a binding site of approximately 30 bp. The binding site within each promoter region is specific for the mitochondrial transcription factor A (mtTFA) required for initiation of transcription (Shoffner and Wallace, 1995a; Jeong-Yu and Clayton, 1996).

The mtTFA initiates transcription by binding and unwinding the mtDNA at the promoter sites of the respective strands. After initiation, transcription of the mtDNA is performed by a mitochondrial RNA (mtRNA) polymerase. Negative supercoiling is induced by unwinding of the mtDNA which enhances the efficiency of the mtRNA polymerase (Larsson and Clayton, 1995). Subsequent to initiation, each strand of the mtDNA is transcribed by the mtRNA polymerase as a polycistronic RNA molecule (Clayton, 1984).

The HSP is responsible for the transcription of 12 mRNAs, 14 tRNAs and two rRNA molecules (Clayton, 1984). The LSP has two functions. Firstly, it transcribes one mRNA and eight tRNAs (Clayton, 1984). Secondly, it creates transcripts which are then utilised as primers for H-strand mtDNA replication (DiMauro and Wallace, 1993; Larsson and Clayton, 1995).

Termination of transcription occurs at the 5'-end of the $tRNA^{Leu\ (UUR)}$ gene. In this region a tridecamer sequence of 5'-TGGCAGAGCCCGG-3' was recognised by Christianson and Clayton (1988). This sequence was suggested to support the 3'-end formation of the RNA transcript and acts on termination of transcription by binding the mitochondrial termination factor (mTERF). The mTERF binds to the mtDNA, physically blocking the mtRNA polymerase from transcribing any further (DiMauro and Wallace, 1993; Shoffner and Wallace, 1995a).

2.3.2.3 Mitochondrial RNA processing

In 1984 Clayton recognised the necessity of post-transcriptional modifications for the genes expressed by the mitochondrial genome. These modifications were later described by DiMauro and Wallace (1993). It was suggested that there are at least four enzymes involved in the processing of mitochondrial transcripts. Due to the compact nature of the polycistronic transcripts, an RNase P, which is able to recognise and process the tRNA sequences in an exact manner, is required to cleave the transcripts at the 5'-ends of the tRNA sequences, while the 3'-ends are cleaved by a

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3'-endonuclease. The third required enzyme is a poly A polymerase which is responsible for polyadenylation of the mRNAs. The polyadenylation of the mtDNA gene transcripts allows formation of the termination codon for the correct processing of mitochondrial proteins. Finally, a tRNA nucleotidyl transferase is responsible for the addition of the –CCA 3'-ends of the tRNA sequences, as these are not encoded within the mtDNA (DiMauro and Wallace, 1993).

High levels of ATP within the mitochondria stimulate termination of transcription, whereas the transcription of full-length H-strand transcripts is favoured by lower levels of ATP which results in a low rRNA: mRNA ratio (Shoffner and Wallace, 1995a). This would explain one of the reasons for the pathogenicity of mutations within the mitochondrial ATPase 6 gene and the role in the expression of the Leigh Syndrome phenotype. As discussed in section 2.2.3.5 complex V is responsible for the transport of ATP out of the mitochondria. Therefore defective subunits of this complex may influence the efficiency of ATP transport, resulting in the accumulation of ATP within the mitochondrial matrix and in the inappropriate termination of mtDNA transcription. This would subsequently lead to decreased transcription of mitochondrial proteins essential for the correct formation of the complexes of the respiratory chain.

2.3.3 Mitochondrial genetics

The mitochondrial genome is unique in its structure and certain characteristics. This plays an important role in the clinical phenotype of the various mitochondrial disorders, as these characteristics contribute towards the high level of clinical heterogeneity observed in mitochondrial disorders (Berg, 1996).

2.3.3.1 Maternal inheritance

In 1980 Giles *et al.* demonstrated that mtDNA exhibits maternal inheritance. This phenomenon is due to the fact that the midpiece of the spermatozoa, the only part of the sperm which contains mitochondria, does not penetrate the ovum during fertilisation. Therefore all the mitochondria present within the fertilised ovum cell are derived from the ovum itself (Berg, 1996; Hanna and Nelson, 1999).

Hanna and Nelson (1999) predicted that most disorder-associated mtDNA point mutations will be transmitted from a female to all her offspring (male and female), regardless of whether she is clinically affected or not. However, the transmission of a point mutation to her offspring does not imply that all her offspring will be clinically affected. The possibility of developing the disorder is dependent on another unique characteristic of the mitochondrial genome, namely the disease penetrance. The important variables which contribute towards the penetrance of a particular disorder include segregation, heteroplasmy levels and the threshold effect associated with the disorder. Each of these factors are described below.

2.3.3.2 Mitotic segregation

Mitotic segregation refers to the fact that both mtDNA replication and mitochondrial division are independent events which are unrelated to the cell cycle or to the timing of nuclear replication. This implies that during cell division a heteroplasmic cell may donate different proportions of mutant mtDNA to its daughter cells, as depicted in Figure 2.12. This potential increase or decrease in the heteroplasmic level of the new daughter cells can cause a phenotypic change (Wallace, 1992; Berg, 1996).

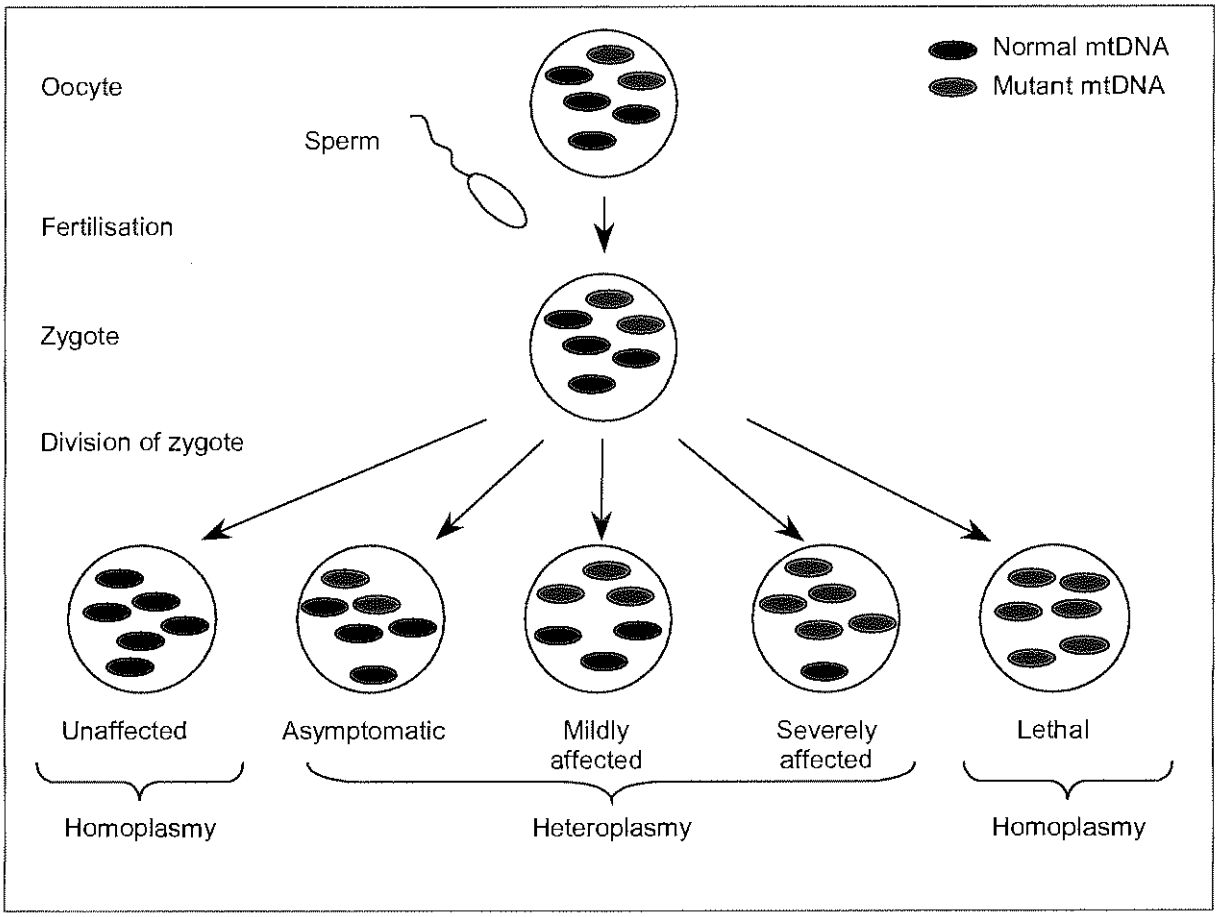
2.3.3.3 Heteroplasmy

Each individual mitochondrion contains two to ten copies of the mtDNA genome, the number depending on the energy demands of the cell in which it is located. Homoplasmy refers to the presence of identical copies of mtDNA, be it normal or affected. In contrast, heteroplasmy is the occurrence of two populations of mtDNA, thus a mixture of normal and mutant mtDNA within the cell.

The percentage of heteroplasmy may vary from greater than 0% to less than 100%. The offspring of a heteroplasmic egg will therefore have certain tissues containing predominantly normal mtDNA, while other tissues and/or organs may contain predominantly affected mtDNA, as illustrated in Figure 2.12. However, different egg cells of a mother may vary dramatically with regard to their heteroplasmic levels. This results in a marked difference between her children with respect to the tissue and/or

organ distribution of affected mtDNA and ultimately the severity and nature of symptoms observed in the phenotype (Wallace, 1997).

Figure 2.12: Schematic illustration of mitotic segregation and heteroplasmy



The presence of heteroplasmy in mtDNA alterations is generally considered to be an indicator of pathogenic mutations. As these severe alterations suppress energy production, the presence of normal mtDNA would be mandatory for the sustainability of life (Wallace, 1997; Hanna and Nelson, 1999). The occurrence of a homoplasmic pathogenic mutation would result in a profound reduction of energy, believed to be incompatible with life (Wallace, 1997; Hanna and Nelson, 1999).

The levels of heteroplasmy may in some instances be directly correlated with the severity of the disorder and also the age of onset. In 1993 DiMauro and Wallace described a direct correlation between higher heteroplasmic levels of the mutant mitochondrial genome and the severity of the observed clinical phenotype. This is especially true for the T8993G mutation associated with both Leigh Syndrome and the NARP phenotype. It has been established that when high concentrations, above 90%,

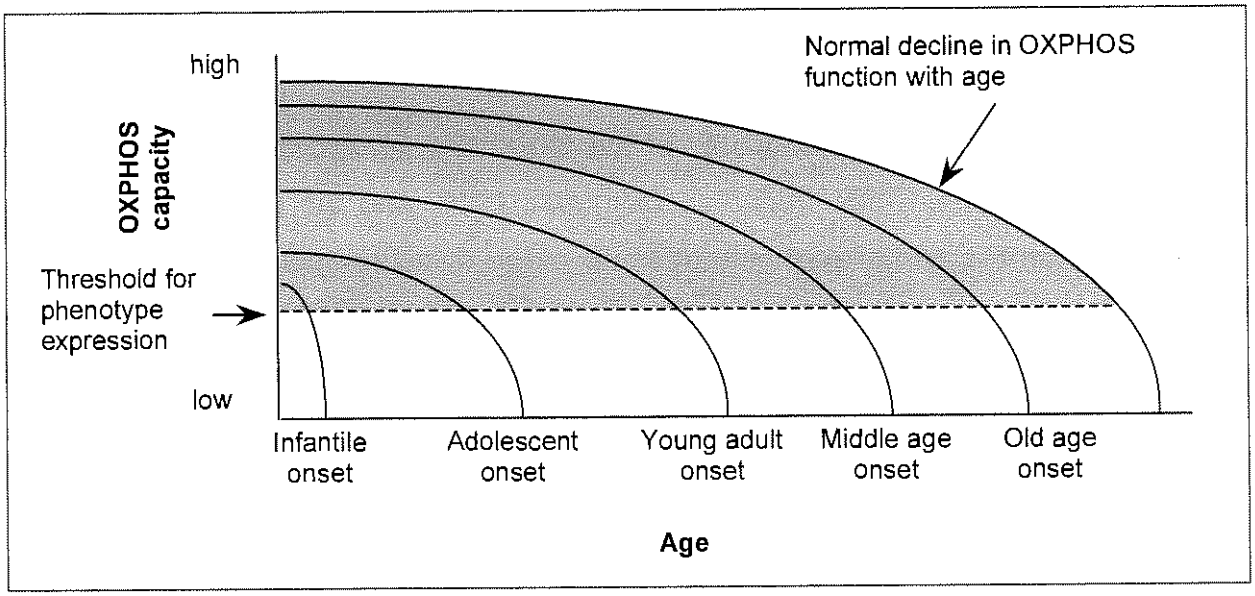
of this mutation are present the LS phenotype is expressed. Lower levels of the mutation result in the NARP phenotype (Tatuch *et al.*, 1992).

2.3.3.4 Threshold effect

The threshold effect of mutated mtDNA is one of the most important determinants of the severity of mitochondrial disorders. It refers to the critical proportion of mutant genomes which must be exceeded to induce presentation of the clinical phenotype. The threshold effect is therefore dependent on the minimum number of mutant mtDNA's required to cause mitochondrial dysfunction in a particular organ, tissue, or cell type, as well as the severity of the OXPHOS defect (Wallace, 1992; Berg, 1996).

Figure 2.13 illustrates the effect of the threshold level on phenotypic expression. The shaded portion of the graph indicates the levels of ATP generation that is able to sustain normal cellular function. The right hand curve represents normal OXPHOS decline while the other five curves to the left denote the decrease in the OXPHOS capacity with mtDNA mutations.

Figure 2.13: Threshold effect of mutant mitochondrial DNA with respect to phenotype expression



OXPHOS = oxidative phosphorylation. Adapted from Schapira and DiMauro (1994).

The heteroplasmy levels of the mutations that are present also play an important role in the attainment of the minimum OXPHOS capacity that is required. Therefore,

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heteroplasmy levels together with the energy threshold levels of particular organs as well as the normal age-related OXPHOS decline all contribute towards expression of a mitochondrial disorder (Schapira and DiMauro, 1994).

2.3.3.5 Mutation rate

The mutation rate of mtDNA is much higher than that of nDNA, evolving 10 to 20 times faster than nDNA (Wallace, 1992). A combination of factors is responsible for this increase in damage to the mitochondrial genome. The mitochondrial genome is an open circular molecule and lacks the protective histones surrounding the nuclear chromosomes. The inefficiency of the mtDNA repair system (discussed below) also plays an important role in the high mutation rate of the mtDNA.

When compared to the nuclear repair pathways that exist the mitochondria have no pathway for nucleotide excision repair (NER). The major repair pathway in the mitochondria is the base excision repair (BER) pathway. The two pathways differ in the former, NER, being a proof reading function of the transcription machinery while the latter, BER, repairs after transcription is completed (Croteau *et al.*, 1999). The lack of introns and the physical proximity of the mtDNA to the respiratory chain within the central matrix, where relatively high levels of oxygen radicals are physiologically produced, also contribute to the raised mutation rate of the mtDNA. (Wallace, 1992).

The clinical phenotype and the severity of the mitochondrial disorder result from the type of mtDNA mutation, the proportion of mutant DNA, and the tissue distribution. Therefore the genetic and clinical heterogeneity of the mitochondrial disorders may be ascribed to the above mentioned characteristics of the mitochondrial genome.

CHAPTER THREE

THE AETIOLOGY OF MITOCHONDRIAL MYOPATHIES

The first human ailment linked to alterations within the mitochondrial genome was only described in 1988 by Wallace *et al.* (1988a). This group was the first to demonstrate that Leber's hereditary optic neuropathy (LHON) resulted from a mutation in the mitochondrial genome. Since then numerous researchers have illustrated the role of the mitochondrial genome in progressive muscle disorders.

3.1 MITOCHONDRIAL MYOPATHY DISEASE CLASSIFICATION

The metabolic and particularly the OXPHOS disorders are most effectively distinguished when classified according to their genetic aetiology rather than on a clinical or biochemical level. In 1995 Manfredi *et al.* divided the mutations responsible for mitochondrial myopathies into two distinct groups, those affecting general mitochondrial protein synthesis and mutations affecting specific respiratory chain complexes. Since then these disorders have been classified according to the type of mutation that was responsible for the observed phenotype. Three subsets of mutations, listed below, have been described to be causative of mitochondrial myopathies (DiMauro, 1996a; Shoubridge, 1998):

- Subset I – mtDNA mutations

  These mutations occur within the mitochondrial genome. Mutations in mtDNA generally cause functional deficiencies in the respiratory chain and these mutations manifest in tissues with high-energy demands. Examples of these mutations are discussed in sections 3.3.3.2, 3.4.2 and 3.5.3, with respect to the disorders they have been associated with.

- Subset II – nDNA mutations

  The second subset of causative mitochondrial myopathy mutations occurs within nDNA genes coding for mitochondrial components, and result in defective oxidative phosphorylation. The genes affected in this subset generally encode for

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structural subunits of the respiratory chain complexes or factors that may be involved in the assembly or maintenance of the complexes within the mitochondrial respiratory chain. Examples of this class of mutations are those discussed in section 3.3.3.3 associated with the expression of Leigh Syndrome phenotype.

- Subset III

  The mutations in this subset are those which result in defective intergenomic communication. These may result in the altered control of translation or expression of mtDNA, ultimately resulting in multiple mtDNA deletions or mtDNA depletion within specific tissues, as discussed in section 3.7.

3.2 CLINICAL PRESENTATION OF MITOCHONDRIAL MYOPATHIES

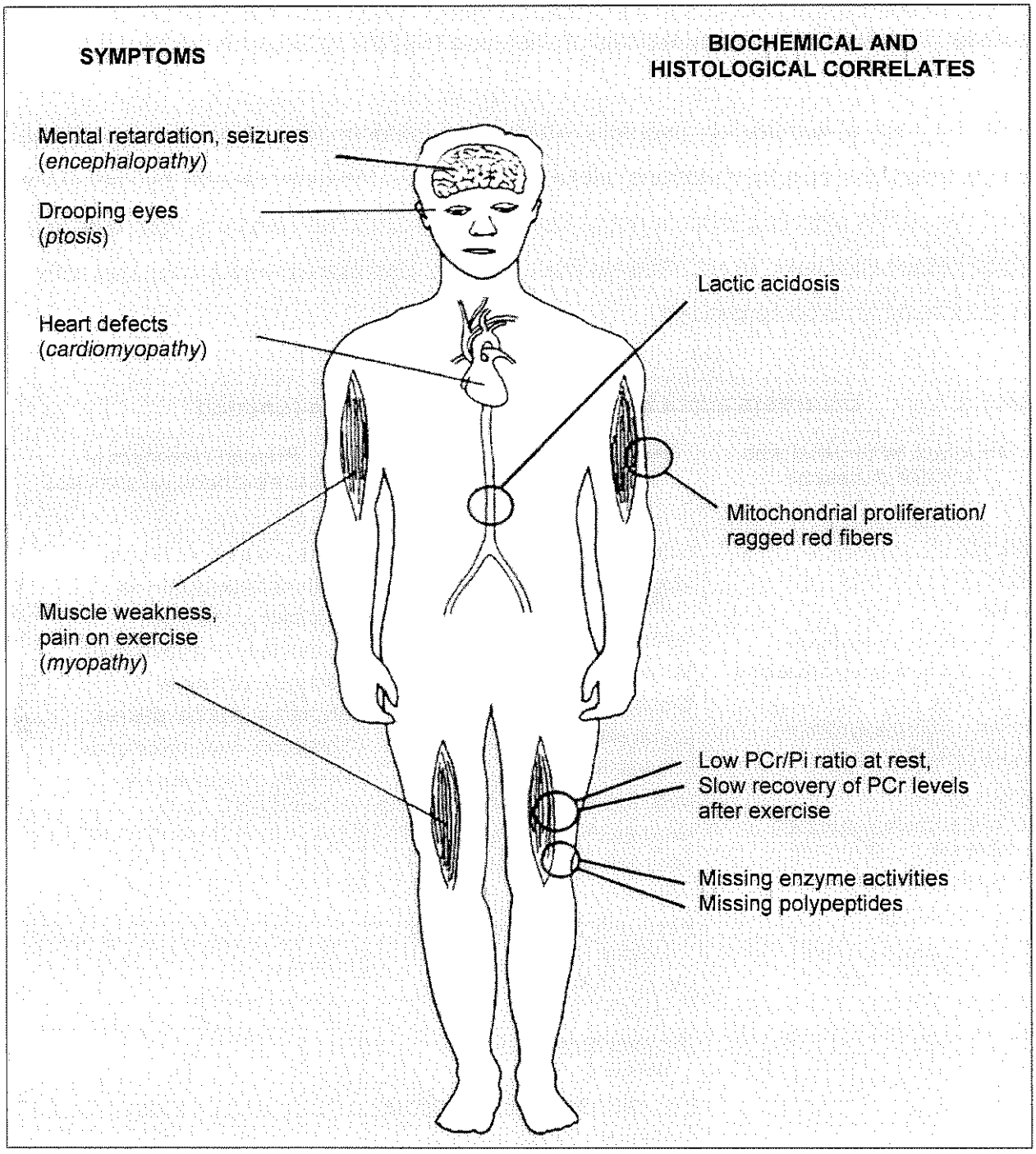
The clinical phenotypes of these disorders are highly variable, with the resulting signs and symptoms being indicative of, but not exclusive to, a particular syndrome. The variability is dependent on the percentage of heteroplasmy present in particular tissues, this being a phenomenon that is unique to the mitochondrial genome, as discussed in section 2.3.3.3. DiMauro and Wallace (1993) described a direct correlation between higher heteroplasmic levels of the mutant mitochondrial genome and the severity of the observed clinical phenotype.

The exclusiveness of specific mitochondrial mutations in causing distinctive phenotypes has been questioned since the early 1990's. In 1992 Silvestri *et al.* described this as being a result of three phenomena that occur. The first is that there are some typical clinical cases, which do not harbour the characteristic mutation. *Vice versa*, the same genetic alteration has been observed in individuals with different clinical phenotypes. Lastly, overlap phenotypes exist, as discussed in section 3.6, in which patients exhibit clinical features of two syndromes but harbour the characteristic mutation of only one (Silvestri *et al.*, 1992).

The phenotype of the particular mitochondrial disorder presents at the clinical level when the individual's mitochondrial ATP-generating capacity declines, resulting in progressive involvement of the CNS, the skeletal muscles, and the heart (Wallace *et al.*, 1988b). The involvement of multiple systems is a hallmark of mitochondrial disorders

and is illustrated in Figure 3.1. The progression of these disorders is also related to the tissue-specific energetic thresholds of the organs or tissue (Shoffner *et al.*, 1990).

Figure 3.1: Schematic representation of the clinical effects defective mitochondria might have



Adapted from Harris (1995).

It has been established that the mitochondrial disorders follow a maternal inheritance pattern and therefore all progeny have an equal chance of being affected (Giles *et al.*, 1980). In MELAS the majority of the mtDNA mutations are single base changes and lie within the mitochondrial $tRNA^{Leu(UUR)}$ gene. The most common point mutations

associated with MERRF syndrome are localised to the mitochondrial $tRNA^{Lys}$ gene while the more rare mutations associated with this disorder occur within the mitochondrial $tRNA^{Leu(UUR)}$ gene. The point mutations associated with MELAS and MERRF are heteroplasmic and there is considerable clinical overlap between the two disorders. Point mutations within the ATPase 6 gene of the mtDNA are associated with neurogenic atrophy, ataxia and retinitis pigmentosa (NARP) or Leigh Syndrome (LS).

Table 3.1 lists a number of disorders which have mitochondrial involvement. These include disorders due to mutations within the mtDNA, disorders caused by mutations within the nDNA affecting mitochondrial function, and disorders resulting from defective mitochondrial enzymes. In all of these disorders the effective energy production within the mitochondria is compromised.

Table 3.1: Disorders associated with mitochondrial dysfunction

Disorders associated with mtDNA mutations	Mendelian (nDNA) disorders involving mitochondrial function	Primary disorders of intramitochondrial enzymes
MELAS	Luft disease	Methylmalonic acidaemia
MERRF	Leigh Syndrome (complex I, COX, PDH)	Erythropoietic porphyria
NARP	Alpers disease	Propionic acidaemia
Myoneurogastrointestinal disorder and encephalopathy (MNGIE)	MCAD, SCAD, VLCAD	Acute intermittent porphyria
Pearson Marrow syndrome	Glutaric aciduria II	Variegate porphyria
Kearns-Sayre-CPEO	Lethal infantile cardiomyopathy	Maple syrup urine disease
LHON	Friedreich ataxia	Nonketotic hyperglycinemia
Aminoglycoside-associated deafness	Maturity onset diabetes of young	Hereditary sideroblastic anaemia
Diabetes with deafness	Malignant hyperthermia	
Leigh Syndrome	Disorders of ketone utilisation	
	mtDNA depletion syndrome	
	Reversible COX deficiency of infancy	
	Various defects of the Krebs cycle	
	Pyruvate dehydrogenase deficiency	
	Pyruvate carboxylase deficiency	
	Fumarase deficiency	
	Carnitine palmitoyl transferase deficiency	

MELAS = mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes; MERRF = myoclonic epilepsy and ragged red muscle fibres; NARP = neurogenic ataxia and retinitis pigmentosa; MNGIE = myoneurogastrointestinal disorder and encephalopathy; CPEO = chronic progressive external ophthalmoplegia; LHON = Leber's hereditary optic neuropathy; COX = cytochrome c oxidase; PDH = pyruvate dehydrogenase; MCAD = medium chain acyl-CoA dehydrogenase; SCAD = short chain acyl-CoA dehydrogenase; VLCAD = very long chain acyl-CoA dehydrogenase. Adapted from Naviaux (1997).

Although there are many disorders associated with defects of the mitochondria, the effects of mitochondrial mutations are still being investigated and some of the fundamental pathways that are affected still need to be elucidated. However, the final outcome of the various mutations is a defective OXPHOS system which culminates in deficient energy production within cells and organs to various levels with regard to particular mutations. The expression of the phenotype within the affected individual is the result of this loss of energy production within the cells. From this it is understandable that patients who are born with mutations in the mtDNA begin life with decreased mitochondrial OXPHOS levels when compared to normal individuals. As they continue with life the residual OXPHOS capacity decreases with age as in all mammalian cells. However, as illustrated in Figure 2.13, in patients the organ specific energy thresholds are crossed at an earlier stage than in unaffected individuals – hence the expression of the mitochondrial myopathy phenotypes we observe in the paediatric population (Shoffner *et al.*, 1990).

3.3 LEIGH SYNDROME

This neurodegenerative disorder of infancy or childhood was first described by Denis Leigh in 1951 and is both clinically and genetically heterogeneous. LS is a fatal multisystem disorder in which the clinical phenotype is dominated by involvement of the CNS with a spectrum of neurological signs (Santorelli *et al.*, 1993; Schapira, 1994). The LS phenotype can segregate via three distinct modes of inheritance: autosomal recessive (Loeffen *et al.*, 1998), X-linked (Brown *et al.*, 1989) and maternally inherited with mutations in the mtDNA (Holt *et al.*, 1990; de Vries *et al.*, 1993; Thyagarajan *et al.*, 1995). The complexity of the aetiology of LS is illustrated in Figure 3.2. Due to the heterogeneous factors which account for LS, it is important that the mode of inheritance and the specific mutations present within the patients be identified. This allows for the correct prognostic report and appropriate counselling of the families involved.

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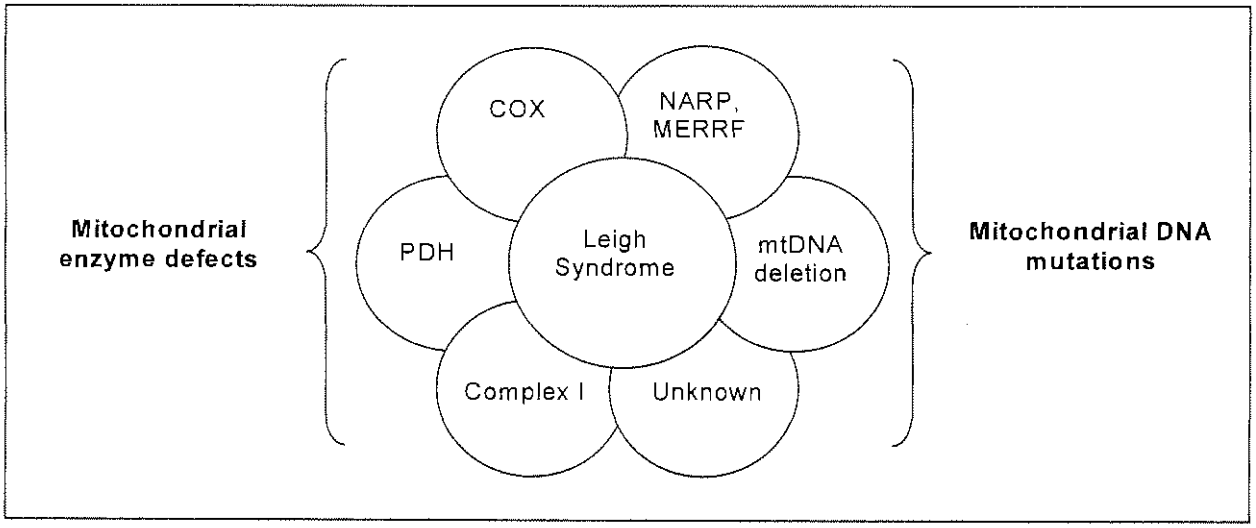
Figure 3.2: Schematic representation of the aetiology of Leigh Syndrome



COX = cytochrome c oxidase; PDH = pyruvate dehydrogenase; NARP = neuropathy, ataxia and retinitis pigmentosa; MERRF = myoclonus epilepsy and ragged red muscle fibres. Adapted from Naviaux (1997).

3.3.1 The clinical phenotype of Leigh Syndrome

LS is a devastating encephalopathy occurring during infancy or childhood with psychomotor regression, brainstem abnormalities and lactic acidosis (Santorelli *et al.*, 1993). Table 3.2 highlights the clinical features which may be observed in patients affected with LS or with the milder phenotype of NARP.

NARP was described by Holt *et al.* in 1990, as a maternally inherited disorder, associated with a point mutation in the mitochondrial ATPase 6 gene. In the family reported on by Holt *et al.* (1990), a positive correlation between clinical severity and the number of mutant mitochondria and thus the level of heteroplasmy, was observed. Genotype-phenotype correlation for NARP has subsequently been confirmed in an independent study (Tatuch *et al.*, 1992). When the heteroplasmic levels of a NARP mtDNA mutation exceed 90% in the affected tissue, a severe form of this disorder is presented, known as LS.

Table 3.2: Clinical features associated with NARP versus Leigh Syndrome

NARP	Leigh Syndrome	
Sensory neuropathy	**Onset:** first year, occasionally later	**Hypotonia**
Cerebellar ataxia	**Episodic:**	**Encephalopathy:**
Retinitis pigmentosa	vomiting	loss of verbal milestones
CNS:	ataxia	**Motor:**
dementia	choreathetosis	spasticity
seizures	hyperventilation	abnormal breathing rhythm
developmental delay	**CNS:**	**Ophthalmologic:**
proximal weakness	Brainstem: hearing loss	visual loss
serum lactate: normal	basal ganglia: dystonia	ophthalmoplegia
	cerebellar: ataxia, nystagmus	**CNS pathology:**
	Lactate: high in CSF > blood	focal, bilaterally symmetric spongiform lesions
	Muscle biopsy:	location: thalamus and brain stem
	normal histology	MRI: symmetrical hyperintense lesions on T2
	COX deficiency: all subunits	**Course:**
	Clinical signs manifesting with stress:	progression: motor and intellectual regression
	intercurrent infection	death often within two years of onset
	carbohydrate intolerance	

CNS = central nervous system; COX = cytochrome *c* oxidase; CSF = cerebrospinal fluid; MRI = magnetic resonance imaging. Adapted from Neuromuscular: Mitochondrial Disorders (2001).

The clinical diagnosis of LS can be confirmed by utilising magnetic resonance imaging (MRI) to demonstrate symmetric abnormalities in the basal ganglia (Davis *et al.*, 1987). In addition, the presence of retinitis pigmentosa (RP) is a useful diagnostic tool for distinguishing the molecular aetiology of LS, because it is not observed in LS due to pyruvate dehydrogenase deficiencies or COX deficiency. However, RP is most commonly associated with LS due to the mtDNA T8993G mutation (Santorelli *et al.*, 1993; Berg, 1996).

3.3.2 The biochemical basis of Leigh Syndrome

The aetiology of LS can be ascribed to deficiencies within the respiratory chain complexes I, II and IV. Defects within the E1α subunit of the PDHC and the

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mitochondrial ATPase subunit 6 have also been ascribed to the clinical expression of Leigh Syndrome (Shoffner *et al.*, 1992; DiMauro and DeVivo, 1996b).

The last component of the respiratory chain, complex IV, catalyses the transfer of reducing equivalents from cytochrome *c* to molecular oxygen (Figure 2.5). Energy, which is released during this exergonic reaction, maintains the transmembrane proton gradient, which is utilised by the complex V to generate ATP (Schapira *et al.*, 1994). When the integrity of the mitochondrial genes coding for these complexes are compromised by mutations, the functioning of the complex is defective, resulting in LS.

3.3.3 The genetic basis of Leigh Syndrome

LS is a highly heterogeneous disorder on the clinical as well as the genetic level (DiMauro and DeVivo, 1996b). The associated defects occur within the enzymes which are involved in aerobic energy metabolism within the mitochondria. Although this mechanism takes place within the mitochondria itself, not all the genetic alterations are based in the mitochondrial genome, as mutations in many nuclear encoded mitochondrial proteins may also result in compromised energy production via the respiratory chain. Therefore, it is noted that all defects associated with LS result in an impaired energy production by affecting terminal oxidative metabolism. It is the effect of this impaired metabolism on developing brains that results in the neuropathological presentations associated with LS (DiMauro and DeVivo, 1996b).

3.3.3.1 Genetic heterogeneity in Leigh Syndrome

The genetic heterogeneity of LS is due to defects within a variety of genes, such as the mitochondrial encoded ATP6 subunit of the ATP synthase complex (Holt *et al.*, 1990; de Vries *et al.*, 1993; Thyagarajan *et al.*, 1995), the E1α subunit of the PDHC localised to the X chromosome (Brown *et al.*, 1989), complex I of the respiratory chain (Loeffen *et al.*, 1998), and the surfeit 1 (SURF-1) gene which is involved in assembly of the cytochrome *c* oxidase (COX) complex (Tiranti *et al.*, 1998; Zhu *et al.*, 1998), as illustrated in Table 3.3. LS has also been associated with a mutation in the tRNA genes of the mitochondrial genome as well as a mutation in the nuclear encoded flavoprotein (F_P) subunit of the succinate dehydrogenase (SDH) complex (Bourgeron *et al.*, 1995).

Table 3.3: Genetic heterogeneity of Leigh Syndrome or Leigh-like disorders

Defect	Frequency[1]	Subunit(s) of affected genes
mtDNA	18 %	ATPase (T8993G/C, T9176C) $tRNA^{Lys}$ (A8344G)
PDHC	10 %	E1α subunit
Complex I	19 %	NDUFS8
COX	14 %	SURF-1
Other	39 %	flavoprotein subunit of complex II mtDNA depletion unidentified loci

PDHC = pyruvate dehydrogenase complex; COX = cytochrome c oxidase; E1α = pyruvate decarboxylase alpha; NDUFS8 = NADH-ubiquinone oxidoreductase FE-S protein 8; SURF-1 = surfeit locus 1. 1 = Rahman *et al.* (1996). Adapted from Dahl (1998).

3.3.3.2 Mitochondrial mutations associated with Leigh Syndrome

As illustrated by Table 3.3 mutations within the mtDNA are responsible for 18% of LS cases (Rahman *et al.*, 1996). The mitochondrial mutations responsible for LS can be classified as missense mutations in which a nucleotide base is changed in the mtDNA leading to the insertion of a different amino acid during translation which in turn compromises the resulting protein's function. The majority of these mutations occur within the mitochondrial ATPase 6 gene. Four missense mutations within this gene have been reported to date, and affect the production of ATP via complex V to various extents (Holt *et al.*, 1990; de Vries *et al.*, 1993; Campos *et al.*, 1997 and Carrozzo *et al.*, 2001).

