Biochemical and molecular genetic characterisation of MADD in a Southern African cohort

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“Do not be afraid, for I am with you.”

Isaiah 43:5

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ABSTRACT

Multiple acyl-coenzyme A dehydrogenase deficiency (MADD) is a heterogeneous group of inborn errors of metabolism caused by pathogenic autosomal recessive variants in *ETFA*, *ETFB*, and *ETFDH*. The latter is mainly affected and encodes for electron transfer flavoprotein-ubiquinone oxidoreductase (ETFQO). MADD presents as three phenotypes with neuromuscular involvement, including two mostly fatal neonatal subtypes (types I and II) and a riboflavin-responsive mild and/or late-onset subtype (type III). While the disorder has been well-studied in other countries, it remains under-recognised and under-diagnosed in ethnically diverse and understudied regions like Southern Africa, due to the paucity of population-specific data. To address these shortcomings, this study was initiated in collaboration with the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD), and aspired to realise two aims: Firstly, to characterise the clinical, metabolic, and genetic properties of a Southern African cohort diagnosed with MADD; and secondly, to evaluate the functional properties of selected pathogenic variants frequently identified in the cohort *in vitro*.

Accordingly, a cohort of 12 unrelated families (12 White and two mixed ethnicity patients) could be recruited via academic medical centres across Southern Africa. For each, the genotype–phenotype association was assessed by extensive clinical evaluations, whole exome sequencing, and Sanger sequencing confirmation. The urinary diagnostic metabolome was profiled before and after treatment, where possible, via targeted gas chromatography–mass spectrometry, and liquid chromatography–triple quadrupole mass spectrometry. This study revealed one novel (c.[287dupA*]) and four known causal variants in *ETFDH*. Based on disease severity and treatment response, three groups emerged: (i) The most severe and fatal phenotypes accompanied the c.[1067G>A];c.[1067G>A] and c.[976G>C];c.[1067G>A] genotypes, which presented at birth as MADD types I and I/II. (ii) The c.[287dupA*];c.[1448C>T], c.[740G>T];c.[1448C>T], and c.[1448C>T];[1067G>A] genotypes displayed moderate early childhood-onset phenotypes of MADD types II/III and III, whereas (iii) the c.[1448C>T];c.[1448C>T] genotype was associated with mild, late-onset MADD type III. All groups exhibited the characteristic organic acids, acylcarnitines and amino acids of MADD (i>ii>iii), which normalised (ii<iii), together with the clinical symptoms, in only MADD type III following riboflavin and/or L-carnitine treatment.

For the two main variants identified (c.[1067G>A] and c.[1448C>T]), the allele frequency was estimated as <0.00034–0.00084% in the four largest South African populations and both revealed a shared haplotype in the region of *ETFDH*, suggesting a founder effect for c.[1067G>A] in the White South African population. The pathogenicity of selected genotypes was further confirmed by investigating the relative quantity of ETFQO and mitochondrial complex I–V, together with respiration in patient fibroblasts. ETFQO was found to be most decreased in c.[287dupA*];c.[1448C>T] (-83%), followed by c.[1067G>A];c.[1448C>T] (-73%) and c.[1448C>T];c.[1448C>T] (-55%). Moreover, two adaptive

responses to mild and severe genotypes of MADD, respectively, are hypothesised: The upregulation of mitochondrial biogenesis, and a shift from oxidative phosphorylation to alternative metabolic pathways. In conclusion, this study provides the first extensive genotype–phenotype, metabolic, and functional evaluation of MADD in the understudied populations of Southern Africa. The data and methods developed will enable timeous screening, genetic counselling, and patient-specific treatment, and support a policy of including MADD in a newborn screening program for Southern Africa.

Keywords: Multiple acyl-CoA dehydrogenase deficiency • MADD • Glutaric aciduria type II • *ETFDH* • Riboflavin • Oroboros Oxygraph-2k • Southern Africa • International Centre for Genomic Medicine in Neuromuscular Diseases • ICGNMD

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LIST OF SYMBOLS AND UNITS

SYMBOLS

%	Percentage
/	Unphased genotype
+	Present and validated; reported
±	Plus-minus
×	Times
↑, ↑↑, and ↑↑↑	Slight, moderate, and substantial increase
↓ and ↓↓↓	Slight and substantial decrease
©	Copyright
®	Registered
AA	Ratio of homozygous samples per total number of samples per population group.
AB	Ratio of heterozygous samples per total number of samples per population group.
p	Allele frequency of variant
™	Trademark
✓	Can be indicative
X	Metabolic control variable
Y_x	Context-dependent. FCE: Background state at low O_2 flux, upon which the metabolic control variable, X, acts; CCE: Specific flux (bc) value prior to addition of CCCP; Specific flux (bc) value prior to addition Cyt c.
Z_x	Context-dependent. FCE: Reference state at high (stimulated by metabolic control variable, X, or uninhibited) O_2 flux; Specific flux (bc) value after addition of CCCP; Specific flux (bc) value after addition Cyt c.
α	Alpha (Greek alphabet)
β	Beta (Greek alphabet)

UNITS

°C	Degrees Celsius	mmol	Millimole
A	Ampere	mol	Mole
bp	Base pairs	ng	Nanogram
cm	Centimetre	nm	Nanometre
cm^2	Square centimetre	nt	Nucleotide
g	Gram	pmol/(s·mL)	O_2 flux per volume
h	Hour(s)	rpm	Revolutions per minute
kb	Kilobases	s	Second(s)

kDa	Kilodalton	U	Enzyme unit
kg	Kilogram	V	Volt
L	Litre	v/v	Volume per volume
Mb	Megabases	w/v	Weight per volume
mg	Milligram	$\times g$	Relative centrifugal force
min	Minute(s)	µg	Microgram
mL	Millilitre	µL	Microlitre
mm	Millimetre	µM	Micromolar
mM	Millimolar	µm	Micrometre
mm^3	Cubic millimetre		

LIST OF ABBREVIATIONS AND LATIN TERMINOLOGY

ABBREVIATIONS

2-HGA	2-Hydroxyglutaric acid
2HGDH	2-Hydroxyglutarate dehydrogenase; EC 1.1.99.39
2-MBGly	2-Methylbutyrylglycine
3'	3’-End of the polynucleotide chain
3’–5'	Reverse DNA strand
4-OH-Pro	4-Hydroxyproline
5'	5’-End of the polynucleotide chain
5’–3’	Forward DNA strand
ACAD9	Acyl-CoA dehydrogenase 9; EC 1.3.8.-
ACDH	Acyl-CoA dehydrogenase
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal dominant
ADP	Adenosine diphosphate
AF	African; allele frequency (context-dependent)
ALT	Alanine aminotransferase
Ama	Oroboros O2k term: antimycin A
AMDIS	Automated Mass spectral Deconvolution and Identification System
AR	Autosomal recessive
AS + I	Asian and Indian
AST	Aspartate aminotransferase
AT	Austria
ATP	Adenosine triphosphate
ATP5A	ATP synthase F1 subunit alpha; Also known as ATP5F1A
ATP5F1A	ATP synthase F1 subunit alpha; Also known as ATP5A
bc	Background-corrected
BCA	Bicinchoninic acid
BCAA	Branched-chain amino acid
BSA	Bovine serum albumin
BVVLS-1	Brown-Vialetto-Van Laere syndrome 1/ Fazio-Londe disease
BVVLS-2	Brown-Vialetto-Van Laere syndrome 2
C	Control
c	Oroboros O2k term: cytochrome c
c.	Variant aligned against coding DNA reference sequence
C0	Free carnitine

C4	Butyric-/isobutyric acid/ butyryl-/isobutyrylcarnitine- or glycine (context-dependent)
c4DC8	*cis*-4-Decenedioic acid
C5	Isovaleric-/2-methylbutyryl-/pivaloyl acid/ isovaleryl-/2-methylbutyryl-/pivaloylcarnitine- or glycine (context-dependent)
C5DC	Glutarylcarnitine
C6	Hexanoic acid/ hexanoylcarnitine- or glycine (context-dependent)
C8	Octanoic acid/ octanoylcarnitine- or glycine (context-dependent)
C10	Decanoic acid/ decanoylcarnitine- or glycine (context-dependent)
C12	Dodecanoic acid/ dodecanoylcarnitine- or glycine (context-dependent)
C14	Tetradecanoic acid
C14:1	Tetradecenoic acid/ tetradecenoylcarnitine- or glycine (context-dependent)
C16	Hexadecanoic acid/ hexadecanoylcarnitine- or glycine (context-dependent)
C16:1	9-Hexadecenoic acid
C18	Octadecanoic acid
C18:1	Octadecenoic acid/ octadecenoylcarnitine- or glycine (context-dependent)
CACT	Carnitine-acylcarnitine translocase
CADD	Combined annotation dependent depletion
CAF	Central Analytical Facilities
Cas	CRISPR-associated protein
Cat. #	Catalogue number
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CCE	Coupling control efficiency
cDNA	Complimentary DNA
CGpDH	Inner mitochondrial membrane flavoprotein-linked L-glycerol 3-phosphate dehydrogenase complex; EC 1.1.5.3
CHM	Centre for Human Metabolomics
chr	Chromosome
CI	Complex I; NADH:ubiquinone oxidoreductase; EC 1.6.5.3
CII	Complex II; succinate:ubiquinone oxidoreductase; EC 1.3.5.1
CIII	Complex III; ubiquinol:ferricytochrome c oxidoreductase; EC 1.10.2.2
CIV	Complex IV; ferrocytochrome-c:oxygen oxidoreductase; EC 1.9.3.1
CK	Creatine kinase
CNS	Central nervous system
CO_2	Carbon dioxide
CoA	Coenzyme A
conc	Concentration
CoQ10	Coenzyme Q10

CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
CPT2	Carnitine palmitoyltransferase 2 gene
CrAT	Carnitine acetyltransferase
CRISPR	Clustered regularly interspaced short palindromic repeats
CV	Complex V; ATP synthase; EC 3.6.1.3
Cyt c	Cytochrome c
Cyt c CE	Cytochrome c control efficiency
D	Oroboros O2k term: Adenosine triphosphate
D and number	Age in days
DBS	Dried blood spot
DC6	Adipic acid
DC8	Suberic acid
DC10	Sebacic acid
DE	Germany
Dig	Oroboros O2k term: Digitonin
DM	Distal muscle
DMEM	Dulbecco's Modified Eagle Medium
DMGDH	Dimethylglycine dehydrogenase; EC 1.5.8.4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DP	Depth
E	European non-Finnish; uncoupled respiration (context-dependent)
EC	Enzyme commission
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N,N*-tetraacetic acid
EMA	Ethylmalonic acid
ETC	Electron transport chain
ETF	Electron transfer flavoprotein
ETFA	Electron transfer flavoprotein subunit alpha gene
ETFB	Electron transfer flavoprotein subunit beta gene
ETFDH	Electron transfer flavoprotein-ubiquinone oxidoreductase gene
ETFQO	Electron transfer flavoprotein-ubiquinone oxidoreductase; EC 1.5.5.1
ETFα	Electron transfer flavoprotein subunit alpha
ETFβ	Electron transfer flavoprotein subunit beta

F	Female; father (context-dependent)
FAD	Oxidised flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FADS	Flavin adenine dinucleotide synthase; EC 2.7.7.2
FAO	Mitochondrial fatty acid β-oxidation
FBS	Foetal bovine serum
FCE	Flux control efficiency
Feb	February
FLAD1	Flavine adenine dinucleotide synthetase 1 gene
FMN	Flavin mononucleotide
FP	Flavoprotein
fwd	Forward strand
G	Oroboros O2k term: glutamate
GA	Glutaric aciduria
GAII	Glutaric aciduria type II
GCDH	Glutaryl-CoA dehydrogenase; EC 1.3.8.6
GC–MS	Gas chromatography–mass spectrometry
GC–MS/MS	Gas chromatography–tandem mass spectrometry
gnomAD	Genome Aggregation Database
Gp	Oroboros O2k term: glycerophosphate
GRCh	Genome Reference Consortium Human Build
GT	Genotype
H3Africa	Human Heredity and Health in Africa
HADHA	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha gene
HCRW	Health and Care Research Wales
HEK293	Human embryonic kidney (HEK) 293
HGly	*N*-Hexanoylglycine
HGVS	Human genome variation society nomenclature
HGVSc	Nucleotide change (according to HGVS) aligned to the coding DNA reference sequence
HGVSp	Amino acid change (according to HGVS) aligned to the protein reference sequence
HIV	Human immunodeficiency virus
HPO	Human phenotype ontology
HRA	Health Research Authority
HRP	Horseradish peroxidase
HYPDH	Hydroxyproline dehydrogenase; EC 1.5.5.3
IBD	Isobutyryl-CoA dehydrogenase; EC 1.3.8.1
iBGly	*N*-Isobutyrylglycine