The most prevalent mutation which affects the ATPase 6 gene is the T8993G mutation (Holt *et al.*, 1990; Santorelli *et al.*, 1993). The other three mitochondrial mutations, T8993C (de Vries *et al.*, 1993), T9176C (Thyagarajan *et al.*, 1995; Campos *et al.*, 1997) and T9176G (Carrozzo *et al.*, 2001), affect the production of ATP via complex V of the electron transport system but to a much milder extent than the T8993G mutation. Other mitochondrial mutations have been associated with various Leigh-like syndromes, these mutations have been observed to be localised to the tRNA genes of the mitochondrial genome. However, the A8344G mutation, most commonly associated with the MERRF phenotype (Shoffner *et al.*, 1990), has also been implicated in LS (Howell *et al.*, 1996).

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3.3.3.2.1 The T8993G mutation

The T8993G mutation was first reported by Holt *et al.* in 1990, and since then it has been recognised to be the mtDNA mutation most frequently associated with LS (Santorelli *et al.*, 1993). The authors described the presence of the mutation in a family with variable clinical phenotypes with a combination of neurological symptoms, including retinitis pigmentosa. The reported family consisted of three generations, in whom the mutation could be detected and in which maternal inheritance was observed (Holt *et al.*, 1990).

The onset of LS as a result of the T8993G mutation is from four and five months of age. The most distinguishing features of the clinical phenotype of this mutation is that 33% of individuals are affected with retinitis pigmentosa or optic atrophy, and 67% of the patients are affected by seizures. This mutation is associated with the most severe form of LS (Santorelli *et al.*, 1993), and also with the NARP phenotype. However, the onset of NARP is generally in the young adult years and the course is slowly progressive compared to LS, which is fatal at an earlier age (Santorelli *et al.*, 1993). In 1992 Tatuch *et al.* demonstrated that a high concentration of mutant mtDNA, affecting more than 90% of the mitochondrial genomes, is responsible for the more severe clinical phenotype of LS.

The thymine to guanine substitution at position 8993 in the mtDNA results in an amino acid change from a hydrophobic leucine to a hydrophilic arginine. The amino acid change is at position 156, near to the c-terminal of the ATPase 6 subunit of the ATP synthase complex (Holt *et al.*, 1990; Tatuch *et al.*, 1992). The leucine codon (CUN), where N represents any of the four bases, is highly conserved across species and occurs within the consistent hydrophobic region of the polypeptide. The authors thus suggested that the substitution of leucine for arginine at this position would affect the structure and function of the ATP synthase holoenzyme (Holt *et al.*, 1990). This thought was substantiated by the investigation of Tatuch *et al.* in 1992.

The F_0 fraction spans the inner mitochondrial membrane (Figure 2.9) in such a manner that subunit-a (encoded by the ATPase 6 gene) has five membrane-spanning helices. These function in alliance with ATPase 9 forming a channel, admitting incoming protons. Both subunits have an internal helix which is crucial for the mechanism of accepting protons effectively. The fourth helix of subunit-a (amino acids 142 – 166 of

the polypeptide) and the second helix of the ATPase 9 subunit (amino acids 42 – 56 of the polypeptide) have been identified as those helices responsible for the acceptance of protons within the respective subunits. The ATPase 9 subunit has a negatively charged glutamate, localised within the channel formed by the two subunits, which functions to absorb the proton energy when ascending through the channel (Tatuch *et al.*, 1992).

The T to G missense mutation at nucleotide position 8993 changes a conserved leucine to a positively charged arginine within subunit-a. This amino acid alteration changes the charge distribution within the channel, potentially interfering with both the entry of protons and ATP formation. The addition of this charged group in the proton channel inhibits the synthesis of ATP (Tatuch *et al.*, 1992; Santorelli *et al.*, 1993). The T8993G mutation in the ATPase 6 gene therefore leads to the energy deprivation and neuronal death that give rise to the symptoms and pathology associated with LS.

The causative nature of the T8993G mutation was affirmed by the presence of heteroplasmy for the mutation, exhibiting a strong correlation between the proportion of mutant mtDNAs and the presence and severity of the neurological disorder observed in the pedigree (Holt *et al.*, 1990). The inheritance pattern observed in the family was also discerned to be a maternal inheritance pattern, an important factor when establishing the aetiology of this disorder.

Since the initial report of the T8993G alteration associated with the NARP and the more severe LS phenotype, many more families were investigated and reported on. However, the clinical features of the patients reported on were highly variable. In 1994 Fryer *et al.* reported on a family which presented with variable neurological symptoms including cerebral palsy. The authors suggested that this alteration should be considered not only in patients with LS (based on autopsy reports) but also in patients with mild neurological dysfunction when pigmentary retinopathy or ophthalmological diseases are described in the family history (Fryer *et al.*, 1994).

The clinical variability of the signs due to the T8993G mutation is not attributed to a variance in the heteroplasmy levels between different tissues. Santorelli *et al.* (1993) demonstrated in 12 LS patients harbouring the T8993G mutation, that the alteration was present in comparable portions in all tissues investigated, thus implying that the varied tissue involvement is due to the various tissue-specific thresholds present for clinical expression. These authors also noted that for the 12 patients, the average

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heteroplasmic levels were 92%, while the proportion of mutant genomes was considerably lower (41-79%) in asymptomatic maternal relatives (Santorelli *et al.*, 1993).

3.3.3.2.2 The T8993C mutation

The T8993C mutation was first reported by de Vries *et al.* in 1993. The inheritance pattern for the LS phenotype was initially considered to be autosomal recessive in the family described by the authors. However, upon further investigation subtle signs were observed in the mother, leading to the identification of a maternal inheritance pattern and the detection of a mitochondrial alteration responsible for the presenting phenotype (de Vries *et al.*, 1993).

The nucleotide alteration at position 8993 of the mtDNA, results in the amino acid change of a leucine being replaced by a proline within the highly conserved helical domain of subunit-a of the ATPase complex, as discussed in section 3.3.3.2.1. This change of amino acid is thought to alter the functioning of the proton channel so that optimal proton translocation is no longer achieved. However, the T8993C mutation does not result in complete failure of the channel, only in decreased efficiency (de Vries *et al.*, 1993).

Pathogenesis for this change was reported by de Vries *et al.* (1993) as well as by Santorelli *et al.* (1994). Both authors observed the mutation in a family, with probands being the most severely affected. Presenting individuals had higher heteroplasmic levels of the mutation in all tissues investigated when compared to that of their maternal relatives. The phenotype resulting from this mutation is much milder than that of the 8993G mutation. The average age of onset is recognised to be four years, with the progression of the disorder being much milder and slower than in individuals harbouring the T8993G mtDNA mutation (Santorelli *et al.*, 1994; Rahman *et al.*, 1996).

3.3.3.2.3 The T9176C mutation

Thyagarajan *et al.* (1995) were the first authors to report this alteration within the ATPase 6 gene of the mtDNA, however, it was not directly associated with LS but with a Leigh-like disorder. It was only in 1997 that the T9176C mutation was confirmed as being associated with Leigh Syndrome, in a report by Campos *et al.* (1997).

The two individuals investigated in the study reported by Thyagarajan *et al.* (1995), presented with bilateral striatal necrosis, but did not display progression of the phenotype, which is characteristic of LS. However, the T9176C mutation observed in the family was reported to be pathogenic as it was heteroplasmic and the proportion of mutant mtDNAs corresponded to the clinical severity observed in the patients (Thyagarajan *et al.*, 1995). Based on autopsy results, the patient reported by Campos *et al.* (1997) had symptoms characteristic of LS. His mother had mild cerebellar atrophy that was observed on MRI, while the other individuals of the family were asymptomatic (Campos *et al.*, 1997).

The T to C transition alters a highly conserved leucine amino acid, which was demonstrated to be conserved across species. The nucleotide change results in the replacement of the leucine amino acid with a proline at codon 270 of the ATPase 6 subunit of ATP synthase. This amino acid is located near the carboxyl end of the ATPase 6 protein and is considered important for proton translocation (Thyagarajan *et al.*, 1995).

The clinical presentation is only observed in individuals with heteroplasmic levels greater than 98%, indicating a threshold effect for this mutation (Thyagarajan *et al.*, 1995; Campos *et al.*, 1997). This was clearly evident in the family described by Campos *et al.* (1997), where the proband and four maternal relatives were screened for the T9176C mutation. The proband was reported to have almost 99% mutant mtDNA, while the patient's mother harboured 93% mutant mtDNA. One of the remaining relatives analysed was shown to harbour 88% heteroplasmy but to be asymptomatic for LS (Campos *et al.*, 1997).

3.3.3.2.4 The T9176G mutation

The T9176G mutation occurring within the ATPase6 gene of the mitochondrial genome was reported by Carrozzo *et al.* in 2001. The alteration was observed in two siblings who demonstrated classical LS features. The presence of the mutation was observed in both siblings as well as maternal relatives and a correlation was noted between the severity of the clinical signs and the percentage of mutant mitochondria (Carrozzo *et al.*, 2001).

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On further investigation the authors demonstrated the importance of this alteration in causing the phenotype by illustrating that all complexes of the OXPHOS pathway were assembled normally except for complex V. This data confirmed the importance of the leucine 217 residue within the complex (Carrozzo *et al.*, 2001). There is still debate around the topic of whether the mutation affects the assembly or functioning of the complex, although it is clear that interference with the energy-driven mechanism of ATP synthesis does occur by decreasing the efficiency of the proton pump.

The clinical features of the T9176G mutation are more severe and debilitating than those observed in patients harbouring the T9176C alteration. It was therefore suggested that, as in the case of the alterations at the nucleotide position 8993, the T to C alteration in both cases has a milder phenotypic effect than the T to G change (Campos *et al.*, 1997; Carrozzo *et al.*, 2001).

3.3.3.2.5 The A8344G mutation

This mutation localised to the mitochondrial tRNA lysine gene is more commonly observed in association with the MERRF phenotype, discussed in paragraph 3.5.3.1. However, in 1996 Howell *et al.* demonstrated that this mutation was responsible for the LS phenotype presenting in the family under investigation. The family exhibited maternal inheritance with diverse neurological abnormalities, the most profound of these being spinocerebellar degeneration and Leigh disease. There was no indication of myoclonus or seizures, thus resulting in an atypical MERRF description.

On molecular analysis Howell *et al.* (1996) observed the presence of the A8344G mutation as the only mtDNA alteration in this family. The mutation was present in three members of the family investigated, while autopsy reports confirmed LS in a further two young individuals who were deceased at the time of the study. The percentage of the mutation varied in the three individuals investigated and correlated positively with the severity of their respective phenotypes.

This report indicated that the A8344G mutation is a causative mutation within the mtDNA which results in a spectrum of neurological manifestations. This mutation is more commonly associated with the typical MERRF phenotype but may have

presentations which are atypical. The authors thus postulated that the A8344G mutation is associated with, but not exclusive to, the MERRF phenotype (Howell *et al.*, 1996). This observation was confirmed by Santorelli *et al.* in 1998, where these authors also demonstrated the presence of the A8344G mutation in a family with LS.

The family reported by Santorelli *et al.* (1998) had previously been reported to have an autosomal dominant trait with atypical association of Friedreich's ataxia and LS. On re-evaluation the deficient mitochondrial function was noted and the samples were re-investigated from paraffin-embedded sections. The investigation revealed high levels (92%) of the mutant mitochondria with the presence of the A8344G mutation in the proband. Unfortunately none of the family members could be re-evaluated, but the aetiology of the disorder observed in the proband clearly resulted from the pathophysiology of the A8344G mutation (Santorelli *et al.*, 1998).

3.3.3.3 Nuclear DNA loci associated with Leigh Syndrome

To date, five nuclear loci have been associated with LS. The majority of the subunits making up the OXPHOS system are encoded by nuclear genes. In LS autosomal recessive and X-linked inheritance patterns have been observed for the mutations within these loci as summarised in Appendix A, Table A.7.

3.3.3.3.1 Cytochrome *c* oxidase (COX) deficiency

According to Tiranti *et al.* (1998) cytochrome *c* oxidase deficiency associated with LS is one of the most common mitochondrial respiratory chain defects which occur in infancy or childhood. This form of LS follows an autosomal recessive pattern of inheritance. Until 1998 no mutations had been described for COX deficiency. In 1998 the SURF-1 gene locus associated with this disorder was identified (Tiranti *et al.*, 1998). Within this gene a number of mutations, as listed in Table 3.4, have since been described, by various authors, to be causative of this form of autosomally inherited LS. According to Tiranti *et al.* (1998) the disease frequency of autosomal recessive COX deficiency is 1 in 100,000.

The clinical features of COX deficiency are similar to that of LS; early onset, rapidly progressive psychomotor regression, generalised hypotonia, truncal ataxia,

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ophthalmoparesis, irregular hyperapnea and high lactate levels. MRI scans reveal symmetric lesions scattered from the basal ganglia to the brain stem. Death usually results from central ventilatory failure (Neuromuscular: Mitochondrial Disorders, 2000).

a) The SURF-1 locus on chromosome 9q34

The SURF-1 gene was described by Zhu *et al.* (1998) to be a housekeeping gene as it is located within a cluster of conserved housekeeping genes on chromosome 9q34. This cluster has been conserved for more than 250 million years of divergent evolution indicating the functional importance of the locus in co-ordinating gene regulation. The authors demonstrated that the SURF-1 protein has a functional role in the assembly or maintenance of the active COX holoenzyme. From their data the authors predicted that the LS phenotype arises as a result of inefficient transfer of electrons between the ubiquinol cytochrome *c* reductase complex and the COX complexes.

In 1998 the SURF-1 gene was localised to chromosome 9q34 by two different groups of researchers (Zhu *et al.*, 1998; Tiranti *et al.*, 1998). This gene has nine exons encompassing 5 kb of genomic sequence and encodes a protein of 300 amino acids (Zhu *et al.*, 1998; Tiranti *et al.*, 1998). Both groups demonstrated that the gene encoded a protein which is not a structural component of the COX complex but is responsible for the maintenance or assembly of the complex. Studies performed on the yeast homologue of SURF-1, *SHY1* (Surf Homolog of Yeast) indicated that mutations within this gene compromised the activity of the COX complex (Mashkevich *et al.* 1997). In this study *SHY1* mutants had decreased levels of COX, increased levels of cytochrome c, and inefficient electron transport between cytochrome b-c_1 and COX (Poyton, 1998). The mutations within the SURF-1 gene are responsible for 14% of the typical Leigh Syndrome phenotype observed in paediatric patients (Rahman *et al.*, 1996).

Table 3.4: Mutations within the SURF-1 gene reported to be associated with Leigh Syndrome

Mutation(s)	Exon(s)	Type(s) of mutation
C765T/337 + 2 T→C[1]	7/i4	stop/splice
326insATdelTCTGCCAGCC/855delCT[1]	4/9	frameshift/frameshift
882insT/882insT[1]	9/9	frameshift/frameshift
37ins17/37ins17[2]	1/1	frameshift/frameshift

Table 3.4: continued ...

Mutation(s)	Exon(s)	Type(s) of mutation
516 + 2T→G/550delAG[2]	i5/6	splice/frameshift
868insT/868insT[2]	9/9	frameshift/frameshift (K290X)
312del10,insAT/...[2]	4/...	frameshift/...
845delCT/845delCT[2]	9/9	frameshift/frameshift
312del10,insAT/845delCT[2]	4/9	frameshift/frameshift
751C→T/751C→T[2]	7/7	stop/stop (Q251X)
845delCT/...[2]	9/...	frameshift/...
772delCC/...[2]	8/...	frameshift/...

1 = Zhu *et al.* (1998); 2 = Tiranti *et al.* (1998); del = deletion; ins = insertion; i4 = intron 4; i5 = intron 5.

The five mutations reported by Zhu *et al.* (1998) all result in the formation of nonsense codons within the gene predicting a truncation of the transcribed protein. These mutations within the SURF-1 gene were demonstrated to be the cause of the LS phenotype observed in the particular patients. The authors supported this notion by demonstrating that functional complementation of the RC deficiency in patient fibroblast cultures only occurred when the region of chromosome 9 containing the SURF-1 locus was reliably transferred. Exclusion mapping with microsatellite markers encompassing the SURF-1 locus also linked the LS phenotype to the genotype. The mutations, which were identified in three pedigrees, were observed to predict a truncation of the SURF-1 protein, furthermore a reduction in the levels of steady-state SURF-1 mRNA was observed with Northern-blot and quantitative RT-PCR. The final proof reported by the authors for the involvement of the SURF-1 locus in the LS phenotype is that the expression of the SURF-1 cDNA was able to restore the COX activity in patient fibroblast cultures.

3.3.3.3.2 Complex I deficiencies

As described and listed in Table 3.3 Rahman *et al.* (1996) suggested that complex I deficiencies are the most common cause of LS. This may be related to the presence of mutations within numerous of the subunits constituting and affecting the holo-complex. Complex I is responsible for the transfer of electrons, and this is made possible by the presence of flavin mononucleotide centres and iron-sulfur clusters within the complex. Deficiency within the complex may arise from defects within the formation of these clusters.

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a) NADH-ubiquinone oxidoreductase FE-S protein 8 (NDUFS8) mutations

The gene encoding the 23 kDa subunit of the NADH-ubiquinone oxidoreductase FE-S protein 8 (NDUFS8) protein has been localised to chromosome 11q13 (Loeffen *et al.*, 1998). The cDNA for this gene consists of an open reading frame of 633 bp and codes for 210 amino acids. The NDUFS8 subunit is a highly conserved entity of complex I.

This protein forms part of the hydrophobic fraction and contains the two iron ferredoxin consensus patterns responsible for binding the iron-sulfur cluster. NDUFS8 is thought to play an important role in reducing ubiquinone while pumping protons across the inner membrane. It has also been demonstrated that this subunit is one of 14 subunits that comprise the minimal structural entity with enzymatic activity thereby illustrating its functional significance (Loeffen *et al.*, 1998).

Mutations within this gene follow an autosomal recessive pattern of inheritance. Clinical features which may be distinct for complex I deficiency as a result of mutations within this gene are the following: onset is at birth, hypotonia, brisk tendon reflexes, seizures, drowsiness and cardiomyopathy. The lactate levels are usually high in the blood as well as in the CSF and the MRI scans reveal hypodensity of white-matter, putamen and mesencephalon. Histological analysis of the muscle biopsy revealed that the number of type I fibres were decreased but there were no ragged red fibres present. On autopsy, the brain pathology may reveal alterations within the spongiform, with capillary proliferation and endothelial swelling. Demyelination and gliosis may also be present (Loeffen *et al.*, 1998).

In the report by Loeffen *et al.* (1998) two compound heterozygote alterations were observed in the NDUFS8 gene. The first mutation was a C236T change and this resulted in an alteration of proline to leucine at position 79. This alteration may affect the architecture of the protein by disturbing an α-helix with a β-sheet. It was stated by the authors that the removal of proline would undoubtedly disturb the secondary or tertiary structure of the protein (Loeffen *et al.*, 1998). The second mutation was a G305A transition and this resulted in an arginine for histidine change at position 102. The new histidine amino acid contains an imidazole side chain which may switch between positively charged and uncharged resulting in the catalysis or breaking of bonds. The authors demonstrated that the region of ca. 24 bp surrounding the arginine amino acid, which was altered in the patient, is 100% conserved across species thereby suggesting

a functional significance for this region (Loeffen *et al.*, 1998). The presence of the compound heterozygote was peculiar and it was therefore suggested that both mutations were necessary for the reduction of activity of complex I. The abnormal NDUFS8 subunit results in an inefficient electron transport chain culminating in the expression of the phenotype observed (Loeffen *et al.*, 1998).

b) NADH:ubiquinon oxidoreductase Fe-S protein 7 (NDUFS7)

The NADH:ubiquinon oxidoreductase Fe-S protein 7 (NDUFS7) gene has been localised to chromosome 19p13 (Hyslop *et al.*, 1996). Complex I deficiency, as a result of mutations within this gene, follows an autosomal recessive mode of inheritance (Triepels *et al.*, 1999). The clinical features which are distinct to this deficiency are onset in the first year of life with repeated vomiting. Hemiparesis, hypotonia and hyperactive tendon reflexes may be observed. Pyruvate and lactate levels are both normal in serum urine and CSF. MRI scans reveal symmetric lesions in the caudate, putamen and dentate regions of the brain. Death may occur within the first decade (Triepels *et al.*, 1999).

The valine to methionine (V122M) amino acid change reported by Triepels *et al.* (1999) is postulated to affect the conformation or incorporation of the NDUFS7 protein within complex I. The valine residue has been observed to be conserved across species indicating its significance within the subunit. The structural defect caused by the amino acid alteration may impair correct organisation of the iron-sulfur clusters, thereby resulting in deficient electron transfer (Triepels *et al.*, 1999).

3.3.3.3.3 Complex II deficiencies

This complex is involved in the oxidation of succinate and facilitates the movement of electrons from FADH to CoQ. All four of the subunits constituting complex II are encoded for by the nuclear genome.

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a) The succinate dehydrogenase 2, flavoprotein subunit (SDHA)

The first mutation within a nuclear encoded gene was the Arg544Trp mutation within the succinate dehydrogenase, subunit A, flavoprotein (F_P) gene described by Bourgeron *et al.* in 1995. The gene was localised to chromosome 5p15 and resulting deficiencies were observed to follow an autosomal recessive pattern of inheritance. The patients investigated were sisters who both presented with Leigh Syndrome.

A C to T alteration was observed at nucleotide 1684 of the SDH gene (F_P subunit), and occurred within a CpG doublet. This region of the gene was noted to be a hot spot for the deamination of 5-methylcytosine to thymine. The amino acid alteration resulting from the mutation is that of a positively charged arginine residue being replaced by a neutral cyclic tryptophan amino acid. The amino acid, which is altered, is localised within a highly conserved region of the protein. The affected domain of the protein is proposed to be involved in the binding of oxaloacetate (OAA) with complex II. It was also suggested that the mutation might alter the redox state or the conformation of the protein thereby instead of directly influencing the binding of OAA it may change the catalysis or response of the enzyme to OAA (Bourgeron *et al.*, 1995).

The mutation was observed in various tissue samples studied, namely skeletal muscle, lymphocytes and fibroblasts. This was in accordance with the variable clinical expression of the disorder as noted in the patients. The pathogenicity of the mutation was further investigated by the expression of the mutant SDH F_P gene in yeast. To validate the causative nature of this mutation its presence was investigated in 120 healthy controls, all of whom were negative (Bourgeron *et al.*, 1995).

3.3.3.3.4 The pyruvate dehydrogenase (PDH) complex deficiency

Deficiency of the PDH complex is a primary cause of congenital lactic acidosis (Robinson *et al.*, 1980). The complex is responsible for the breakdown of pyruvate to acetyl-CoA in the mitochondria, which is then further metabolised in the tricarboxcylic acid cycle. Of the three enzymatic subunits of the complex, E1α is the most frequently affected in LS (Hansen *et al.*, 1991).

a) The pyruvate dehydrogenase alpha 1 locus on Xp22.1

The X-linked form of LS is associated with mutations in the pyruvate decarboxylase (E1α) subunit of the PDHC. The pyruvate dehydrogenase alpha 1 (PDHA1) gene coding for the alpha chain of the E1 subunit, has been mapped to chromosome Xp22.2 - Xp22.1 (Brown *et al.*, 1989). Defects in the other two enzymatic subunits, dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3), have been identified as being inherited in a Mendelian fashion. (Scherer *et al.*, 1991; Thekkumkara *et al.*, 1988).

PDH E1α deficiency can be classified as either an X-linked recessive or an X-linked lethal disorder, depending on the mutation inherited. As described by Brown *et al.* (1989) the fact that there are equal numbers of male and female patients affected by PDH deficiency is unusual for an X-linked disorder. This can however be explained by the fundamental role the PDH complex plays in energy metabolism. In female patients the severity of the disorder depends on the pattern of X inactivation in the various tissues within the body. The most severely affected organ in female PDH deficient patients is generally the CNS, which is particularly susceptible to any dysfunction within the metabolic cycles and especially decreased activities of the PDH complex (Brown *et al.*, 1989). Within the brain, even mutations which cause only slight reductions in PDH activity result in limited normal function. Defects with a more severe decrease in PDH activity result in brain cell degeneration and death, resulting in the prominent feature of the LS phenotype. The distribution of these lesions vary as a result of the various X-inactivation patterns within the precursor cells of the different regions of the brain (Brown *et al.*, 1989; Chen *et al.*, 1995).

In male patients the clinical manifestation depends on the functional severity of the mutation. Mutations which have little or no resultant enzyme activity cause foetal death in-utero as the mutation is present in all cells of the body. This phenomenon results in a lower number of affected males than expected with an X-linked disorder (Brown *et al.*, 1989; Chen *et al.* 1995).

The carboxy terminal end of the subunit has been identified as being a hotspot for mutations within the PDHA1 gene (Hansen *et al.*, 1991). According to Marsac *et al.* (1997) over 52 mutations with the PDHA1 gene have been described as causative of

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PDH deficiency and associated with LS phenotype. The numerous mutations which have been reported within the PDHA1 gene are listed in Appendix B.

The clinical phenotype of PDH deficiency associated with LS is highly heterogeneous. This heterogeneity may be explained by the fact that several factors influence the phenotype. These are the chromosome X inactivation pattern in females, the proportion of cells expressing the normal gene in female patients, tissue-variable expression of the mutant E1α protein, the brain's dependency on PDH E1α activity, the energy requirement of neurological organs during foetal growth and development, and the severity of the mutation (Chen *et al.*, 1995).

3.4 MITOCHONDRIAL ENCEPHALOMYOPATHY, LACTIC ACIDOSIS AND STROKE-LIKE EPISODES (MELAS)

MELAS was first described in 1975 as a phenotype which could be identified by stroke-like episodes, lactic acidaemia and ragged-red fibres (Shapira *et al.*, 1975). However, the acronym of MELAS was only ascribed to the disorder by Pavlakis *et al.* in 1984 when these authors recognised that the distinctive phenotype of mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes was a mitochondrial disorder associated with the onset of symptoms between the ages of three and eleven years. MELAS is currently reported to be the most common mitochondrial disorder (Tanahashi *et al.*, 2000).

3.4.1 The clinical features of MELAS

The ultimate goal of investigating the molecular basis of mitochondrial myopathies in a group of patients is elucidation of the phenotype genotype correlation. Patients with MELAS present with normal early development, followed by devastating progressive encephalomyopathy and stroke-like episodes. Variable clinical features associated with MELAS are presented in Table 3.5. The majority of patients have an early onset during childhood and all affected individuals are symptomatic before the age of 40 years (DiMauro and Wallace, 1993).

Table 3.5: The clinical features of MELAS

Onset:	mean = 10 years (range 2 – 40 years)	
	migraine-like attacks: headaches and vomiting	
	occasional seizures	
Encephalopathy:	often episodic (may be increased by valporate)	
Generalised:	headache and vomiting (90%)	
	loss of consciousness (85%)	
	seizures (85%)	
	dementia or mental retardation (50-60%)	
Focal:	hemiplegia	
	cortical visual defect (occipital blindness or hemiplegia)	
	basal ganglia calcifications	
Hearing loss	---	
Systemic features:	pigmentary retinopathy (10-20%)	
	cardiomyopathy (15%)	
	short stature	
Myopathy:	exercise intolerance and weakness	
Lactic acidosis:	blood and CSF	
Pathology:		
Muscle:	ragged red fibres (RRF):	COX positive
		SDH positive
	mitochondrial proliferation in blood vessels	
CNS:	abnormal cellular metabolism	
	regional changes:	basal ganglia calcification (26%)
	cerebral:	focal necrosis
		cortical atrophy
		white matter gliosis
	cerebellum:	cortical atrophy
		white matter gliosis
		Purkinje dendrite cactus formations with increased number of mitochondria (60%)

CSF = cerebrospinal fluid; COX = cytochrome c oxidase; SDH = succinate dehydrogenase; CNS = central nervous system. Adapted from Neuromuscular: Mitochondrial Disorders (2000).

The diagnosis of MELAS has been further defined by Hirano and Pavlakis in 1994. The most distinctive are the presence of stroke-like episodes before the age of 40 years, the presentation of an encephalopathy characterised by seizures and dementia, the presence of lactic acidosis, and ragged-red fibres upon investigation. The authors noted that although there is a distinction between the MELAS and MERRF phenotypes (discussed in section 3.5), individual symptoms were not exclusively indicative of one disorder (Hirano *et al.*, 1992).

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3.4.2 The genetic basis of MELAS

The MELAS phenotype is heterogeneous on both a clinical as well as a genetic level. The three most common mtDNA mutations occur within the mitochondrial $tRNA^{Leu(UUR)}$ gene at nucleotide positions 3243, 3271 and 3291. The A3243G mutation is responsible for 80% of all MELAS cases (Goto *et al.*, 1991). Although it is the most common mtDNA mutation, it has been widely reported that the A3243G transition within the $tRNA^{Leu(UUR)}$ gene is not the only genetic defect associated with the MELAS phenotype (Ciafaloni *et al.*, 1992). Thus far ten mtDNA point mutations have been associated with MELAS, each of which are discussed below.

3.4.2.1 The G583A mutation

In 1998 Hanna *et al.* reported the first MELAS associated mutation within the mtDNA tRNA gene for the amino acid phenylalanine. The transition occurs at position 583 in the aminoacyl acceptor stem of the $tRNA^{Phe}$. The guanine nucleotide at this position was observed to be highly conserved across species. The authors considered the mutation to be causative of the phenotype as it was observed to be heteroplasmic in the affected individual. The transition occurred at a highly conserved position within the tRNA structure and this change was not present in any of the controls. (Hanna *et al.*, 1998).

3.4.2.2 The G1642A mutation

The G1642A mutation occurs within the $tRNA^{Val}$ gene of the mitochondrial genome (Taylor *et al.*, 1996). This was the first mutation within this gene to be associated with the MELAS phenotype, although the patient harbouring the mutation did not have a myopathy. The most striking clinical feature of the patient was the progressive encephalopathy associated with stroke-like episodes (Taylor *et al.*, 1996).

The guanine for alanine substitution within the valine tRNA molecule occurs within the anticodon stem, disrupting the tertiary structure of the molecule and in so doing altering its function. This base alteration leads to changes within the synthesis of respiratory chain polypeptides encoded by the mtDNA (Taylor *et al.*, 1996).

The pathogenicity of the G1642A mutation was demonstrated by the presence of heteroplasmy within the tissues analysed. In muscle there was 94% mutant mtDNA while in brain and blood the heteroplasmic levels were 95% and 54% respectively, allowing a correlation to be shown between the tissues worst affected and the heteroplasmic levels. Furthermore, this alteration was the only rearrangement within the patients' mtDNA. It was also not observed in normal controls or in other mitochondrial patients (Taylor *et al.*, 1996).

3.4.2.3 The A3243G mutation

In 1990 Goto *et al.* reported the presence of the MELAS A3243G mutation in 26 of 31 MELAS patients. At this time the authors stated that the 3243 mutation seemed to be the major cause of MELAS, this having since been confirmed by recent studies in Finland and England (Majamaa *et al.*, 1998; Chinnery *et al.*, 2000). Majamaa *et al.* (1998) investigated the frequency of the A3243G mutation in the adult Finnish population with the inclusion criteria of diabetes mellitus, sensorineural hearing impairment, cardiomyopathy and brain infarct. These authors reported that the A3243G mutation has a prevalence of 16 in 100,000 in adults (Majamaa *et al.*, 1998). This figure is however an underestimation of the prevalence of the mutation within the population as no children were included in the study.

The A3243G mutation was reported as the cause for the MELAS phenotype observed in the patients described by Goto *et al.* (1990), as heteroplasmy was illustrated. However, five individuals, of the patient population investigated, did not harbour the mutation, despite the fact that these patients displayed clinical and histological features of MELAS. This suggests that there was another molecular cause for the MELAS phenotype observed in the other five patients. The authors further stated that the A3243G mutation is specific but not exclusive to the MELAS phenotype (Goto *et al.*, 1990). During the same year Kobayashi *et al.* (1990) reported the presence of the A3243G mutation in a Japanese MELAS patient, thereby contributing towards the confirmation of this change as an aetiology for the clinical phenotype observed by both groups.

The 3243 mutation is a transition from A to G and occurs within the dihydro-uridine loop of the mitochondrial $tRNA^{Leu(UUR)}$ gene. More specifically, the adenine base at position

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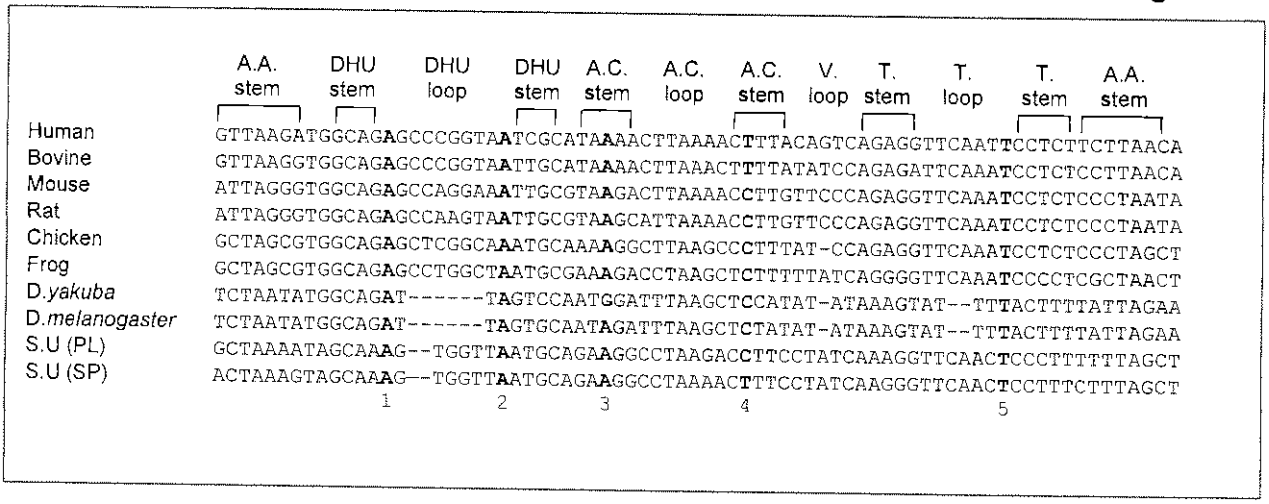
3243 is the first nucleotide of the dihydro-uridine loop and is highly conserved across species, as illustrated in Figure 3.3. This nucleotide is responsible for forming a tertiary hydrogen bond with the thymine residue of the amino-acid acceptor stem positioned around a turn of the L-shape structure. Therefore, according to Goto *et al.* (1990), any variation at this nucleotide position would greatly affect the tRNA function. A transition within a tRNA gene results in defects within the respiratory-chain enzymes, especially within the NADH coenzyme Q reductase and cytochrome *c* oxidase complexes (Goto *et al.*, 1990).

Kobayashi *et al.* (1990) suggested that the pathological nature of this mutation arises from the fact that the alteration occurs within the sequences necessary for transcription termination, thereby impairing mitochondrial transcriptional regulation. This line of thought was substantiated by Hess *et al.* (1991) who reported their findings from an investigation on the effects of the A3243G mutation on the binding of the mTERF. The authors demonstrated that the mutation causes the template sequence to be ineffective in transcription termination, as it is no longer recognised by the termination factor. The assumption is made that the molecular defect, resulting from the A3243G MELAS mutation, is the inability to produce the correct quantity and type of rRNA species relative to the other mitochondrial gene products (Hess *et al.*, 1991).

Figure 3.3: Interspecies conservation of the mitochondrial tRNA$^{Leu(UUR)}$ gene



A.A. = amino acid; DHU = dihydro-uridine; A.C. = anti-codon; V. = variable; T. = TψC loop; D. *yakuba* = *Drosophila yakuba*; D. *melanogaster* = *Drosophila melanogaster*; S.U. = sea urchin (PL = *Paracentrotus lividus* and SP = *Strongylocentrotus purpuratus*); 1 = nt 3243; 2 = nt 3252; 3 = nt 3260; 4 = nt 3271; 5 = nt 3291. Adapted from Goto *et al.* (1990).