ICGNMD	International Centre for Genomic Medicine in Neuromuscular Diseases
IEM	Inborn error of metabolism
Ig	Immunoglobulin
IMD	Inherited Metabolic Disease
INFORM	International Network for Fatty Acid Oxidation Research and Management
IVD	Isovaleryl-CoA dehydrogenase; EC 1.3.8.4
iVGly	*N*-Isovalerylglycine
JP	Japan
LCAD	Long-chain acyl-CoA dehydrogenase; EC 1.3.8.8
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
M	Mixed; male; mother (context-dependent)
M and number	Age in months
M.1	Oroboros O2k term: Malate (non-saturating concentration)
M.2	Oroboros O2k term: Malate (saturating concentration)
MADD	Multiple acyl-CoA dehydrogenase deficiency
MADD-DS3	Multiple acyl-CoA dehydrogenase deficiency-disease severity 3
MADD-M	MADD-mild
MADD-S	MADD-severe
MCAD	Medium-chain acyl-CoA dehydrogenase; EC 1.3.8.7
MFT	Mitochondrial folate/FAD transporter
MiR05	Substrate-limited mitochondrial respiration (medium)
MM	Metabolic myopathy
MPC	Mitochondrial pyruvate carrier
MPV17	Mitochondrial inner membrane protein MPV17
mRNA	Messenger ribonucleic acid
MTCO1	Cytochrome c oxidase subunit I; EC 7.1.1.9
MTHF	Methyl-tetrahydrofolate
MW	Molecular weight
N	Normal
n	Normal; number of patients (context-dependent)
n.a	Not applicable
n.d	Not determined
n.r	Not reported
NAD^+	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NBS	Newborn screening
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit

NGS	Next generation sequencing
NHLS	National Health Laboratory Service
NL	The Netherlands
NMD	Neuromuscular disorder
NWU	North-West University
NWU-HREC	Health Research Ethics Committee of the Faculty of Health Sciences, North-West University
O_2	Oxygen
O2k	Oxygraph-2k
Oct	Oroboros O2k term: octanoylcarnitine
OH	Hydroxy
OMIM	Online Mendelian Inheritance in Man (www.omim.org/)
ox	Oxidised
OXPHOS	Oxidative phosphorylation
P	Patient; pyruvate; coupled respiration (context-dependent)
p.	Variant aligned against a protein reference sequence
P/S	Penicillin:streptomycin
P5C	Δ^1-Pyrroline-5-carboxylate
PBS	Phosphate buffered saline
PCR	Polymerase-chain reaction
PCR–RFLP	Polymerase-chain reaction-restriction fragment length polymorphism
P_i	Inorganic phosphate
PLIEM	Potchefstroom Laboratory for Inborn Errors of Metabolism
PM	Proximal muscle
PNS	Peripheral nervous system
PO_4	Phosphate
Pro	Proline
PRODH	Proline dehydrogenase; EC 1.5.5.2
PVDF	Polyvinylidene difluoride
Rb	Riboflavin
RC	Respiratory chain
RDSA	Rare Disease South Africa
red	Reduced
rev	Reverse strand
RFK	Riboflavin kinase
RFVT1−3	Riboflavin transporter 1−3
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species

Rot	Oroboros O2k term: Rotenone
RR-MADD	Riboflavin-responsive MADD
rsID	Reference single nucleotide polymorphism cluster identification
RU-MADD	Riboflavin-unresponsive MADD
rxn	Reaction (replicate)
S	Oroboros O2k term: succinate; sibling (context-dependent)
s	Number of available samples
SA	South Africa(n)
SASHG	Southern African Society for Human Genetics
SBAH	Steve Biko Academic Hospital
SBCAD	Short branched-chain acyl-CoA dehydrogenase; EC 1.3.99.12
SCAD	Short-chain acyl-CoA dehydrogenase; EC 1.3.8.1
SDH	Sarcosine dehydrogenase; EC 1.5.8.3
SDHB	Succinate dehydrogenase (ubiquinone) iron-sulfur subunit; EC 1.3.5.1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLC25A32	Solute carrier family 25 member 32 gene
SLC52A1−3	Solute carrier family 52 member 1−3 gene
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
SSIEM	Society for the Study of Inborn Errors of Metabolism
SU	Stellenbosch University
SUIT	Substrate-Uncoupler-Inhibitor Titration
T_a	Annealing temperature
TBH	Tygerberg Hospital
TBS	Tris buffered saline
TCA	Tricarboxylic acid
TEMED	*N,N,N′,N′*-Tetramethylethylenediamine
THF	Tetrahydrofolate
Tr	Treated
U	Unknown
U*	Oroboros O2k term: CCCP at multiple titrations
UCL	University College London
UCT	University of Cape Town
UK	United Kingdom
Untr	Untreated
UP	University of Pretoria

UQCRC2	Cytochrome *b*-*c*1 complex subunit 2
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet light
v	Version
VLCAD	Very-long-chain acyl-CoA dehydrogenase; EC 1.3.8.9
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing
Y and number	Age in years

LATIN TERMINOLOGY

In silico	In silicon; performed by computer simulation
In trans	Across; on different chromosomes
In vitro	In the glass; a process outside of a living organism
In vivo	In life; a process within a living organism
Per annum	Yearly
Post-mortem	After death

Chapter 1

Introduction

1.1 BACKGROUND, RATIONALE, AND PROBLEM STATEMENT

Multiple acyl-coenzyme A (CoA) dehydrogenase deficiency (MADD; ORPHAcode 26791) is an inborn error of metabolism (IEM) with an estimated global and ethnically variable birth prevalence of 1:200,000 (Orphanet, 2014). It is primarily caused by defects in the genes encoding for the α and β subunits of electron transport flavoprotein (ETF; *ETFA* and *ETFB*) and ETF-ubiquinone oxidoreductase (ETFQO; *ETFDH*) (Goodman & Frerman, 1984; Grünert, 2014). These proteins play a central role in mitochondrial metabolism by re-oxidising reduced flavin adenine dinucleotide ($FADH_2$), following the donation of electrons to the electron transport chain (ETC). In its oxidised form, flavin adenine dinucleotide (FAD) functions as a cofactor to at least 90 cellular flavoproteins (Ghisla & Thorpe, 2004; Lienhart *et al.*, 2013). Along with ETF and ETFQO, 14 other flavoproteins—nine of which are acyl-CoA dehydrogenases—are known to be compromised during MADD (Mosegaard *et al.*, 2020; Ostrander *et al.*, 2009; Summitt *et al.*, 2015). This leads to impaired mitochondrial fatty acid β-oxidation (FAO), branched-chain amino acid (BCAA) catabolism, amino acid catabolism, and choline metabolism.

Depending on the time of onset and disease severity, MADD may be divided into three phenotypes: two severe neonatal-onset subtypes that present with or without congenital features, respectively (types I and II), and a heterogenous mild and/or later-onset subtype (type III) (Goodman & Frerman, 1984). Clinically, MADD is characterised by multi-system involvement, including liver dysfunction, cardiomyopathy, encephalopathy (types I, II, and III), and muscle weakness (type III) or hypotonia (types I and II). Additionally, MADD may be accompanied by recurrent episodes of lethargy and/or vomiting (type III), hypoglycaemia, metabolic acidosis (types I, II, and III), and/or hyperammonaemia (types I and II). Since MADD results in the ineffective re-oxidation of $FADH_2$, genetic defects affecting proteins involved in FAD

Selected glossary

Terms that may be ambiguous, are used throughout this thesis as follows:

1. **MADD** refers to the IEM caused by variants in *ETFA*, *ETFB* and *ETFDH,* and may present as MADD types I–III.
2. **MADD-like disorders** refer to a group of IEMs caused by variants in *SLC52A1−3*, *FLAD1*, and *SLC25A32*, namely RFVT1−3, FADS and MFT deficiency, which may present with clinical phenotypes and diagnostic metabolites similar to MADD (see Table 2.1).
3. **Disorders of FAD or riboflavin metabolism** collectively refer to MADD and MADD-like disorders.
4. **Southern Africa** refers to countries located in Southern Africa (including SA and Namibia).
5. **SA (South Africa)** refers to the country, South Africa.
6. **Mixed ethnicity** refers to SA individuals of mixed ethnic (including African, Chinese, Khoisan, Malay, and White) origin.

metabolism have also been shown to produce MADD-like phenotypes. These proteins include riboflavin transporter 1−3 (RVFT1−3, encoded by *SLC52A1−3*, respectively), FAD synthase (FADS, encoded by *FLAD1*), and mitochondrial folate/FAD transporter (MFT, encoded by *SLC25A32*) and bring about RFVT1−3, FADS and MFT deficiency, respectively; collectively called MADD-like disorders (Bosch *et al.*, 2012; Green *et al.*, 2010; Haack *et al.*, 2012; Ho *et al.*, 2011; Johnson *et al.*, 2010; Johnson *et al.*, 2012; Mosegaard *et al.*, 2020; Olsen *et al.*, 2016; Schiff *et al.*, 2016; Taylor *et al.*, 2014). A diagnosis of MADD(-like disorders) is typically made by broad-spectrum metabolic testing/genetic screening, followed by genetic confirmation. The latter also enables the identification of the associated phenotype, which facilitates optimal therapy. Notably, MADD(-like disorders) is amenable to treatment, with the exception of only three fatal phenotypes (MADD type I, selected cases of MADD type II, and severe FADS deficiency).

In understudied populations, such as Southern Africa, MADD(-like disorders) remains under-recognised and under-diagnosed. While contributing factors include Southern Africa's socio-economic obstacles and high burden of communicable and non-communicable disease, it is the limited expertise in the required, specialised clinical and diagnostic fields due to the paucity of data which constitutes the largest limitation (Raga *et al.*, 2022). By contrast to most well-studied countries, Southern Africa does not provide a compulsory newborn screening (NBS) programme (Conradie *et al.*, 2021). Instead, IEMs are diagnosed on an elective basis. This service is currently offered by only two facilities in South Africa (SA): (i) the Potchefstroom Laboratory for Inborn Errors of Metabolism [PLIEM; Centre for Human Metabolomics (CHM), North-West University (NWU), Potchefstroom] and (ii) the National Health Laboratory Service (NHLS) Inherited Metabolic Disease (IMD) molecular laboratory (University of Cape Town; UCT). Here, diagnoses are made by means of comprehensive metabolic analyses. However, genetic screening/confirmation is much less common, due to the absence of population-specific genetic information. Although metabolic testing is effective, it may not detect diagnostic markers if conducted on samples collected during the anabolic state. Consequently, without a genetic component to aid diagnosis, many cases of MADD(-like disorders) may be overlooked or misdiagnosed. Indeed, a study by Waters *et al.* (2018), has estimated a case fatality rate of 33% in all IEM occurring in understudied countries, versus a rate of 2−23% reported in well-studied countries.