3.4.2.4 The A3252G mutation

Morten *et al.* (1993) described an A to G transition at position 3252 in a patient with mitochondrial encephalomyopathy, pigmentary retinopathy, dementia, hypothyroidism and diabetes mellitus. The mutation was observed as heteroplasmic in both blood (30%) and muscle (76%) samples of the proband. The mutation was also observed to segregate in the family following a maternal inheritance pattern. The A3252G mutation was not observed in any controls investigated but was present in other members of the proband's family with various levels of heteroplasmy (Morten *et al.*, 1993).

The mutation occurs within a highly conserved region of the mitochondrial $tRNA^{Leu(UUR)}$ within the dihydro-uridine (DHU) stem. This change also lies within close proximity to a cluster of transitions at nucleotide 3250 (Goto *et al.,* 1992a) and 3251 (Sweeney *et al.,* 1993) as well as the 3243 mutation which has been closely associated MELAS (Goto *et al.*, 1990). The authors suggested that the 3252 mutation could affect the termination of transcription and/or tRNA functioning in a similar fashion to that of the A3243G transition (Morten *et al.*, 1993).

3.4.2.5 The A3260G mutation

Zeviani *et al.* (1991a) reported this mutation after observing the A3260G change in a single large pedigree with hereditary cardiomyopathy. In this pedigree the mutation was found to be heteroplasmic in maternal relatives. The amount of mutant mtDNA in the muscle of the individuals affected in the pedigree correlated with the severity of their clinical presentation (Zeviani *et al.*, 1991a). The same nucleotide substitution was associated with MELAS in a Japanese family (Nishino *et al.,* 1996).

In the Japanese family the proband had a significantly higher level of mutant mitochondria (87% in muscle) while the heteroplasmic levels in the asymptomatic maternal relatives was markedly lower, thereby suggesting the pathogenicity of the alteration (Nishino *et al.*, 1996).

The adenine to guanine change at position 3260 of the mitochondrial $tRNA^{Leu(UUR)}$ molecule occurs within the anticodon stem. This base forms part of a four base pair palindrome sequence that is highly conserved across species (Goto *et al.*, 1990). The

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transition creates a mismatch in the stable anticodon stem, thereby destroying the stability of the structure. The mutations within the transfer RNA function in the same destructive manner by decreasing the efficiency of the mitochondria in translating its own genes necessary for the respiratory complexes (Zeviani *et al.*, 1991a). It is suggested that the A3260G mutation may be responsible for both the mitochondrial cardiomyopathy and the MELAS phenotypes observed (Zeviani *et al.*, 1991a; Nishino *et al.*, 1996).

3.4.2.6 The T3271C mutation

The T3271C mutation was reported in 1991 by Goto *et al.* The authors reported this mutation in three patients in whom the A3243G mutation was not observed. This novel mutation was observed to be heteroplasmic in all three patients and no pedigree analysis was reported in this study (Goto *et al.*, 1991).

The nucleotide change occurs within the anticodon stem of the mitochondrial $tRNA^{Leu(UUR)}$ molecule. Although the nucleotides within the anticodon stem are not conserved across species, they are complementary to each other and are therefore termed preserved nucleotides (Goto *et al.*, 1991). The novel transition of the thymidine for cytosine at nucleotide position 3271 is predicted to have a similar effect to the transition at nucleotide 3243, which alters the structure. As a result the function of the tRNA molecule will be compromised (Goto *et al.*, 1991).

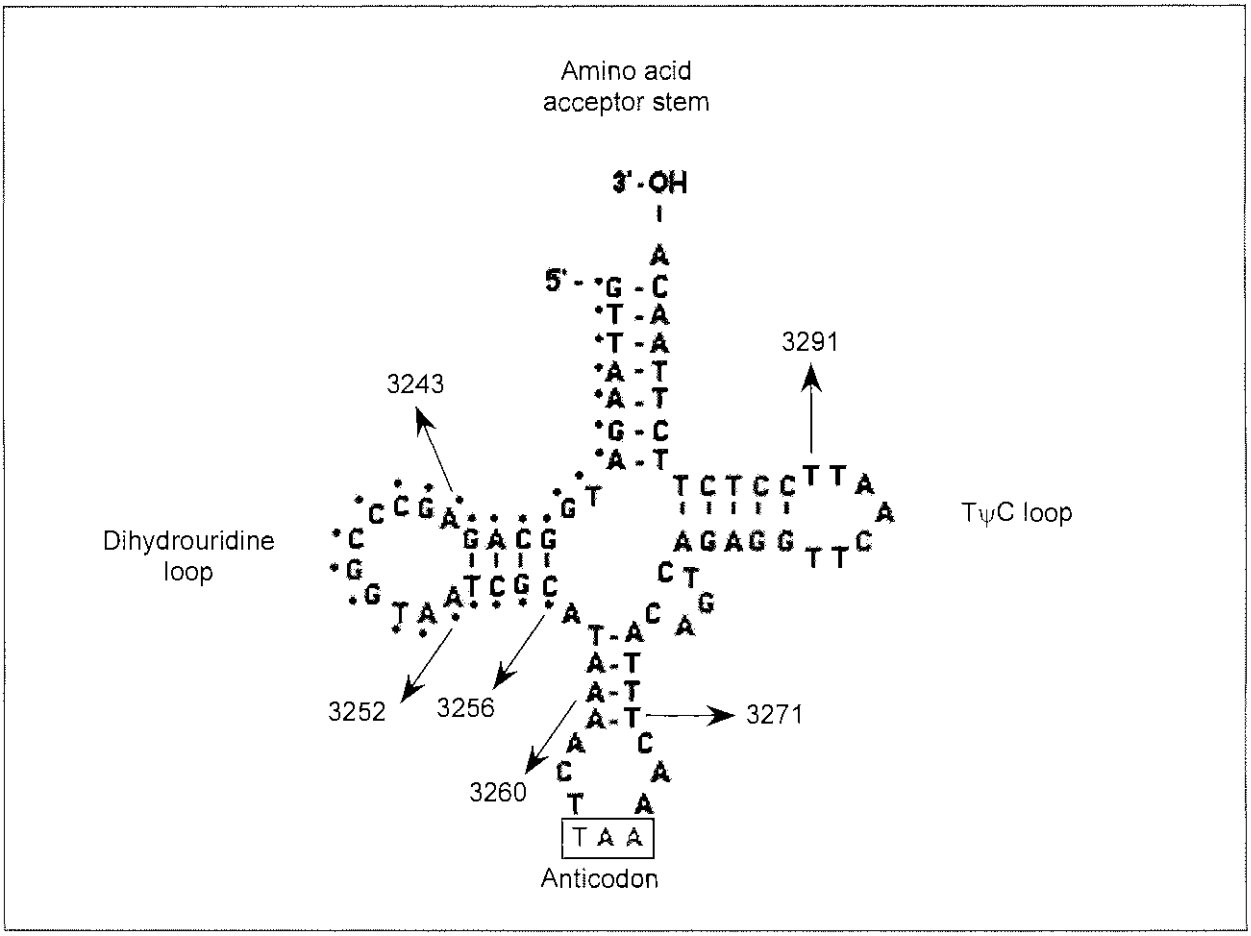
3.4.2.7 The T3291C mutation

In 1994 Goto *et al.* (1994) reported the presence of the T3291C mutation within a Japanese MELAS patient. Heteroplasmy was observed for this mutation with proportions of mutant being 86% in muscle and 30% in whole blood samples of the patient (Goto *et al.*, 1994).

The 3291 thymidine residue is the last nucleotide of the TΨC loop in the mitochondrial $tRNA^{Leu(UUR)}$ molecule. This site is highly conserved across species and forms part of the three dimensional structure which is associated with a protein-recognition system involved in discriminating between the tRNAs. This site is therefore essential for effective tRNA functioning (Goto *et al.*, 1994).

In the majority of the mutations associated with the MELAS phenotype the heteroplasmic levels are observed to correlate with the severity of the symptoms indicated in the patients. This is especially true for the most common mutation, the MELAS A3243G transition. Although many of the base alterations within the mitochondrial $tRNA^{Leu(UUR)}$ gene are associated with the MELAS phenotype, there are mutations within this gene which are not specific to this syndrome (Ciafaloni *et al.*, 1992). Figure 3.4 is a diagrammatic representation of the tertiary structure of the $tRNA^{Leu(UUR)}$ molecule and depicts the positions of the mutations discussed above.

Figure 3.4: Tertiary structure of the mitochondrial $tRNA^{Leu(UUR)}$ molecule



Dots next to nucleotides indicate the position of the transcription termination sequence. Adapted from MITOMAP (2001).

3.4.2.8 The A5814G mutation

The adenine to guanine residue alteration at mtDNA nucleotide position 5814 was reported by Manfredi *et al.* (1996). This is the first mutation within the mitochondrial $tRNA^{Cys}$ gene to be associated with a MELAS-like syndrome.

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The A5814G mutation was present in heteroplasmic levels of greater than 90% in both muscle and blood samples analysed from the patient. This alteration was absent from the normal healthy controls as well as from other mitochondrial myopathy patients investigated. The mutation would alter the base pair within the D-stem of the $tRNA^{Cys}$ molecule, which is highly conserved across species, advocating the pathogenicity of the A5814G mutation (Manfredi *et al.*, 1996).

3.4.2.9 The T9957C mutation

The first mutation within a polypeptide-coding gene associated with the MELAS phenotype was described by Manfredi *et al.* (1995). This T9957C alteration within the gene encoding subunit III of the cytochrome *c* oxidase complex (COX III) of the mitochondrial genome was observed in a patient with classical MELAS features. The presence of the mutation was noted to be heteroplasmic in the proband, with 81% mutant mtDNAs in a muscle sample and 60% in a blood sample. The mutation was also detected within a blood sample from the proband's mother (Manfredi *et al.*, 1995).

The T to C alteration at nucleotide position 9957 within the mtDNA results in an amino acid change from phenylalanine to leucine at position 251 in the COX III subunit. The causative nature of this alteration could be emphasised by the fact that the phenylalanine amino acid at position 251 is highly conserved across species, as is the C-terminal region of the protein, where this residue is located (Manfredi *et al.*, 1995). The function of the COX III subunit has not been fully elucidated and the exact mechanism for the defect caused by the mutation is therefore unknown. However, it is possible to speculate that the functioning or assembly of the holoenzyme is influenced by the mutation in such a way as to result in the MELAS phenotype (Manfredi *et al.*, 1995).

3.4.2.10 The G13513A mutation

The G13513A mutation was reported as the first mutation within the mitochondrial ND5 gene associated with MELAS (Santorelli *et al.*, 1997b). ND5 is the fifth subunit of complex I. Other mutations have been localised to this gene but they are associated with LHON (Wallace *et al.*, 1988a). The mutation was observed to be heteroplasmic

within a patient with classical MELAS features except that onset was later than is generally the case for MELAS. The percentage of mutant mtDNAs was proportional to the tissue involvement. Brain samples were the most severely affected, harbouring 73% mutant mitochondria (Santorelli *et al.*, 1997b).

The guanine to adenine alteration at mtDNA nucleotide position 13513 results in the amino acid change from a conserved aspartic acid to asparagine at position 393 in the ND5 protein. Although the amino acid change does not affect the structural organisation of the protein, it does alter the charge of the peptide, resulting in impairment of the activity of the entire complex (Santorelli *et al.*, 1997b).

A second mutation within the ND5 gene was reported in 2001 by Corona *et al.* These authors observed a nucleotide substitution at mtDNA position 13514, one base pair from the G13513A mutation reported by Santorelli *et al.* (1997b). The mutation at nucleotide position 13514 involves an adenine for guanine substitution, resulting in the same aspartic acid at position 393 being altered to glycine. The presence of this novel mutation in two MELAS patients emphasises the importance of the D393 amino acid within the ND5 subunit (Corona *et al.*, 2001).

3.5 MYOCLONIC EPILEPSY AND RAGGED RED MUSCLE FIBRES (MERRF)

This disorder was first described in 1975 by Shapira *et al.* who described the presence of ragged-red fibres in a patient affected with a neuromuscular disorder. It was only in 1980 that Fukuhara *et al.* distinguished this group of mitochondria myopathies from the generalised group when he named it with the acronym MERRF.

3.5.1 The clinical features of MERRF

The clinical features of MERRF may be defined by the presence of myoclonus or myoclonic epilepsy, ataxia and myopathy with ragged-red fibres (Silvestri *et al.*, 1992). In 1988, Wallace *et al.* (1988b) demonstrated the hierarchy of the clinical phenotypes with regard to the tissue-specific energy thresholds. The most sensitive manifestation was that of the CNS, followed by the formation of ragged-red fibres in type I muscle, then sensorineural hearing loss and finally, myoclonus. Dementia, cerebellar ataxia,

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hypoventilation and cardiomyopathy were observed as being the least sensitive of the manifestations associated with the MERRF syndrome. In accordance with Wallace *et al.* (1988b), the symptoms and the clinical signs noticeable in differently affected individuals are listed in Table 3.6. The onset of MERRF may occur in childhood or adulthood and the course of the disorder varies from progressively slow to rapid degeneration (Berg, 1996).

Shoffner *et al.* (1990) reported that individuals younger than 20 years of age generally require more than 95% mutant mtDNAs for the clinical expression of the MERRF phenotype. Individuals of the same age group harbouring 85% of the mutant mtDNA are clinically normal. However, older individuals between 60 and 70 years of age may be severely affected with 85% mutant mtDNAs while those harbouring 63% mutant mtDNAs may be mildly affected with the MERRF phenotype (Shoffner *et al.*, 1990).

Table 3.6: Synopsis of the clinical signs associated with MERRF

Severely affected individuals	Frequency
uncontrolled myoclonic epilepsy (periodic jerking)	60%
mitochondrial myopathy (ragged red fibres)	N/A
epilepsy	45%
polyneuropathy	20%
hearing loss	40%
optic atrophy	20%
short stature	10%
lipomata	10%

Severely affected maternal relatives	Less severely affected maternal relatives
brain electrophysiology aberrations	electrophysiological aberration
neurosensory hearing loss	mitochondrial myopathy
myoclonus	deafness
dementia	myoclonus
respiratory failure	dementia
dilated cardiomyopathy	respiratory failure
renal dysfunction	cardiomyopathy

N/A = not available. Adapted from Neuromuscular: Mitochondrial Disorders (2000) and Wallace (1992).

3.5.2 The biochemical features of MERRF

The MERRF phenotype is observed as a result of a biogenesis mutation within the mtDNA. The mutations responsible for causing defective biogenesis within the mitochondria are localised to the tRNA genes of the mitochondrial genome. Where there is an alteration in the structure or functioning of the tRNAs the mitochondrial protein synthesis is severely compromised. In the case of the mutations associated with the MERRF phenotype the $tRNA^{Lys}$ is defective in the majority of cases. The MERRF phenotype can therefore be described as being a pleiotropic OXPHOS defect, with complexes I and IV being primarily affected (Wallace *et al.*, 1988b). According to Wallace *et al.* (1988b) the severity of the clinical phenotype is directly proportional to the OXPHOS defect. Complexes I and IV are the OXPHOS complexes which contain the most mitochondrial encoded proteins and are therefore preferentially affected by the reduction of mitochondrial protein synthesis (Wallace *et al.*, 1988b; Wallace, 1992).

3.5.3 The genetic basis of MERRF

Although this disorder was first described by Shapira *et al.* in 1975 the genetic basis was only elucidated in 1988 by Wallace *et al* (1988b). Until that time the pedigrees of the patients affected by the MERRF phenotype were not large enough for the inheritance pattern to be elucidated. In their study, Wallace *et al.* (1988b) investigated 12 members of a MERRF family on a detailed clinical, physiological and biochemical level. Although this study did not detect the mtDNA alteration it did pave the way for the studies which followed by suggesting that the alteration could possibly be in one of the genes encoding the subunits of complex I or IV. Another suggestion proposed by the authors was that the alteration might be in one of the tRNA or rRNA genes. This was thought to be the most likely as both complexes were seen to be affected in the disorder (Wallace *et al.*, 1988b). The mitochondrial aetiology of this disorder was evident from the study by Wallace *et al.* (1988b) indicating that the syndrome followed a maternal pattern of inheritance. They also revealed the association of the clinical phenotype with a defect in the OXPHOS system and proved that this deficiency was variable among family members, in whom the clinical signs were also different (Wallace *et al.*, 1988b).

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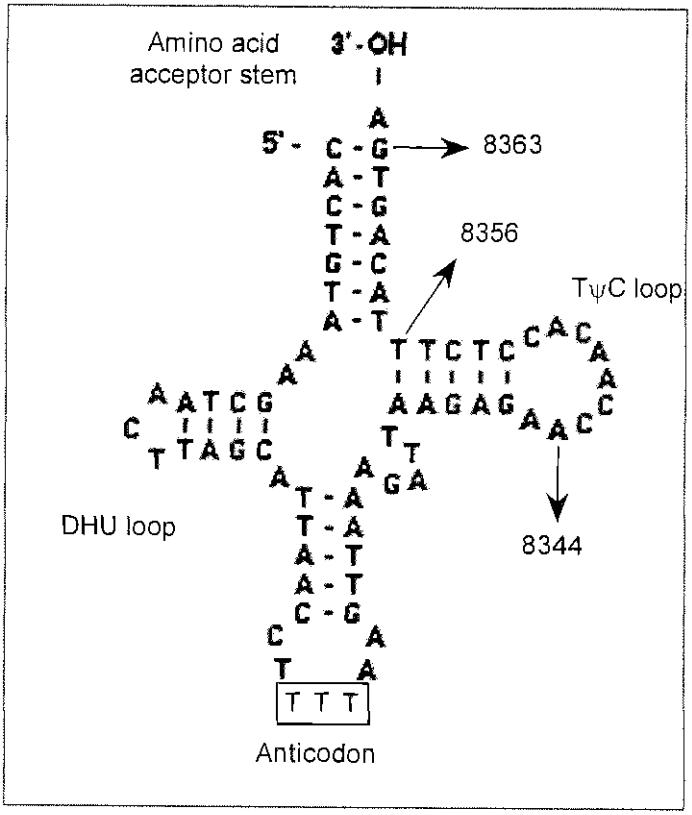
The first mtDNA mutation to be ascribed to the MERRF phenotype was never indicated to be the only alteration responsible for the syndrome (Shoffner *et al.*, 1990). Since 1990 two other mutations, also localised to the mitochondrial $tRNA^{Lys}$ gene, have been reported to be associated with the MERRF phenotype (Silvestri *et al.*, 1992; Ozawa *et al.*, 1997). The A8344G mutation is responsible for approximately 80% of all MERRF cases, thereby implying genetic heterogeneity for the disorder (Silvestri *et al.*, 1992), as is now observed to be common for the majority of mitochondrial disorders.

3.5.3.1 The A8344G mutation

The first mutation associated with the MERRF phenotype was the A8344G alteration within the mitochondrial $tRNA^{Lys}$ gene (Shoffner *et al.*, 1990). The mutation was observed in three independent families investigated and absent in the controls as well as in patients with other mitochondrial myopathies. The A8344G mutation was confirmed as causative of the MERRF phenotype by Seibel *et al.* in 1991, on investigation of the alteration in a family with clinical signs indicative of MERRF.

The A to G transition at position 8344 occurs within the TΨC stem of the tRNA gene of the amino acid lysine, as depicted within the tertiary structure of the tRNA molecule in Figure 3.5 (Shoffner *et al.*, 1990). It is presumed that the alteration in this area of the molecule will affect the binding of the tRNA to the ribosome (Seibel *et al.*, 1991).

Figure 3.5: Tertiary structure of the mitochondrial $tRNA^{Lys}$ molecule



DHU = dihydro-uridine. Adapted from Shoffner *et al.* (1990).

The functional significance of this position was investigated by the authors via comparisons of the sequence of the $tRNA^{Lys}$ gene across species as indicated in Figure 3.6. From this it was concluded that the adenine

nucleotide at position 8344 was functionally conserved, and that a substitution at this position would thus affect the function of the $tRNA^{Lys}$ molecule. The pathogenic nature of the A8344G mutation was suggested by Shoffner *et al.* (1990) as the alteration complied with the following implications. Firstly, the A8344G mutation was one of two alterations observed in the mtDNA of individuals affected with MERRF, which was responsible for altering conserved regions of a gene product. Secondly the segregation of the mutation correlated perfectly with the observed MERRF phenotype. The third line of evidence for pathogenicity was the fact that the substitution would manipulate the mtDNA tRNA, thereby inhibiting mitochondrial protein synthesis. In the fourth instance this change occurred at a conserved region of the TΨC stem of the tRNA and was observed to be heteroplasmic in the investigated individuals, thereby indicating pathogenicity. Lastly, the percentage of mutant mtDNAs could be correlated to the severity of the clinical phenotype and the decline in the oxidative work capacity, anaerobic threshold, of the skeletal muscle (Shoffner *et al.*, 1990).

Figure 3.6: Interspecies homology of the mitochondrial $tRNA^{Lys}$ gene

	A.A. stem	DHU stem	DHU loop	DHU stem	A.C. stem	A.C. loop	A.C. stem	V. loop	T. stem	T. loop	T. stem	A.A. stem
Human	CACTGTAAAGCTA-	ACT	-----	TAGC	ATTAA	CCTTTTAAGTTAAAG-	ATTAAGAGA	A	CCAACAC-	CTCTT	TACAGTGA
Bovine	CACTAAGAAGCTA-	TA	------	TAGC	ACTAA	CCTTTTAAGTTAGAG-	ATTGAGAG-	-	CCATATA-	CTCTC	CTTGGTGA
Mouse	CACTATGAAGCT-	AAG	-------	AGCG	TTAA	CCTTTTAAGTTAAAG-	-	TT-	AGAG-	A	CCTTAAAA-	TCTCCATAGTGA
Rat	CATTGCGAAGCTT-	AG	-------	AGCG	TTAA	CCTTTTAAGTTAAAG-	-	TT-	AGAG-	AC	-AACAA-	ATCTCCACAATGA
Hamster	CACTATGAAGCTC-	AG	-------	AGCG	TTAA	CCTTTTAAGTTAAA-	-	ATTGAGAG-	A	CTTTCTA-	GTCTCCATGGTGA
Chicken	CATTAAGAAGCT-	-	ATGCACC-	-	AGCA	CTAGCCTTTTAAGCTAGAG-	A	-	GAGGGG	A	CACCCT-	-	CCCCCCTTAATGA
Xenopus	CACTAAGAAGCTAAATAGGCATTAGCGACAGCCTTTTAAGCTGTAG-	ATTG-	GTG-	A	CTCCCAACCAC-	CCTTAATGA
Cod	CACTAAGAAGCTAATATGGGTTAAGCACCAGCCTTTTAAGCTGGAA-	GCAG-	GTG-	A	CTCCCAACCAC-	CCTTAATGA
Honey bee	CATTAGATGTCT-	-	GAATTAT-	-	AGAGTTGATCTTTTAAATCAAAT-	ATAGTAT-	-	A	TT-	-	TTAATACTTCTAATGA
Drosophila	CATTAGATGACT-	-	GAAAGCA-	-	AGTACTGGTCTCTTAAACCA-	TTTTATAGTAA-	A	TT-	AGCACTTACTTCTAATGA
S.U (PL)	CCTTAATTAGCTT-	ATTTA-	-	-	AAGCCCTAAACTCTTAATTTAAAA-	GAAAATAGTTAA	-	-	-	AACCTATTATTAAGGA
S.U (SP)	CCTTAATTAGCTT-	ATTTTA-	-	-	AAGCTTTAGACTCTTAATTTAAAA-	GAAATTAGCTAA	-	-	-	TACCTATTATTAAGGA
										1	2	3

A.A. = amino acid; DHU = dihydro-uridine; A.C. = anti-codon; V. = variable; T. = TΨC loop; S.U. = sea urchin (PL = *Paracentrotus lividus* and SP = *Strongylocentrotus purpuratus*); 1 = nt 8344; 2 = nt 8356; 3 = nt 8363. Adapted from Shoffner *et al.* (1990).

Although this change was causative of the MERRF phenotype observed in the three pedigrees investigated, Shoffner *et al.* (1990) stated that this specific mtDNA mutation does not eliminate the function of the $tRNA^{Lys}$ completely. This was demonstrated by the fact that one of the probands described by the authors displayed trace amounts of the alanine nucleotide and could therefore be considered to be homoplasmic for the A8344G mutation. However, she did not present with overt clinical symptoms until her early teenage years, thus illustrating the protective effect a small proportion of normal

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mtDNAs could have on the clinical phenotype of this particular individual (Shoffner *et al.*, 1990).

The segregation and manifestation of the A8344G mutation was investigated by Larsson *et al.* in 1992. These authors identified the presence of the mutation in three pedigrees, determined the levels of heteroplasmy between the family members and then correlated this information with the transmission of the mutation. They concluded that a large proportion (>92%) of the mtDNA should harbour the mutation before dysfunction of the respiratory chain occurs, as was indicated by Shoffner *et al.* (1990). Furthermore, it was noted that the risk of transmission to offspring increases with higher levels of the mutation. Above the heteroplasmic level of 35 – 40% mutation it is anticipated that all offspring will harbour the mutation (Larsson *et al.*, 1992).

Zeviani *et al.* (1991b) presented further evidence for the causative nature of the A8344G mutation being responsible for the MERRF phenotype, when these authors demonstrated the presence of this mutation in seven Italian MERRF pedigrees. The presence of this mutation associated with the MERRF phenotype in different ethnic groups indicates that it could be considered to be the most frequent, although not exclusive cause of MERRF (Zeviani *et al.*, 1991b). The A8344G mutation, while causative of the MERRF phenotype, has also been associated with other mitochondrial myopathies as discussed in paragraph 3.3.3.2.5 for LS. This is substantiated by the study reported by Silvestri *et al.* (1993), revealing that the mutation may also be associated with mitochondrial encephalomyopathies, limb-girdle myopathy with ragged-red fibres and PEO (Silvestri *et al.*, 1993).

3.5.3.2 The T8356C mutation

The T to C change at position 8356 was reported by Silvestri *et al.* in 1992. This alteration was observed in a MERRF family in whom the A8344G mutation had been excluded (Silvestri *et al.*, 1992). The proband of the family presented with classical MERRF signs and her sister and mother both exhibited similar symptoms with RRF being present in muscle biopsies from all three individuals (Silvestri *et al.*, 1992).

The authors reported this T8356C alteration within the mtDNA as causative for MERRF, since it complied with the guidelines of evidence for pathogenicity; firstly, the change

had never been observed in normal individuals; secondly, it was observed to be heteroplasmic in the individual identified and finally, the transition disrupts a highly conserved base pair in the TΨC stem of the mitochondrial tRNA encoding for the amino acid lysine (Silvestri *et al.*, 1992).

The T to C substitution is located at the 8356 position in the $tRNA^{Lys}$ proposed secondary structure. The thymine nucleotide is generally bound to an adenine nucleotide at position 8338, 18 base pairs upstream from the mutation site. These nucleotides are part of a 5-bp palindrome which forms the TΨC stem of the $tRNA^{Lys}$ cloverleaf. As evident from Figure 3.6 the 8356 nucleotide position itself is not conserved during evolution, however, the T-A pair is moderately conserved through species. The presence of the transition creates a mismatch within the stem structure, thereby affecting the stability of the TΨC stem. The mutation therefore, affects the efficiency of mitochondrial protein synthesis with relation to the incorporation of lysine amino acids (Silvestri *et al.*, 1992; Zeviani *et al.*, 1993).

The identification of this mutation together with the A8344G mutation allows the speculation that alterations occurring within the $tRNA^{Lys}$ gene result in the expression of the MERRF phenotype (Silvestri *et al.*, 1992). However, the T8356C mutation was confirmed to be pathogenic, but not specific for the MERRF syndrome, as it was observed in a different disorder by Zeviani *et al.* (1993). In this report the substitution of thymine for cytosine at position 8356 in the lysine tRNA gene, was observed in a patient who presented with the clinical features of both the MERRF and MELAS syndromes, suggesting an overlap syndrome (Zeviani *et al.*, 1993).

3.5.3.3 The G8363A mutation

The G to A nucleotide change at position 8363 within the mtDNA was first described by Santorelli *et al.* in 1996 within two families. The clinical presentations of the individuals included maternally inherited cardiomyopathy and hearing loss but none displayed classical MERRF signs (Santorelli *et al.*, 1996). However, in these families the mutation displayed the appropriate pathogenicity criteria of being heteroplasmic with various levels in the patients versus asymptomatic individuals. The mutation was also not observed in any of the controls investigated (Santorelli *et al.*, 1996).

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However, it was a year later before the G8363A mutation was associated with the MERRF phenotype when Ozawa *et al.* (1997) reported their observation of this change within two independent Japanese families with MERRF. As depicted in Figure 3.5 the guanine residue of the mtDNA nucleotide position 8363 is located at the 3'end of the aminoacyl acceptor stem of the $tRNA^{Lys}$ molecule. This nucleotide is highly conserved across species, as illustrated in Figure 3.6. The alteration of an adenine for guanine residue at this position abolishes the conserved base pair in the aminoacyl stem of the tRNA molecule. Santorelli *et al.* (1996) proposed that the alteration would disrupt the double helix of the stem structure and thereby transform the secondary and/or tertiary structure of the molecule. The authors also suggested that the absence of the base pair at the 3'-end would impair the peptidyl-tRNA hydrolase activity and the correct coupling of amino acids to their affiliated tRNA (Santorelli *et al.*, 1996).

It was then postulated by Ozawa *et al.* (1997) that the G8363A mutation was causative of the MERRF phenotype but it was not specific for this syndrome because of its association with the cardiomyopathy and hearing loss reported by Santorelli *et al.* in 1996. However, in 1999 Arenas *et al.* published their findings of the mutation in association with a MERRF pedigree. These authors reported the presence of the G8363A mutation in a proband and two oligosymptomatic relatives. The heteroplasmy levels of the alteration were as high as 95% in the muscle of the proband compared to the muscle samples containing 40% to 70% of the mutation in his relatives (Arenas *et al.*, 1999).

The pathogenicity of the G8363A mutation was confirmed in this pedigree by a maternal inheritance pattern and the fact that the mutation was absent in more than 100 control samples and had never been observed in the normal population. This was compounded by the proportion of mutant mtDNA equating with the biochemical defect and the clinical expression of the signs observed in the proband and his relatives (Arenas *et al.*, 1999).

3.6 MELAS/MERRF OVERLAP SYNDROME

The complexity of the two disorders is compounded by the fact that there are many cases which have been reported in which the clinical signs are indicative of the MELAS phenotype but the presence of the mutations associated with MERRF are detected and *vice versa*.

In 1995 Folgerø *et al.* were the first investigators to report the presence of the A3243G MELAS mutation in a MERRF pedigree. The affected individuals from the two generation family were classified as MERRF using stringent clinical criteria. The MELAS phenotype was specifically ruled out on the basis that none of the individuals displayed stroke-like episodes. However, molecular data generated revealed the absence of the A8344G MERRF mutation. The presence of the A3243G mutation exemplifies the diversity of the clinical manifestations of the mutation (Folgerø *et al.*, 1995).

Before this time, the overlap syndrome between MELAS and MERRF was described as the syndrome in which clinical features were characteristic of both disorders, as was noted by Zeviani *et al.* in 1993. In this report the novel alteration of T8356C was demonstrated in a three generation pedigree of whom the individuals had myoclonus epilepsy, neural deafness, ataxia and variable association of stroke-like episodes (Zeviani *et al.*, 1993), as discussed in paragraph 3.5.3.2.

3.7 MITOCHONDRIAL DISORDERS ASSOCIATED WITH INTERGENOMIC SIGNALLING

The evolution of the mitochondrial genome from a symbiotic relationship between an aerobic prokaryotic bacteria and anaerobic host explains the structure and presence of DNA within the mitochondrion (DiMauro and Wallace, 1993). However the fact that the mitochondria rely on the nuclear genome for the majority of its structural components illustrates the co-dependence of the two genomes. It is this co-dependence that is reliant on the correct and efficient intergenomic signalling between the mtDNA and nDNA. Primary genetic defects of the nDNA result in a breakdown of the intergenomic communication and result in quantitative or qualitative alterations of the mtDNA (DiMauro, 1996a).

3.7.1 Quantitative alterations of the mitochondrial genome

Quantitative alterations of the mtDNA are also referred to as mtDNA depletion. This was first reported by Moraes *et al.* (1991) in patients with almost negible amounts of mtDNA in their affected tissues, muscle and hepatocytes. mtDNA depletion is characterised by

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a substantial decrease in the concentration of mtDNA within a specific tissue. Depletion of mtDNA follows an autosomal recessive form of inheritance and is reported to be caused by mutations within the nuclear genes encoding factors involved in the replication of the mtDNA. Larsson *et al.* (1994) demonstrated that low levels of mtTFA were observed in tissues demonstrating mtDNA depletion, this however still remains to be confirmed.

3.7.2 Qualitative alterations of the mitochondrial genome

Qualitative alterations of mtDNA are characterised by multiple deletions within the mitochondrial genome (Zeviani *et al.*, 1989; Servidei *et al.*, 1991). Deletions of mtDNA follow both autosomal recessive as well as autosomal dominant inheritance patterns. The most common phenotype associated with multiple mtDNA deletions is progressive external ophthalmoplegia (Suomalainen *et al.*, 1992). Although these disorders clearly follow an autosomal inheritance pattern no nDNA defect has been elucidated for the mtDNA depletion syndromes.

3.8 THE DIAGNOSIS OF MITOCHONDRIAL DISORDERS

The complex nature of the mitochondrial disorders requires that a definitive diagnosis of a particular syndrome be made with the assistance of various analyses. When a precise clinical presentation is observed molecular analysis may be the only investigation necessary to confirm the presence of a known mtDNA mutation. However, many patients present with complex phenotypical expressions, it is in these cases that various analysis processes be employed to enable an informative diagnosis. To this end biochemical, histological as well as molecular investigations are performed (Adams and Turnbull, 1996).

3.8.1 Biochemical analysis of the mitochondrial disorders

The fact that the mitochondrial respiratory chain is under dual genetic control makes the molecular diagnosis of metabolic disorders complex. More than one hundred genes are

involved in the structural and functional maintenance of the OXPHOS system (Chretien *et al.*, 1994). However, biochemical analysis allows the isolation of the deficient complex thereby delimiting the molecular analysis to particular genes (Lowerson *et al.*, 1992).

Biochemical analyses can be performed on a variety of different sample materials. However, muscle samples are preferred for the analysis of mitochondrial disorders. As this is the tissue most commonly affected it is assumed that the probability of detecting an enzymatic deficiency via biochemical analysis is most likely in this particular tissue sample. However it has been established that cultured fibroblast cells can also be utilised for detecting respiratory chain deficiencies (Lowerson *et al.*, 1992).

There are limitations when performing biochemical analyses. Firstly, only a small amount of sample material is usually available for analysis when utilising muscle samples. The availability of controls and the scattered RC enzyme activities within the control population presents a further challenge when determining the base line for particular enzymes. In addition, the technical accuracy for these tests within various sample materials is of great importance and therefore standardised methods and techniques need to be employed (Chretien *et al.*, 1994).

The measurement of the activities of the respiratory chain complexes can be performed separately or in groups. The biochemical assays are performed on a mitochondrial enriched fraction obtained from the muscle sample. All assays are performed at 30 degrees centigrade (°C) and measured spectrophotometrically. Cytochrome *c* oxidase analysis is the initial test and is performed in an iso-osmotic medium, preserving the mitochondrial membrane. The rate of cytochrome *c* oxidation in this medium is measured spectrophotometrically. Thereafter a detergent, lauryl maltoside, is added to the medium to permeate the external membrane allowing the oxidation process to continue freely. The integrity of the external membrane is accessed by comparing the cytochrome *c* rate of oxidation in the absence and presence of the detergent (Rustin *et al.*, 1994; Birch-Machin *et al.*, 1994).

Complex I and III activities are measured utilising their sensitivity to the inhibitory factor, rotenone. The activity of the rotenone-sensitive complex I is estimated by measuring the rotenone-sensitive NADH-dependent cytochrome *c* reduction. Measuring the rotenone-sensitive NADH oxidation in the presence of decyl ubiquinone allows the estimation of complex I activity only (Birch-Machin *et al.*, 1994). Both these assays are performed on

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mitochondria incubated in distilled water which results in mitochondria burst (Rustin *et al.*, 1994).

The activity of complex II is assayed by measuring the rate of succinate-dependent reduction of dichlorophenol indophenol in the presence of decyl ubiquinone. In this assay the electrons from complex II reduce decyl ubiquinone which reacts with the dichlorophenol indophenol. The succinate dehydrogenase activity may also be attained by utilising phenazine-methosulfate instead of the decyl ubiquinone (Rustin *et al.*, 1994; Birch-Machin *et al.*, 1994).

Complex V is the final complex of the RC. The activity of this complex is measured utilising a coupling assay with lactate dehydrogenase and pyruvate kinase as the coupling enzymes. The oligomycin-sensitive ATP hydrolysis, catalysed by complex V, is coupled to the pyruvate kinase reaction. This reaction is performed in the presence of the substrate, phosphoenolpyruvate, phosphorylates ADP and creates pyruvate. The pyruvate is then utilised by lactate dehydrogenase to oxidise NADH to produce lactate (Rustin *et al.*, 1994).

Comparisons of the activities of the respiratory chain complexes when measured separately and in their defined groups allow the characterisation and quantification of particular defects. Accurate biochemical characterisation of defects in the RC relies on the assessment of the activities of the RC complexes in normal controls (Rustin *et al.*, 1994). The challenge of obtaining age-matched controls for the RC enzyme studies has been minimised by the findings of Chretien *et al.* (1998). In this report it was demonstrated that the biochemical parameters of the RC enzymes did not vary with age, deeming it unnecessary to have age-matched controls for diagnosis of metabolic disorders (Chretien *et al.*, 1998).

3.8.2 Histological investigations for the mitochondrial disorders

The histological studies for mitochondrial disorders included staining muscle sections of biopsies with specific reagents to investigate the presence of particular characteristics indicative of mitochondrial disorders. The Gomori trichrome stain is the most popular staining technique to indicate the presence of ragged red fibres (RRF) within the muscle

section (Engel and Cunningham, 1963; Olson *et al.*, 1972). The RRF is formed by the aggregation of abnormal mitochondria and therefore a very useful indication of mitochondrial pathology (Romero *et al.*, 1996).

Histochemistry can also be utilised to enable a definitive diagnosis testing the activity of specific complexes within the section under investigation. COX activity is indicated by brown stain while fibres deficient in COX activity will remain pale after staining. The activity of complex II can also be evaluated by SDH staining revealing a blue stain if normal activity is present (Romero *et al.*, 1996).

3.8.3 Molecular analysis for mitochondrial disorders

In the past biochemical assays and histological investigations provided a means for the diagnosis of mitochondrial disorders. However, with the advent of more mtDNA mutations being identified as associated with particular mitochondrial disorders molecular analyses offers a more definitive diagnosis than in the past (Adams and Turnbull, 1996).

When the clinical phenotype is indicative of a mitochondrial disorder with known mtDNA mutations it is suggested that molecular analysis be performed initially (Adams and Turnbull, 1996). The molecular diagnosis of metabolic disorders is further enhanced by the discovery of mutations within the nuclear genes associated with either structure or function of the mitochondrial OXPHOS system.

The molecular diagnosis of mitochondrial disorders is enhanced when an inheritance pattern may be discerned within a particular family. This allows the establishment of whether the genetic defect may be localised to the mitochondrial genome or be attributed to nuclear genes.

3.9 GENETIC COUNSELLING FOR MITOCHONDRIAL DISORDERS

Genetic counselling in Mendelian disorders is usually unequivocal and prenatal diagnosis can be performed in many instances. However, this issue is complicated

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when regarding the disorders of mitochondrial energy metabolism, as heterogeneity is observed on a clinical, biochemical and genetic level (Ruitenbeek *et al.*, 1996).

The complexity of mitochondrial genetics as discussed in section 2.3.3 is the biggest hurdle with regard to genetic counselling for the mitochondrial disorders. This is especially difficult when no known mutation has been identified on the molecular level. In 1996 DiMauro described counselling to be a "dismal problem" and to date there is still few bright prospects in this area for patient care.

Genetic counselling is complicated by the fact that the segregation of the mtDNA point mutations cannot be predicted in the developing foetus. It is therefore impossible to foretell which tissues will be affected. To date, the only correlations which have been made with regard to amniocytes and chorionic villus samples are for the T8993G Leigh Syndrome mutation (Harding *et al.*, 1992). The results are only preliminary but it has been reported that the heteroplasmy levels of the chorionic villus samples are a moderate indication of the heteroplasmy levels of the developing foetus (Harding *et al.*, 1992). This is, however, not true for any of the mtDNA mutations occurring within the mitochondrial tRNA genes (DiMauro, 1996).

Although counselling is difficult in these cases it is vital. Even though no predictive testing can be offered and the chances of having an affected child cannot be estimated it is still important to know the risks. This is especially true for the reproductive choices of the asymptomatic female family members who harbour a mtDNA mutation.

3.10 OBJECTIVES OF THE STUDY

The objective of this study was to screen twenty five clinically well characterised mitochondrial myopathy patients, who had been referred to the centre, for the presence of the most commonly associated mtDNA mutations of the MELAS, LS and MERRF syndromes. Of the mutations described above the following ten mutations were investigated in this study: A3243G, A3252G, C3256T, A3260G, T3271C, T3291C, A8344G, T8356C, T8993C and the T8993G. The analysis of these mutations would be performed on a molecular level utilising standard protocols.

CHAPTER FOUR

MATERIALS AND METHODS

The study reported in this thesis has been reviewed by the Ethics Committee of the Faculty of Medicine of the University of Pretoria and Pretoria Academic Hospitals. The approval number 91/98 was assigned to the study entitled "Mitochondrial DNA (mtDNA) mutations in patients with a suspected mitochondrial disorder in the South African context". Written informed consent was obtained from the patients, or their legal guardians, prior to the collection of blood or tissue samples.

All the chemicals that were used were analar grade products of Promega® [1] unless stated otherwise. The protocols followed during the study were carried out according to the published instructions or those acquired with the respective kits, any modifications to the protocols are indicated throughout.

4.1 PATIENT POPULATION

Twenty-five South African individuals were referred to our national referral centre for molecular screening of mutations causing mitochondrial myopathies. All 25 patients included in the investigation were clinically evaluated by Dr. I. Smuts (Department of Paediatrics, University of Pretoria), clinical co-ordinator of the mitochondrial myopathy research programme. Subsequent to clinical evaluation patients were referred for molecular screening of the most appropriate mitochondrial mutations reported to date. The clinical diversity presented by mitochondrial myopathies necessitates the evaluation of all the patients by one clinician. The clinical criteria utilised for this study are listed in Appendix C, and states that for inclusion into the study the patient should present with involvement of two or more of the described systems. Table 4.1 lists the most apparent clinical signs observed in the patients included in this study and indicates which clinical phenotype (MELAS, LS or MERRF) was suggested as the most likely diagnosis for the particular individuals.

[1] Promega® is the registered trademark of the Promega Corporation, Madison, WI, U.S.A.

Table 4.1: The clinical phenotype of the mitochondrial myopathy patients

		Patients												
Patient number		356	366	384	385	386	387	388	389	390	397	398	399	400
Age		10	8	†	3	2	3	3	4	3	10	5	11	2
Gender		M	F	F	F	F	F	F	F	F	F	M	M	F
Ethnicity		C	C	A	A	C	C	C	C	A	C	C	A	C
Tissue	**Symptom/Sign**													
CNS	Seizures													
	Ataxia													
	Myoclonus													
	Psychomotor delay													
	Psychomotor regression													
	Hemiparesis													
	Blindness cortical													
	Migraine like headaches													
	Dystonia													
	Apnoea													
	Long tract signs													
PNS	Peripheral neuropathy													
Muscle	Hypotonia													
	Hypertonia													
	Ptosis													
Kidney	Fanconi syndrome													
	Chronic renal failure													
Heart	Conduction block													
	Cardiomyopathy													
Endocrine	Short stature													
	Diabetes mellitus													
Eye	Pigment retinopathy													
	Strabismus													
	Optic atrophy													
	Ptosis													
	Cataracts													
	External opthalmoplegia													
ENT	Sensorineural deafness													
Laboratory	Lactic acidosis													
	Metabolic profile*													
Radiology	Basal ganglia involved													

Table 4.1: continued ...

		Patients											
Patient number		403	404	406	407	409	410	411	412	413	425	426	433
Age		2	†	3	12	10	1	†	8	2	1	†	5
Gender		M	M	M	M	M	M	F	F	M	M	M	F
Ethnicity		A	A	A	A	C	C	A	C	A	C	I	C
Tissue	**Symptom/Sign**												
CNS	Seizures	■					■	■	▒				▒
	Ataxia												
	Myoclonus								▒				▒
	Psychomotor delay	■	■	■	▒	■	■		▒				
	Psychomotor regression												▒
	Hemiparesis									▒			
	Blindness cortical	■											
	Migraine like headaches							■					
	Dystonia				▒								
	Apnoea											■	
	Long tract signs					■	■						
PNS	Peripheral neuropathy												
Muscle	Hypotonia	■	■	■	▒		■	■				■	▒
	Hypertonia									▒			
	Ptosis												
Kidney	Fanconi syndrome												
	Chronic renal failure												
Heart	Conduction block												
	Cardiomyopathy											■	
Endocrine	Short stature												
	Diabetes mellitus					■							
Eye	Pigment retinopathy							■					
	Strabismus					■							
	Optic atrophy												
	Ptosis												
	Cataracts												
	External opthalmoplegia								▒			■	
ENT	Sensorineural deafness												
Laboratory	Lactic acidosis		■				■	■		▒			
	Metabolic profile*	■		■	▒		■	■			■		
Radiology	Basal ganglia involved									▒			

The age of the patients is indicated in years with respect to the date of this thesis. M = male; F = female. The ethnicity of the patients is indicated by A = black African, C = Caucasian, I = Indian. ▒ = clinically indicative of MERRF; ■ = clinically indicative of MELAS; ▒ = clinically indicative of Leigh Syndrome; *Metabolic profile: indicative of a mitochondrial disorder.

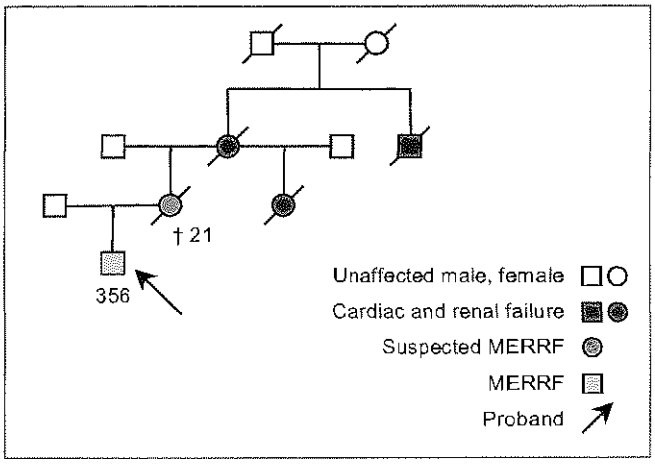
4.1.1 Pedigrees for selected patients

Many of the patients were the first individual within the family in whom a positive mitochondrial myopathy diagnosis has been made. The majority of the families were small and in many instances the pedigrees were not informative. In some instances no family history could be obtained, therefore only seven selected pedigrees are provided below. The molecular investigation included all the patients listed in Table 4.1 and additional maternal relatives as indicated by a number in the pedigrees. Thus all individuals indicated by a patient number in any of the pedigrees, were included in this study. All 25 probands included in the study were analysed for all ten of the reported mtDNA mutations associated with MELAS, Leigh Syndrome and MERRF (as listed in section 4.3).

4.1.1.1 Patient 356

Patient 356 is a ten year old male Caucasian. He presented shortly after birth with episodes of myoclonus. His mother died at the age of 21 years and had clinical features suggestive of MERRF. The autopsy of the mother revealed atrophy of the proximal and distal muscles, and the muscle samples displayed ragged red fibres. Maternal inheritance was suggestive in this pedigree as the maternal relatives of the proband's mother died of cardiac and renal failure (Figure 4.1). These signs could be associated with a mitochondrial myopathy but cannot be unequivocally attributed to alterations within the mtDNA in these individuals as detailed clinical records are not available. The maternal relatives were also never investigated on a molecular level for any of the mitochondrial myopathies.

Figure 4.1: Pedigree for patient 356

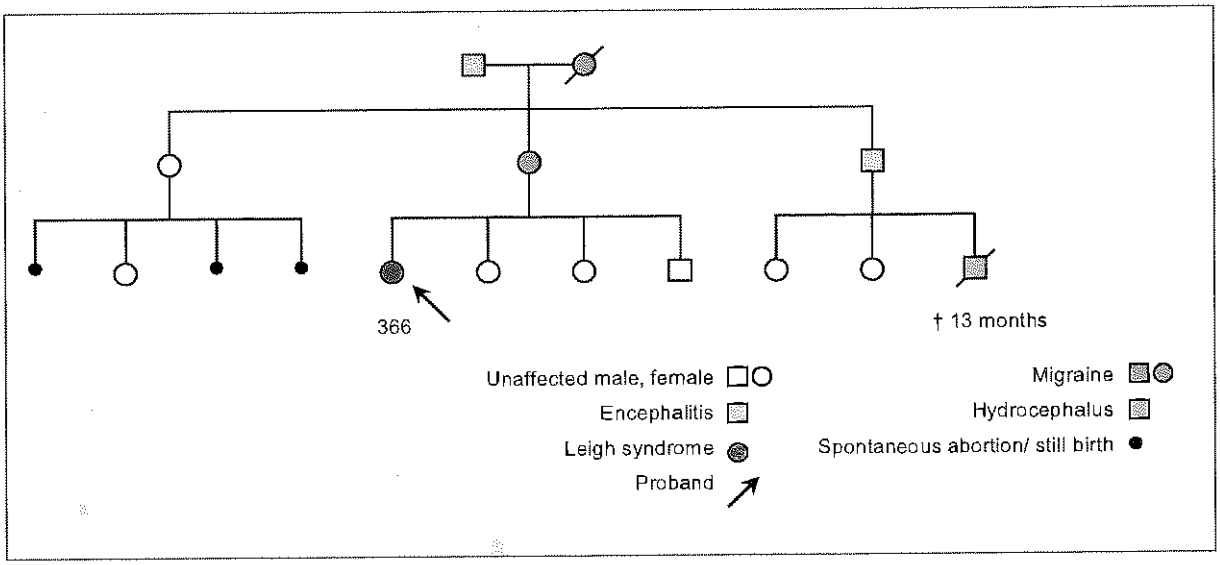


† 21 = deceased at the age of 21 years.

4.1.1.2 Patient 366

Patient 366 is an eight year old female Caucasian patient. She first presented with seizures at the age of six. The family history and pedigree presented in Figure 4.2, has a marked maternal inheritance trend enhancing the probability of a mitochondrial aetiology. The number of spontaneous miscarriages within the proband's immediate family further supports the notion of a mitochondrially inherited disorder.

Figure 4.2: Pedigree for patient 366

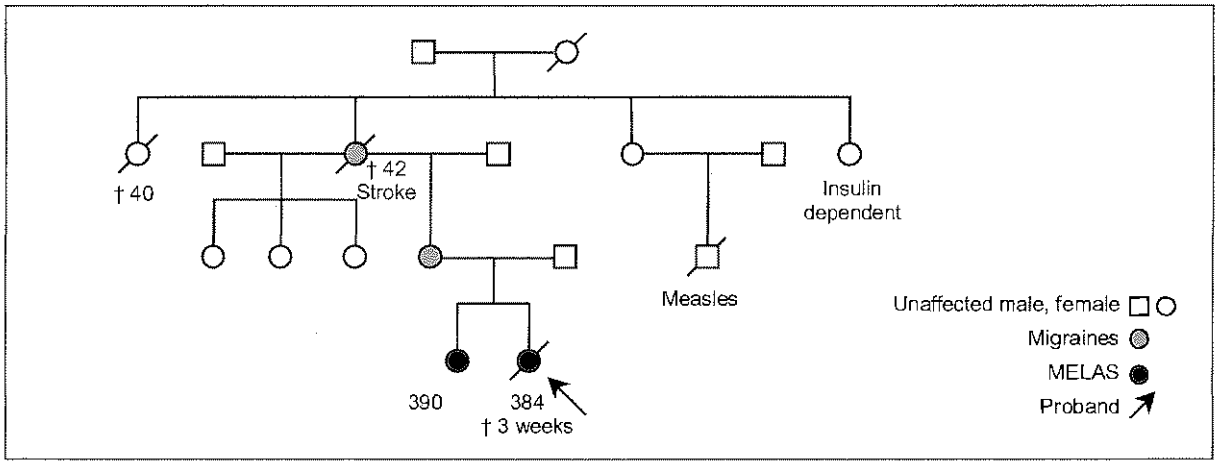


† 13 months = deceased at the age of 13 months.

4.1.1.3 Patients 384 and 390

As illustrated in Figure 4.3 the above mentioned two patients are sisters. The elder sister (390) presented with similar symptoms to her younger sibling (384), but displayed a much less aggressive progress of symptoms. Individual 384 presented at birth with hypotonia and contractures. She also had a cardiomyopathy and unexplained seizures.

Figure 4.3: Pedigree for patients 384 and 390

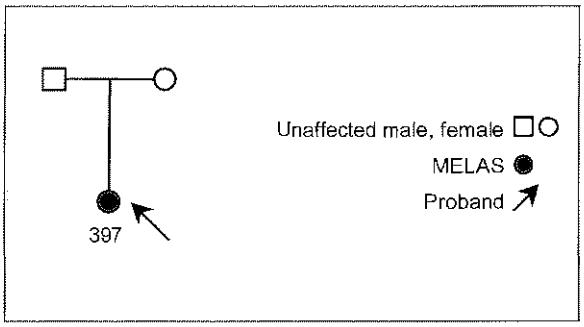


† 40 = deceased at the age of 40 years; † 42 stroke = deceased at the age of 42 years from a stroke; † 3 weeks = deceased at the age of three weeks.

4.1.1.4 Patient 397

Patient 397 was eight years of age when she initially presented with sudden onset of progressive bilateral sensorineural deafness. Her developmental milestones were also delayed. As depicted in Figure 4.4 there is no apparent family history of mitochondrial myopathies in this family and her case appears to be sporadic.

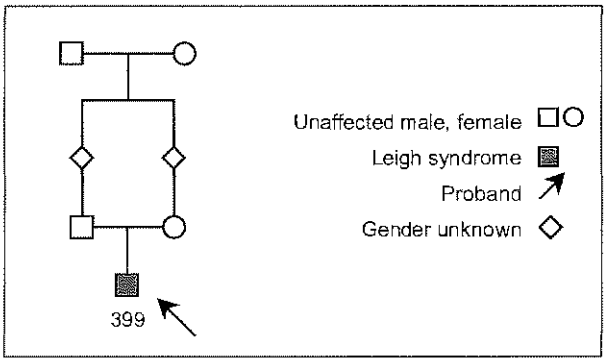
Figure 4.4: Pedigree for patient 397



4.1.1.5 Patient 399

As illustrated in Figure 4.5 consanguinity is present within the family of individual 399. This patient presented with delayed milestones and hypotonia during infancy. Neuroradiological findings indicated low density areas within the bilateral periventricular regions

Figure 4.5: Pedigree for patient 399

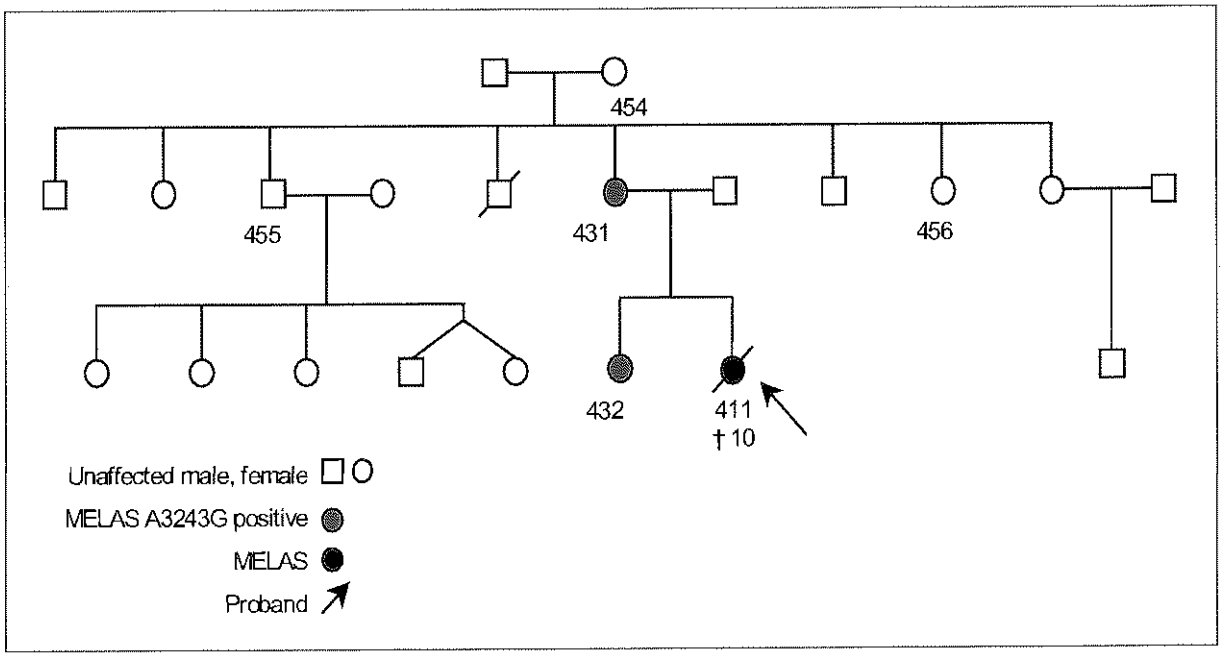


surrounding the frontal horns within the basal ganglia and thalami, suggestive of a Leigh Syndrome diagnosis.

4.1.1.6 Patient 411

The proband (411) first presented with symptoms indicative of a mitochondrial disorder at the age of 8 years. She had a lactic acidosis, high serum glucose and severe recurrent episodic migraines. Initially it was suspected that the patient was afflicted with a Leigh-like disorder as no stroke-like episodes were present. Extensive molecular analysis was performed on this family including three other maternal relatives (454, 455, 456) depicted in Figure 4.6. For illustrative purposes the molecular findings, which will be discussed in chapter 5, are presented in this pedigree.

Figure 4.6: Pedigree for patient 411

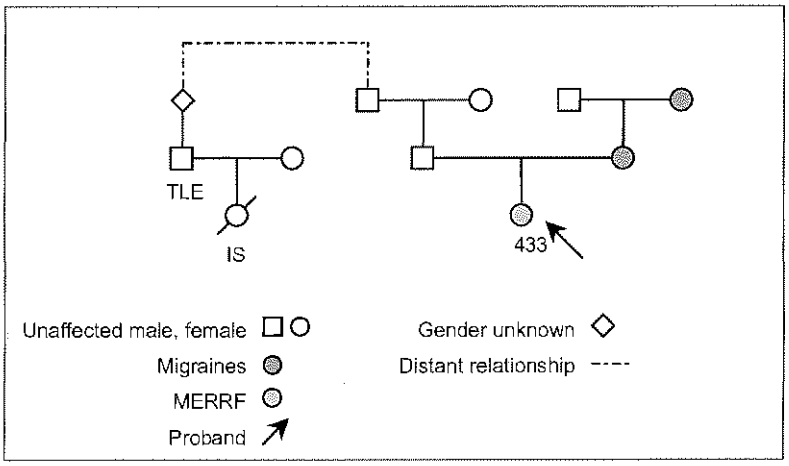


† 10 = deceased at the age of ten years.

4.1.1.7 Patient 433

Patient 433 presented with the first clinical signs of epilepsy at the age of 9 months. Her mother and grandmother both suffered from migraines as indicated in Figure 4.7. From the clinical data of the patient a phenotype suggestive of MERRF was diagnosed.

Figure 4.7: Pedigree for patient 433



TLE = temporal lope epilepsy; IS = infantile spasms.

4.2 ISOLATION OF DNA

Genomic DNA (gDNA) was extracted from whole blood or fibroblast samples. gDNA was isolated from the various tissue samples following the protocols stated by the Wizard®[1] Genomic DNA Purification kit (Promega®).

4.2.1 Isolation of DNA from whole blood

The Wizard® Genomic DNA Purification Kit is designed to allow the isolation of gDNA from various tissue types and small sample volumes such as 300 microlitres (µl). The procedure for DNA isolation was a four-step protocol starting with the lysis of the red blood cells by the addition of the Cell Lysis Solution, leaving the white blood cells intact. The 300 µl whole blood sample was added to 900 µl of the Cell Lysis Solution in a sterile 1.5 millilitre (ml) eppendorf-type tube. The lysis of red blood cells took place during incubation at room temperature for ten minutes during which time the solution was mixed twice by inversion. The sample was then centrifuged at 13 000 x gravity (x g)

[1] Wizard® is the registered trademark of the Promega Corporation, Madison, WI, U.S.A.

for 30 seconds (sec). A white pellet formed and the supernatant was discarded. The pellet was resuspended in the eppendorf tube by vigorous vortexing.

Once the white cells were completely resuspended cell lysis was performed by the addition of 300 µl Nuclei Lysis Solution. This solution was added to the resuspended white blood cells and mixing occurred by pipetting a few times to ensure efficient lysis. The sample was incubated at 37 degrees centigrade (°C) for 30 minutes (min). After incubation the samples were allowed to cool down to room temperature by leaving them on the bench for 15 minutes.

After nuclei lysis, protein precipitation occurred by the addition of 100 µl of Protein Precipitation Solution to the room temperature nuclear lysate. The sample was vortexed vigorously as the sample becomes extremely viscous and mixing of the lysate and solution is difficult if not assisted. Once the sample was mixed it was centrifuged at 13 000 x g for three minutes, where after the cellular protein formed a visible pellet at the bottom of the tube.

The supernatant containing the high molecular weight gDNA was removed from the protein pellet and transferred to a new sterile eppendorf tube containing 300 µl isopropanol at room temperature. The sample was mixed gently to ensure complete precipitation of the DNA. Once DNA precipitation was complete the sample was centrifuged at 13 000 x g for one minute to aggregate the DNA into a white pellet. The supernatant was removed and discarded, and 300 µl of a 70 percent (%) ethanol (EtOH) solution was added to the DNA pellet to wash out all remaining salts. Subsequently, the solution was centrifuged at 13 000 x g for one minute and the supernatant removed. Care was taken while discarding the supernatant in order not to disturb the fairly loose pellet. Once all the EtOH was removed the DNA pellet was air dried for 15 minutes by inverting the eppendorf tube on a sterile towel.

During the final step in the isolation procedure the gDNA pellet was rehydrated into solution. The DNA Rehydration Solution was utilised for this purpose and 100 µl of the solution was added to the tube containing the gDNA pellet. The DNA Rehydration Solution consisted of a TE buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA]. Generally the samples were rehydrated at 4°C overnight. Once the samples were rehydrated they were stored as a concentrated stock DNA sample at 4°C.

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4.2.2 Isolation of DNA from cultured cells

For the extraction of gDNA from tissue culture cells, the protocol of the Wizard® Genomic DNA Purification Kit for whole blood samples was slightly modified. The tissue culture cells were first harvested from the culture flasks via treatment with Active Trypsin Versene (ATV) [pH 7.6]. The culture medium was discarded prior to the addition of 2 ml of ATV which was utilised to rinse the culture flask, after which the medium was discarded. Another 2 ml of ATV was added to the culture flask and the sample was heated for 3 minutes on a hot plate. The sides of the flask where then rinsed with the sample by pipetting of the solution. The trypsinised cells were harvested and transferred to two clean 1.5 ml centrifuge tubes. A cell pellet was obtained by centrifugation of the sample at 13 000 x g for 3 minutes. After centrifugation the cells were visible as a yellowish pellet at the bottom of the tube. The supernatant was discarded and the cells were resuspended. For this protocol no Cell Lysis Solution was added, instead the resuspended cells were treated with 300 µl of the Nuclei Lysis Solution. Thereafter the isolation of the gDNA from the tissue culture cells was performed as for whole blood samples. In this modified protocol for tissue culture cells the RNase digestion was compulsory. Three microlitres of the RNAse A (4 $mg.ml^{-1}$) solution was added to the nuclear lysate and then incubated at 37°C for half an hour. The sample was then allowed to cool down to room temperature before the Protein Precipitation Solution was added. The remaining steps of the procedure were identical to the protocol for whole blood samples.

The yield of gDNA isolated from whole blood or tissue culture cells varied between 50 $ng.\mu l^{-1}$ and 1250 $ng.\mu l^{-1}$. The average yield via the Wizard® Genomic DNA Purification Kit was approximately 250 $ng.\mu l^{-1}$ with an A_{260}/A_{280} ratio of 1.8 to 2.0. The optical density (OD) of the samples were analysed using the A_{260} and A_{280} values that were generated via spectrophotometry. The concentration of the gDNA was calculated using the formula: [DNA] = A_{260} x (50 $ng.\mu l^{-1}$ x dilution factor) as described by Sambrook *et al.* (1989). Generally the dilution factor was 100 X, as a result of diluting 10 µl of concentrate sample in 990 µl ddH_2O for analyses purposes. Once the concentration of the samples was determined working dilutions of 50 $ng.\mu l^{-1}$ were prepared and utilised to perform all experiments. The gDNA stock solutions were stored at -20°C while the working dilutions were stored at 4°C.

4.3 THE POLYMERASE CHAIN REACTION

Amplification of DNA via the polymerase chain reaction (PCR) was performed according to a modified version of the PCR protocol described by Mullis *et al.* (1986 and 1987). Specific regions of the mitochondrial genome, surrounding the respective mutations to be analysed, were amplified via PCR.

For optimisation and direct sequencing the PCR reactions were prepared in a total reaction volume of 12.5 µl. Reaction volumes were adjusted for restriction endonuclease digestion analysis to a final volume of 25.0 µl.

The PCR reactions contained the following: 1 X PCR buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100® [1]], 1.5 mM $MgCl_2$, 200 µM of each nucleotide [2'-deoxyadenosine-5'triphosphate (dATP), 2'-deoxycytidine-5'-triphosphate (dCTP), 2'-deoxyguanosine-5'-triphosphate (dGTP) and 2'-deoxythymidine-5'-triphosphate (dTTP)], 10 pmol each of the respective forward and reverse primers, 1 U Taq DNA polymerase and 100 ng gDNA in a final reaction volume of 12.5 µl.

Amplification of the desired fragments was performed in a Hybaid Touchdown™ [2] thermocycler. The calculated annealing temperature of each primer set was determined by utilising the formula $T_m = 2(A + T) + 4(G + C)$ as described by Thein and Wallace in 1986.

The cycling parameters as listed in Table 4.2 for all ten mutations were kept constant with the annealing temperature being the only variable, and dependent on the primer set. All PCR amplification conditions were optimised before mutation analysis was performed. The amplification of all the amplicons for the various mutations, therefore, utilised the following PCR cycling conditions:

[1] Triton X-100® is a registered trademark of Rohm & Haas Company, Philadelphia, PA, U.S.A.
[2] TouchDown™ is a trademark of Hybaid Limited, Ashford, Middlesex, United Kingdom.

Table 4.2: PCR conditions for amplification of mitochondrial DNA regions

PCR step	# of cycles	Description	Temperature	Duration
1	1	denaturation	94°C	10 min
2	30	denaturation	94°C	30 sec
		annealing	T_a°C	30 sec
		extension	72°C	30 sec
3	1	extension	72°C	7 min

Tables 4.3, 4.4 and 4.5 summarise the primers, their respective sequences, and the calculated annealing temperatures which were utilised to amplify the specific amplicons for each of the respective mutations.

4.3.1 Mutations within the mitochondrial tRNA$^{Leu(UUR)}$ gene

Six mutations localised to the tRNA$^{Leu(UUR)}$ gene were analysed during this study. Analysis of the different mutations was performed on amplicons generated for the specific detection of mutations via restriction fragment length polymorphism (RFLP) analysis. The primers, annealing temperatures and amplicon sizes for the six mutations within the tRNA$^{Leu(UUR)}$ gene that were analysed, are listed in Table 4.3.

Table 4.3: Primers utilised for amplification of the mutations localised to the tRNA$^{Leu(UUR)}$ gene

Mutation	Primer name	Primer sequence	T_m	T_a	size
A3243G[1]	F: ND1-FOR-B	5'-ccc gat ggt gca gcc gc-3'	60	55	364
	R: 3.3-REV	5'-gca tta gga atg cca ttg cg-3'	60		
A3252G[2]	F: 2851FOR	5'-aca tgc taa gac ttc acc ag-3'	58	55	425
	R: 3276REV	5'-tgt aaa gtt tta agt ttt atg tga-3'	58		
C3256T[3]	F: 3237FOR	5'-tgg cag agc ccg gta aac g-3'	62	55	509
	R: 3745REV	5'-cta ggg tga ctt cat atg ag-3'	58		
A3260G[4]	F: 3242FOR	5'-gag ccc ggt aat cgc tta-3'	56	55	476
	R: 16SREV	5'-ggc tac tgc tcg cag tg-3'	56		

Table 4.3: continued ...

Mutation	Primer name	Primer sequence	T_m	T_a	size
T3271C[5]	F: ND1-FOR-B	5'-ccc gat ggt gca gcc gc-3'	60	55	286
	R: 3272-REV	5'-gaa ttg aac ctc tga ctc taa-3'	58		
T3291C[6]	F: 3266FOR	5'-aaa act tta cag tca gag gtt cga t-3'	68	55	493
	R: 3758REV	5'-agt aga atg atg gct agg gtg ac-3'	68		

T_m = calculated annealing temperature for each primer; T_a = calculated optimal annealing temperature for the primer set; F = forward primer; R = reverse primer. Wallace (1999). Size indicated in base pairs. Mutations reported by: 1 = Goto *et al.* (1990); 2 = Morten *et al.* (1993); 3 = Moraes *et al.* (1993); 4 = Zeviani *et al.* (1991); 5 = Goto *et al.* (1991); 6 = Goto *et al.* (1994).

4.3.2 Mutations within the mitochondrial $tRNA^{Lys}$ gene

Two mutations within the $tRNA^{Lys}$ gene were analysed during this study. Although the mutations lie within a close distance of each other two different amplicons were generated for the RFLP analysis of the mutations. The primers utilised for the generation of the two amplicons are listed in Table 4.4, as well as the annealing temperatures and amplicons sizes.

Table 4.4: Primers utilised for amplicon generation of the mutations localised to the $tRNA^{Lys}$ gene

Mutation	Primer name	Primer sequence	T_m	T_a	size
A8344G[1]	F: 8155FOR	5'-ggt ata cta cgg tca atg ctc t-3'	64	55	212
	R: Ban2-REV	5'-ttt cac tgt aaa gag gtg tgg g-3'	64		
T8356C[2]	F: 8155FOR	5'-ggt ata cta cgg tca atg ctc t-3'	64	55	226
	R: 8380REV2	5'-att tag ttg ggg cat ttc act cta-3'	66		

T_m = calculated annealing temperature for each primer; T_a = optimal annealing temperature for the primer set; F = forward primer; R = reverse primer. Wallace (1999). Size indicated in base pairs. Mutations reported by: 1 = Shoffner *et al.* (1990); 2 = Silvestri *et al.* (1992).

4.3.3 Mutations within the mitochondrial ATPase 6 gene

During this study the two mutations at nucleotide 8993 of the ATPase 6 gene were analysed. RFLP analysis of the two mutations was performed on the same amplicon generated via PCR. Detection of the two mutations was dependent on the digestion of the amplicon with two different restriction endonucleases, each specific for one of the

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mutations. The primers required for the amplification of the region surrounding the 8993 mutations are listed in Table 4.5, as well as the annealing temperatures utilised for PCR.

Table 4.5: Primers utilised for amplicon generation of the mutations localised within the ATPase 6 gene

Mutation	Primer name	Primer sequence	T_m	T_a	size
T8993C[1]	F: 952x10BF	5'-cct agc cat ggc cat cc-3'	56	55	1031
T8993G[2]	R: 9859-REV	5'-cag ata gtg agg aaa gtt ga-3'	56		

T_m = calculated annealing temperature for each primer; T_a = optimal annealing temperature for the primer set; F = forward primer; R = reverse primer. Wallace (1999). Size indicated in base pairs. Mutations reported by: 1 = de Vries *et al.* (1993); 2 = Holt *et al.* (1990).