Since variants that cause rare disease, including MADD(-like disorders), tend to be population-specific (Bustamante *et al.*, 2011), a comprehensive description of a disease cohort is required to identify the pathogenic variants leading to MADD in Southern Africa. The diagnosis of MADD further hinges on knowledge regarding its clinical presentation—an aspect that has not yet been widely explored in Southern Africa. As the clinical symptoms associated with MADD(-like disorders) are often heterogeneous, the lack of phenotype data contributes to the already challenging clinical diagnosis of the disorder. Collectively, these factors increase the morbidity and mortality of MADD(-like disorders) in Southern Africa by delaying

its diagnosis and comprehensive treatment, including counselling. This is especially problematic for neonatal/infant cases, for whom early diagnosis and treatment is essential for survival. In addition, this poses a problem for late-onset cases of MADD(-like disorders), in which apt treatment would vastly improve quality of life.

Previously, a study by Chaya *et al.* (2018) reported on the first case of RFVT3 deficiency in Southern Africa in a patient of White ethnicity (*SLC52A3* variants c.[639C>G], p.[Tyr213Ter] and c.[374C>A], p.[Thr125Asn]). Shortly thereafter, our group identified one novel (c.[1067G>A], p.[Gly356Glu]) and one known (c.[1448C>T], p.[Pro483Leu]) *ETFDH* variant in three patients of White SA ethnicity (van der Westhuizen *et al.*, 2018). Based on these early case reports, as well as anecdotal observations, it was suggested that in Southern Africa, the prevalence of MADD may be in the same order as that of a more prevalent group of IEMs called organic acidaemias. Moreover, it was proposed that the occurrence of MADD may be more prevalent in the White SA population, and that the *ETFDH* c.[1067G>A] variant could be the result of a founder effect.

To investigate these findings and to address the problems described in the diverse populations of Southern Africa, this study was initiated under the auspices of the larger International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD) study (Wilson *et al.*, 2023). The ICGNMD, headed by the University College London (UCL), Cambridge University and Newcastle University (United Kingdom; UK), comprises a five-year project (initiated on 1 June 2019) focused on establishing a transcontinental genomics research and capacity-building partnership concerning neuromuscular disorders [NMDs; which include MADD(-like disorders)]. The overall aim of this larger project is to elucidate the genetic aetiology of NMDs in five understudied countries (SA, Zambia, Brazil, India, and Turkey), with the purpose of better understanding their disease mechanisms and improving the countries' genetic diagnostic capacities. In SA, collaborators of the ICGNMD study, hereafter called 'centres', include four participating universities and four corresponding academic state (i.e., public) hospitals: NWU (Potchefstroom), University of Pretoria (UP; Steve Biko Academic Hospital, Pretoria), UCT (Red Cross War Memorial Children's Hospital and Groote Schuur Hospital, Cape Town), and Stellenbosch University (SU; Tygerberg Hospital, Stellenbosch). Furthermore, each centre has at least one collaborating clinical paediatric or adult neurologist.

Considering the limitations discussed above, an extensive investigation is required to elucidate not only the clinical phenotypes and molecular genotypes associated with MADD(-like disorders) in Southern Africa, but also the metabolic profile prior to and following treatment. In addition, any potential pathogenic variants identified will need to be assessed to determine their pathogenicity, prevalence, and the possibility of founder effects. Functional assays of patient fibroblasts have also been shown to yield valuable insights into the mechanisms involved in MADD (van Rijt *et al.*, 2019). This thesis reports on the outcome of a study design following this approach, with the purpose of characterising MADD in the understudied populations of Southern Africa.

1.2 AIMS AND OBJECTIVES

1.2.1 Aims

This study has two related aims. The first was to characterise the clinical and metabolic properties, as well as the molecular genetics, of a Southern African cohort diagnosed clinically and biochemically with MADD. The second aim was to evaluate the functional properties of selected pathogenic variants frequently identified in the cohort *in vitro*.

1.2.2 Objectives

To achieve these aims, the following Objectives were formulated:

The Objectives pertaining to Aim 1 were to:

1.1. Select and recruit a cohort of Southern African patients that had been diagnosed with MADD at four diagnostic centres in SA.
1.2. Collect relevant pre-existing or new samples, and generate clinical and biochemical (metabolic) data of the study cohort.
1.3. Investigate genes known to be affected in MADD, using whole exome sequencing (WES).
1.4. Identify and confirm existing and/or novel putative pathogenic genetic variants from the study cohort and compare the results to the data obtained in Objective 1.2.
1.5. Determine the allele frequency and haplotype of selected prevalent variants in the four largest population groups of SA.

The Objectives pertaining to Aim 2 were to:

2.1. Acquire primary fibroblasts from patient (where available) and control skin biopsies.
2.2. Characterise selected relevant molecular properties of these *in vitro* models, including the:
    a. Steady state protein level of the affected enzyme(s).
    b. Steady state protein level of mitochondrial respiratory chain (RC) enzymes, complex I–V (CI–CV).
2.3. Investigate the oxidation of relevant substrates in permeabilised primary fibroblasts.
2.4. Propose a list of pathogenic variants and their clinical and biochemical characteristics in the Southern African population that can be translated to national diagnostic services.

1.3 STRUCTURE OF THESIS

This thesis is presented in chapter format and adheres to the conditions of the NWU for the completion of the degree *Doctor of Philosophy in Science with Biochemistry*. The thesis consists of five chapters, which are presented as a hybrid of the traditional thesis and journal publication-based formats. It should be noted that published results are presented with minor alterations[1] to better fit the format of the thesis.

Chapter 1: Introduction

The background and rationale of the study are presented, together with the problem statement. Subsequently, the aims and objectives are laid out, along with the structure of the thesis. The chapter concludes with the study's research outputs, and the contributions and signed declarations of the author and co-authors.

Chapter 2: Literature review

This chapter comprises the first manuscript resulting from the thesis, in which a literature review is given on acyl-CoA dehydrogenases, the ETF−ETFQO system, and the associated inherited error of metabolism, MADD. The metabolic pathways affected, clinical and genetic features, diagnostic approaches, treatment options, and prognosis of the disorder are examined. The chapter concludes with additions to the manuscript, including the current limitations of the diagnosis of MADD and its genetic aetiology in Southern Africa.

Chapter 3: Clinical, biochemical, and genetic spectrum of MADD in a Southern African cohort: an ICGNMD study

This chapter includes the second manuscript resulting from the thesis, in which the methods and results pertaining to Aim 1 (Objectives 1.1–1.5) are given, followed by a critical discussion, short conclusion, and additions to the allele frequency assays. The chapter provides information on the recruitment and sampling, clinical phenotypes, metabolic findings, and genotypes of the Southern African MADD cohort, along with the haplotype data and allele frequencies of the most frequently encountered pathogenic variants in Southern Africa.

[1] This includes changes to the table and figure numbering and referencing style, the omission of published abstracts, keywords and declarations, literature updates since the date of publication, as well as some stylistic changes.

Chapter 4: Functional evaluation of Functional evaluation of MADD in patient fibroblasts

In this chapter, the methods and results of Aim 2 (Objectives: 2.1–2.4) are presented, followed by a critical discussion and short conclusion. This chapter comprises the acquirement of patient fibroblasts, the evaluation of steady state protein levels (ETFQO and mitochondrial RC CI–CV), as well as the effect of the identified pathogenic variants on respiration.

Chapter 5: Summary and conclusions

The final chapter provides a summary of the findings (explained in relation to the rationale, aims and objectives), followed by the strengths, limitations, and future prospects of the study.

References

All references (including those of Chapters 2 and 3) are listed at the back of the thesis, in the NWU–Harvard style. The reference style used in Appendix A is as requested by the publishing journal.

Supplementary information:

Additional information/data and research outputs are given in:

- Appendix A: Peer-reviewed article i and Supplementary Material: Chapter 2
- Appendix B: Peer-reviewed article ii and Supplementary Material: Chapter 3
- Appendix C: Additional methods and results from Chapter 3
- Appendix D: Language certificates

1.4 RESEARCH OUTPUTS

1.4.1 Published peer-reviewed articles

- Mereis, M., Wanders, R.J.A., Schoonen, M., Dercksen, M., Smuts, I. & van der Westhuizen, F.H. 2021. Disorders of flavin adenine dinucleotide metabolism: MADD and related deficiencies. *Int J Biochem Cell Biol*, 132:105899.

The first article (doi: 10.1016/j.biocel.2020.105899) is discussed in Chapter 2 and was published as an invited review in the International Journal of Biochemistry and Cell Biology (https://www.sciencedirect.com/journal/the-international-journal-of-biochemistry-and-cell-biology), with a five-year impact factor of 4.5 (2022). The scope of the journal focuses on original research articles, in-focus articles, and invited reviews in all areas of biomedical, and cell and molecular biology research.

Author guidelines may be accessed at: https://www.elsevier.com/journals/the-international-journal-of-biochemistry-and-cell-biology/1357-2725/guide-for-authors. The published manuscript may be found in Appendix A.

- Bisschoff, M., Smuts, I., Dercksen, M., Schoonen, M., Vorster, B.C., van der Watt, G., Spencer, C., Naidu, K., Henning, F., Meldau, S., McFarland, R., Taylor, R.W., Patel, K., Fassad, M.R., Vandrovkova, J., The ICGNMD Consortium, Wanders, R.J.A. & van der Westhuizen, F.H. 2024. Clinical, biochemical, and genetic spectrum of MADD in a South African cohort: an ICGNMD study. *Orphanet J Rare Dis*, 19(1):15.

The second article (doi: 10.1186/s13023-023-03014-8) is discussed in Chapter 3 and was published in the Orphanet Journal of Rare Diseases (https://ojrd.biomedcentral.com/), with a five-year impact factor of 4.4 (2022). The journal publishes high-quality reviews of specific rare diseases, reports of clinical trial outcomes as well as articles regarding public health issues on the subject of rare diseases and orphan drugs. Author guidelines may be accessed at: https://ojrd.biomedcentral.com/submission-guidelines/preparing-your-manuscript/research. Supplementary Material of the published manuscript may be found in Appendix B.

Other peer-reviewed publications from the author on the data reported in this thesis, include:

- Wilson, L.A., Macken, W.L., Perry, L.D., Record, C.J., Schon, K.R., Frezatti, R.S.S., Raga, S., Naidu, K., Köken, Ö.Y., Polat, I., Kapapa, M.M., Dominik, N., Efthymiou, S., Morsy, H., Nel, M., Fassad, M.R., Gao, F., Patel, K., Schoonen, M., Bisschoff, M., Vorster, A., Jonvik, H., Human, R., Lubbe, E., Nonyane, M., Vengalil, S., Nashi, S., Srivastava, K., Lemmers, R.J.L.F., Reyaz, A., Mishra, R., Töpf, A., Trainor, C.I., Steyn, E.C., Mahungu, A.C., van der Vliet, P.J., Ceylan, A.C., Hiz, A.S., Çavdarlı, B., Semerci Gündüz, C.N., Ceylan, G.G., Nagappa, M., Tallapaka, K.B., Govindaraj, P., van der Maarel, S.M., Narayanappa, G., Nandeesh, B.N., Wa Somwe, S., Bearden, D.R., Kvalsund, M.P., Ramdharry, G.M., Oktay, Y., Yiş, U., Topaloğlu, H., Sarkozy, A., Bugiardini, E., Henning, F., Wilmshurst, J.M., Heckmann, J.M., McFarland, R., Taylor, R.W., Smuts, I., van der Westhuizen, F.H., Sobreira, C.F., Tomaselli, P.J., Marques, W.J., Bhatia, R., Dalal, A., Srivastava, M.V.P., Yareeda, S., Nalini, A., Vishnu, V.Y., Thangaraj, K., Straub, V., Horvath, R., Chinnery, P.F., Pitceathly, R.D.S., Muntoni, F., Houlden, H., Vandrovkova, J., Reilly, M.M. & Hanna, M.G. 2023. Neuromuscular disease genetics in under-represented populations: increasing data diversity. *Brain*: awad254.

This collaborative article (doi: 10.1093/brain/awad254) was published in Brain (https://academic.oup.com/brain), with a five-year impact factor (2022) of 14.6, and gives a broad oversight of the genotype–phenotype findings of the larger, transcontinental ICGNMD study, including those reported in this thesis. The scope of the journal is broad and ranges from studies that elucidate disease

mechanisms, to those of novel clinical trials for brain disorders. Author guidelines may be accessed at: https://academic.oup.com/brain/pages/General_Instructions. The published manuscript is not given or discussed in this thesis.