4.4 RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

The mtDNA mutations were detectable via RFLP analyses as they either create or abolish specific restriction sites within the amplicons. This allowed for the identification of these mutations based on the detection of fragments of specific lengths subsequent to electrophoresis of the digested amplicons.

All RFLP reactions were performed in a total volume of 30 µl. The restriction digestion reagents made up a volume of 20 µl and 10 µl of the PCR product was added. The restriction digestion reagents were utilised as prescribed by the manufactures of the restriction endonuclease enzymes, but in general, these included a buffer that was compatible with the specific restriction endonuclease, and when required bovine serum albumin (BSA) was added to the reaction.

4.4.1 Mutations within the mitochondrial tRNA$^{Leu(UUR)}$ gene

The six mutations within the mitochondrial tRNA$^{Leu(UUR)}$ gene were analysed via RFLP after amplification of the various amplicons as stated in paragraph 4.3.1. Digestion for RFLP analysis was performed utilising a particular restriction endonuclease for each mutation.

4.4.1.1 The A3243G mutation

The mutation, A3243G, was detected within the $tRNA^{Leu(UUR)}$ gene via digestion of a 364 bp PCR amplified fragment. The primers listed in Table 4.3 were utilised for the amplification of the region surrounding the mutation. In Table 4.6 the position of the primers, as well as that of the A3243G mutation is indicated.

Table 4.6: Partial sequence of the mitochondrial $tRNA^{Leu(UUR)}$ gene encompassing the 3243 mutation

Nucleotide	Sequence
3001	ggacatcccg atggtgcagc cgctattaaa ggttcgtttg ttcaacgatt aaagtcctac
3061	gtgatctgag ttcagaccgg agtaatccag gtcggtttct atctaccttc aaattcctcc
3121	ctgtacgaaa ggacaagaga aataaggcct acttcacaaa gcgccttccc ccgtaaatga
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agAgccggt aatcgcataa aacttaaaac tttacagtca gaggttcaat tcctcttctt
3301	aacaacatac ccatggccaa cctcctactc ctcattgtac ccattctaat cgcaatggca
3361	ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). Nucleotide position 3243 is indicated in capital letter while the block indicates the position of the *Hae* III restriction site created by the mutation, the arrow at the digestion point indicates the other restriction sites. Sequence adapted from MITOMAP (2001).

For RFLP analysis the amplicon was digested with 10 U of the restriction endonuclease *Hae* III. This restriction endonuclease has the following recognition site sequence 5'-GG↓CC-3', as illustrated in Table 4.6. The buffer utilised in this digestion reaction was 1 X buffer C composed of 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). BSA was added to a final concentration of 0.1 $mg.\mu l^{-1}$. The digestion reaction was incubated at 37°C overnight. Digested products were electrophoresed either on a 2% agarose gel or a 10% polyacrylamide gel, as described in paragraph 4.5.

4.4.1.2 The A3252G mutation

The A3252G mutation associated with MELAS is amplified from the $tRNA^{Leu(UUR)}$ gene of the mtDNA via PCR with primers which have been modified for RFLP analysis. A fragment of 425 bp is generated via amplification of the region surrounding the A3252G mutation utilising the primers listed in Table 4.3.

The reverse primer, 3276REV, was modified to contain a mismatch at nucleotide position 3255. This C to T mismatch creates a *Mae* III recognition site (5'-↓GTNAC-3') in the presence of the A3252G mutation. Table 4.7 illustrates the primer positions and the restriction sites utilised for RFLP analysis.

Table 4.7: Partial sequence of the mitochondrial tRNA$^{Leu(UUR)}$ gene encompassing the 3252 mutation

Nucleotide	Sequence
2821	cctcggagca gaacccaacc tccgagcagt acatgctaag acttcaccag tcaaagcgaa
2881	ctactatact caattgatcc aataacttga ccaacggaac aagttaccct agggataaca
2941	gcgcaatcct attctagagt ccatatcaac aatagggttt acgacctcga tgttggatca
3001	ggacatcccg atggtgcagc cgctattaaa ggttcgtttg ttcaacgatt aaagtcctac
3061	gtgatctgag ttcagaccgg agtaatccag gtcggtttct atctaccttc aaattcctcc
3121	ctgtacgaaa ggacaagaga aataaggcct acttcacaaa gcgccttccc ccgtaaatga
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agagcccggt aATcgcataa aacttaaaac tttacagtca gaggttcaat tcctcttctt

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The introduced mismatch at position 3255 is indicated in bold. The position of nucleotide 3252 is indicated by a capital letter, the block indicates the created *Mae* III restriction site, and the arrow indicates the other restriction site. Sequence adapted from MITOMAP (2001).

For RFLP analysis the 425 bp amplicon was digested with 2 U of the restriction endonuclease *Mae* III (Boehringer Mannheim). The buffer utilised in this digestion reaction was a 2 X *Mae* III buffer (40 mM Tris-HCl [pH 8.2], 550 mM NaCl, 12 mM $MgCl_2$, 14 mM mercaptoethanol) diluted in the total volume of 30 µl to yield a final concentration of 1 X. The RFLP digestion reaction was performed at 55°C overnight. The digested products were electrophoresed on a 2% agarose gel.

4.4.1.3 The C3256T mutation

The primers listed in Table 4.3 were utilised to amplify a 509 bp fragment surrounding the MERRF C3256T mutation. The forward primer was modified to facilitate RFLP analysis for the presence of the mutation, as illustrated in Table 4.8. The forward primer, 3237FOR, contains a T to A mismatch at the 3253 position. This modification of the primer creates an additional *Mae* II recognition site (5'-A↓CGT-3') within the amplicon.

Table 4.8: Partial sequence of the mitochondrial $tRNA^{Leu(UUR)}$ gene encompassing the 3256 mutation

Nucleotide	Sequence
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agagcccggt aatcgCataa aacttaaaac tttacagtca gaggttcaat tcctcttctt
3301	aacaacatac ccatggccaa cctcctactc ctcattgtac ccattctaat cgcaatggca
3361	ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac
3421	gttgtaggcc cctacgggct actacaaccc ttcgctgacg ccataaaact cttcaccaaa
3481	gagcccctaa aacccgccac atctaccatc accctctaca tcaccgcccc gaccttagct
3541	ctcaccatcg ctcttctact atgaaccccc ctccccatac ccaaccccct ggtcaacctc
3601	aacctaggcc tcctatttat tctagccacc tctagcctag ccgtttactc aatcctctga
3661	tcagggtgag catcaaactc aaactacgcc ctgatcggcg cactgcgagc agtagcccaa
3721	acaatctcat atgaagtcac cctagccatc attctactat caacattact aataagtggc
3781	tcctttaacc tctccaccct tatcacaaca caagaacacc tctgattact cctgccatca

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The mismatch introduced at position 3253 is indicated in bold. The C3256T mutation is indicated in capital letter, the block indicates the created *Mae* II restriction site, and the arrow indicates the other restriction site. Sequence adapted from MITOMAP (2001).

RFLP analysis was performed on the 509 bp amplicon utilising 2 U of the *Mae* II restriction endonuclease (Boehringer Mannheim). The reaction buffer supplied with the enzyme was a 2 X concentration buffer containing 100 mM Tris-HCl (pH 8.8), 440 mM NaCl, 12 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 200 $\mu g.ml^{-1}$ BSA. This reaction buffer was utilised in the digestion reaction in a 1 X final concentration. The digestion reaction was performed at 50°C overnight. The digested fragments are electrophoresed on a 2% agarose gel.

4.4.1.4 The A3260G mutation

The A3260G MELAS associated mutation is detected via amplification of a 476 bp fragment with the use of the specific primers listed in Table 4.3. The forward primer for this mutation has been modified at position 3257 to allow mutation detection via RFLP. A mismatch of A to T has been introduced at the 3' end of the forward primer, 3242FOR, to create an additional *Dde* I restriction endonuclease site (5'-C↓TNAG-3') in the presence of the mutation as illustrated in Table 4.9.

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Table 4.9: Partial sequence of the mitochondrial tRNA$^{Leu(UUR)}$ gene encompassing the 3260 mutation

Nucleotide	Sequence
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agagcccggt aatcgcataA aacttaaaac tttacagtca gaggttcaat tcctcttctt
3301	aacaacatac ccatggccaa cctcctactc ctcattgtac ccattctaat cgcaatggca
3361	ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac
3421	gttgtaggcc cctacgggct actacaaccc ttcgctgacg ccataaaact cttcaccaaa
3481	gagcccctaa aacccgccac atctaccatc accctctaca tcaccgcccc gaccttagct
3541	ctcaccatcg ctcttctact atgaaccccc ctccccatac ccaaccccct ggtcaacctc
3601	aacctaggcc tcctatttat tctagccacc tctagcctag ccgtttactc aatcctctga
3661	tcagggtgag catcaaactc aaactacgcc ctgatcggcg cactgcgagc agtagcccaa
3721	acaatctcat atgaagtcac cctagccatc attctactat caacattact aataagtggc

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The mismatch introduced at position 3257 is indicated in bold. The A3260G mutation is indicated by a capital letter. The block indicates the created *Dde* I recognition site, the arrow indicates the other site. Sequence adapted from MITOMAP (2001).

The 476 bp amplicon was digested by the addition of 10 U of *Dde* I restriction endonuclease to the digestion reaction. Promega buffer D was utilised as the reaction buffer, and as a 1 X buffer it contains 6 mM Tris-HCl (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT. BSA was also added to a final concentration of 0.1 mg.ml^{-1}. The reaction was incubated at 37°C overnight to achieve complete digestion and the digested products were electrophoresed on a 2% agarose gel.

4.4.1.5 The T3271C mutation

The T3271C mutation is detected via the use of a modified reverse primer for the amplification of a 286 bp fragment. The primers utilised during this detection protocol are listed in Table 4.3. The mismatch G to C, which was introduced at position 3275, creates an additional *Dde* I restriction recognition site of 5'-C↓TNAG-3' within the amplicon when the mutation is present as illustrated in Table 4.10.

Table 4.10: Partial sequence of the mitochondrial tRNA$^{Leu(UUR)}$ gene encompassing the 3271 mutation

Nucleotide	Sequence
3001	ggacatcccg atggtgcagc cgctattaaa ggttcgtttg ttcaacgatt aaagtcctac
3061	gtgatctgag ttcagaccgg agtaatccag gtcggtttct atctaccttc aaattcctcc
3121	ctgtacgaaa ggacaagaga aataaggcct acttcacaaa gcgccttccc ccgtaaatga
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agagcccggt aatcgcataa aacttaaaac Tttacagtca gaggttcaat tcctcttctt

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The introduced mismatch at position 3275 is indicated in bold. The T3271C mutation is indicated by a capital letter. The block indicates the created *Dde* I restriction site, while the arrows indicate the other recognition sites. Sequence adapted from MITOMAP (2001).

Digestion of the amplicon surrounding the T3271C mutation was performed by using 10 U of the restriction endonuclease *Dde* I. The supplied buffer D was utilised as the reaction buffer. This 1 X buffer contained 6 mM Tris-HCl (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT. BSA was added to a final concentration of 0.1 mg.ml^{-1}. The digestion reaction was incubated at 37°C overnight and electrophoresis of the digested products was performed on an 8% polyacrylamide gel.

4.4.1.6 The T3291C mutation

The region surrounding the T3291C mutation located within the tRNA$^{Leu(UUR)}$ gene of the mitochondria was amplified via modified primers to facilitate RFLP analysis. The primers listed in Table 4.3 were utilised to amplify a fragment of 493 bp. The forward primer, 3266FOR, contained a mismatch of A to G at position 3288 at the 3' end enabling the creation of an additional *Mbo* I restriction endonuclease recognition site (5'-↓GATC-3') in the presence of the T3291C mutation, as illustrated in Table 4.11.

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Table 4.11: Partial sequence of the mitochondrial tRNA$^{Leu(UUR)}$ gene encompassing the 3291 mutation

Nucleotide	Sequence
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agagcccggt aatcgcataa aacttaaaac tttacagtca gaggttcaat Tcctcttctt
3301	aacaacatac ccatggccaa cctcctactc ctcattgtac ccattctaat cgcaatggca
3361	ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac
3421	gttgtaggcc cctacgggct actacaaccc ttcgctgacg ccataaaact cttcaccaaa
3481	gagcccctaa aacccgccac atctaccatc accctctaca tcaccgcccc gaccttagct
3541	ctcaccatcg ctcttctact atgaaccccc ctccccatac ccaaccccct ggtcaacctc
3601	aacctaggcc tcctatttat tctagccacc tctagcctag ccgtttactc aatcctctga
3661	tcagggtgag catcaaactc aaactacgcc ctgatcggcg cactgcgagc agtagcccaa
3721	acaatctcat atgaagtcac cctagccatc attctactat caacattact aataagtggc

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The introduced mismatch at position 3288 is indicated in bold. The T3291C mutation is indicated by a capital letter. The block indicates the created *Mbo* I restriction site, and the arrows indicate the other recognition sites. Sequence adapted from MITOMAP (2001).

RFLP analysis utilised 10 U of the restriction endonuclease *Mbo* I to digest the 493 bp amplicon. The reaction buffer C was added to the digestion reaction. In a final concentration of 1 X this buffer contained 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT. BSA was also added to a final concentration of 0.1 mg.ml^{-1}. The digestion was incubated at 37°C overnight and the digested products were electrophoresed on a 2% agarose gel.

4.4.2 Mutations within the mitochondrial tRNA^{Lys} gene

Two mutations within the mitochondrial tRNA^{Lys} gene were analysed in this study. An RFLP mutation screening strategy was employed after the amplification of the various amplicons as stated in paragraph 4.3.1. Both analyses rely on the technique of utilising a modified primer to create a restriction site in the presence of a particular mutation, thereby allowing detection of the mutation by analysing the restriction pattern after digestion.

4.4.2.1 The A8344G mutation

The region encompassing the A8344G mutation was amplified by the utilisation of modified primers as listed in Table 4.4. The modification of the reverse primer, Ban2REV, enabled the detection of the mutation via RFLP analysis. As illustrated in Table 4.12 the reverse primer contains a mismatch at position 8347 of a T to G, allowing for the detection of the mutation via digestion of the 212 bp amplicon with the *Ban* II restriction endonuclease. An additional recognition site, 5'-G(A/G)GC(T/C)↓C-3', for *Ban* II is created in the presence of the mutation.

Table 4.12: Partial sequence of the mitochondrial tRNA^{Lys} gene encompassing the 8344 mutation

Nucleotide	Sequence
8101	agatgcaatt cccggacgtc taaaccaaac cactttcacc gctacacgac cgggggtata
8161	ctacggtcaa tgctctgaaa tctgtggagc aaaccacagt ttcatgccca tcgtcctaga
8221	attaattccc ctaaaaatct ttgaaatagg gcc↓cgtattt accctatagc acccccctcta
8281	cccccctctag agcc↓cactgt aaagctaact tagcattaac cttttaagtt aaagattaag
8341	a↓gaAcc**a**aca cctctttaca gtgaaatgcc ccaactaaat actaccgtat ggcccaccat

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The introduced mismatch at position 8347 is indicated in bold. The A8344G mutation is indicated by a capital letter. The block indicates the created *Ban* II restriction site while the arrows indicate the other restriction sites. Sequence adapted from MITOMAP (2001).

The amplicon generated via PCR was digested with 10 U of the *Ban* II restriction endonuclease. This enzyme requires buffer E to be added for optimal functioning. As a 1 X concentration buffer E contained 6 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT. BSA in a final concentration of 0.1 mg.ml^{-1} was added to the digestion reaction. Digestion was performed overnight at 37°C and electrophoresis of the products was performed utilising a 12% polyacrylamide gel.

4.4.2.2 The T8356C mutation

Amplification of the region surrounding the 8356 mutation was performed via PCR utilising the primers listed in Table 4.4. The reverse primer, 8380REV2, contains a G to C mismatch at position 8359 so as to create an additional recognition site (5'-T↓CTAG-3') for the restriction endonuclease *Xba* I as demonstrated in Table 4.13.

5'-C↓PyCGPuG-3'. Ten units of *Ava* I were utilised together with the reaction NEBuffer 4 (New England Biolabs® [1]). The final concentration of the NEBuffer 4 was a 1 X and contained 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate and 1 mM DTT (pH 7.9 at 25°C). The digestion reaction was performed at 37°C overnight. The digested products were electrophoresed on a 2% agarose gel.

4.5 GEL ELECTROPHORESIS

Two types of gel electrophoresis systems were utilised to separate the digested fragments after digestion was completed. The choice between the two systems was made dependent on the size of the fragments to be separated. In general, agarose gels were utilised for fragments larger than 150 bp while polyacrylamide gels were preferred when digested fragments to be analysed were smaller than 150 bp.

4.5.1 Agarose gel electrophoresis

Two percent mini submarine agarose gels were utilised to electrophorese the PCR amplified fragments. These gels had a final volume of 25 ml and contained 0.5 grams (g) of SeaKem® [2] LE agarose [FMC], 2.5 ml of 10 X TBE buffer (89.15 mM Tris® [3][pH 8.1], 88.95 mM boric acid, 2.498 mM Na_2EDTA), and 0.5 $\mu g.ml^{-1}$ ethidium bromide (EtBr) [Sigma®]. Five microlitres of the PCR amplified product was mixed with 2 µl of a 10 X loading buffer (0.04% Orange G and 50% glycerol) prior to loading of the gels. The agarose gels were electrophoresed for 30 minutes in 1 X TBE buffer at 100 volts (V), unless stated otherwise. The amplified gDNA fragments were visualised via ultra violet (UV) fluorescence and photographed by a video-documentation system.

[1] NEB® is the registered trademark of New England Biolabs, Inc. Beverly, MA, U.S.A.
[2] SeaKem® is a registered trademark of FMC BioProducts, Rockland, ME, U.S.A.
[3] Tris® is a registered trademark of Rohm & Haas Company, Philadelphia, PA, U.S.A.

4.5.2 Non-denaturing polyacrylamide gel electrophoresis

Depending on the product size of the restriction endonuclease digested amplicons polyacrylamide gels were utilised to separate the resulting fragments. The various percentage polyacrylamide (PA) gels were prepared in a final volume of 40 ml and consisted of the desired volume of 40% PA gel stock (19:1 acrylamide: bis-acrylamide [United States Biochemicals (USB)]), 2 ml 10 X TBE buffer, 40 µl of the polymerisation catalyst N,N,N',N'-Tetramethylethylenediamine (TEMED) and 350 µl of the initiator, 10% ammonium persulphate (APS). The 40% PA stock with 5% crosslink (% C) was made up to a final volume of 125 ml with 47.5 g acrylamide and 2.5 g bis-acrylamide. Thus for a 12% PA gel, 12 ml of the 40% PA stock was added to 2 ml 10 X TBE buffer and 26 ml of ddH_2O.

The PA gels were generally electrophoresed at 250 V for 2 hours in 0.5 X TBE buffer. After electrophoresis the fragments were visualised by either EtBr staining or SYBR® [1] Gold staining. A 1 X SYBR® Gold staining solution was made in a final volume of 250 ml 0.5 X TBE buffer. For EtBr staining the staining solution was also a final volume of 250 ml, and consisted of 0.5 X TBE buffer containing 0.5 $\mu g.ml^{-1}$ EtBr. The staining time was generally 30 minutes at room temperature.

4.6 DIRECT SEQUENCING

Direct di-deoxynucleotide chain termination sequencing was performed according to the principle described by Sanger *et al.* (1977). Radioactive nucleotides, α ^{35}S-dATP and α ^{32}P-dCTP, were obtained from Amersham Life Science in the United Kingdom (UK). Sequencing was performed on the three PCR products encompassing the three genes of interest, namely the mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$ and ATPase 6 genes. Table 4.15 indicates which primers were utilised for the amplification of these amplicons and their respective sizes. PCR fragments were sequenced in both directions by utilising the forward and reverse primers in separate sequencing reactions.

[1] SYBR® is a registered trademark of Molecular Probes, Inc., Eugene, Oregon, U.S.A.

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Table 4.15: Primers utilised for the amplification of regions encompassing the mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$ and ATPase 6 genes.

Region	Primer	Sequence	T_m[1]	T_a[2]	size[3]
$tRNA^{Leu(UUR)}$	F[4]: ND1-FOR-B	5'-ccc gat ggt gca gcc gc-3'	60	55	356
	R[5]: 3.3-REV	5'-gca tta gga atg cca ttg cg-3'	60		
$tRNA^{Lys}$	F: 8155FOR	5'-ggt ata cta cgg tca atg ctc t-3'	64	55	324
	R: 8474REV	5'-gag gga ggt agg tgg tag ttt gtg-3'	74		
ATPase6	F: 952x10BF	5'-cct agc cat ggc cat cc-3'	56	55	222
	R: LM9031R	5'-cca att agg tgc atg agt ag-3'	58		

1 = T_m, the calculated annealing temperature for each primer. 2 = T_a, the optimal annealing temperature for the primer set. 3 = size of the amplicon indicated in base pairs. 4 = F, the forward primer. 5 = R, the reverse primer. Wallace (1999).

Sequencing was performed using the T7 Sequenase™ [1] Version 2.0 (Sequenase) kit from USB. All chemicals were supplied with the kit. Five microlitres of the PCR amplified product was pre-treated with 10 U of Exonuclease I (*Exo* I) and 2 U of Shrimp Alkaline Phosphatase (SAP) in a final volume of 7 µl. The pre-treatment reaction was performed at 37°C for 15 minutes, subsequently the enzymes were inactivated at 80°C for a further 15 minutes. After pre-treatment of the PCR products the samples were allowed to cool down to room temperature before addition of the primer. Once the samples were at room temperature 10 pmol of the relevant primer and distilled water were added to a final volume of 10 µl together with the pre-treated samples. Primer annealing was then performed at 98°C for three minutes. This was followed by snap cooling of the samples on ice. The samples remained on ice until the labelling reaction was to be performed.

During the sample pre-treatment process, 0.5 ml tubes or 96 well plates were labelled and filled with 2.5 µl of the termination mixtures (ddNTPs). Four tubes are required for each sample, namely ddA, ddC, ddG and ddT. These tubes were covered and utilised for the termination reaction procedure.

Internal labelling was performed by the addition of 5 µCi α^{35}S-dATP or α^{32}P-dCTP as required and the labelling mix (7.5 µM dGTP, 7.5 µM dCTP and 7.5 µM dTTP) supplied with the kit. When α^{32}P-dCTP was utilised the labelling mix in the kit could not be used as this solution contains no dATPs. Therefore instead of adding the labelling mix, 3µM of each of the following dNTPs (dATP, dGTP, dTTP) were added separately to the

[1] Sequenase™ is a trademark of United States Biochemical Corporation, Cleveland, Ohio, U.S.A.

labelling reaction from solutions provided in the kit. When the isotope, α^{35}S-dATP, was used for labelling, the labelling reaction consisted of 10 µl of the annealed DNA mixture, 0.6 X Sequenase reaction buffer, 0.1 µmol DTT, 1:5 diluted labelling mix, 5 µCi α^{35}S-dATP and 3.2 U of the Sequenase DNA polymerase. When the radioisotope α^{32}P-dCTP was utilised to label the sequencing fragments the labelling reaction was composed of the following: 10 µl of the annealed DNA mixture, 0.6 X Sequenase reaction buffer, 0.1 µmol DTT, 5 µCi α^{32}P-dCTP, 3 pmol each of dATP, dGTP, dTTP, as well as 3.2 U of the Sequenase DNA polymerase.

The labelling and elongation reaction was incubated at room temperature for 3 minutes, after which the termination reactions were performed by the addition of 3.5 µl of the labelled sample to each of the four di-deoxynucleotide (ddNTP) tubes. Incorporation of the ddNTPs was performed at 37°C for 7 minutes. Subsequently, the sequencing reactions were stopped by the addition of 4 µl of a 2 X stop solution that contained 95% formamide, 20 mM EDTA, 0.05% bromophenol blue (BPB) and 0.05% xylene cyanol FF (XC). The reactions were then stored at 4°C until electrophoresis.

4.6.1 Mutations within the mitochondrial tRNA$^{Leu(UUR)}$ region

The six mutations within the mitochondrial tRNA$^{Leu(UUR)}$ gene were analysed via sequencing of the 356 bp amplicon. Both the forward and the reverse primers were utilised as sequencing primers. The positions of the mutations localised within the mitochondrial tRNA$^{Leu(UUR)}$ gene are illustrated below in Table 4.16.

Table 4.16: Partial sequence of the mitochondrial tRNA$^{Leu(UUR)}$ region

Nucleotide	Sequence
3001	ggacatcccg atggtgcagc cgctattaaa ggttcgtttg ttcaacgatt aaagtcctac
3061	gtgatctgag ttcagaccgg agtaatccag gtcggtttct atctaccttc aaattcctcc
3121	ctgtacgaaa ggacaagaga aataaggcct acttcacaaa gcgccttccc ccgtaaatga
3181	tatcatctca acttagtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agAgcccggt aAtcgCataA aacttaaaac Tttacagtca gaggttcaat Tcctcttctt
3301	aacaacatac ccatggccaa cctcctactc ctcattgtac ccattctaat cgcaatggca
3361	ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The six mutations within this region are indicated in bold capital letters. Sequence adapted from MITOMAP (2001).

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Sequencing of this amplicon was optimised when utilising the reverse primer as the sequencing primer. Under these conditions the sequence reaction was labelled for three minutes at room temperature and the termination reactions were performed at 37°C for seven minutes.

When utilising the reverse primer as the sequencing primer, the sequencing reactions were electrophoresed for one and three quarter hours at 60 W in 1 X TBE buffer. This electrophoresis time period was sufficient to visualise all six mutation positions after exposure of the gel to X-ray film. If the forward primer was utilised as the sequencing primer the sequence reactions were electrophoresed for a longer time period (three hours). For this reason the reverse primer was selected as the sequencing primer for this amplicon.

4.6.2 Mutations within the mitochondrial $tRNA^{Lys}$ region

The two mutations analysed during this study localised within the mitochondrial $tRNA^{Lys}$ gene were investigated via sequencing of the 324 bp amplicon. This amplicon, however, differs from that used in RFLP analysis. The reverse primers utilised in the RFLP analysis for both mutations were modified to enable the creation of particular restriction sites in the presence of the specific mutations. Neither of these two reverse primers could therefore be used for sequence analysis as they were situated too close to the mutation sites. For this reason a new reverse primer (8474REV) corresponding to nucleotides 8455-8478 was made and utilised for PCR for sequencing of the $tRNA^{Lys}$ region (Wallace, personal communication).

Both the forward and the new reverse primer were utilised as sequencing primers. The positions of the mutations localised within the mitochondrial $tRNA^{Lys}$ gene are illustrated below in Table 4.17.

Table 4.17: Partial sequence of the mitochondrial tRNA^{Lys} region

Nucleotide	Sequence
8101	agatgcaatt cccggacgtc taaaccaaac cactttcacc gctacacgac cgggggtata
8161	ctacggtcaa tgctctgaaa tctgtggagc aaaccacagt ttcatgccca tcgtcctaga
8221	attaattccc ctaaaaatct ttgaaatagg gcccgtattt accctatagc acccccctca
8281	ccccctctag agcccactgt aaagctaact tagcattaac cttttaagtt aaagattaag
8341	agaAccaaca cctctTtaca gtgaaatgcc ccaactaaat actaccgtat ggcccaccat
8401	aattaccccc atactcctta cactattcct catcacccaa ctaaaaatat taaacacaaa
8461	ctaccaccta cctccctcac caaagcccat aaaaataaaa aattataaca aaccctgaga

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The two mutations are indicated in bold capital letters. Sequence adapted from MITOMAP (2001).

Optimisation of this sequencing reaction required the addition of manganese (Mn) buffer as suggested in the Sequenase kit protocol to prevent the formation of compressions within the sequence. Both forward and reverse primers were optimised as sequencing primers. However, sequencing with the reverse primer consistently generated results that were more reproducible. The sequencing reaction was therefore optimal when utilising the reverse primer as the sequencing primer. The labelling of the sequence reactions were performed at room temperature for three minutes after which the Mn buffer was added to each sample. Thereafter the termination reactions were conducted at 37°C for seven minutes.

When utilising the reverse primer as the sequencing primer the reactions were electrophoresed for two hours at 60 W in 1 X TBE buffer. The forward primer as sequencing primer required longer electrophoresis time which was extended to three and a quarter hours to allow for the visualisation of the same region as the reverse primer after only two hours. To be time efficient it was decided that the reverse primer would be utilised through the investigation of the patient samples.

4.6.3 Mutations within the mitochondrial ATPase 6 region

The two mutations at position 8993 in the ATPase 6 gene can be investigated via direct sequencing. As presented in Table 4.15 above the amplicon created for sequencing is shorter than that used during the RFLP analysis for the same mutations. In this instance the reverse primer utilised for the RFLP analysis has been replaced with a

reverse primer situated closer to the mutation site. It was therefore decided to create a primer in closer proximity to the mutation so that both forward and reverse primers could be utilised for the sequencing reactions.

The new reverse primer, LM9031R, corresponds to mitochondrial nucleotides 9031-9050 and was designed utilising the Net Primer software (PREMIER Biosoft International, 2000) available on the internet. The new reverse primer together with the forward primer results in a 222 bp amplicon which is remarkably shorter than the 1031 bp amplicon utilised for the RFLP analyses. The position of the mutation with respect to the shorter amplicon is illustrated in Table 4.18.

Table 4.18: Partial sequence of the mitochondrial ATPase6 region

Nucleotide	Sequence
8821	tctataaacc tagccatggc catcccctta tgagcgggca cagtgattat aggctttcgc
8881	tctaagatta aaaatgccct agcccacttc ttaccacaag gcacacctac accccttatc
8941	cccatactag ttattatcga aaccatcagc ctactcattc aaccaatagc ccTggccgta
9001	cgcctaaccg ctaacattac tgcaggccac ctactcatgc acctaattgg aagcgccacc

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The nucleotide 8993 is indicated by a capital letter. Sequence adapted from MITOMAP (2001).

Optimal sequencing was obtained when utilising the forward primer as the sequencing primer. No additional modifications were necessary to the sequencing protocol. The sequence reactions were labelled for three minutes at room temperature after which the termination reactions were performed for seven minutes at 37°C.

The utilisation of the forward primer as the sequencing primer required the electrophoresis to be conducted for three hours at 60 W in 1 X TBE buffer. If the reverse primer was utilised as the sequencing primer the electrophoresis time could be cut down to one hour but these sequencing reactions were not reproducible and it was therefore decided rather to perform the analysis with the forward primer as sequencing primer.

4.7 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Denaturing polyacrylamide gel electrophoresis (PAGE) was utilised for visualisation of the sequencing products. These gels contain urea and are heated during electrophoresis to ensure complete denaturation of the fragments.

A six-percent denaturing PA gel with five percent cross-link was utilised to separate the fragments of the sequencing reaction. The gel mixture was prepared in a total volume of 75 ml, containing 19:1 (acrylamide: bis-acrylamide), 7 M urea, 50 µl TEMED and 500 µl 10% APS. Samples were denatured at 80°C for 3 minutes and placed on ice, prior to loading of the gel.

Two different gel systems, Owl and Hoeffer, were utilised for the electrophoresis of the PA gels. The polyacrylamide gels were electrophoresed at 60 watts (W) for the Owl apparatus and 45 W in the Hoeffer system until sufficient separation of the fragments was achieved. After electrophoresis the gels were transferred to 3 MM [Whatman® [1]] filter paper, covered in Cling Wrap and vacuum dried for 45 min at 80°C. Once the gels were dry the Cling Wrap was removed and the gels dusted with Johnson's® [2] baby powder before exposure. The powder prevented the gels from sticking to the film if they were still slightly damp. The dried gels were exposed to X-ray film [Fuji RX-U] until adequate exposure was obtained.

[1] Whatman® is a registered trademark of Whatman Scientific Ltd., Kent, England.
[2] Johnson's® is a registered trademark of Johnson & Johnson Professional Products (Pty.) Ltd., Halfway House, South Africa.

CHAPTER FIVE

RESULTS AND DISCUSSION

The mitochondrial genomes of the patients referred to the centre were mined utilising conventional methods such as RFLP and sequencing analysis, in order to determine the underlying genotype causing expression of the clinical phenotype observed in each of the patients. Although each individual had been clinically identified as exhibiting clinical characteristics of either of the three mitochondrial myopathies investigated, namely MELAS, LS and MERRF, the patients were screened for the ten most common mutations associated with the three disorders.

5.1 ISOLATION OF DNA

The Wizard® Genomic DNA Purification Kit was utilised to isolate gDNA from the samples obtained with informed consent from the patients or their parents. The kit provides a fast and accurate isolation procedure and allows for the isolation of gDNA from small volumes of sample material (300 μl) within two hours. The purity of the gDNA isolated depended on efficient mixing of the protein solution with the lysate so as to remove the maximum amount of cellular proteins from the solution. To this end the gDNA extraction was performed on fresh blood samples where possible so as to maximise the gDNA yield. The gDNA obtained with this procedure was clean, and the concentrations were sufficient to perform the subsequent PCR and sequencing analysis required.

The feature of being able to extract gDNA from such small volumes of blood was essential as the amount of blood collected from paediatric patients is generally less than 10 ml. The protocol supplied with the kit also made it possible to adapt the kit for gDNA isolation from various sample sources. Therefore it was possible to utilise cultured fibroblast cells for DNA isolation. The protocol could also be adapted for isolating DNA from hair samples, however, DNA from this tissue type was not investigated in this study.

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5.2 THE POLYMERASE CHAIN REACTION

All PCR experiments were initially performed on control gDNA for optimisation of the protocols discussed in paragraph 4.3 before patient gDNA was analysed. Table 5.1 summarises the respective primers and their optimised annealing temperatures utilised for the amplification of the PCR fragments required for restriction digestion of the various mutations. As evident from Table 5.1 all the PCR reactions were optimised at an annealing temperature of 55°C, which was the first annealing temperature investigated. The amplification of the regions of the mitochondrial genome were easily optimised and amplified with ease. No additional reagents (DMSO or formamide) were thus required for optimal amplification.

Table 5.1: Summary of PCR amplicons for mitochondrial mutations analysed via restriction fragment length polymorphism

Mutation	Forward Primer	Reverse Primer	T_a[1]	Amplicon Size[2]
A3243G	ND1-ForB	3.3-Rev	55	364
A3252G	2851For	3276Rev	55	425
C3256T	3237For	3745Rev	55	509
A3260G	3242For	16S-Rev	55	476
T3271C	ND1-ForB	3272Rev	55	286
T3291C	3266For	3758Rev	55	493
A8344G	8155For	Ban2-Rev	55	212
T8356C	8155For	8380Rev2	55	226
T8993C/G	952x10Bfor	9859-Rev	55	1031

1 = T_a is the optimised annealing temperature for the primer set. 2 = Amplicon size is indicated in base pairs.

All PCR amplicons were electrophoresed on a 2% mini or midi submarine agarose gel to verify that the correct size amplicon was generated during PCR. The amplicons were sized against an appropriate molecular weight marker during electrophoresis. The mini submarine agarose gels were electrophoresed at 100 V for 30 min compared with the midi submarine agarose gels, which were electrophoresed at 200 V for one hour. Visualisation of the amplicons was performed by the addition of EtBr into the agarose gels prior to electrophoresis allowing fluorescence upon exposure to UV light. A permanent record of the gel was then retained via a photo-documentation system.

5.3 MUTATION DETECTION VIA RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

The optimal detection strategy was experimentally determined and for some of the mutations may differ from the reported protocols. For the ten mutations discussed in this study the analysis strategy included RFLP analysis or sequence determination of the region surrounding the nucleotides associated with the various phenotypes. All samples were analysed in duplicate and if a change was observed the particular sample was analysed in duplicate via both RFLP and direct sequencing to confirm the presence of the alteration. Negative control samples were also included in all mutation analyses.

As far as possible a RFLP strategy which created an additional restriction endonuclease recognition site in the presence of the particular mutation was utilised. This strategy allows for the restriction of the PCR amplicon in the absence of the mutation and further restriction in the presence of the mutation, serving as an internal control for the activity of the restriction enzyme. When no additional restriction site is created by the presence of a mutation it is advisable to include a positive control sample into the RFLP strategy.