1.4.2 Conference outputs and awards

Poster presentations and awards on the data reported in this thesis, include:

- Bisschoff, M., Smuts, I., Dercksen, M., Schoonen, M., Vorster, B.C., Naidu, K., Spencer, C., van der Watt, G., Henning, F., Patel, K., Fassad, M.R., Vandrovkova, J., Taylor, R.W., McFarland, R., The ICGNMD Consortium, Wanders, R.J.A. & van der Westhuizen, F.H. 2023, 27-28 August. *MADD in Southern Africa: a clinical, biochemical, and genetic spectrum.* Poster presented at the International Network for Fatty Acid Oxidation Research and Management (INFORM) 2023 Meeting, Jerusalem, Israel.
- INFORM 2023 Best Junior Researcher Poster Award.

Conference proceedings on the data reported in this thesis, include:

- Mereis, M., Dercksen, M., van der Westhuizen, F.H., Schoonen, M. & Vorster, B.C. 2019. Multiple acyl-CoA dehydrogenase deficiency: clinical and biochemical heterogeneity in the Caucasian South African population. *In*: Baumgartner, M., Patterson, M., Rahman, S. & Zschocke, J., *eds. J Inherit Metab Dis*. Annual Society for the Study of Inborn Errors of Metabolism (SSIEM) Symposium, Rotterdam, the Netherlands. New Jersey: Wiley-Blackwell. p 166.
- Bisschoff, M., Smuts, I., Schoonen, M., Dercksen, M., Vorster, B.C., van der Watt, G., Jassen, A., Naidu, K., Henning, F., McFarland, R., Patel, K., Vandrovkova, J., Morsy, H., The ICGNMD Consortium, Wanders, R.J.A & van der Westhuizen, F.H. 2023. The clinical, genetic, and biochemical spectrum of MADD in a Southern African cohort: an ICGNMD sub-study. *In*: Pitceathly, R.D.S., *ed. J Neuromuscul Dis.* Sixteenth UK Neuromuscular Translational Research Conference, London, United Kingdom. Amsterdam: IOS Press. p S86.
- Bisschoff, M., Smuts, I., Dercksen, M., Schoonen, M., Vorster, B.C., Naidu, K., Spencer, C., van der Watt, G., Henning, F., Patel, K., Fassad, M.R., Vandrovkova, J., Taylor, R.W., McFarland, R., The ICGNMD Consortium, Wanders, R.J.A. & van der Westhuizen, F.H. 2023. A Southern African MADD cohort: clinical, biochemical, and genetic spectrum. *In*: Spiekerkoetter, U., *ed*. *J Inherit Metab Dis*. Annual Society for the Study of Inborn Errors of Metabolism (SSIEM) Symposium, Jerusalem, Israel. New Jersey: Wiley-Blackwell. p 243.

Other conference proceedings from the author on the data reported in this thesis, include:

- Fassad, M.R., Patel, K., Schoonen, M., van der Westhuizen, F.H., Bisschoff, M., Human, R., Lubbe, E., Nonyane, M., Vorster, A., The ICGNMD Consortium, Vandrovkova, J., Smuts, I., Taylor, R.W. & McFarland, R. 2023. Virtual Panel versus exome-first analysis in neuromuscular diseases: outcome of a single centre within ICGNMD consortium. *In*: Pitceathly, R.D.S., *ed. J Neuromuscul Dis*. Sixteenth UK Neuromuscular Translational Research Conference, London, United Kingdom. Amsterdam: IOS Press. p S91.

1.5 AUTHOR AND CO-AUTHOR CONTRIBUTIONS

The following section lists the contributions of the author and various co-authors to the presented body of work. Contributions regarding the obtaining of ethics approval and patient recruitment may be found in Appendix C.1 and C.2, respectively.

1.5.1 Peer-reviewed article i presented in Chapter 2

M. Bisschoff (née Mereis) and F.H van der Westhuizen were responsible for the conceptualisation and study design. Supervision was provided by F.H van der Westhuizen, R.J.A. Wanders, I. Smuts, and M. Dercksen. The literature review and manuscript writing were performed by M. Bisschoff, with assistance by F.H. van der Westhuizen. Data acquisition and analysis comprised a systematic review of all reported MADD-related pathogenic variants, and was conducted by M. Bisschoff, with assistance by F.H. van der Westhuizen. Variant Combined Annotation Dependent Depletion (CADD) scores, reference single nucleotide polymorphism cluster identifications (rsIDs), and genetic loci were obtained by M. Schoonen. Intellectual input regarding the lesser-known metabolic alterations, differential diagnosis, and clinical aspects of MADD, were provided by R.J.A. Wanders, M. Dercksen, and I. Smuts, respectively. Data interpretation was done by M. Bisschoff. All authors were involved in the review, editing, and final approval of the manuscript.

1.5.2 Peer-reviewed article ii presented in Chapter 3

M. Bisschoff, F.H. van der Westhuizen, and I. Smuts were involved in the conceptualisation and study design. Supervision was provided by F.H van der Westhuizen, R.J.A. Wanders, I. Smuts, and M. Dercksen. Clinical evaluation of, and sample collection from, the study cohort were performed by I. Smuts (Steve

Biko Academic Hospital, UP), G. van der Watt and C. Spencer (Red Cross War Memorial Children's Hospital, UCT), and F. Henning and K. Naidu (Tygerberg Hospital, SU). Where necessary, additional clinical records and pre-existing samples were provided by the CHM and UCT. Dried blood spot (DBS) and deoxyribonucleic acid (DNA) samples of healthy controls were provided by the CHM and UCT. Sample preparation for genetic and protein, and metabolic analyses was conducted by M. Bisschoff and the CHM, respectively. Data generation and analysis were performed by M. Bisschoff (DNA isolation, Sanger validation, and allele frequency assays), M. Schoonen and K. Patel (WES Bioinformatics), the CHM and UCT (metabolic profiling), J. Vandrovkova (haplotyping), and S. Meldau (allele frequency assays). M. Bisschoff, M. Schoonen, K. Patel, M. Dercksen, and J. Vandrovkova were responsible for data interpretation. I. Smuts provided intellectual input regarding the clinical presentation of the cohort and M. Bisschoff, M. Schoonen, M. Dercksen, J. Vandrovkova, and F.H. van der Westhuizen were involved in manuscript writing. All authors contributed to the review, editing, and final approval of the manuscript.

1.5.3 Data presented in Chapter 4

Cell culturing and the generation and interpretation of the steady state protein levels and respiration data were performed by M. Bisschoff.

1.5.4 Figures and Tables

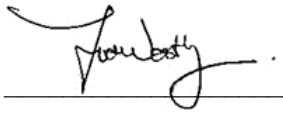
Figures 3.1 and 3.2 presented in Chapter 3 were generated by J. Vandrovkova. The visualisation of all other data depicted in this thesis was done by M. Bisschoff.

1.5.5 Declaration by co-authors

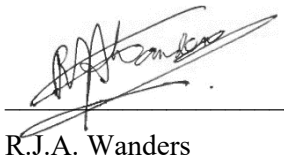
All authors involved have signed the declaration below:

“As co-author/researcher, I hereby approve and give consent that the mentioned articles and data can be used for the PhD of M. Bisschoff. I declare that my role in the study, as indicated above, is a representation of my actual contribution.”

M. Bisschoff



F.H. van der Westhuizen



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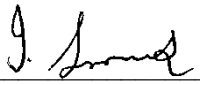

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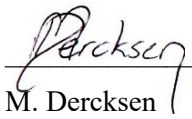

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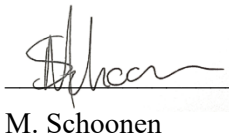

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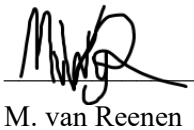

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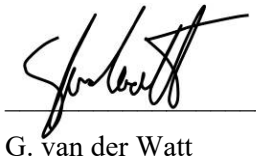

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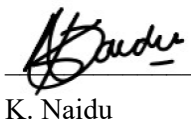
C. Spencer

F. Henning



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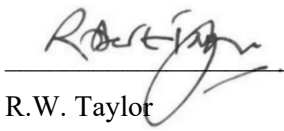

R.W. Taylor

K. Patel

K. Patel

J. Vandrovkova

Chapter 2

Literature review:

Disorders of flavin adenine dinucleotide metabolism: MADD and related deficiencies

Mereis, M., Wanders, R.J.A., Schoonen, M., Dercksen, M., Smuts, I. & van der Westhuizen, F.H. 2021. Int J Biochem Cell Biol, 132:105899.

2.1 CHAPTER INTRODUCTION

This chapter provides an overview of the fundamental concepts addressed in this thesis and comprises the first journal publication originating from this study[2] [see Appendix A for the journal publication and Supplementary Material 1 (Tables A.1 and A.2)], with relevant literature updates[3] (this publication was printed in 2021). An abstract and bulleted list of highlights of the publication are given, followed by an introduction to acyl-CoA dehydrogenases and their relation to the ETF–ETFQO system. The focus then shifts to the main study topic, namely the inherited metabolic disorder, MADD. Key aspects relating to MADD and other disorders of FAD/riboflavin metabolism (MADD-like disorders) are examined, including the metabolic pathways involved, clinical and genetic features, diagnostic approaches, therapeutic intervention, and disorder prognosis. The chapter concludes with a second addition to the manuscript (Section 2.10), addressing the diagnosis and research of IEMs and MADD in the Southern African context.

2.2 ABSTRACT

Multiple acyl-coenzyme A dehydrogenase deficiency (MADD), or glutaric aciduria type II (GAII), is a group of clinically heterogeneous disorders caused by mutations in electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO)—the two enzymes responsible for the re-oxidation of enzyme-bound flavin adenine dinucleotide ($FADH_2$) via electron transfer to the respiratory chain at the level of coenzyme Q10. Over the past decade, an increasing body of evidence has further coupled mutations in FAD metabolism (including intercellular riboflavin transport, FAD biosynthesis and FAD transport) to MADD-like phenotypes. In this review we provide a detailed description of the overarching and specific metabolic pathways involved in MADD. We examine the eight associated genes (*ETFA, ETFB, ETFDH, FLAD1, SLC25A32* and *SLC52A1–3*) and clinical phenotypes, and report 740[4] causative mutations following a systematic literature review. Finally, we focus attention on the value and shortcomings of current diagnostic approaches, as well as current and future therapeutic options for MADD and its phenotypic disorders.

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[2] This journal publication has been included in this thesis with permission from the copyright holder, Elsevier.

[3] This includes an updated list (up until October 2023) of suspected and identified genetic variants that cause MADD(-like disorders) (Appendix A).

[4] Throughout this chapter, the number of MADD-related variants have been updated (up until October 2023). See Appendix A.2 and A.3.

2.3 FAD METABOLISM AND MADD FACTS

- Flavoproteins are intricately involved in the mitochondrial metabolism and require oxidised FAD as cofactor.
- The re-oxidation of $FADH_2$ is mediated by the ETF–ETFQO system, while the synthesis and transport of FAD depends on the combined action of RFVT1–3, FADS and MFT.
- Genetic defects in *ETFA*, *ETFB* and *ETFDH* lead to a potentially fatal mitochondrial myopathy called multiple acyl-CoA dehydrogenase deficiency (MADD).
- Disorders associated with mutations in *FLAD1*, *SLC25A32* and *SLC52A1–3* also frequently present with MADD-like phenotypes and -biochemistry.
- The effective diagnosis of MADD, resulting from ~740 mutations, is challenging and involves a combination of biochemical investigations, enzyme/flux assays and next generation sequencing.
- Apart from MADD types I and II and severe FADS dysfunction, the majority of MADD(-like) phenotypes are very amendable to treatment by riboflavin.