5.3.1 Mutations within the mitochondrial $tRNA^{Leu(UUR)}$ gene

Nucleotides 3230 to 3304 in the mitochondrial genome code for the $tRNA^{Leu(UUR)}$ molecule. To date fourteen mutations associated with mitochondrial disorders have been identified within this gene. Initially RFLP analyses was performed for the six mutations under investigation within the $tRNA^{Leu(UUR)}$ gene. However, this strategy was altered to a direct sequencing approach which was more time and cost effective. The region encompassing the six mutations under investigation and eight additional mutations were therefore amplified and analysed via sequencing and are discussed in paragraph 5.4.1. Results obtained from the initial RFLP analyses are discussed below.

5.3.1.1 The A3243G mutation

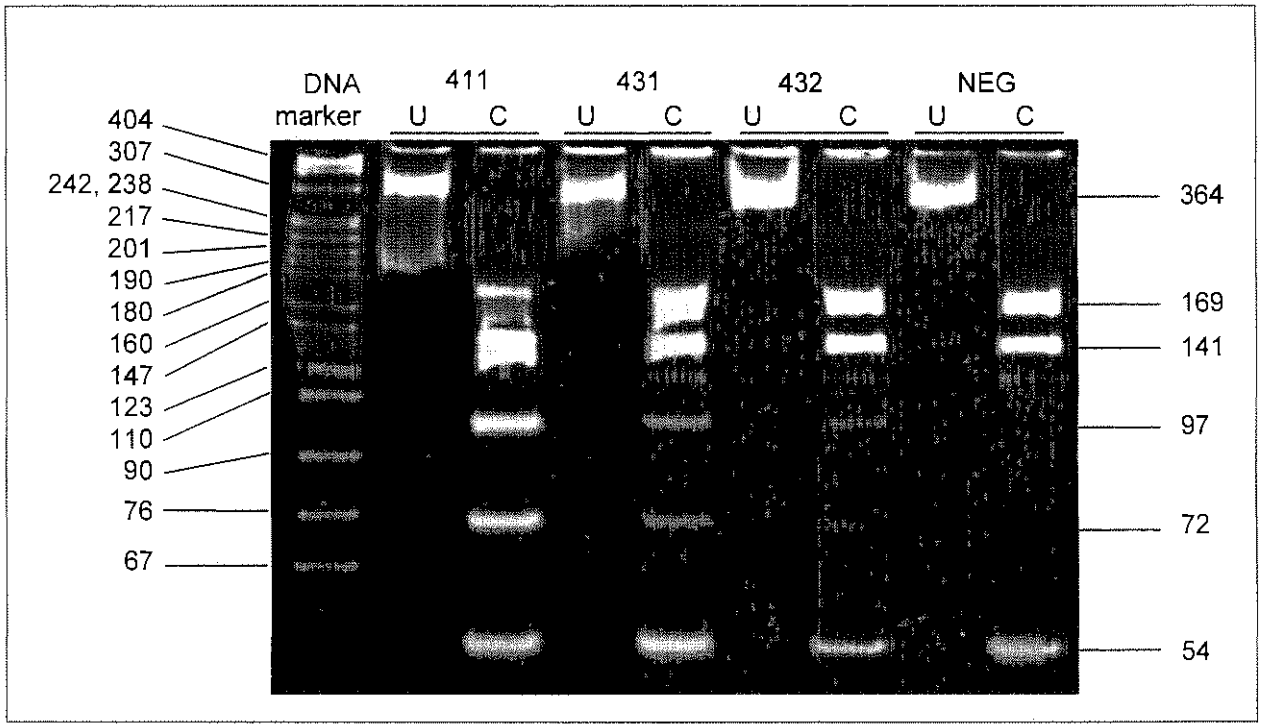
The most common MELAS mutation, A3243G, is detected within the mitochondrial $tRNA^{Leu(UUR)}$ gene via digestion of the 364 bp PCR generated amplicon. This fragment extends on either side beyond the tRNA gene therefore encompassing the gene. The

364 bp amplicon contains two *Hae* III recognition sites. The detection of the mutation relies on the presence of the A3243G mutation to create an additional restriction site for the *Hae* III restriction endonuclease, as illustrated in Table 4.6.

Upon electrophoresis the presence of three fragments, of 169, 141 and 54 bp respectively, will represent the sample of an unaffected individual. In the presence of the mutation the 169 bp fragment is digested into two fragments with sizes of 97 and 72 bp respectively, therefore resulting in the presence of four fragments (141, 97, 72 and 54 bp).

Of the 25 individuals analysed for the A3243G mutation via RFLP and sequencing only one patient was observed to harbour this alteration. The change was detected via RFLP analysis as well as direct sequencing in patient number 411. Five maternal relatives of the proband were then included in the analyses for the mutation, as indicated in the pedigree (see section 4.1). Of the five additional individuals investigated, only two, the mother and sister of the proband, were observed to harbour the mutation. The heteroplasmy levels of the mutation are considerably lower in the two individuals who present with extremely mild (431) to no clinical signs (432) respectively, than in the affected individual (411).

Figure 5.1: Photographic representation of the A3243G mutation analysis



12% polyacrylamide gel elctrophoresed at 250 V for 2 hours and 30 minutes in 1 X TBE buffer. Stained with SYBR® Gold for 30 min. DNA marker = pBR322 digested with *Msp* I. U= uncut, C = cut. NEG = negative control.

Although the presence of the mutation can be observed and various levels of heteroplasmy noted in Figure 5.1 the exact percentage of heteroplasmy could not be calculated for the proband or her relatives. The absence of the mutation within the remaining relatives of the proband may be a function of the detection assay rather than a true indication of the absence of the mutation within these individuals. This may be assumed as blood samples were utilised for analysis and the sensitivity of the assay might not be accurate enough to detect minute amounts of mutation.

Ideally a muscle sample would be more appropriate to exclude the possibility of low levels in blood samples but the mutation being present in muscle tissue. However, it is also apparent that heteroplasmic levels, which are required for the presentation of mitochondrial myopathies on a clinical level, will be detected with the assay utilised in this investigation.

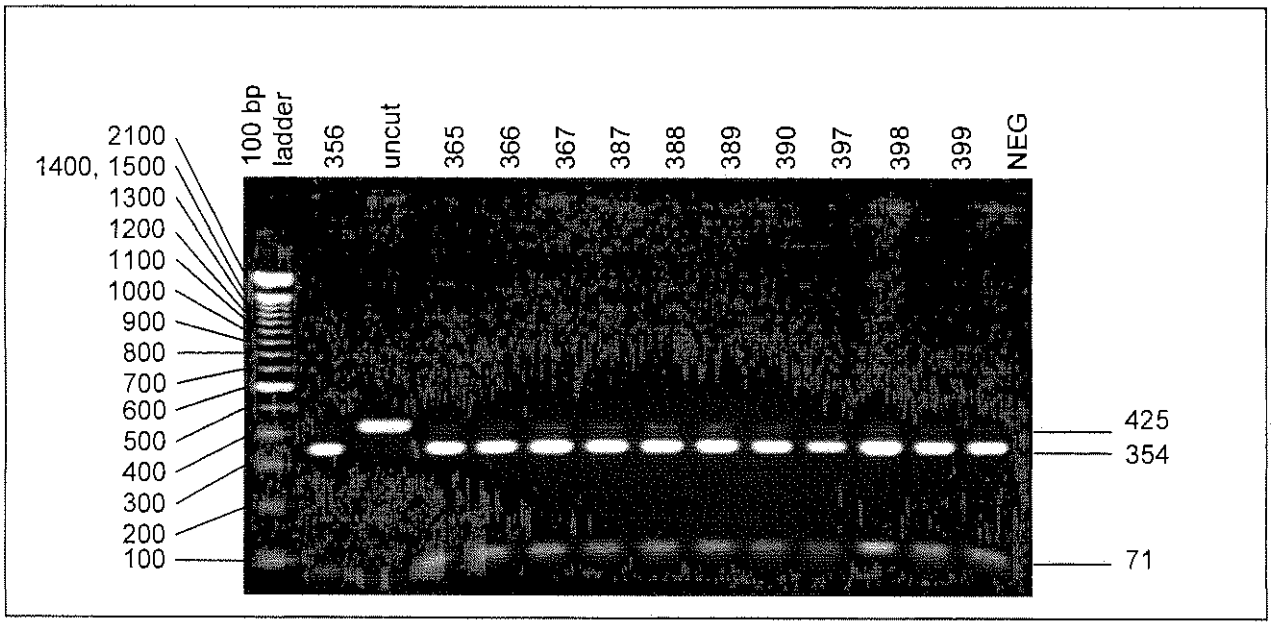
The detection of the A3243G mutation within this family is unique as the proband presented with atypical MELAS symptoms, the most striking of which was that she did not have any stroke-like episodes. However, molecular characterisation of the patient's DNA yielded a definitive diagnosis and proper counselling for the family. The counselling for this family was especially important for the mother and elder sister of the proband. These individuals although not clinically affected did harbour the MELAS A3243G mutation, therefore their offspring are at risk of being affected. In the same instance that the mother had two children, one affected and the other not affected, her unaffected daughter has the same chances of having either severely affected children or unaffected children who harbour the mutation. This risk can however not be calculated and should therefore be taken into consideration when the individual is faced with reproductive choices. With the uncle of the proband counselling was also important in order for him to understand the causes of the genetic defect present in his immediate family. Even though this screening method was unable to detect the presence of the mutation in this individual, and despite the fact that he does not stand a risk of transmitting the disorder, providing appropriate counselling is still important in order for him to be able to support his family.

5.3.1.2 The A3252G mutation

The A3252G mutation associated with MELAS is amplified from the $tRNA^{Leu(UUR)}$ gene of the mtDNA via PCR with primers which have been modified for RFLP analysis as presented in Table 5.1. A fragment of 425 bp is generated via amplification.

As illustrated in Table 4.7 the 425 bp amplicon has only one *Mae* III recognition site. In the presence of the mutation the reverse primer creates an additional recognition site in the amplicon. Upon electrophoresis of the digested fragments the presence of two fragments, 354 and 71 bp, therefore indicates the absence of the mutation. The mutation is indicated by the presence of three fragments (330, 71 and 24 bp). This mutation was, however, not observed in the patient population investigated as illustrated in Figure 5.2.

Figure 5.2: Photographic representation of the A3252G mutation analysis



2% agarose gel electrophoresed for 45 minutes at 100 V in 1 X TBE buffer. 100 bp ladder supplied by Gibco BRL. NEG = negative control.

In Figure 5.2 undigested PCR fragments of the size 425 bp can be noted in all the digested samples, indicating that incomplete digestion of the PCR fragment by the restriction enzyme *Mae* III occurred. In this instance this phenomenon does not hamper the correct interpretation of the molecular analyses results. The presence of the mutation is indicated by the existence of three fragments after digestion, resulting from the 354 bp fragment containing an additional recognition site for the enzyme. However, if the mutation had been present within a sample and incomplete digestion occurred the

heteroplasmic levels observed would not be a true indication of those present. The 354 bp fragment would be incompletely digested indicating a lower level of the mutation than what is truly present. One possibility to overcome this discrepancy would be to increase the amount of enzyme utilised for digestion, prior to the latter amendment the activity of the enzyme should be verified. In this investigation a sequencing strategy was performed in lieu of optimising the RFLP strategy.

The fragment in the uncut lane which is smaller than 100 bp represents the primer dimers formed during the PCR reaction. It is however unclear why the alleged 71 bp restriction fragment appears to segregate below the 100 bp fragment of the marker in individual 356, as it should, while in the remaining individuals it segregates above the 100 bp fragment. However, all patients were confirmed not to harbour this mutation by performing direct sequence analysis (Figure 5.11).

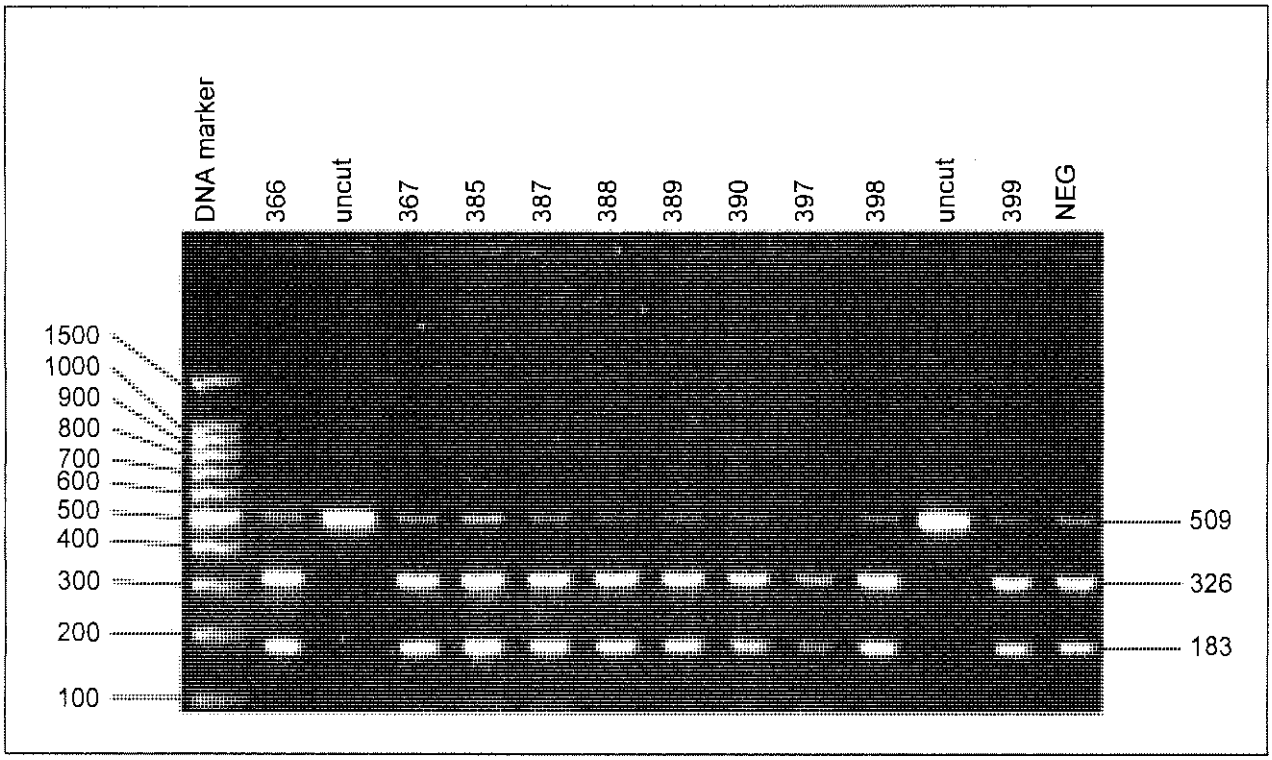
5.3.1.3 The C3256T mutation

The C3256T mutation has been associated with MERRF, unlike most mutations in the mitochondrial $tRNA^{Leu(UUR)}$ gene which are involved in the expression of the MELAS phenotype. An amplicon of 509 bp surrounding the C3256T mutation is generated via PCR utilising the primers listed in Table 5.1.

In an unaffected sample the 509 bp fragment harbours only one *Mae* II recognition site. However, in the presence of the mutation the mismatch introduced into the amplicon by the forward primer creates an additional recognition sequence for this enzyme. Therefore, an unaffected sample will be recognisable by the presence of two fragments of 326 and 183 bp respectively. The mutation will be evident by the existence of three fragments (326, 166 and 17 bp).

As depicted in Figure 5.3 the C3256T mutation was not observed in any of the patients investigated. Incomplete digestion was, however, noted with this RFLP analysis procedure, but did not influence the diagnosis of the mutation. This could also be confirmed by the fact that a negative control sample was included in all the RFLP analyses.

Figure 5.3: Photographic representation of the A3256G mutation analysis



2% agarose gel electrophoresed at 200 V for 1 hour in 1 X TBE buffer. DNA marker is 100 bp ladder from Promega. NEG = negative control.

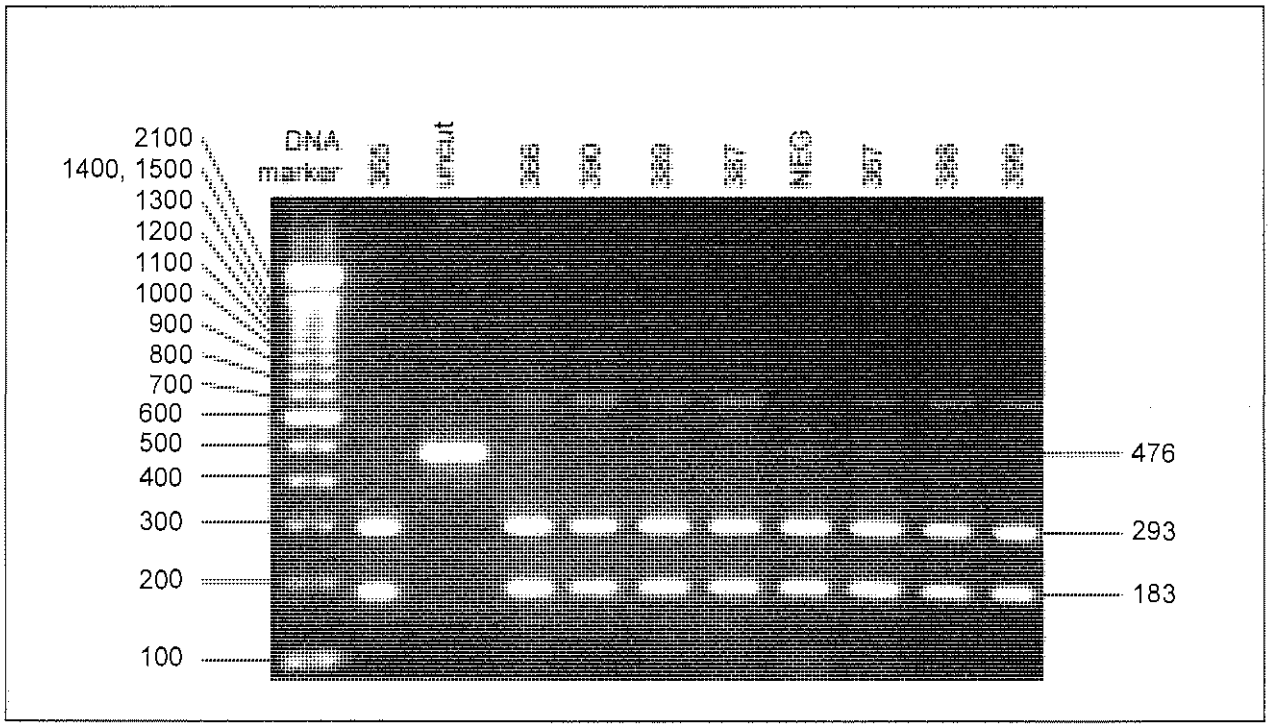
Although the incomplete digestion observed in Figure 5.3, does not interfere with the molecular diagnosis in this case it may, however, cause a misinterpretation of the heteroplasmy levels with samples harbouring the mutation as previously discussed (section 5.3.1.2). Figure 5.3 represents the results obtained from digestion of the PCR fragment with the *Mae* II enzyme. As can be noted there is a difference in fragment intensity between fragments in all the digested lanes vs. 397, this is due to a sample specific difference in PCR amplification was not optimal for individual 397. However, it may be argued that the 183 bp fragment is less intense as it has been further digested into the fragments indicative of the presence of the mutation (166 and 17 bp). This is however not accurate as the intensity of the 326 bp fragment which is unaltered by the presence of the mutation is also decreased in patient 397 when compared to patient 390 or the negative control. The absence of this mutation was verified for all the individuals under investigation by sequence analysis as discussed in section 5.4.1.

5.3.1.4 The A3260G mutation

RFLP analysis of the A3260G MELAS associated mutation is detected via digestion of the 476 bp fragment generated by PCR. The modified forward primer indicated in Table 5.1 enabled detection of the mutation utilising RFLP analysis.

The 476 bp amplicon contains a recognition site for the enzyme *Dde* I. Electrophoresis of the amplicon revealed two fragments (293 and 183 bp) after digestion, indicating the absence of the mutation. However, the introduction of the A to T mismatch in the forward primer created an additional restriction site for this enzyme when the mutation is present. The mutation will therefore be observed by the visualisation of three fragments (277, 183 and 16 bp) upon electrophoresis of the digested amplicon. As represented in Figure 5.4 none of the patients investigated harboured the A3260G mutation and all results were verified with direct sequencing.

Figure 5.4: Photographic representation of the A3260G mutation analysis



2% Agarose gel electrophoresed at 100 V for 45 minutes in 1X TBE buffer. DNA marker is 100 bp ladder supplied by Gibco BRL. NEG = negative control.

The feint ca. 650 bp fragment observed in the lanes of the digested samples cannot be explained. As these fragments are not present in the undigested sample they do not represent non-specific amplification products. They do also not represent incomplete

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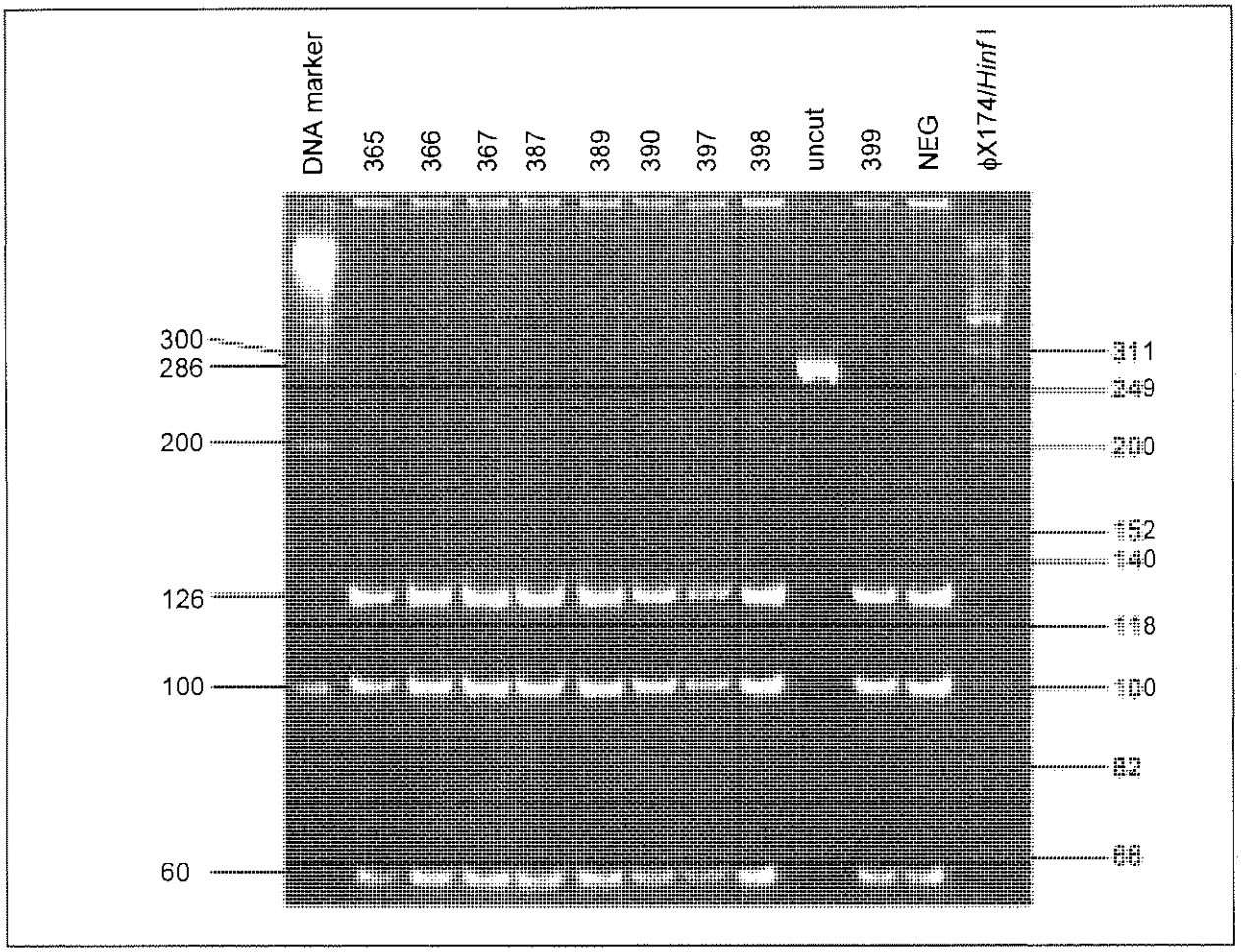
digestion as they are larger in size than the PCR amplicon. The only possible explanation is that the *Dde* I restriction enzyme digests the whole mitochondrial genomes still present in the RFLP reaction. However, this is thought to be highly unlikely as digestion of whole mtDNA would result in a number of fragments which would present on an agarose gel as a smear. This is not true in this case as predominantly only one fragment of ca. 650 bp is observed and the same enzyme was utilised for the detection of the T3271C mtDNA mutation. In Figure 5.5 the 650 bp fragment was not observed, thereby excluding the possible explanation of whole genome digestion.

5.3.1.5 The T3271C mutation

The T3271C mutation is the second most common mitochondrial $tRNA^{Leu(UUR)}$ mutation associated with MELAS. The modified reverse primer utilised for the amplification of the 286 bp fragment, allows the detection of the mutation via restriction digestion. The introduced mismatch creates an additional *Dde* I restriction site within the amplicon in the presence of the T3271C mutation (illustrated in Table 4.10).

In an unaffected sample the amplicon has two recognition sites for the *Dde* I enzyme. The 286 bp amplicon is thus digested into three fragments of 126, 100 and 60 bp respectively in the absence of the mutation. The creation of the additional restriction site only occurs with the presence of the mutation. Therefore amplicons from a sample harbouring the mutation will have three recognition sites for the *Dde* I restriction enzyme, resulting in four fragments (126, 79, 60 and 21 bp) after digestion.

Figure 5.5: Photographic representation of the T3271C mutation analysis



8% polyacrylamide gel electrophoresed at 250 V for 2 hours in 0.5 X TBE buffer. Stained with EtBr for 30 minutes. The DNA marker is the 100 bp ladder from Promega. NEG = negative control.

Figure 5.5 is a representation of the results obtained from digestion of the PCR product with the *Dde* I enzyme. Complete digestion of the amplicon was achieved for this mutation analysis. Although the intensity of the fragments of the ϕX174/*Hinf* I DNA marker is not sufficient for scoring the digested fragments accurately the 100 bp marker on the left of the gel could be utilised to determine the 100 bp digested fragment for all the samples. The presence of the mutation is evident by the digestion of the 100 bp fragment into 79 and 21 bp, and the 79 bp fragment would be visualised between the 60 and 100 bp fragments. It was therefore concluded together with RFLP analysis and direct sequencing that all individuals investigated were negative for the T3271C mutation.

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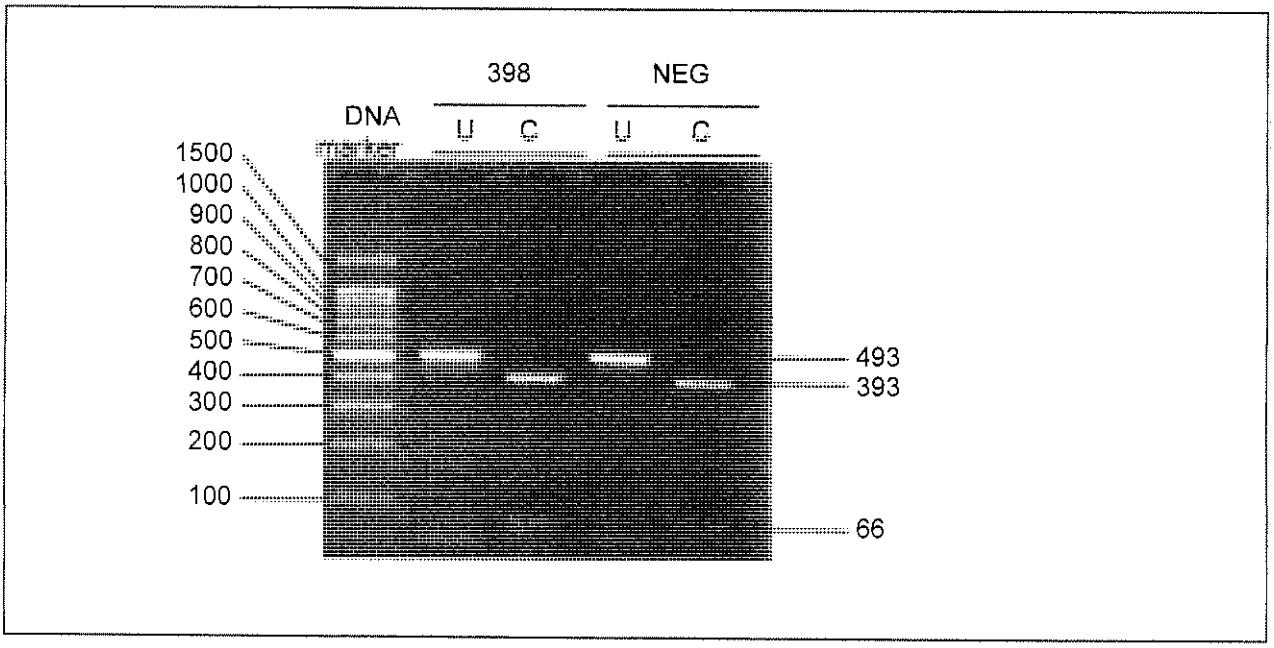
5.3.1.6 The T3291C mutation

The T3291C mutation is associated with MELAS, although it is reported to be one of the rarer MELAS mutations. The region surrounding this mutation, located within the $tRNA^{Leu(UUR)}$ gene of the mitochondria, can be amplified via ACRS to facilitate RFLP analysis.

The forward primer contains a mismatch at its 3'-end enabling the creation of an additional *Mbo* I recognition site in the presence of the T3291C mutation (illustrated in Table 4.11). The 493 bp amplicon therefore has two recognition sites for the *Mbo* I restriction endonuclease. In an unaffected sample the amplicon will be digested into three fragments of 393, 66 and 34 bp respectively. However, in the presence of the T3291C mutation the amplicon is digested into four fragments (371, 66, 34, and 22 bp). Figure 5.6 represents the results obtained from an RFLP analysis for the T3291C mutation in our patient population.

Figure 5.6: Photographic representation of the T3291C mutation analysis



2% agarose gel electrophoresed at 200 V for 1 hour in 1 X TBE buffer. DNA marker = 100 bp ladder supplied by Promega. U = uncut; C = cut; NEG = negative control.

Complete digestion of the 493 bp amplicon was obtained. The 66 bp fragment generated after digestion could not be observed utilising 2% agarose gel detection. However, the presence of the mutation would be evident from digestion of the 393 bp fragment into a 371 and 22 bp fragments. From Figure 5.6 it is evident that no 371 bp fragments were detected, implying that the sample does not harbour the mutation. None

of the individuals investigated in this study harboured the T3291C mtDNA mutation, and RFLP analysis results were verified by direct sequencing.

5.3.2 Mutations within the mitochondrial $tRNA^{Lys}$ gene

To date seven mutations have been localised to the mitochondrial $tRNA^{Lys}$ gene associated with various mitochondrial myopathies, as listed in Appendix A. Both point mutations investigated in this study are associated with the MERRF phenotype.

5.3.2.1 The A8344G mutation

The A8344G mutation is located within the lysine tRNA gene of the mtDNA. The region surrounding this mutation is amplified via PCR with a modified reverse primer that enables detection of the mutation via an amplification created restriction site (ACRS).

As illustrated in Table 4.12 the reverse primer contains a mismatch at its 3'-end allowing for the detection of the mutation by digestion with the *Ban* II enzyme. *Ban* II has two recognition sites within the 212 bp amplicon. Therefore, in an unaffected sample the 212 bp amplicon will be digested into three fragments of 99, 72 and 41 bp. The presence of the A8344G mutation creates an additional restriction enzyme recognition site together with the mismatch introduced by the reverse primer during amplification. The amplicon of an affected sample will thus contain three *Ban* II recognition sites and will be digested into four fragments (99, 52, 41 and 20 bp). Figure 5.7 is a representation of the results generated via RFLP analysis for the A8344G mutation. All RFLP analysis results were confirmed by direct sequencing.

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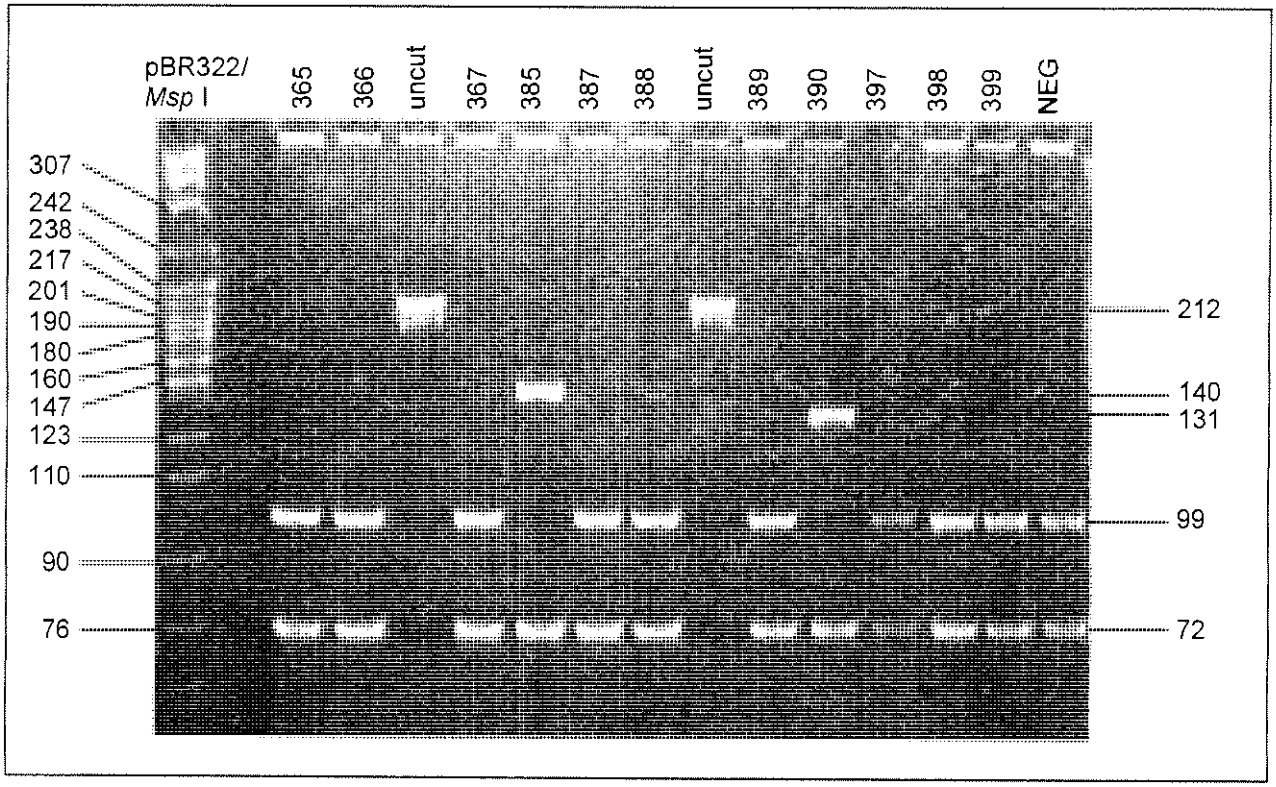
Figure 5.7: Photographic representation of the A8344G mutation analysis



10% polyacrylamide gel electrophoresed at 250 V for 2 hours in 0.5 X TBE buffer. Visualisation of the fragments was achieved by SYBR® Gold staining. NEG = negative control.

Three different results are depicted in Figure 5.7. The first was observed in the majority of the lanes, and was the presence of two fragments 99 and 72 bp in size. This digestion pattern represents samples which are negative for the A8344G mutation, with the 41 bp fragment not visible on this PAGE detection system. The 72 bp fragment in the patient lanes segregates very closely to the 76 bp fragment of the DNA marker. A higher percentage polyacrylamide gel may have provided better resolution between these two fragments. The second digestion pattern which was observed is depicted in lane 385 and is indicative of a sample harbouring a reported polymorphism at position 8249 resulting in a *Ban* II site loss. This site loss results in the observation of two fragments, now 140 and 72 bp in size, in an unaffected individual. The presence of this particular polymorphism within individual 385 was confirmed via sequencing as is depicted in Figure 5.16.