2.4 INTRODUCTION

Acyl-coenzyme A (CoA) dehydrogenases (ACDHs), a family of homologous mitochondrial flavoproteins that contain flavin adenine dinucleotide (FAD) as a non-covalently-bound prosthetic group, are key players in mitochondrial metabolism (Battaile *et al.*, 2002; Henriques *et al.*, 2010). Of the 11 ACDHs currently identified, a total of nine partake in: (i) mitochondrial fatty acid β-oxidation (FAO), (ii) branched chain amino acid (BCAA) catabolism and (iii) amino acid catabolism (Ghisla & Thorpe, 2004; Zhang *et al.*, 2019). These include: (i) acyl-CoA dehydrogenase 9 (ACAD9), short-, medium-, long-, and very-long-chain acyl-CoA dehydrogenase (SCAD, MCAD, LCAD and VLCAD), (ii) isovaleryl-CoA-, isobutyryl-CoA-, and short branched-chain acyl-CoA dehydrogenase (IVD, IBD and SBCAD) and (iii) glutaryl-CoA dehydrogenase (GCDH). To enable the sustained operation of these enzymes, their reduced FAD groups require continuous re-oxidation by the concerted action of electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO) (Ghisla & Thorpe, 2004). In addition, three other mitochondrial dehydrogenases rely on ETF and ETFQO as redox partners, including 2-hydroxyglutarate- (2HGDH), dimethylglycine- (DMGDH; involved in amino acid metabolism), and sarcosine dehydrogenase (SDH; involved in choline metabolism), while FAD is also required for the function of hydroxyproline- (HYPDH) and proline dehydrogenase (PRODH) (Ostrander *et al.*, 2009; Summitt *et al.*, 2015; Toplak *et al.*, 2019; Zhang *et al.*, 2019).

The importance of the ETF–ETFQO system is exemplified by the existence of a variety of different inborn errors of metabolism (IEMs), collectively called multiple acyl-CoA dehydrogenase deficiency (MADD) (Goodman & Frerman, 1984; Grünert, 2014). MADD, also known as glutaric aciduria type II (GAII), may

be classified as a combined FAO- and protein metabolism disorder resulting in lipid storage myopathy and is historically caused by mutations in the genes coding for ETF and ETFQO (Vasiljevski *et al.*, 2018; Wanders *et al.*, 2010). More recently, mutations in the genes coding for enzymes involved in the metabolism of riboflavin—the precursor of FAD—have also been shown to present as MADD-like phenotypes (Bosch *et al.*, 2012; Green *et al.*, 2010; Haack *et al.*, 2012; Ho *et al.*, 2011; Johnson *et al.*, 2010; Johnson *et al.*, 2012; Mosegaard *et al.*, 2020; Olsen *et al.*, 2016; Schiff *et al.*, 2016; Taylor *et al.*, 2014). In this review, we present the current state of knowledge on the metabolic pathways which are affected in MADD and the genes involved in the disorders of FAD metabolism from the perspective of both the ETF–ETFQO system as well as riboflavin transport and metabolism. We provide an overview of the phenotypes and diagnostic procedures used to identify these disorders and discuss available and future therapeutic avenues.

2.5 PATHWAYS

In its fully oxidised state, FAD functions by accepting hydrogen ions and electrons from the substrates of mitochondrial flavoproteins. In order to maintain mitochondrial flavoproteome homeostasis, the resulting $FADH_2$ needs to be re-oxidised by the action of ETF, which is a heterodimer comprised of a 30 kDa ETFα and 28 kDa ETFβ subunit located in the mitochondrial matrix (Frerman & Goodman, 2001; Ruzicka & Beinert, 1975). This protein in turn, is re-oxidised by ETFQO, a 64 kDa monomer embedded in the inner mitochondrial membrane, which feeds the transferred electrons into the respiratory chain at the level of coenzyme Q10 (CoQ10) (Frerman, 1987). Together, ETF and ETFQO couple various metabolic pathways to the oxidative phosphorylation (OXPHOS) system for the production of adenosine triphosphate (ATP) (Figure 2.1).

In humans, FAD and its precursor flavin mononucleotide (FMN) are produced from riboflavin, a water-soluble vitamin (B2) which itself cannot be synthesised in humans, but instead is obtained from dietary sources and in part from intestinal microflora (Figure 2.1) (Barile *et al.*, 2016). From the small (and less so, the large) intestine, free riboflavin is transported into the enterocyte via the action of riboflavin transporter (RFVT) 3, and released into the blood by RFVT1 and 2. From here, riboflavin (bound to albumin/immunoglobulin) can be transferred to various tissues and transported into cells by RFVT1−3, which are expressed in a tissue-specific manner (see Barile *et al.*, 2016 for review). Once inside the cytoplasm, riboflavin is phosphorylated to FMN by riboflavin kinase (RFK), and subsequently adenylated to FAD by FAD synthase (FADS) (Brizio *et al.*, 2006; Henriques *et al.*, 2010; Leone *et al.*, 2018). A similar process takes place in the mitochondrion following the import of riboflavin. The enzymatically active cofactor (FAD) can then be transported to and from the mitochondrion via the mitochondrial folate/FAD transporter (MFT), and incorporated into apo-flavoproteins.

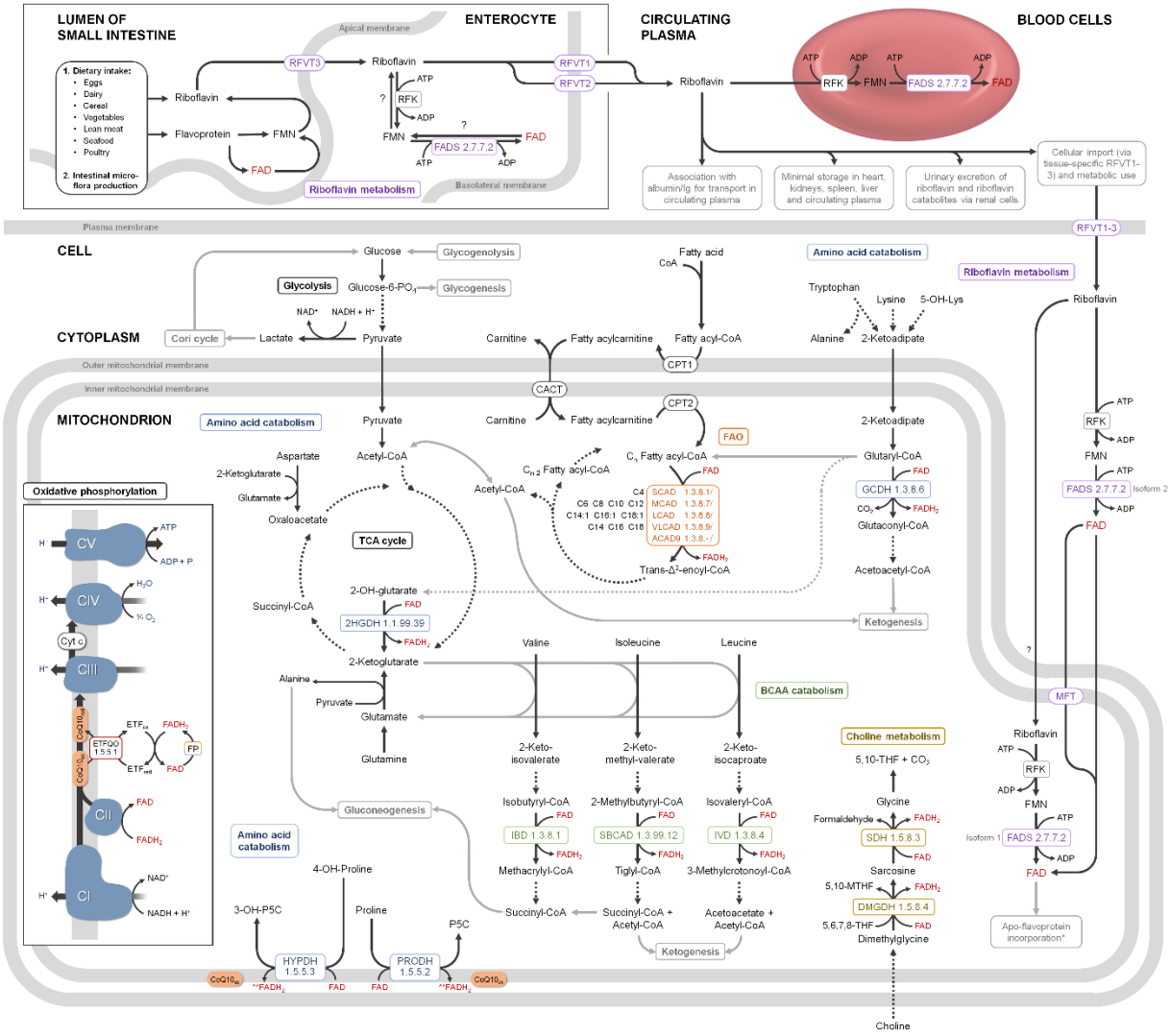

Figure 2.1: Riboflavin metabolism and flavoproteins affected in MADD(-like disorders)

In the small intestine, flavoproteins are denatured, thereby releasing FMN and FAD, which can be converted into riboflavin via non-specific hydrolases. Riboflavin is then absorbed into the blood and subsequently taken up by different blood cells (including erythrocytes and leukocytes) or bound to albumin/immunoglobulins to ensure transport through the circulation and tissue-uptake via cell-type specific RFVTs for cellular use or urinary excretion. Once inside the cell, riboflavin can be converted to the different enzymatically active cofactors (FMN and FAD) within the cytoplasm and mitochondrion. A deficiency of FAD, caused by a genetic defect in ETF, ETFQO, RFVT1–3, FADS or MFT may affect at least 14 flavoproteins, resulting in the accumulation of their associated metabolites. Dashed lines indicate omitted metabolic steps. Question marks refer to presently undefined hydrolases and mitochondrial import-export mechanisms. * Hypothesised to occur in the mitochondrion and cytoplasm via protein-protein interactions between FADS and apo-flavoproteins; ** Hypothesised to donate electrons directly to $CoQ10_{ox}$. Abbreviations: 2HGDH: 2-hydroxyglutarate dehydrogenase; ACAD9: acyl-CoA dehydrogenase 9; ADP and ATP: adenosine diphosphate and -triphosphate (respectively); CACT: carnitine-acylcarnitine translocase; CI–CV: mitochondrial respiratory chain complexes I–V; CoA: coenzyme A; CPT1 and CPT2: carnitine palmitoyltransferase 1 and 2; CoQ10: coenzyme Q10; Cyt c: cytochrome c; DMGDH: dimethylglycine dehydrogenase; ETF: electron

transfer flavoprotein; ETFQO: ETF-ubiquinone oxidoreductase; FAD and $FADH_2$: flavin adenine dinucleotide (oxidised and reduced, respectively); FADS: FAD synthase; FAO: mitochondrial fatty acid β-oxidation; FMN: flavin mononucleotide; FP: flavoprotein; GCDH: glutaryl-CoA dehydrogenase; HYPDH: hydroxyproline dehydrogenase; IBD: isobutyryl-CoA dehydrogenase; Ig: immunoglobulin; IVD: isovaleryl-CoA dehydrogenase; LCAD: long-chain acyl-CoA dehydrogenase; MCAD: medium-chain acyl-CoA dehydrogenase; MFT: mitochondrial FAD transporter; MTHF: methyl-tetrahydrofolate; NAD^+ and NADH: nicotinamide adenine dinucleotide (oxidised and reduced, respectively); OH: hydroxy; $_{ox}$: oxidised; P5C: Δ^1-pyrroline-5-carboxylate; P_i: inorganic phosphate; PO_4: phosphate; PRODH: proline dehydrogenase; $_{red}$: reduced; RFK: riboflavin kinase; RFVT1−3: riboflavin transporter 1−3; SBCAD: short branched-chain acyl-CoA dehydrogenase; SCAD: short-chain acyl-CoA dehydrogenase; SDH: sarcosine dehydrogenase; TCA: tricarboxylic acid; THF: tetrahydrofolate; VLCAD: very-long-chain acyl-CoA dehydrogenase.

Moreover, MFT is involved in the mitochondrial export of FAD for the maintenance of flavin homeostasis and FAD-dependant cell signalling processes (Giancaspero *et al.*, 2015; Vergani *et al.*, 1999). Since humans have a very low storage capacity for riboflavin, any surplus will be excreted in urine, a factor which contributes to riboflavin's low toxicity, even at pharmacological doses.

Due to the central catalytic function of FAD in ETF, ETFQO, RFVT1−3, FADS and MFT, as well as its role in CoQ10 reduction, at least 14 flavoproteins (nine ACDHs, 2HGDH, DMGDH, SDH, HYPDH and PRODH), distributed across a multitude of interconnected secondary metabolic pathways, may be affected in MADD(-like disorders) (Frerman & Goodman, 2001; Henriques *et al.*, 2010). Under normal physiological conditions, ATP is produced via glycolysis and FAO, of which the latter is the main energy pathway in the heart, skeletal muscle and kidney. However, in MADD the ACDH-catalysed reactions within the FAO and BCAA catabolic pathways are deficient and metabolism shifts to glycolysis, followed by glycogenolysis in order to cope with the energy demand. Accordingly, pyruvate (and subsequently lactate) increases, while acetyl-CoA production declines and various fatty acyl-CoA species accumulate, resulting in the secondary accumulation of the corresponding acylglycine and -carnitine conjugates. The decrease in acetyl-CoA limits hepatic ketogenesis and results in the proteolytic degradation of muscle tissue to supply glucogenic and ketogenic substrates for energy production. The decreased availability of FAD further reduces the catabolism of BCAAs and other amino acids (lysine, 5-hydroxylysine, tryptophan, alanine, glutamate, aspartate, sarcosine, proline and 4-hydroxyproline) in the muscle, leading to the accumulation of their related intermediates and to a decrease in the ketogenic substrate, acetoacetate. While MADD generally presents without ketonuria, it should be noted that exceptions have been reported, owing to variants that result in adequate residual levels of the affected ACDHs in FAO and BCAA catabolism (Cairns & Connell., 2018; Chandra *et al.*, 2018). MADD caused by mutations in *ETFDH* may also be associated with secondary CoQ10 deficiency, which, together with the metabolic stress, will lead to a decreased activity of the respiratory chain complexes (Gempel *et al.*, 2007). Consequently, depending on

the severity of the genetic variant, MADD may give rise to varying degrees of disturbed energy homeostasis, as well as the accumulation of toxic metabolites and cellular starvation.

2.6 CLINICAL AND GENETIC FEATURES

Deficiencies of those flavoproteins that rely on ETF and ETFQO and their cofactor (FAD and thus riboflavin) lead to a number of disease phenotypes that are generally recognised as metabolic myopathies (Gaspar *et al.*, 2019). These disorders have overlapping clinical features and are biochemically characterised by a set of metabolite abnormalities typical for MADD, as described in more detail in the diagnosis section. The deficiencies and resulting phenotypes associated with the genes involved in this metabolism are summarised in Table 2.1. Table A.1 provides a list of 590 published pathogenic genetic variants (mutations) that have been associated with these phenotypes, as well as 150 additional potential pathogenic variants from the Ensembl Genome Browser (https://www.ensembl.org/). Table A.2 provides an overview of the distribution of the variants in the gene structures. It should be noted that most of these 740 variants where not scrutinised using current classification guidelines to determine pathogenicity (Richards *et al.*, 2015).

2.6.1 Multiple acyl-CoA dehydrogenase deficiency (MADD/GAII)

The first case of MADD/GAII was described over 40 years ago (Przyrembel *et al.*, 1976). The disorder presents with a heterogeneous and multi-system phenotype which may be divided into three clinical classes including types I and II—two ultimately fatal, neonatal-onset subtypes presenting with or without congenital features [MADD-severe (S)], and type III—a mild and/or later-onset form [MADD-mild (M)]. As the disease severity correlates well with MADD's response to high-dose riboflavin treatment, it has been suggested to classify these phenotypes as riboflavin-unresponsive (RU-MADD) or -responsive (RR-MADD) (Yıldız *et al.*, 2019).

Together, MADD has a general prevalence of 1 to 9:1,000,000 and an estimated birth prevalence of 1:200,000 (https://www.orpha.net/; ORPHAcode:26791), with wide variation reported between different countries and ethnicities. Historically, MADD is caused by autosomal recessive mutations in the *ETFA* (47), *ETFB* (29) and *ETFDH* (347) genes, which encode the alpha and beta subunits of ETF, and ETFQO, respectively (Table A.1). The majority of these reported mutations are private and more or less equally distributed in both MADD types I and II (Table A.1), while type III is most frequently caused by mutations in *ETFDH* (Grünert, 2014).

Table 2.1: Clinical phenotypes of genetic disorders associated with ETF, ETFQO and FAD metabolism.

Deficiency	ETF and ETFQO			Riboflavin transporters			FAD synthase	MFT
OMIM	#231680/608053/130410/#231675			607883/#615026	607882	613350/#211500/#211530	610595/#255100	610815
Gene*	*ETFA*, *ETFB*, *ETFDH* for classification as glutaric aciduria II (GA2)A, GA2B, GA2C, respectively			*SLC52A1*	*SLC52A2*	*SLC52A3*	*FLAD1*	*SLC25A32*
Protein	ETFα, ETFβ, ETFQO			RFVT1	RFVT2	RFVT3	FADS	MFT
Clinical phenotypes**	MADD Type I (MADD-S, RU-MADD)	MADD Type II (MADD-S, RU-MADD)	MADD Type III (MADD-M, RR-MADD)	Riboflavin deficiency	BVVLS-2	Fazio-Londe disease/BVVLS-1	Lipid storage myopathy	Riboflavin-responsive exercise intolerance
Inheritance	AR	AR	AR	AD, possibly haplo-insufficiency, maternal	AR	AR	AR	AR
Onset	neonatal, severe	neonatal	mild and/or late-onset	neonatal/ pregnancy	all ages	all ages	all ages	early age
Clinical and biochemical features	• with congenital anomalies • multi-system involvement • hypotonia • cardiomyopathy • hepatomegaly • nonketotic hypoglycemia • metabolic acidosis • hyperammonemia *(see text for detailed metabolic profile)*	• without congenital anomalies	• high variability • recurrent episodes of lethargy, vomiting • muscle, cardiac, liver involvement • hypoglycemia • metabolic acidosis *(see text for detailed metabolic profile)*	• transient neonatal riboflavin deficiency • lethargy • hypotonia • poor peripheral circulation • hypothermia • lactic acidosis • MADD-type metabolites	• ponto-bulbar palsy • sensory ataxia • optic atrophy • early-onset muscle weakness of upper limbs and axial muscles • hearing loss • cranial nerve deficits • respiratory symptoms • feeding difficulties • MADD-type metabolites possible	• ponto-bulbar palsy • muscle weakness in upper limbs and axial muscles • hearing loss (BVVLS-1 only) • cranial nerve deficits • respiratory symptoms • feeding difficulties • sensory symptoms • MADD-type metabolites possible	• hypotonia • muscle weakness • swallowing and speech difficulties • respiratory symptoms • lipid storage myopathy • acylcarnitine abnormalities or ethylmalonic- and/or adipic aciduria	• recurrent exercise intolerance • acylcarnitine abnormalities
Effective Treatment***	diet (low fat and protein)	diet (low fat and protein)	riboflavin, carnitine, glycine, CoQ10	riboflavin	riboflavin	riboflavin	riboflavin	riboflavin
Prognosis (with treatment)	fatal	fatal	good	good	good	good	mostly fatal	good
References	Frerman & Goodman, 2001; Grünert, 2014; van Rijt *et al.*, 2019			Chiong *et al.*, 2007; Mosegaard *et al.*, 2017	Bosch, 2020; Foley *et al.*, 2014; Jaeger & Bosch, 2016	Bosch, 2020; Bosch *et al.*, 2011; Jaeger & Bosch, 2016	Bosch, 2020; Olsen *et al.*, 2016	Bosch, 2020; Schiff *et al.*, 2016

*See Table A.1 for variants; ** Recognised by OMIM; *** Variability should be recognised. Abbreviations: AD: autosomal dominant; AR: autosomal recessive; BVVLS-1 and -2: Brown-Vialetto-Van Laere syndrome 1 and -2; ETFα and -β: electron transfer flavoprotein subunit alpha and beta, respectively; ETFQO: ETF-ubiquinone oxidoreductase; FADS: flavin adenine dinucleotide synthase; MADD-M and -S: MADD-mild and -severe; MFT: mitochondrial folate/flavin adenine dinucleotide transporter; RFVT1−3: riboflavin transporter 1−3; RR- and RU-MADD: riboflavin-responsive and -unresponsive MADD.

In addition, MADD types I and II display a strong genotype–phenotype correlation, with the former most often being associated with homozygous null mutations and the latter resulting from mutations often leading to amino acid substitutions which allow some level of residual enzyme activity. Conversely, while individuals with MADD type III have at least one missense mutation and do not display a significantly reduced activity of the affected enzyme(s), the genotype–phenotype correlation is poor (Grünert, 2014).

2.6.2 Riboflavin transporter deficiencies

Although dietary deficiency of riboflavin has been well-documented (Powers, 2003), genetic disorders of the three riboflavin transporters (RFTV1−3) have only been recognised in the past two decades. Of these, the least frequently reported is a deficiency of RFVT1, which has only been described in five cases of dominantly inherited, adult-onset or neonatal (transiently expressed) riboflavin deficiency (Chiong *et al.*, 2007; Ho *et al.*, 2011; Mosegaard *et al.*, 2017; Kang *et al*., 2020). More frequently, two related autosomal recessive disorders of RFVT2 [Brown-Vialetto-Van Laere syndrome (BVVLS)-2] and RFVT3 (Fazio-Londe disease/BVVLS-1) have been described, with BVVLS-2 showing distinguishing features of ponto-bulbar palsy, ataxia, and optic atrophy (Bosch *et al.*, 2011; Cornelius *et al.*, 2012; Foley *et al.*, 2014; Ho *et al.*, 2011; Olsen *et al.*, 2016; Schiff *et al.*, 2016). At present, only 10 mutations have been published for *SLC52A1* (coding for RFVT1), whereas 68 mutations have been published for *SLC52A2* and *3* (coding for RFVT2 and 3), each (see Table A.1).

2.6.3 FAD synthase and transporter deficiencies

Two additional, mostly riboflavin-responsive phenotypes, resulting from dysfunction in the synthesis or transport of FAD, have been identified. A lipid storage myopathy with a variable age of onset and a mostly fatal outcome, is associated with FADS deficiency, resulting from mutations in *FLAD1*, of which 15 have been reported (Table A.1). Furthermore, six mutations have hitherto been identified in *SLC25A32*, which cause a deficiency of the inner mitochondrial membrane carrier MFT and result in a milder, recurrent exercise intolerance phenotype. To date, however, no mutations have been identified in human RFK (involved in FAD biosynthesis) (Ryder *et al.*, 2019). Owing to the many unsolved cases of riboflavin deficiency, it is very likely that the number of affected genes will increase in the future as research advances (Olsen *et al.*, 2016).

2.7 CLINICAL AND GENETIC FEATURES

Dysfunction of ETF, ETFQO and the riboflavin metabolism, together with the associated secondary flavoprotein deficiencies, affects a myriad of metabolic pathways—as summarised in Figure 2.1. These deficiencies are expressed as phenotypes that are recognised in a clinical setting through their varied neonatal- or late-onset MADD clinical presentations, and via newborn screening (NBS) programmes in which they are included (Frerman & Goodman, 2001; Houten *et al.*, 2016; Sahai *et al.*, 2014).

2.7.1 Conventional diagnostic approach

In the event of a MADD-related clinical presentation (symptomatic patients) or abnormal NBS profile (asymptomatic patients), the differential diagnosis of MADD(-like disorders), as illustrated in Figure 2.2, typically proceeds via broad-spectrum metabolite analyses. This may be followed by functional (enzymatic/flux) assays, with a range of options for genetic analyses essential to confirm the diagnosis.