A further polymorphism, which may be present in unaffected as well as affected individuals, is that of a 9 bp deletion at positions 8271 to 8281. This deletion is associated with a specific mitochondrial haplogroup, namely haplogroup L, which is African specific. The presence of fragments of 99 bp, 72 bp and 32 bp will indicate the presence of the deletion in an unaffected individual. However, individual 390 presents

with only two fragments one of 72 bp and the other being smaller than 140 bp. This digestion pattern is explained by the presence of the *Ban* II site loss at nucleotide 8249 occurring simultaneously with the presence of the 9 bp deletion within the sample. This creates a fragment which is smaller than the 140 bp fragment by 9 nucleotides, and implies that the larger of the two digested fragments in lane 390 is therefore 131 bp in size. The presence of these two polymorphisms was confirmed via sequencing of individuals 384 and 390.

5.3.2.2 The T8356C mutation

A second mutation within the mitochondrial $tRNA^{Lys}$ gene associated with MERRF occurs at position 8356. The amplification of the region surrounding this mutation is performed via ACRS PCR.

The reverse primer allowed the creation of an additional recognition site for the restriction endonuclease *Xba* I (illustrated in Table 4.13) in a sample harbouring the mutation. In an unaffected sample the 226 bp amplicon contains only one recognition site for the *Xba* I enzyme. The amplicon will therefore be digested into two fragments of 132 and 94 bp respectively in the absence of the mutation. The presence of the mutation together with the mismatch introduced by the reverse primer creates an additional recognition site for this enzyme. In an affected sample, the amplicon thus contains two recognition sites and is digested into three fragments (132, 73 and 21 bp). As represented in Figure 5.8 the T8356C mutation is absent in all the individuals investigated. Direct sequencing was utilised to verify these results.

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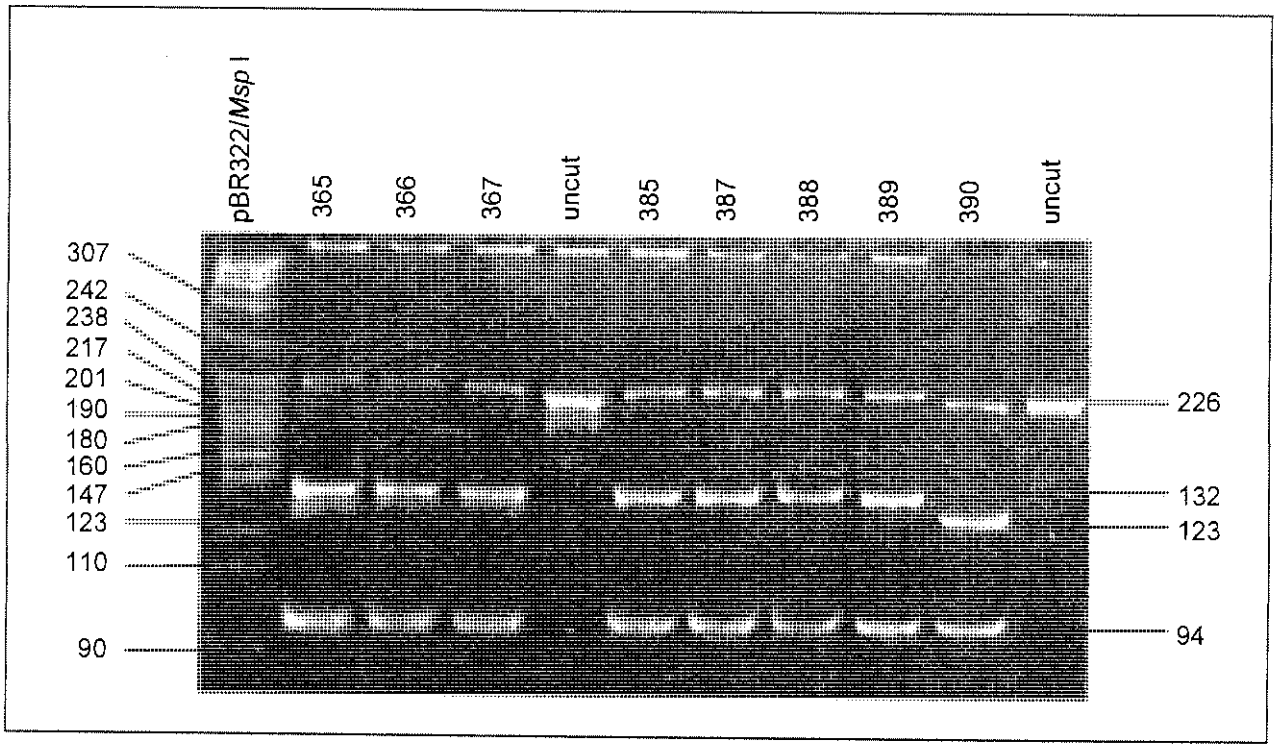
Figure 5.8: Photographic representation of the T8356C mutation analysis



10% polyacrylamide gel electrophoresed at 250V for 2 hours in 0.5 X TBE buffer. Visualisation of the fragments was achieved by SYBR gold staining.

Although incomplete digestion of the 226 bp amplicon can be observed in Figure 5.8, this does not interfere with the molecular diagnosis for the T8356C mutation. However, it may cause misinterpretation of the heteroplasmy levels with samples harbouring the mutation as discussed in section 5.3.1.2.

The 9 bp deletion polymorphism between nucleotides 8271 and 8281, as discussed in section 5.3.2.1, may also be detected via the RFLP analysis of this amplicon. In Figure 5.8 the polymorphism is observed in individual 390 by the presence of the 123 bp fragment instead of the 132 bp. If the polymorphism was present in a sample together with the T8356C mutation the presence of four fragments (123 bp, 94 bp, 73 bp and 21 bp) would be observed. The presence of the 9 bp deletion polymorphism was confirmed in individual 390 by the RFLP analysis for both the A8344G and T8356C mutations.

5.3.3 Mutations within the ATPase 6 gene

The ATPase 6 gene encodes the ATPase 6 subunit of the ATP synthase complex responsible for the translocation of electrons across the inner mitochondrial membrane

for the formation of ATP. Mutations within this gene are associated with disorders of energy depletion, namely Leigh Syndrome.

5.3.3.1 The T8993C and T8993G mutations

Two mutations occur at the same position within the ATPase 6 gene but have different phenotypic expressions. The T8993G mutation is the mutation most commonly associated with LS. The T8993C mutation is associated with the milder NARP phenotype and later onset of the disorder.

The two mutations are analysed via RFLP analysis of the same amplicon, however, different restriction enzymes were utilised for the detection of the mutations. Unmodified primers are utilised for the amplification of a 1031 bp fragment as described in Table 4.14.

5.3.3.1.1 The T8993C mutation

The 1031 bp amplicon contains one *Hpa* II restriction site, and the absence of the T8993C mutation is therefore indicated by the digestion of the amplicon into two fragments (567 and 463 bp). The presence of the T8993C mutation creates an additional *Hpa* II restriction site. Therefore, the observation upon electrophoresis of fragments of 567, 299 and 164 bp indicates the presence of the T8993C mutation. Figure 5.9 represents the results obtained after digestion of the amplicon with the *Hpa* II enzyme and all individuals under investigation were negative for the T8993C mutation. The results from the RFLP analysis were confirmed via direct sequencing.

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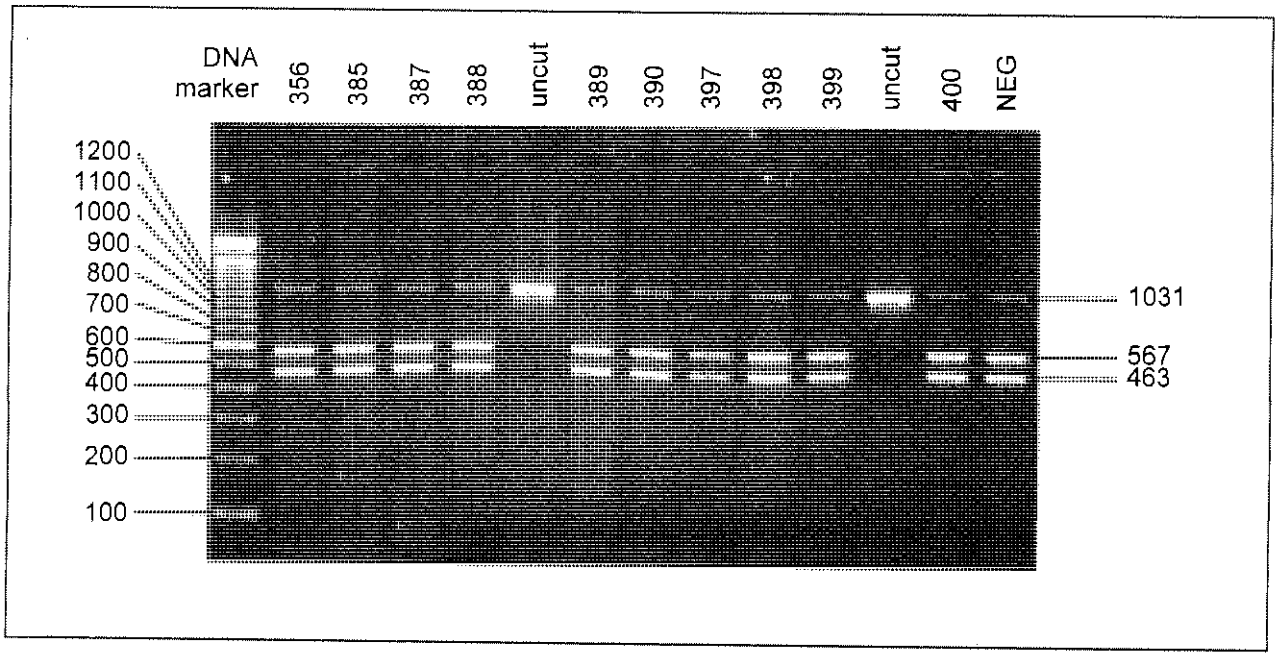
Figure 5.9: Photographic representation of the T8993C mutation analysis



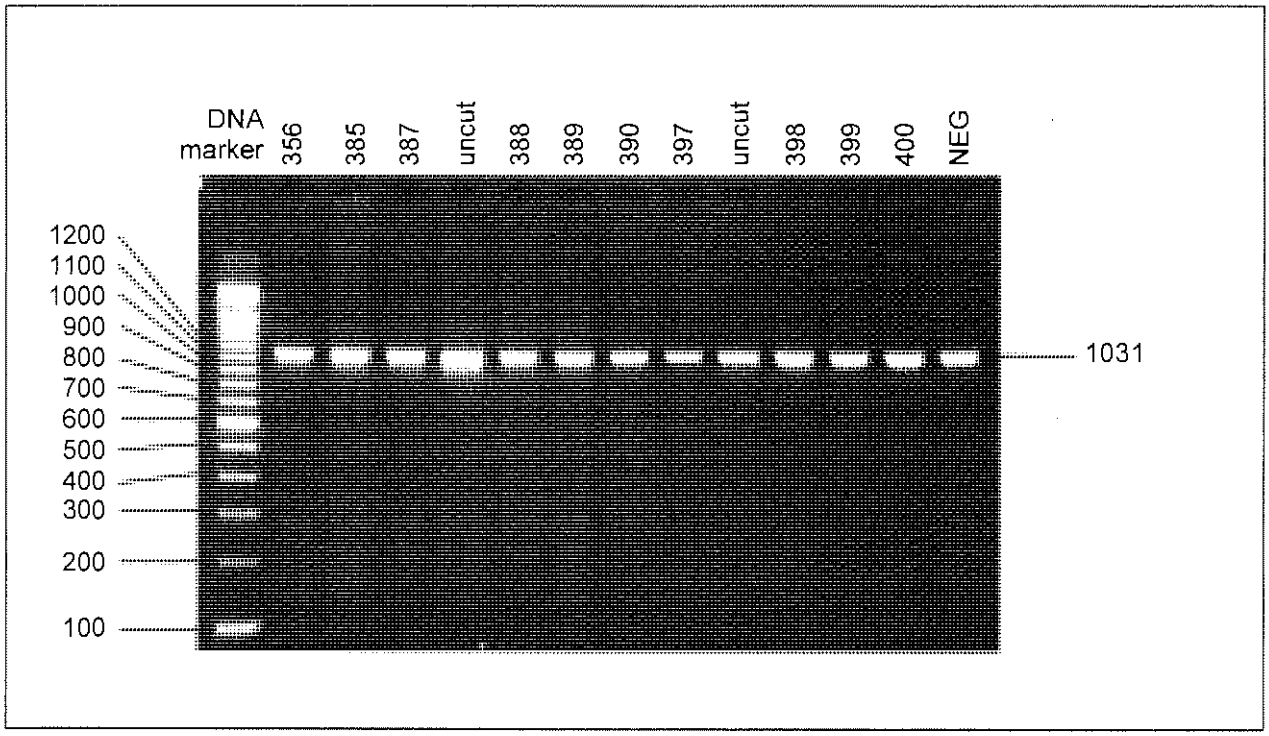
2% agarose gel electrophoresed at 200 V for 1 hour in 1 X TBE buffer. DNA marker = 100 bp ladder from Gibco BRL. NEG = negative control .

Incomplete digestion is observed in Figure 5.9. This does not interfere with the molecular diagnosis. In the presence of the mutation the 463 bp fragment would be digested further into fragments of 299 and 164 bp. These fragments were not observed in the individuals under investigation. The incomplete digestion of the 1031 bp fragment may, however, cause misinterpretation of the heteroplasmy levels in samples had they harboured the mutation as discussed in section 5.3.1.2.

5.3.3.1.2 The T8993G mutation

The detection of the T8993G mutation relies on the creation of a restriction site for the *Ava* I enzyme in the presence of the mutation. In an unaffected sample the amplicon generated via PCR does not contain a restriction site for *Ava* I and remains undigested. However, in an affected sample a recognition site is created by the mutation and the 1031 bp amplicon is digested into two fragments of 869 and 162 bp respectively. Figure 5.10 depicts the results generated from restriction digestion of the 1031 bp amplicon with *Ava* I.

Figure 5.10: Photographic representation of the T8993G mutation analysis



2% agarose gel electrophoresed at 200 V for 1 hour in 1 X TBE buffer. DNA marker = 100 bp ladder from Gibco BRL. NEG = negative control.

No digestion of the PCR amplicons is observed in the individuals under investigation indicating that none of the patients analysed harboured the T8993G mutation. The RFLP strategy for the T8993G mutation is the only strategy utilised in this study which relies on the presence of the mutation for digestion to occur. The RFLP strategies for the other mutations relied on the presence of an additional recognition site for the respective enzymes in the presence of the mutation. In these strategies the recognition site for the enzyme in the absence of the mutation serves as a control with respect to enzyme activity. Therefore, for the T8993G mutation the activity of the *Ava* I enzyme should ideally be verified by the analyses of a positive control. However, a positive control for the T8993G mutation was not available during this investigation. For reliable diagnosis of this change a sequence strategy was therefore utilised as discussed in section 5.4.3.

5.4 DIRECT SEQUENCING

Initially direct di-deoxynucleotide chain termination sequencing was employed to confirm nucleotide changes observed upon RFLP analysis. This would have been conducted as explained in section 4.6. Sequence analysis for the various mutations also

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served as a control to verify that the correct region of the mtDNA had been amplified during the PCR reaction.

Early in this study it was noted that sequencing of three regions of the mitochondrial genome could replace ten RFLP analyses. It was subsequently decided that the three regions encompassing the mitochondrial $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$ and the ATPase6 genes would be sequenced to detect the known reported mutations described in paragraph 4.3. The three fragments were sequenced in both directions by utilising the forward and reverse primers in separate sequencing reactions. Upon detection of a mutation by direct sequencing the sample would be subjected to RFLP analysis to confirm and indicate the presence of heteroplasmy.

The sequencing of these regions not only allowed the detection of the ten mutations described in paragraph 4.3 but also facilitated the investigation of additional mtDNA mutations associated with other mitochondrial myopathies, as listed below in Table 5.2.

Table 5.2: Additional mtDNA mutations associated with mitochondrial myopathies

Nucleotide	Mitochondrial Myopathy	Reference
T3250C	myopathy	Goto *et al.* (1992b)
A3251G	PEO, myopathy, sudden death	Sweeney *et al.* (1993)
C3254G	MIMyCa	Kawarai *et al.* (1997)
T3264C	diabetes, ataxia, PEO	Suzuki *et al.* (1997/8)
ΔTnt-3271	mitochondrial encephalomyopathy	Shoffner *et al.* (1995b)
A3288G	myopathy	Hadjigeorgiou *et al.* (1999)
A3302G	myopathy	Bindoff *et al.* (1993)
C3303T	MIMyCa	Silvestri *et al.* (1994)
A8296G	diabetes, deafness	Kameoka *et al.* (1998)
	hypertrophic cardiomyopathy	Akita *et al.* (2000)
G8313A	gastrointestinal, encephaloneuropathy	Verma *et al.* (1997)
G8328A	mitochondrial encephalomyopathy	Houshmand *et al.* (1999)
G8342A	PEO, myoclonus	Tiranti *et al.* (1999)
G8363A	multisystem cardiomyopathy	Santorelli *et al.* (1996)
	MERRF	Ozawa *et al.* (1997)
T9101C	LHON	Lamminen *et al.* (1995)

Table 5.2: continued ...

Nucleotide	Mitochondrial Myopathy	Reference
T9176C	bilateral striatal necrosis	Thyagarajan *et al.* (1995)
	NARP, MILS	Campos *et al.* (1997)

PEO = progressive external ophthalmoplegia; MIMyCa = mitochondrial myopathy and cardiomyopathy; MERRF = myoclonus epilepsy and ragged red muscle fibres; LHON = Leber's hereditary optic neuropathy; NARP = neuropathy, ataxia and retinitis pigmentosa; MILS = maternally inherited Leigh Syndrome; ΔTnt = deletion of T nucleotide.

The primers utilised for the RFLP strategies usually created a restriction site and were therefore in close proximity to the mutation. The majority of these primers were not suitable for sequencing, and two new primers were designed for amplification of the fragments used in the sequence analysis. The primers utilised for the amplification of the three regions for the sequencing strategy are discussed below.

5.4.1 Direct sequence analysis of the mitochondrial tRNA$^{Leu(UUR)}$ region

To date fourteen mutations within the mitochondrial tRNA$^{Leu(UUR)}$ gene have been reported, through direct sequencing all fourteen mutations were investigated for all the individuals analysed. The positions of the mutations localised within the mitochondrial tRNA$^{Leu(UUR)}$ gene region are illustrated below in Table 5.3.

Table 5.3: Partial sequence of the mitochondrial tRNA$^{Leu(UUR)}$ region

Nucleotide	Sequence
3001	ggacatcccg atggtgcagc cgctattaaa ggttcgtttg ttcaacgatt aaagtcctac
3061	gtgatctgag ttcagaccgg agtaatccag gtcggtttct atctaccttc aaattcctcc
3121	ctgtacgaaa ggacaagaga aataaggcct acttcacaaa gcgccttccc ccgtaaatga
3181	tatcatctca acttaGtatt atacccacac ccacccaaga acagggtttg ttaagatggc
3241	agAgcccggT AAtCgCataA aacTtaaaac Tttacagtca gaggttcAat Tcctcttctt
3301	aACaacatac ccatggccaa cctcctactc ctcattgtac ccattctaat cgcaatggca
3361	ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). The mutations detectable within this region are indicated in bold capital letters. Sequence adapted from MITOMAP (2001).

These alterations were verified upon sequencing of the mtDNA tRNA$^{Leu(UUR)}$ region. A 6% denaturing polyacrylamide gel was utilised as discussed in section 4.7 to electrophorese the sequenced products. Both forward and reverse primers were utilised in the sequencing strategy. Figure 5.11 is an autoradiographic representation of the

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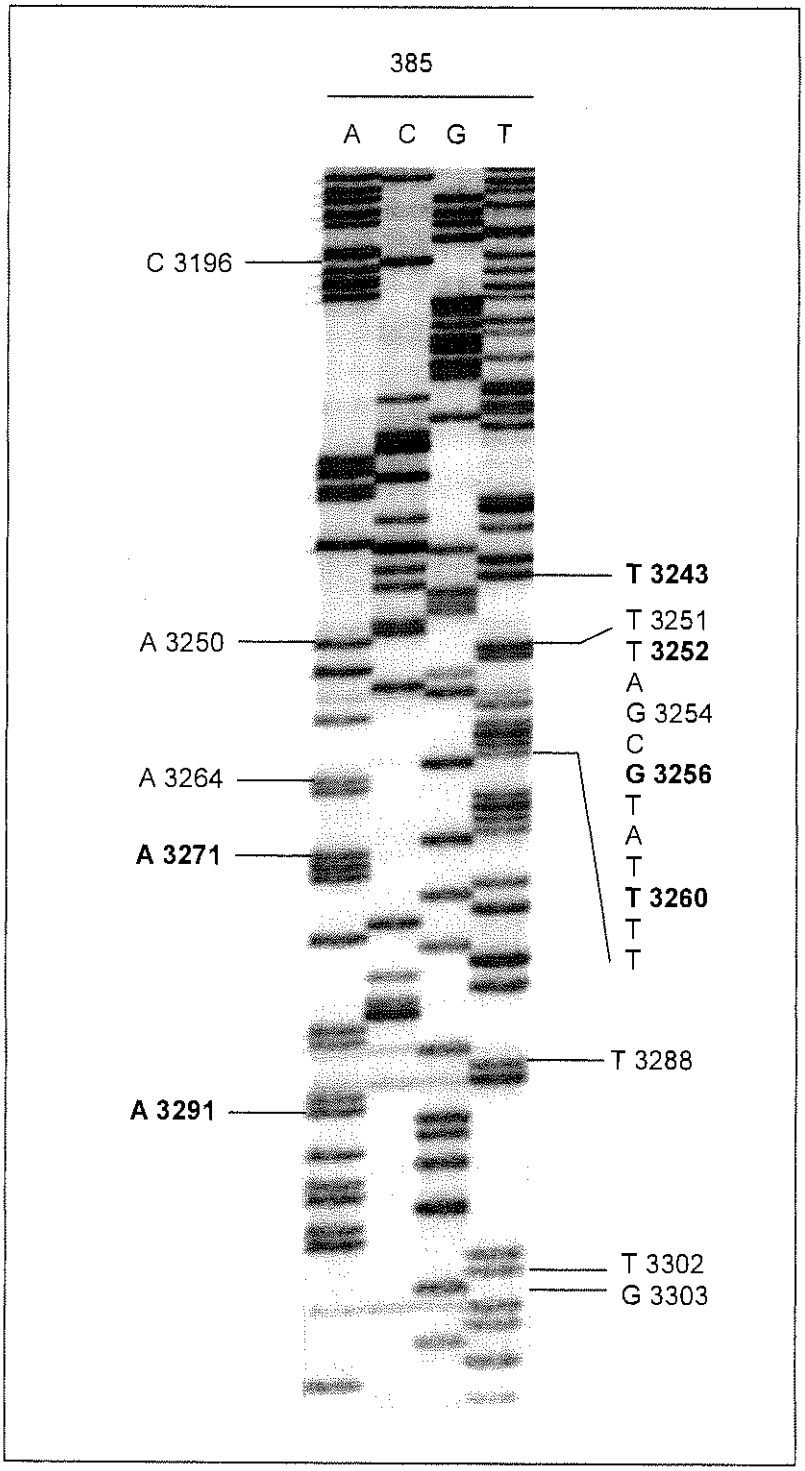
sequencing results obtained after electrophoresis of the 6% PAGE gel for one and three quarters of an hour, when utilising the reverse primer as the sequencing primer, at 60 W in 1 X TBE buffer.

Figure 5.11: Representative autoradiograph of the mitochondrial tRNA$^{Leu(UUR)}$ region



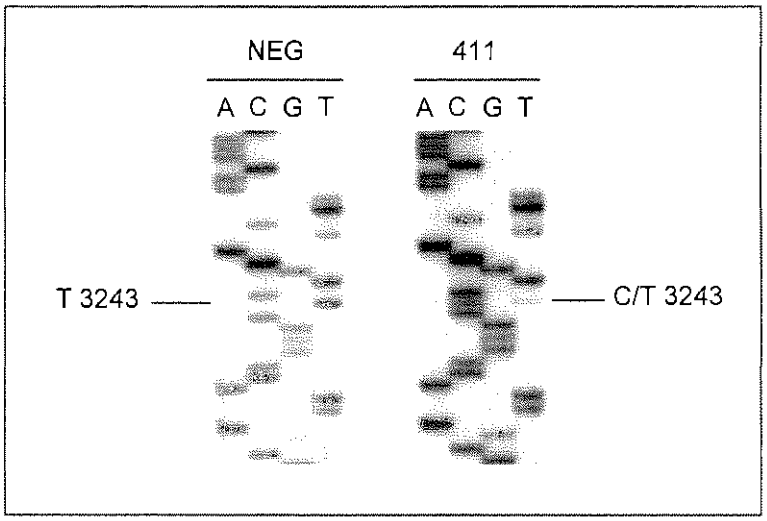
6% polyacrylamide gel electrophoresed at 60 W for 1 hour and 45 minutes in 1 X TBE buffer. The reverse primer was utilised as the sequencing primer. Possible mutations are indicated by their nucleotide number. Nucleotides and numbers in **bold** represent the six mutations which were analysed in this study.

As illustrated in Figure 5.11 all fourteen nucleotides that could possibly be altered resulting in a mitochondrial myopathy were investigated via one sequencing reaction. This strategy allowed all individuals to be investigated for all fourteen possible mutations within the mtDNA $tRNA^{Leu(UUR)}$ region.

5.4.1.1 The A3243G mutation

In section 5.3.1.1 it was reported that the A3243G mutation had been observed in a single patient. This mutation was observed on sequence analysis and confirmed via the RFLP as discussed. Figure 5.12 below illustrates the presence of the A3243G mutation via sequence analysis within patient 411.

Figure 5.12: Autoradiographic representation of the A3243G mutation within the $tRNA^{Leu(UUR)}$ region of the mitochondrial genome



6% polyacrylamide gel electrophoresed at 60 W for 1 hour and 45 minutes in 1 X TBE buffer. The reverse primer was used as the sequencing primer. NEG = negative control.

The reverse primer was used as the sequencing primer and therefore the mutation is observed as a T to C alteration (A to G with the forward primer). The mutation is observed to be heteroplasmic on sequence analysis in the patient when compared to the negative control. The diagnosis of the A3243G mutation was confirmed via RFLP and direct sequencing analyses for all six individuals from the family of proband 411. The presence of the mutation, and its heteroplasmic state, was only revealed in the proband upon sequencing analysis. This was confirmed via RFLP analysis (Figure 5.1).

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However, sequence analyses of the maternal relatives did not reveal the presence of the A3243G mutation, but its presence within the mother and sister of the proband was confirmed with RFLP analysis (Figure 5.1).

From this observation it may be argued that mutation analysis via direct sequencing will not be effective in detecting all samples harbouring specific mutations. This argument may be countered by the fact that the patients referred for mutation analysis are severely affected and would therefore harbour the specific mutations at high heteroplasmic levels, as in the case of proband 411. Mutation screening via sequencing of the maternal relatives of an affected proband is not recommended without RFLP analysis to confirm results. In the maternal relatives the same mutation may be present, but as in this family, the members might harbour very low heteroplasmic levels of the mutation.

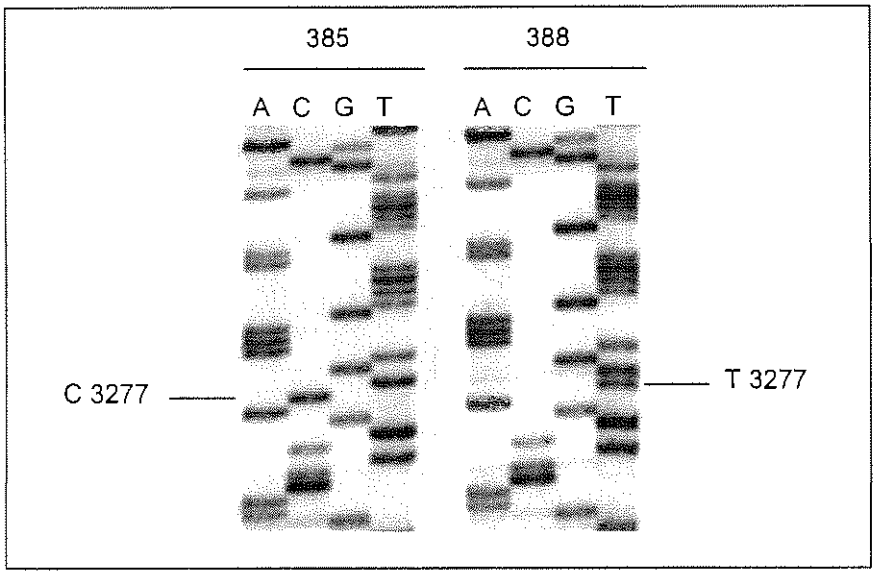
The absence of the A3243G mutation in the other three maternal relatives of proband 411 may not be truly correct. The analysis was performed on blood samples from these individuals and an extremely low percentage of heteroplasmy may be undetectable utilising these methods of analysis. However, the greatest possible chance of detection was ensured by utilising SYBR® Gold staining instead of EtBr. The SYBR® Gold stain is 10X more sensitive than EtBr (Molecular Probes product information, 1997). For absolute confirmation of the absence of the mutation in the other maternal relatives it would be advised to perform the mutation analysis on muscle samples. However, these are invasive as well as expensive, and many individuals are reluctant to submit young children to such a procedure. An alternative to muscle samples was suggested by Sue *et al.* (1998) who reported that gDNA could be extracted from hair follicles and these samples could be used for the analysis of mtDNA mutations. According to these authors the "mtDNA analysis on hair follicles is as sensitive as muscle in detecting this mutation" (referring to the MELAS A3243G mutation). However, to date no hair samples have been used for the isolation of gDNA for mitochondrial disorder analysis in this study.

5.4.1.2 The G3277A mutation

Further analysis of the patient population for the $tRNA^{Leu(UUR)}$ region of the mitochondrial genome revealed an alteration at nucleotide 3277. At this position in individual 388 the guanine base is altered to an adenine (G3277A). As depicted in Figure 5.13 the

alteration is observed by the C to T change at position 3277, since the reverse primer was utilised as the sequencing primer.

Figure 5.13: Autoradiographic representation of the G3277A alteration within the tRNA$^{Leu(UUR)}$ region of the mitochondrial genome



6% polyacrylamide gel electrophoresed at 60 W for 1 hour and 45 minutes in 1 X TBE buffer. The reverse primer was utilised as the sequencing primer.

Of all the patients investigated this alteration was only observed in individual 388. This change represents a novel change within the tRNA$^{Leu(UUR)}$ gene and to date has not been reported in any literature nor as an unpublished alteration on the MITOMAP database.

The G3277A alteration observed in patient 388 occurs within the variable loop of the leucine tRNA, refer to Figures 3.3 and 3.4. It is therefore unknown as to what the effect of this alteration will have on the secondary structure of the tRNA molecule as a whole or towards its functioning. From Figure 5.13 above it is not apparent that there is any heteroplasmy for this change. However, before this can be stated unequivocally RFLP analysis will need to be performed. If it is confirmed that this change is indeed homoplasmic it may be assumed that the change is a polymorphism. The confirmation of this alteration in the mtDNA of individual 388 will still be performed outside the scope of this study and any further investigation of this novel change will be completed.

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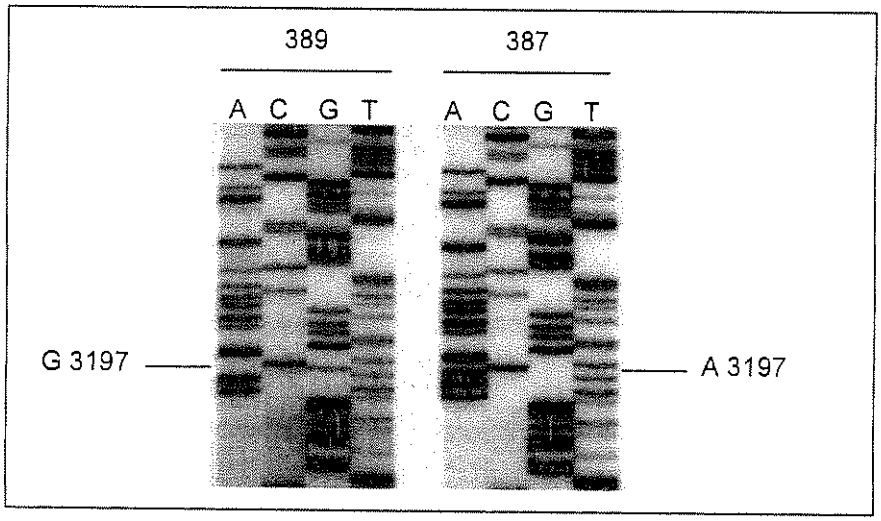
5.4.1.3 The T3197C polymorphism

The other alteration which was observed upon sequencing of the mtDNA $tRNA^{Leu(UUR)}$ region was a reported polymorphism of a T to C change at nucleotide 3197. This change was observed in two patients under investigation, namely 389 and 412. Figure 5.14 illustrates the presence of this polymorphism in individual 389. This particular alteration has been reported on the database of MITOMAP as published and confirmed (unpublished) polymorphism. Hess *et al.* (1995) were the first to report the observation of this change it was later confirmed by Shields and Bridge in 1999.

Figure 5.14: Autoradiographic representation of the T3197C alteration within the $tRNA^{Leu(UUR)}$ region of the mitochondrial genome



6% polyacrylamide gel electrophoresed at 60 W for 1 hour and 45 minutes in 1 X TBE buffer. The reverse primer was utilised as the sequencing primer.

Since the reverse primer was utilised to obtain the sequence presented in Figure 5.14 the polymorphism is represented by an A to G alteration in individual 389. The same observation was noted in patient 412 (results not shown). Although this change does occur within the mtDNA $tRNA^{Leu(UUR)}$ region it is, however, not localised within the mitochondrial $tRNA^{Leu(UUR)}$ gene. The nucleotide position 3197 occurs within the gene encoding the mitochondrial 16S rRNA. The T3197C change within this gene does not alter an amino acid, and was observed in a homoplasmic state. For these reasons this alteration was regarded to be a polymorphism.

5.4.2 Direct sequence analysis of the mitochondrial tRNA^{Lys} region

The mitochondrial tRNA^{Lys} gene harbours seven possible alterations which could result in the expression of various phenotypes. Three of the possible nucleotide changes are associated with the MERRF phenotype. The seven mutations, which may be verified via sequencing of the mtDNA tRNA^{Lys} gene, are illustrated below in Table 5.4.

Table 5.4: Partial sequence of the mitochondrial tRNA^{Lys} region

Nucleotide	Sequence
8101	agatgcaatt cccggacgtc taaaccaaac cactttcacc gctacacgac cgggggtata
8161	ctacggtcaa tgctctgaaa tctgtggagc aaaccacagt ttcatgccca tcgtcctaga
8221	attaattccc ctaaaaatct ttgaaatagg gcccgtattt accctatagc acccccctca
8281	ccccctctag agcccActgt aaagctaact taGcattaac cttttaaGtt aaagattaag
8341	aGaAccaaca cctctTtaca gtGaaatgcc ccaactaaat actaccgtat ggcccaccat
8401	aattaccccc atactcctta cactattcct catcacccaa ctaaaaatat taaacacaaa
8461	ctaccaccta cctccctcac caaagcccat aaaaataaaa aattataaca aaccctgaga

The position of the forward primer is indicated by underlined text (xxx) and the position of the reverse primer is indicated by double underlined text (yyy). Additional mutations are indicated in **bold** capital letters. Sequence adapted from MITOMAP (2001).

The primers utilised for the amplification of the mitochondrial tRNA^{Lys} region differed from those discussed in section 4.3.2. The reverse primer utilised during RFLP analyses for the tRNA^{Lys} region was situated too close to the mutations of interest. Therefore, a new reverse primer was designed further away from the site of the mutations as illustrated in Table 4.6, which depicts the primer locations and mutations which may be investigated upon sequencing of this fragment.

The results of the sequence analysis for the tRNA^{Lys} region of the mitochondrial genome is illustrated by the autoradiograph in Figure 5.15. In this figure all seven nucleotide positions for the possible mutations are observed. None of these mutations were present in the patient population investigated.