At present, metabolic investigations remain the cornerstone of diagnosis and involve the analysis of urinary organic acids (GC–MS) along with plasma/serum amino acids (GC–/LC–MS/MS) and acylcarnitines (LC-MS/MS). Urinary organic acids suggestive of MADD include a combination of elevated glutaric-, ethylmalonic-, adipic-, suberic-, sebacic-, dodecanedioic-, 2-hydroxyglutaric-, 3-hydroxyisovaleric-, and 5-hydroxyhexanoic acid, in conjunction with C4 and C5 glycine conjugates. Moreover, elevated concentrations of proline, 4-hydroxyproline (neonatal-onset) and sarcosine (late-onset) are common biochemical features (Frerman & Goodman, 2001; Grünert, 2014). Acylcarnitine profiling, which is also used in NBS, is highly predictive and reflects the intra-mitochondrial accumulation of various acyl-CoAs as well as the corresponding acylcarnitines generated by carnitine palmitoyltransferase 2 (CPT2) and carnitine acetyltransferase (CrAT). A typical MADD-specific acylcarnitine profile would include C4, C5, C5DC, C6, C8, C10, C12, C14:1, C16 and C18:1 acylcarnitine species (Grünert, 2014; Houten *et al.*, 2016; Violante *et al.*, 2013; Wanders *et al.*, 2010). Important, however, is that abnormal metabolite profiles may, in some instances, only be observed during symptomatic events and may improve or even normalise when clinical symptoms are absent (Chaya *et al.*, 2018). For metabolites to be most indicative, samples should be obtained during the catabolic state or metabolic crisis, or before the next feeding (for neonates), following minimal medication intake.

Although metabolic profiling can be highly predictive for a particular IEM, enzymatic and/or flux analyses have, in general, been shown to be paramount in pinpointing the enzyme defect with certainty and disclosing the level of the deficiency. However, in the case of MADD, direct measurement of ETFα, ETFβ and ETFQO activity has been notoriously difficult to perform routinely, mainly due to the requirement of purified ETF, which is not commercially available.

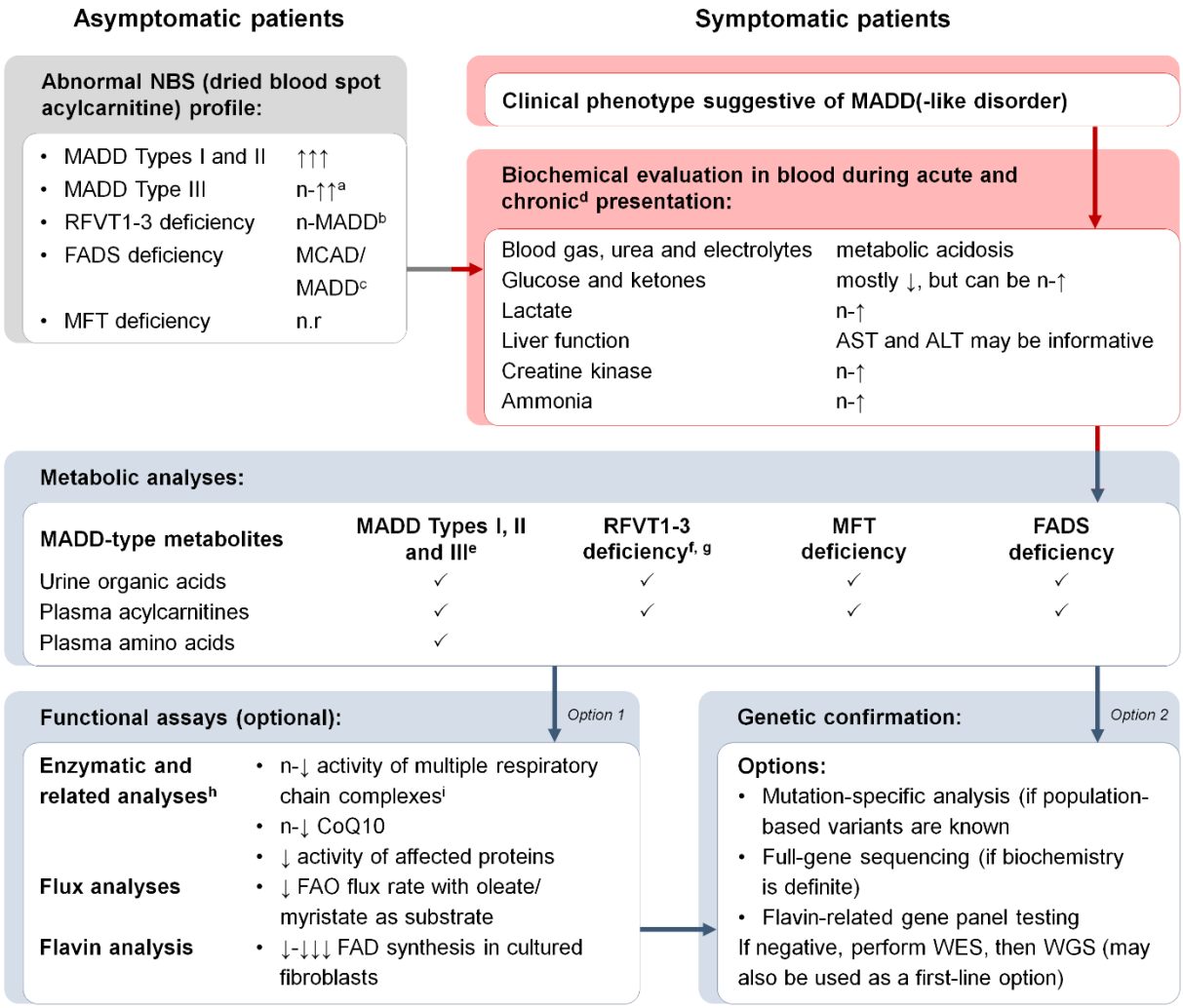

Figure 2.2: Overview of the differential diagnosis for MADD(-like disorders)

[a] Low birth weight and/or total parenteral nutrition may result in a MADD-like acylcarnitine profile (Shai *et al.*, 2008); [b] NBS is not feasible for the diagnosis of RFVT2 and 3 deficiencies (due to suspected compensation by maternal riboflavin), whereas RFVT1 deficiency has displayed normal or abnormal (MADD) NBS profiles in the reported cases (Bosch *et al.*, 2011; Ho *et al.*, 2011; Mosegaard *et al.*, 2017); [c] NBS has only been reported in three cases (Muru *et al.*, 2019; Ryder *et al.*, 2019; Yamada *et al.*, 2019); [d] Biochemical parameters may normalise and abnormal liver function is more to likely to occur later in the disease progression; [e] Metabolic analysis is most indicative during catabolic status/metabolic crisis, as the profile may be normal otherwise; [f] Some genotypes may have normal metabolic profiling; [g] Metabolite analysis can be indicative, but may be normal in some patients (molecular analysis of RFTV1−3 is required for definite diagnosis) (Jaeger & Bosch, 2016); [h] Should enzymatic assays be unavailable, diagnosis may proceed directly to genetic analyses or a combination of flux- and genetic assays; [i] Only CII activity has been analysed in MFT deficiency (Hellebrekers *et al.*, 2017). ✓, can be indicative; ↓, ↓↓↓, slight and substantial decrease; ↑, ↑↑ and ↑↑↑, slight, moderate, and substantial increase. Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; CoQ10: coenzyme Q10; FAD: flavin adenine dinucleotide; FADS: FAD synthase; FAO: mitochondrial fatty acid β-oxidation; MADD: multiple acyl-CoA dehydrogenase deficiency; MFT: mitochondrial

folate/FAD transporter; n: normal; n.r: not reported; NBS: newborn screening; RFVT: riboflavin transporter; WES: whole exome sequencing; WGS: whole genome sequencing.

As reviewed by Wanders *et al.* (2010), radio- and stable isotope-labelled FAO flux analyses, which are based on methods developed in the 1990s in fibroblasts and lymphocytes, have proven to be of great value to distinguish MADD from other FAO disorders (Manning *et al.*, 1990; Niezen-Koning *et al.*, 1992; Olpin *et al.*, 1992). In addition, FAO flux analysis using oleate as substrate in patients' fibroblasts has been found to correlate with the clinical phenotype as determined using a recently proposed MADD-disease severity scoring system (MADD-DS3) (van Rijt *et al.*, 2019). Considering the clinical and biochemical variability that makes MADD prone to misdiagnosis when using a differential diagnosis with limited assays (Frerman & Goodman, 2001; Vasiljevski *et al.*, 2018; Yıldız *et al.*, 2018), the study of van Rijt *et al.* (2019) illustrated the extensive nature of the investigations required to obtain markers for early prediction of disease severity that would allow preventative and follow-up treatment.

2.7.2 Postgenomic era diagnostic approach

With the expanding ability, lowered cost, and advantages of whole exome- (WES) and whole genome sequencing (WGS) to study complex inherited diseases, the application of these genomic tools has become an increasingly popular option to include earlier in the diagnostic process at several laboratories (Haack *et al.*, 2012; Lieber *et al.*, 2013; Neveling *et al.*, 2013; Wortmann *et al.*, 2015; Wortmann *et al.*, 2017). Altogether, genetic approaches have resulted in the rapid expansion of gene and mutation identification, as summarised in Table A.1. Of the eight genes and ~590 reported mutations, as well as other potential variants involved in MADD(-like disorders), five genes and the vast majority of mutations have been identified using next generation sequencing (NGS). With a limited number of genes involved in this group of disorders, gene panels have proven to be exceptionally useful, particularly when run in parallel with functional (flux) assays. Nevertheless, the value of biochemical and enzymatic/flux analyses, as well as the use of disease expression systems in unravelling the impact of genetic variants, diagnostic variability and the pathophysiology and treatment of MADD, should not be underestimated (Henriques *et al.*, 2009; Tolomeo *et al.*, 2020).

2.8 TREATMENT AND PROGNOSIS

As a first-line treatment, early oral supplementation of riboflavin at a high dose (100−400 mg/day) is advised for all MADD(-like) phenotypes (Mosegaard *et al.*, 2020; Ryder *et al.*, 2019). This vitamin has

been shown to dramatically improve the clinical and biochemical abnormalities associated with MADD type III (especially in cases of *ETFDH* deficiency), RFVT1−3 defects, MFT deficiency and milder cases of FADS dysfunction without major side effects (Auranen *et al.*, 2017; Jaeger & Bosch, 2016; Ryder *et al.*, 2019; Schiff *et al.*, 2016). Studies on *ETFDH* deficiency have attributed the molecular mechanism of riboflavin's response to the chaperone function of FAD, which promotes folding of the variant protein or otherwise stabilises its folding intermediates or mature form (Cornelius *et al.*, 2012; Henriques *et al.*, 2009). However, this improvement, the extent of which depends on the nature of the variant, usually does not completely correct for the variant-induced structural defect(s). Other possible explanations for the beneficial effect of riboflavin include the increase of flavin content to compensate for the loss of FAD, and the induction of flavoprotein-transcription and -translation by FAD (Henriques *et al.*, 2010; Nagao & Tanaka, 1992).

In MADD(-like disorders), riboflavin supplementation is often combined with a high-caloric diet reduced in fat and protein, the strict avoidance of fasting, and/or oral supplementation with glycine and L-carnitine to promote the export of acylglycine and -carnitine conjugates (Gordon, 2006). The latter is especially important since acyl-CoA esters cannot traverse the mitochondrial- or plasma membrane, but should be approached with caution as L-carnitine may worsen symptoms, at least in some cases (Gempel *et al.*, 2007). Riboflavin may further be combined with CoQ10 to decrease the oxidative stress and treat MADD type III with secondary CoQ10 deficiency, whereas bezafibrate has been shown to improve MADD in the absence of riboflavin, although its *in vivo* application requires further research (Cornelius *et al.*, 2014; Gempel *et al.*, 2007; Xi *et al.*, 2014; Yamada *et al.*, 2017; Yamaguchi *et al.*, 2012). Moreover, the efficacy and safety of D,L-3-hydroxybutyric acid (100−2,600 mg/kg/day, administered orally or via a nasogastric/gastrostomy tube) to treat MADD by bypassing the disturbed ketogenesis was recently demonstrated, with the authors reporting a longer survival rate (notably in MADD type II) and clinical improvement in 70% of the patients (including MADD types II and III and RFVT3 deficiency) (van Rijt *et al.*, 2020).