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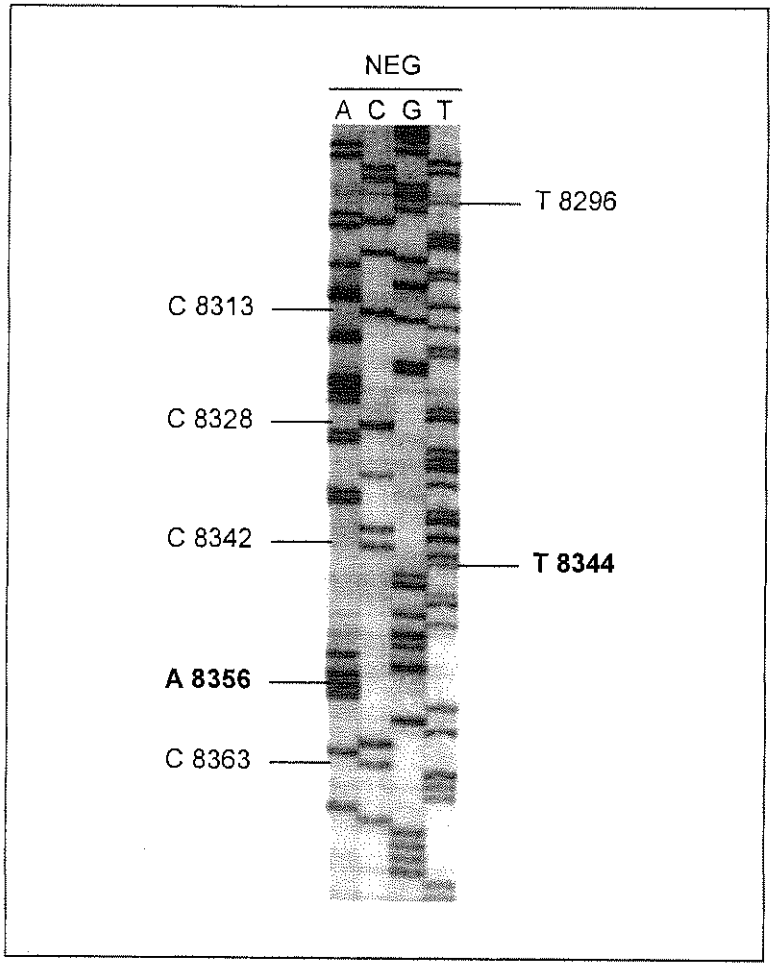
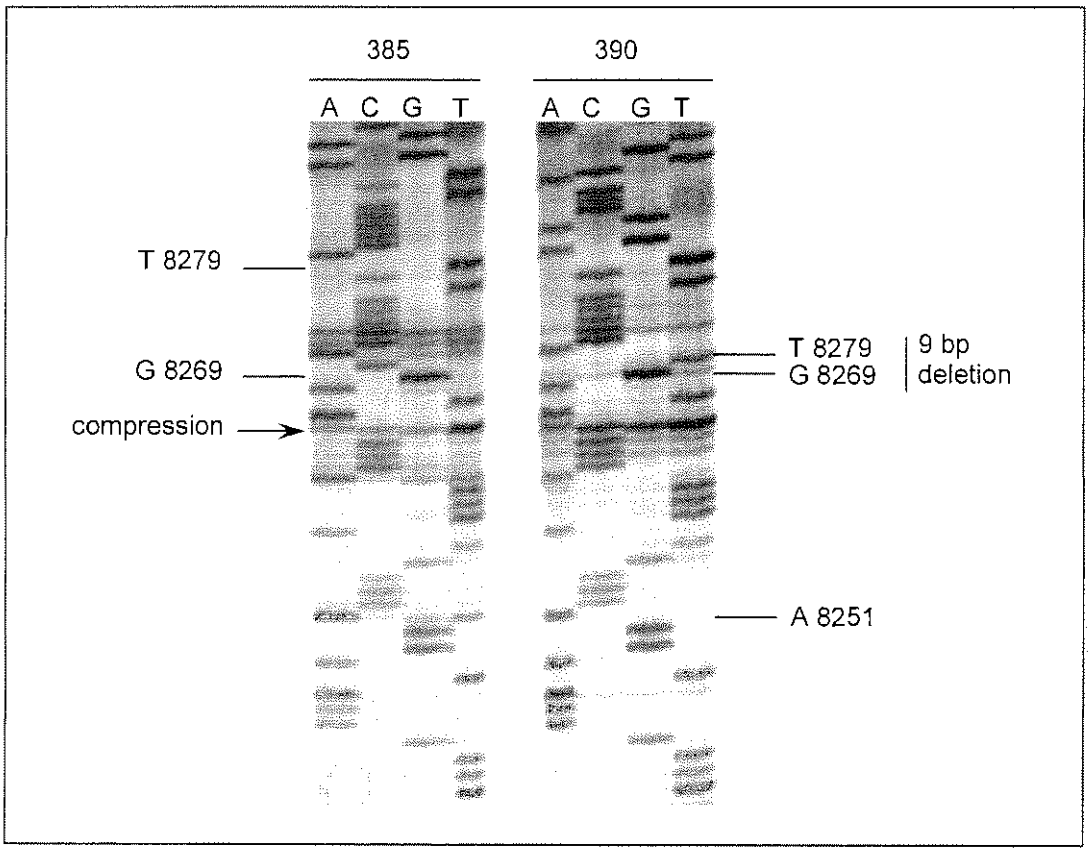
Figure 5.15: Autoradiographic representation of the tRNA^{Lys} region of the mitochondrial genome



6% polyacrylamide gel electrophoresed at 45 W for 2 hours in 1 X TBE buffer. The reverse primer was utilised as the sequencing primer. Mutations analysed in the original investigation are indicated in **bold**. NEG = negative control.

The sequencing of this region was optimised by the addition of manganese (Mn) buffer as suggested in the Sequenase kit protocol, when the reverse primer was utilised as the sequencing primer. The addition of this buffer removed compressions from the sequence. These compressions can be observed in Figure 5.16, which represents sequence results obtained utilising the forward primer as sequencing primer and before optimisation of the protocol for this region.

Figure 5.16: Autoradiographic representation of the 9 bp deletion within the tRNA^{Lys} region of the mitochondrial genome



6% polyacrylamide gel electrophoresed at 60 W for 2 hours in 1 X TBE buffer. The forward primer was utilised as the sequencing primer.

The two polymorphisms noted in the RFLP analyses (section 5.3.2.1) were confirmed with sequence analysis as illustrated above in Figure 5.16. The *Ban* II site loss noted in the RFLP analyses was confirmed in both patients (390 and 385). It was reported that a recognition site at position 8249 was lost for the enzyme. As depicted above, these two patients harboured a G8251A alteration which destroys the *Ban* II recognition site and results in the digestion pattern observed in Figure 5.7. Of all the patients included in the study four (356, 384, 385 and 390) were found to harbour this particular polymorphism.

The 8251 nucleotide is localised within the cytochrome c oxidase II gene and the G to A change does not alter the encoded amino acid. This polymorphism is considered to be specific for a particular haplogroup, namely L1 (Chen *et al.*, 1995). In lane 385 at position 8251 an A as well as a T fragment is observed instead of the normal G nucleotide at this position. The G to A alteration results from the presence of the G8251A polymorphism within this individual which destroys the *Ban* II restriction endonuclease recognition site, as presented in Figure 5.7. The G to T alteration at this nucleotide position is, however, not related to this polymorphism and awaits confirmation. Alternatively, the presence of the T fragment at this position may have

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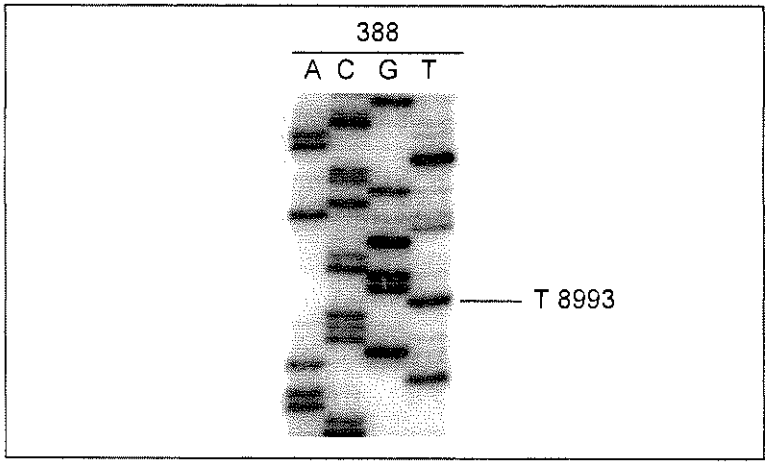
been due to the compressions noted in the sequence data generated for this region of the mitochondrial genome, as illustrated in Figure 5.16.

The 9 bp deletion polymorphism is also depicted in Figure 5.16 in lane 390. This polymorphism is most commonly associated with the African specific haplogroup L and was observed in four individuals in this study. Sequencing of the $tRNA^{Lys}$ region of the mtDNA therefore confirmed RFLP analysis results for these two polymorphisms. Sequence data generated from this region of the mtDNA was also helpful in distinguishing between the different haplogroups within the South African population.

5.4.3 Direct sequence analysis of the mitochondrial ATPase6 region

The two mutations within the ATPase6 region of the mtDNA may be investigated simultaneously by sequence analysis. The amplification of the fragment surrounding the T8993C/G mutations was altered for sequencing by the generation of a new reverse primer so as to amplify a shorter amplicon during PCR. The sequence generated by utilising the forward primer as the sequencing primer is illustrated in Figure 5.17.

Figure 5.17: Autoradiographic representation of the ATPase6 region of the mitochondrial genome



6% polyacrylamide gel electrophoresed at 60 W for 3 hours in 1 X TBE buffer. The forward primer was utilised as the sequencing primer.

As represented by Figure 5.17 above the T8993C/G mutations were absent in all the individuals investigated. Although there have been other mutations reported within this region of the mitochondrial genome, this sequence investigation was not appropriate for

the screening of additional mutations. It would therefore be an advantage to design a primers so as to incorporate the T9176C mutation in the PCR product and then upon sequencing utilise either two different forward primers so as to maximise the efficiency of the detection strategy. However, unlike the mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ genes the mutations harboured within the ATPase6 gene are further apart. It will therefore be necessary to use two distinct sequencing primers.

The results obtained for the patients included in this study were performed in duplicate with the correct controls in place. All RFLP analyses results were confirmed with direct sequencing. It is envisaged that for the A3277G mutation a RFLP strategy will be implemented to establish whether or not the mutation is indeed heteroplasmic or homoplasmic within patient 388.

As previously discussed only one patient, individual 411, was observed to harbour a reported mutation. The A3243G mutation observed in this proband was also present in her mother (431) and sister (432) but not in other maternal relatives investigated (454, 455, 456). Apart from this mutation none of the other nine mutations analysed were observed in the patient population under investigation.

Beyond the scope of this study additional information was attained by the development of the alternative screening strategy. This served to incorporate fifteen additional mutations in the sequencing strategy, allowing a diagnosis of more than the ten originally mentioned mtDNA mutations. The study was therefore extended to screen each individual for 25 mtDNA mutations associated with particular mitochondrial disorders besides the MELAS, MERRF and Leigh Syndrome mutations as originally intended. This new strategy further enhanced the probability of offering a more reliable diagnosis. However, the absence of the 25 mtDNA mutations within a particular patient does not indicate that the patient does not harbour a mitochondrial disorder. This may only be verified by screening the entire mitochondrial genome.

CHAPTER SIX

CONCLUSION

This study presents the first extensive mitochondrial DNA analysis for paediatric patients within the South African population. The molecular investigation for the presence of reported mitochondrial mutations was performed on 25 probands and seven maternal relatives of two probands.

Overlap syndromes occur within the group of mitochondrial disorders, and it was therefore decided that all the patients, irrespective of their particular clinical diagnoses, would be screened for all ten mutations analysed in this study. This approach of molecular analysis was substantiated with the observation of the MELAS A3243G mutation in patient 411 who initially presented with atypical MELAS signs, most notably with the absence of stroke-like episodes. If molecular analysis were based solely on clinical indications this patient would not have been screened for the MELAS mutations. The results obtained in this case argues in favour of including as many as possible mitochondrial mutations in the molecular analysis of patients with suspected mitochondrial disorders.

In the group of well characterised patients analysed only one patient (411) was observed to harbour the reported MELAS A3243G mutation. However, patient 411 did not display the typical stroke-like episodes as a clinical feature associated with MELAS. This same phenotype was observed amongst the disease control group of individuals investigated by Ciafaloni *et al.* in 1992. These authors reported that five individuals within their disease control population harboured the A3243G MELAS mutation but could not be clinically classified as having the MELAS disorder as they did not present with stroke-like episodes. The presence of the A3243G mutation in patient 411 confirms that stroke-like episodes are not always associated with the presence of the A3243G mutation, as initially reported by Ciafaloni *et al.* (1992). This may represent a new group of patients which should possibly be referred to as MELA, as no stroke-like episodes are present.

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Although only one reported mutation was observed in this paediatric population the absence of the mtDNA mutations analysed is of great significance. The absence of the reported mtDNA mutations within this well characterised group of patients lead to the postulation that either novel or reported mutations, not included in the screening strategy, are responsible for the phenotypes observed.

The novel alteration (G3277A) observed in patient 388 lends credence to this postulate. This observation has far reaching implications for the molecular diagnosis of mitochondrial myopathies in South Africa. We observed that patients with mitochondrial myopathies in South Africa presented with the same, or similar, clinical phenotypes as reported in other international studies. However, it became clear that the genetic aetiology of our South African patient population is different. It would therefore be incorrect to make a negative diagnosis when only screening for the reported mutations within the SA population, especially when presented with a well characterised phenotype suggestive of a mitochondrial disorder. These findings emphasise the uniqueness of the South African population with regard to the aetiology of mitochondrial disorders.

In this study one novel change was observed in a patient (388) whose clinical phenotype was indicative of LS, although the alteration was observed in the mitochondrial $tRNA^{Leu}$ gene as presented in Figure 5.13. The fact that none of the reported mutations were observed in this patient suggests that the G3277A alteration represents the first of the novel changes observed within the South African population. This novel change needs to be characterised in order to ascertain its pathological role in the expression of the phenotype observed in this patient. To this end a RFLP analysis should be constructed to allow determination of the presence of heteroplasmy. Confirmation of heteroplasmy for this alteration will give an indication of the pathogenicity of the mutation, in accordance with the requirements for pathogenicity of mtDNA mutations. Once heteroplasmy has been confirmed for the alteration it is envisaged that functional studies will be conducted to investigate the role this mutation plays in the expression of the phenotype.

The population investigated in this study included individuals from various ethnic backgrounds. When comparing the sequence data generated with that of the Cambridge reference sequence (Anderson *et al.*, 1981) numerous differences were noted. Many of these were confirmed to be African specific polymorphisms. However,

an additional polymorphism, T3197C, was also noted in patients 389 and 412, with both these patients being of Caucasian decent. This polymorphism was previously reported (Hess *et al.*, 1995) and confirmed in the MITOMAP database (Shields and Bridge, 1999).

In this study only one reported mutation and one novel alteration were observed in the limited regions (ca. 2%) of the mitochondrial genome investigated. It would therefore be worthwhile extending the screening strategy to sequencing of the entire mitochondrial genome of patients with mitochondrial disorders to elucidate the aetiology of the clinical phenotype. To be effective with respect to time and cost implications it would be advisable to initially screen the regions mentioned in this study via a sequencing strategy, and subsequently including the rest of the genome. The presence of any of the mutations described in this study will thus be uncovered in the initial screening phase of the investigation.

The patients referred to the centre for molecular analysis are an extremely well characterised group of neurological paediatric patients with phenotypes indicative of mitochondrial disorders. The molecular aetiology of these phenotypes, however, remains to be elucidated. Determination of the molecular basis for these disorders is essential for the management of the patients, especially with regard to counselling. Genetic counselling for any disorder is complex and requires highly trained professionals who are able to counsel the affected families in a non-directive manner. In mitochondrial disorders the complexity is compounded by the fact that the chance of bearing affected offspring can not be ascertained. This is because the heteroplasmic levels vary between the different egg cells and subsequent somatic segregation of the mutant mtDNA can not be predicted for the developing tissue. Although this does not simplify counselling, identification of a mtDNA mutation within a family generally rules out the possibility of other genetic alterations, and allows the correct inheritance pattern to be established, thereby enabling informative counselling.

Although enzyme studies are available in South Africa for metabolic disorders, the service has been restricted to certain areas of the country as a result of transport restraints. In addition, particular tests are required to be performed within a certain time period after collection of the sample. Due to the distance between certain collection points and the testing site, the option of enzyme analysis is not always feasible. It is envisaged that in the near future this valuable service will be more readily available as

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new systems are put into place to utilise this critical resource optimally. Ultimately a complete service is foreseen which will offer both molecular and biochemical analysis for patients suspected of harbouring metabolic disorders with a mitochondrial aetiology.

The future of mitochondrial genetics has acquired a bright gleam with the development of mouse models and the utilisation of microarrays for molecular analyses. In 2000 two mouse models were developed for mitochondrial disorders. The first was developed with a single nucleotide substitution within the mtDNA (Sligh *et al.*, 2000) while the second "mito mouse" had a large deletion of the mitochondrial genome (Inoue *et al.*, 2000). Both mouse models were observed to display maternal transmission of the mtDNA defect and displayed stable inheritance of the mutations over several generations. The utilisation of oligonucleotide microarrays for the detection of single nucleotide alterations within the mitochondrial genome (Erdogan *et al.*, 2001) have broadened the horizons for mitochondrial research programmes. This technology will enable a screening strategy to be employed that utilises a single microarray for analysis of the entire mitochondrial genome.

At present, the pathogenicity of mtDNA mutations are investigated via cybrid cell analysis. Here cells containing the nucleus of an unaffected donor are fused with cells containing patient mitochondria (rho zero), to investigate the contribution of the patient (affected) mitochondria against several different nuclear backgrounds. This strategy allows the functional effects of a mutation to be studied *in vivo* (Zeviani *et al.*, 1996). The mouse models created for single nucleotide alterations (Sligh *et al.*, 2000) and mtDNA deletions (Inoue *et al.*, 2000) have paved the way for further mouse models in order to investigate the pathological role of various mtDNA alterations *in vivo*.

In the future it is envisaged that whole mitochondrial genome screens for patients with mitochondrial disorders will reveal novel alterations responsible for the observed pathophysiology. It is also foreseen that the nuclear components of the OXPHOS system will be investigated. It was with this thought in mind that gDNA was isolated from samples obtained from the patients included in this study. This gDNA could thus be used to address questions regarding the molecular aetiology with regard to both the mitochondrial and nuclear genomes.

The uniqueness of the South African population was evident from this molecular investigation. Even though the clinical phenotypes observed were similar to others

reported internationally, the genetic aetiology was not the same. From this study it can be inferred for the South African, as well as, other populations that the absence of common or reported mutations does not necessarily exclude a mitochondrial aetiology. A negative diagnosis may therefore only be confirmed if a whole mitochondrial genome screen has been performed and no alterations noted. The data generated through this investigation substantiates the postulate that novel mitochondrial mutations are responsible for the majority of clinical phenotypes observed in the South African paediatric population.

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APPENDIX A

GENETIC HETEROGENEITY OF THE MITOCHONDRIAL MYOPATHIES

Table A.1: Disorders associated with mutations in the mitochondrial $tRNA^{Leu(UUR)}$ gene

Mutation	Disorder	Inheritance	Reference
A3243G	MELAS	maternal	Goto *et al.* (1990) Kobayashi *et al.* (1990)
	diabetes/ deafness	maternal	van den Ouweland *et al.* (1992)
	Pearson's s./multisystem PEO	maternal	Moraes *et al.* (1993b)
	myopathy/ painful stiffness	sporadic?	Deschauer *et al.* (1999)
	myopathy/ dystonia	maternal	Sudarsky *et al.* (1999)
	infantile encephalopathy	maternal	Sue *et al.* (1999)
A3243T	mitochondrial encephalomyopathy	sporadic	Shaag *et al.* (1997)
T3250C	myopathy	maternal	Goto *et al.* (1992b)
A3251G	PEO, myopathy, sudden death	maternal	Sweeney *et al.* (1993)
A3252G	MELAS	maternal	Morten *et al.* (1993)
C3254G	MIMyCa	maternal	Kawarai *et al.* (1997)
C3256T	Multisystem/PEO	maternal	Moraes *et al.* (1993a)
A3260G	MIMyCa	maternal	Zeviani *et al.* (1991a)
	MELAS	maternal	Nishino *et al.* (1996)
T3264C	diabetes/ ataxia/ PEO	maternal	Suzuki *et al.* (1997)
T3271C	MELAS	maternal	Goto *et al.* (1991)
ΔTnt-3271	mitochondrial encephalomyopathy	sporadic	Shoffner *et al.* (1995b)
A3288G	myopathy	maternal	Hadjigeorgiou *et al.* (1999)
T3291C	MELAS	maternal	Goto *et al.* (1994)
A3302G	myopathy	maternal	Bindoff *et al.* (1993)
C3303T	MIMyCa	maternal	Silvestri *et al.* (1994)

Δ = deletion. Adapted from Servidei (2001).

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Table A.2: Disorders associated with mutations in the mitochondrial $tRNA^{Lys}$ gene

Mutation	Disorder(s) associated	Inheritance	Reference
A8296G	diabetes / deafness	maternal	Kameoka *et al.* (1998)
	Hypertrophic cardiomyopathy	maternal	Akita *et al.* (2000)
G8313A	gastrointestinal / encephalonueropathy	sporadic	Verma *et al.* (1997)
G8328A	mitochondrial encephalomyopathy	sporadic	Houshmand *et al.* (1999)
G8342A	PEO / myoclonus	sporadic	Tiranti *et al.* (1999)
A8344G	MERRF	maternal	Shoffner *et al.* (1990)
	Spino-cerebellar / Leigh Syndrome	maternal	Howell *et al.* (1996)
T8356C	MERRF	maternal	Silvestri *et al.* (1992)
	MERRF/MELAS	maternal	Zeviani *et al.* (1993)
G8363A	multisystem cardiomyopathy	maternal	Santorelli *et al.* (1996)
	MERRF	maternal	Ozawa *et al.* (1997)

Adapted from Servidei (2001).

Table A.3: Disorders associated with mutations in the mitochondrial ATPase 6 gene

Mutation	Disorder	Inheritance	Reference
T8851C	bilateral striatal necrosis	maternal	De Meirlier *et al.* (1995)
T8993G	NARP	maternal	Holt *et al.* (1990)
	MILS	maternal	Tatuch *et al.* (1992) Shoffner *et al.* (1992)
	NARP / MILS	maternal	Fryer *et al.* (1994)
T8993C	NARP / MILS	maternal	de Vries *et al.* (1993)
	MILS	maternal	Santorelli *et al.* (1994)
T9101C	LHON	maternal	Lamminen *et al.* (1995)
T9176C	bilateral striatal necrosis	maternal	Thyagarajan *et al.* (1995)
	NARP / MILS	maternal	Campos *et al.* (1997)

Adapted from Servidei (2001).

Table A.4: Mitochondrial DNA mutations associated with MELAS

Mutation	Gene	Inheritance	Reference
G583A	$tRNA^{Phe}$	presumed sporadic	Hanna *et al.* (1998)
G1642A	$tRNA^{Val}$	maternal	Taylor *et al.* (1996)
A3243G	$tRNA^{Leu}$	maternal	Goto *et al.* (1990) and Kobayashi *et al.* (1990)
A3252G	$tRNA^{Leu}$	maternal	Morten *et al.* (1993)
A3260G	$tRNA^{Leu}$	maternal	Nishino *et al.* (1996)
T3271C	$tRNA^{Leu}$	maternal	Goto *et al.* (1991)
T3291C	$tRNA^{Leu}$	maternal	Goto *et al.* (1994)
A5814G	$tRNA^{Cys}$	maternal	Manfredi *et al.* (1996)
T9957C	COX III	maternal	Manfredi *et al.* (1995)
G13513A	ND5	maternal	Santorelli *et al.* (1997b)
Single large deletion		sporadic	Campos *et al.* (1995)

Adapted from Servidei (2001).

Table A.5: Mitochondrial DNA mutations associated with MERRF

Mutation	Gene	Inheritance	Reference
A8344G	$tRNA^{Lys}$	maternal	Shoffner *et al.* (1990)
T8356C	$tRNA^{Lys}$	maternal	Silvestri *et al.* (1992)
G8363A	$tRNA^{Lys}$	maternal	Ozawa *et al.* (1997)

Adapted from Servidei (2001).

Table A.6: Mitochondrial DNA mutations associated with Leigh Syndrome or NARP

Mutation	Gene	Inheritance	Reference
A8344G	$tRNA^{Lys}$	maternal	Howell *et al.* (1996)
T8993G	ATPase 6	maternal	Holt *et al.* (1990) Fryer *et al.* (1994)
T8993C	ATPase 6	maternal	de Vries *et al.* (1993)
T9176C	ATPase 6	maternal	Campos *et al.* (1997)

Adapted from Servidei (2001).

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Table A.7: Nuclear gene mutations associated with Leigh Syndrome due to respiratory chain deficiency

Symbol (gene product)	Gene location	Respiratory chain defect	Mode of inheritance	reference
NDUFS7 (NADH-ubiquinone oxidoreductase FE-S protein 7)	19p13	complex I	AR	Triepels *et al.* (1999)
NDUFS8 (NADH-ubiquinone oxidoreductase FE-S protein 8)	11q13	complex I	AR	Loeffen *et al.* (1998)
SDHA (succinate dehydrogenase 2, flavoprotein subunit)	5p15	complex II	AR	Bourgeron *et al.* (1995)
SURF-1	9q34	complex IV	AR	Tiranti *et al.* (1998b) Zhu *et al.* (1998)

Adapted from Servidei (2001).

APPENDIX B

GENETIC HETEROGENEITY WITHIN THE PYRUVATE DEHYDROGENASE COMPLEX

Discrepancies noted in the literature pertaining to reported mutations, are indicated in this table by an asterisk (*). No alterations were made for the purpose of this thesis and mutations appear in the table below exactly as originally reported.

Table B.1: Mutations in the PDHA1 gene encoding the E1α subunit of the pyruvate dehydrogenase complex

Mutation	Exon	Result
*G134C[23]	1	R10P
*A131G[18]	3	H44R
G265A[24]	3	G89S
*C214T[25]	3	R43C
C319T[27]	3	R72C
C367A[29]	3	R88S
C484G[16]	4	R127W
C442G[27]	4	H113D
G499A[10]	5	V148M
G589A[27]	5	G162R
G523A[25]	6	deletion of exon 6
A691G[10]	7	T202A
G595A[10]	7	A170T
G628C[15]	7	A175P
T832A[20]	7	Y243N
C693T[21]	7	P188L
C615A[25]	7	F176L
A671G[28]	7	M181V
*C829G[9]	8	R234G
*C787G[10, 25]	8	R234G
A795C[13]	8	D258A
*G788A[19]	8	Arg263Gln
*C892G[27, 29]	8	R263G
*A875T[10]	9	H263L
927del7[10]	9	deletion of 7 bp (AGTAAGA) tandem repeat deletion
A949C[20]	9	M282L
972Tins[20]	9	R288ins, causing frameshift and premature termination

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Table B.1: continued ...

Mutation	Exon	Result
G871A[24]	9	G291R
I307ins[17]	9/10	33 bp duplication of 13 bp 3'end exon 9 and 20 bp of 5'end exon 10 – results in elongated protein
R302fs[11]	intron9/ exon10	5 bp duplication of GTTAC at intron 9 junction with exon 10
1032del7[3]	10	deletion of 7 bp, causing frameshift
1042del3[4]	10	deletion of 3 bp at nt 1042, results in deletion of K313
C1008T[7, 27]	10	R302C
21bp ins[8]	10	in frame 21 bp insertion between codons 305 and 306
948del2[10]	10	deletion of 2 bp results in premature termination
Ser[300]del20[12]	10	S300del, causing frameshift and premature termination
G949A[20]	10	D315A
C1052T[22]	10	P316L
927del7[25]	10	deletion of 7 bp, causing premature termination
*931del3[25]	10	removes R282
981ins13[25]	10	insertion of 13 bp, causing premature termination
1038del4[26]	10	deletion of 4 bp, causing premature termination
1069ins15[27]	10	insertion of 15 bp results in a frameshift at D322
*974del3[28]	10	deletion of R282
1156del4[1]	11	deletion of 4 bp causing frameshift
1167del4[2]	11	deletion of 4 bp, causing frameshift resulting in elongated protein
1264del2[4]	11	deletion of two adenines results in a frameshift at Lys[387]
G1248A[4, 20]	11	R378H
1145ins4[5]	11	insertion of 4 bp, premature termination
1144del20[6]	11	deletion of 20 bp, results in a frameshift, creating a premature stop codon
1146ins4[10]	11	4 bp (ATCA) insert creates premature stop codon
1126ins2[10]	11	insertion of 2 bp (TT) results in an extended protein
1251ins4[14]	11	Q382fs, results in a premature stop codon
G1133A[25]	11	R349H
1078ins46[25]	11	insertion of 46 bp, causing premature termination
1163ins4[25]	11	insertion of 4 bp, causing premature termination
1264ins39[27]	11	insertion of 39 bp results in a frameshift at K387
1217ins13[27]	11	insertion of 13 bp results in a frameshift at S371
1202ins16[28]	11	insertion of 16 bp, causing premature termination
1247del8[29]	11	deletion of 8 bp, causing premature termination

1 = Endo *et al.* (1989a); 2 = Endo *et al.* (1989b); 3 = Dahl *et al.* (1990); 4 = Hansen *et al.* (1991); 5 = Endo *et al.* (1991); 6 = Chun *et al.* (1991); 7 = Dahl *et al.* (1992); 8 = De Meirleir *et al.* (1992); 9 = Wexler *et al.* (1992); 10 = Chun *et al.* (1993); 11 = Hansen *et al.* (1993); 12 = Matthews *et al.* (1993a); 13 = Matthews *et al.* (1993b); 14 = Takakubo *et al.* (1993a); 15 = Takakubo *et al.* (1993b); 16 = Fujii *et al.* (1994); 17 = Hansen *et al.* (1994); 18 = Naito *et al.* (1994); 19 = Awata *et al.* (1994); 20 = Matthews *et al.* (1994); 21 = Hemalatha *et al.* (1995); 22 = Takakubo *et al.* (1995a); 23 = Takakubo *et al.* (1995b); 24 = Matsuda *et al.* (1995); 25 = Chun *et al.* (1995); 26 = Lissens *et al.* (1995); 27 = Lissens *et al.* (1996); 28 = Tripatara *et al.* (1996); 29 = Marsac *et al.* (1997). fs = frameshift.

APPENDIX C

CLINICAL INFORMATION SHEET

The following list of clinical criteria was compiled by Dr. I. Smuts, Department of Paediatrics, University of Pretoria. It is reproduced in this thesis with her permission.

Patient information

Patient Data	
Name:	
Hospital number:	
Date of birth:	
Age at presentation:	
Sex:	
Contact number:	
Address	

History

Family history

Family tree

Clinical features

CNS	Seizures	
	Ataxia	
	Myoclonus	
	Psychomotor retardation	
	Psychomotor regression	
	Hemiparesis/hemianopia	
	Cortical blindness	
	Migrainelike headache	
	Dystonia	
	Apnoea	
PNS	Peripheral neuropathy	
Muscle	Myopathic	
	Hypertonia	
	Hypotonia, atrophy	
	Recurrent myoglobinuria	
	Ptosis	
Blood	Sideroblastic anemia. Neutropenia, Thrombocytopenia	
Kidney	Fanconi syndrome	

Eye	Pigmentary neuropathy	
	Optic atrophy	
	Cataracts	
	Opthalmoplegia	
Endocrine	Short stature	
	Hypoparathyroidism	
	Diabetes mellitus	
Heart	Conduction block	
	Cardiomyopathy	
Liver	Enlarged	
	Hepatic failure	
GI	Pancreatic dysfunction	
	Pseudoobstruction	
	Recurrent vomiting	
	Chronic diarrhea	
ENT	Sensorineural deafness	

Special Investigations

Date				
Na				
K				
Cl				
CO_2				
AG				
Urea				
Creatinine				
Uric acid				
Ca				
Mg				
PO_4				
Osmol				
Protein				
Albumin				
Bilirubin				
c-Bilirubin				
ALP				
GGT				
ALT				
AST				
LD				
Glucose				
Cholesterol				
Lactate				
Pyruvate				
L:P				
NH_3				
CK				
Aldolase				
CSF prot				
CSF Cl				
CSF gl				
CSF Lactate				
CSF pyruvate				
CSF L:P				
u-Dipstick				
u-Org. acids				
Alanine P,S				
OH Butyrate				
Acetoacetate				
f-Carnitine				
Hb				
RBC				
MCV				
PLT				
WBC				
Neutro.				
Lymph.				

Neuroradiology

Neurophysiology	
EEG:	
EMG:	
ERG:	
VEP:	
ABR:	

Muscle Biopsy

Heart	
ECG:	
Sonar:	

Biochemistry

Molecular genetics

Other

APPENDIX D

CONFERENCES AND MEETINGS AT WHICH RESEARCH CONTAINED IN THIS THESIS WERE PRESENTED

Parts of the research contained in this thesis were presented at the following international and national meetings as D. Prosser (nèè Cawood). The name of the presenting author in each case is underlined.

D.1 RESEARCH PRESENTED AT INTERNATIONAL MEETINGS

D.1.1 **51st Annual meeting of the American Society of Human Genetics:** San Diego, U.S.A., October 2001.

Olckers A., Prosser D., Wallace D.C., Brown M.D. and Smuts I. Mitochondrial myopathies: a South African perspective.

D.1.2 **5th Congress of the World Muscle Society:** White River, South Africa, June 2000.

Cawood D, Hosseini S.H., Smuts I., Brown M.D., Wallace D.C. and Olckers A. Mitochondrial genome screening of five South African mitochondrial myopathy paediatric patients: novel changes.

D.1.3 **4th European meeting on mitochondrial pathology (Euromit4):** Cambridge, United Kingdom, September 1999.

Cawood D., Smuts I., Engelbrecht S., Wallace D.C. and Olckers A. Mitochondrial encephalopathies: molecular investigation of causative mutations within the South African population.

D.2 RESEARCH PRESENTED AT NATIONAL MEETINGS

D.2.1 **Ninth biennial congress of the South African Society of Human Genetics:** Kruger National Park, South Africa, August 2001.

Olckers A., Prosser D., Wallace D.C., Brown M.D. and Smuts I. Mitochondrial myopathies in the South African paediatric population.

D.2.2 **Annual congress of the Neurological association of South Africa**, Wild Coast Sun, KwaZulu-Natal, March 2001.

Prosser D., Smuts I., Brown M.D., Wallace D.C. and Olckers A. Molecular analysis of mitochondrial myopathies within South Africa.

D.2.3 **Millipaed 2000 Congress (South African Paediatric Association):** Sun City, Pilanesberg, May 2000.

Cawood D., Smuts I., Engelbrecht S., Wallace D.C. and Olckers A. Molecular investigation of mitochondrial encephalopathies within the South African population.

D.3 RESEARCH PRESENTED AT THE FACULTY OF MEDICINE, UNIVERSITY OF PRETORIA

D.3.1 Faculty Day 2000.

Cawood D., Smuts I., Hosseini S.H., Brown M.D., Wallace D.C. and Olckers A. Molecular investigation of the mitochondrial genome in South African mitochondrial myopathy paediatric patients.

D.4 PUBLISHED ABSTRACTS IN INTERNATIONAL PEER-REVIEWED JOURNALS

D.4.1 Olckers A., Prosser D., Wallace D.C., Brown M.D. and Smuts I. Mitochondrial myopathies: a South African perspective (abstract), *Am. J. Hum. Genet.*, **69** (4), p 490, 2001.

D.4.2 Cawood D., Hosseini S.H., Smuts I., Brown M.D., Wallace D.C. and Olckers A. Mitochondrial genome screening of five South African mitochondrial myopathy paediatric patients: novel changes (abstract), *Neuromusc. Disord.* **10** (4,5), p 347-348, 2000.