Despite these treatment options, mortality remains high in severe MADD(-like) phenotypes (most notably MADD types I and II) (van Rijt *et al.*, 2020). More effective treatment strategies are thus still desperately needed and, consequently, future research in enzyme replacement-, gene- and gene expression therapies hold great promise in MADD as the phenotype can often be rescued by even a slight increase in the activity of the variant protein (Ørngreen & Vissing, 2017). Likewise, disease expression models are of immeasurable value in the study of therapeutic options (Tolomeo *et al.*, 2020).

2.9 CONCLUSION

FAD and the flavoproteins it supports, participate in a number of catabolic pathways which all converge on the mitochondrion. Deficiencies of FAD's synthesis, transport and dedicated redox machinery are thus expected to have a severe impact on a wide range of metabolic functions. In this article, we have provided a succinct overview of these processes and the clinical phenotypes resulting from genetic deficiencies of the FAD metabolism, which, at present, include eight genes with at least 590 reported mutations that lead to a range of different MADD(-like) phenotypes. *ETFDH* remains the gene with the most (59%) reported mutations followed by *SLC52A2* and *3* (12% each), while the other genes each exhibit less than 8% of the reported mutations. With the exception of MADD types I and II, an important feature of these disorders is the possibility of effective treatment. At present, these disorders are included in few NBS programmes and an effective early metabolic diagnosis relies predominantly on disease expression (catabolic state) (Austin & Dawkins, 2017; Baynam *et al.*, 2020). Together, this warrants the development of novel diagnostic approaches—notably with the focus on finding more successful and direct avenues for diagnostics in a more inclusive global population. Over the past decade genomic approaches have, alongside biochemical investigations, proven their value in the diagnosis of MADD(-like disorders). Considering the limited number of genes involved, the earlier inclusion of mutational analysis in diagnosis, and the development of targeted gene therapy should therefore be considered in earnest.

2.10 IEMS AND MADD IN THE SOUTHERN AFRICAN CONTEXT

In Southern Africa, metabolic testing for IEMs is currently offered by two facilities: These include (i) the CHM[5] (since 1981; NWU), and (ii) the NHLS IMD molecular laboratory (UCT) (NMP, 2023; UCT, 2023). Per annum, the former tests clinically referred samples from approximately 2,634 infants, children, and adults (metabolic testing) and 5,282 newborns (elective NBS)[6] across the country[7]. Within the scope of IEMs tested for at these facilities, MADD is diagnosed only as a secondary condition (Vorster & Meldau, 2023, personal communication). Considering the number of recorded live births in SA (1,175,776 per year), as well as the global incidence of MADD (1:200,000 births), the expected referral rate of MADD should

[5] The CHM offers its services via (i) PLIEM, which focuses on infant, child, and adult patients, and (ii) the NBS Laboratory, of which the patients are exclusively newborns (NMP, 2023).

[6] NBS of both symptomatic and asymptomatic cases is performed by the CHM, whereas only symptomatic newborns are tested by the NHLS IMD molecular laboratory (NMP, 2023; UCT, 2023).

[7] The numbers reported represent a four-year mean (2019–2021). No data is available for the NHLS IMD molecular laboratory.

translate to 5–6 patients annually (Statistics South Africa, 2023). However, with only 2–3 diagnoses being made in Southern Africa per year, the current referral rate is significantly lower than this projection (Vorster & Meldau, 2023, personal communication).

These missed- or non-diagnoses may be attributed to various pre-existing difficulties faced by IEM diagnostics in Southern Africa. This includes the absence of a compulsory NBS programme, which should be capable of detecting neonatal-onset MADD types I and II (Conradie *et al*., 2021). In Southern Africa, IEMs are diagnosed on an elective basis following the request of a parent/guardian or a clinical referral. In addition, the associated cost of metabolic testing is not currently covered by most medical aid schemes and not available in the public sector (Vorster & Meldau, 2023, personal communication). As a result, <1% of newborns in Southern Africa have access to NBS (Conradie *et al*., 2021). Southern Africa, like the rest of the African continent, is also faced by multiple competing burdens of disease (Baynam *et al.*, 2020; Raga *et al*., 2022). Owing to Southern Africa being a hub of communicable and non-communicable disease, IEMs are prioritised to a lesser extent by the national health sector and national support structures. A consequence of this shift in medical diagnostic and research focus, is the resulting shortage of healthcare providers and pathologists (in both the private and public sector) who are trained in recognising and treating IEMs (Conradie *et al*., 2021). Other factors limiting the diagnosis of IEMs in Southern Africa, are the severe burden of socio-economic obstacles (which precludes adequate diagnosis, medical care, and timely treatment), and an often, inapt infrastructure (e.g., challenges in sample transportation and the current national crises in electricity provision, which negatively impact sample viability and contribute to the potential down-time of instrumentation).

In SA and its neighbouring countries, patients with an IEM include a unique diversity of African, mixed, Asian (including Indian), and White populations (Raga *et al*., 2022). Since variants that cause rare disease, including MADD(-like disorders), tend to be population-specific, knowledge of the genetic cause of disease will greatly contribute to the advancement of diagnostics (Bustamante *et al.,* 2011). However, in Southern Africa, population-specific knowledge regarding the aetiology of, and the subsequent genetic screening for, IEMs are limited to some extent and often absent. In addition, many patients are lost to follow up, as the importance of genetics for research purposes is not emphasised or understood (Vorster & Meldau, 2023, personal communication). This not only delays the confirmation of diagnoses, but also prevents the development of more timely genetic screening assays, and contributes to the paucity of population genomic data, which in turn hampers prevalence studies (Baynam *et al*., 2020). Prior to the onset of this study, only four variants of MADD(-like disorders) had been identified in single case studies in Southern Africa (Table 2.2). These included two *SLC52A3* variants, which were identified in a patient of White ethnicity—the first reported case of RFVT3 (BVVLS-1) deficiency in sub-Saharan Africa (Chaya *et al.*, 2018). Moreover, our group identified one novel (c.[1067G>A]) and a previously documented (c.[1448C>T]) *ETFDH* variant in three patients of White SA ethnicity who were diagnosed with MADD types I and III

(van der Westhuizen *et al.,* 2018). Considering the biochemical data collected from previous metabolic testing and the limited period in which the latter three patients had been diagnosed, it was postulated that, in Southern Africa, MADD may occur at a frequency similar to that of a more prevalent group of IEMs called organic acidaemias (van der Westhuizen *et al.,* 2018). Furthermore, since the patients all shared the same ethnicity, it was proposed that MADD may be more prevalent in the White SA population. This, in addition to the recurrent incidence of the novel c.[1067G>A] variant, also suggested the possibility of a founder effect.

Table 2.2: Known pathogenic variants of MADD(-like deficiency) in Southern Africa in 2020.

Variant	1	2	3	4
Affected gene	NM_033409.3(*SLC52A3*)	NM_033409.3(*SLC52A3*)	NM_004453.4(*ETFDH*)	NM_004453.4(*ETFDH*)
Exon	2	3	9	11
Nucleotide change (position)	chr20:765401G>T	chr20:763932G>C	chr4:158699081G>A	chr4:158706351C>T
Nucleotide change (allele call)	c.[374C>A]	c.[639C>G]	c.[1067G>A]	c.[1448C>T]
Amino acid change (predicted from cDNA)	p.[Thr125Asn]	p.[Tyr213*]	p.[Gly356Glu]	p.[Pro483Leu]
rsID	rs767263985	rs778363575	n.a	rs377656387
CADD score	27	0	n.a	27
ACMG classification	VUS	pathogenic	likely pathogenic	pathogenic
Reference	Chaya *et al.*, 2018	Chaya *et al.*, 2018	van der Westhuizen *et al.*, 2018	van der Westhuizen *et al.*, 2018

Abbreviations: ACMG: American College of Medical Genetics and Genomics (Richards *et al.,* 2015); cDNA: complimentary deoxyribonucleic acid; chr: chromosome; n.a, not applicable; rsID: reference single nucleotide polymorphism (SNP) cluster ID; VUS: variant of unknown significance.

To address these and other hypotheses, which, at present, are founded only on case reports and anecdotal observations, a comprehensive study of a disease cohort is required. This will not only facilitate the identification of pathogenic variants leading to MADD in Southern Africa, but also the metabolic and clinical presentations involved, the possibility of genotype–phenotype correlations, as well as subsequent natural history studies and the estimation of prevalence. Considering the challenges faced by IEM diagnostics in Southern Africa, it is this paucity of data that constitutes the largest limitation (Raga *et al.*, 2022). By addressing these shortcomings in the understudied populations of Southern Africa, diagnostic and treatment delay (including counselling) can be greatly improved, thereby decreasing the morbidity and mortality associated with MADD(-like disorders).

Chapter 3

Clinical, biochemical, and genetic spectrum of MADD in a South African cohort: an ICGNMD study

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3.1 CHAPTER INTRODUCTION

This chapter consists of the second journal publication resulting from this thesis[8] and focuses on the first aim of this study namely, to characterise the clinical and metabolic properties, and molecular genetics of MADD in a Southern African cohort of clinically and biochemically diagnosed patients. Firstly, an abstract of the publication and background to the study are given. This is followed by a description of the methods applied, the results obtained, and a discussion thereof. Topics addressed, include recruitment of the MADD cohort and sampling (Objectives 1.1–1.2), clinical and biochemical (metabolic) investigations (Objective 1.2), genetic analyses and genotype–phenotype correlations (Objectives 1.3–1.4), as well as allele frequency estimation and haplotyping (Objective 1.5). The chapter closes with a concluding summary. The final allele frequency estimations, calculated after the inclusion of additional samples to the study (but following publication), are given in Section 3.7. The journal publication, along with Supplementary Materials 1 and 2 (Tables B.1–6 and Figure B.1) may be found in Appendix B, whereas Appendix C (unpublished) provides additional supporting methodology and results[9].

3.2 ABSTRACT

Background: Multiple acyl-CoA dehydrogenase deficiency (MADD) is an autosomal recessive disorder resulting from pathogenic variants in three distinct genes, with most of the variants occurring in the electron transfer flavoprotein-ubiquinone oxidoreductase gene (*ETFDH)*. Recent evidence of potential founder variants for MADD in the South African (SA) population, initiated this extensive investigation. As part of the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD) study, we recruited a cohort of patients diagnosed with MADD from academic medical centres across SA over a three-year period. The aim was to extensively profile the clinical, biochemical, and genomic characteristics of MADD in this understudied population.

Methods: Clinical evaluations and whole exome sequencing were conducted on each patient. Metabolic profiling was performed before and after treatment, where possible. The recessive inheritance and phase of the variants were established via segregation analyses using Sanger sequencing. Lastly, the haplotype and allele frequencies were determined for the two main variants in the four largest SA populations.

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[8] This journal publication has been included in this thesis with permission from the copyright holder, BioMed Central.

[9] The unpublished supporting information includes the ethics approval and consent/assent documentation, recruitment strategy and sampling, DNA isolation and sequencing, sequence validation and segregation analyses, as well as allele frequency assays.

Results: Twelve unrelated families (ten of White SA and two of mixed ethnicity) with clinically heterogeneous presentations in 14 affected individuals were observed, and five pathogenic *ETFDH* variants were identified. Based on disease severity and treatment response, three distinct groups emerged. The most severe and fatal presentations were associated with the homozygous c.[1067G>A];c.[1067G>A] and compound heterozygous c.[976G>C];c.[1067G>A] genotypes, causing MADD types I and I/II, respectively. These, along with three less severe compound heterozygous genotypes (c.[1067G>A];c.[1448C>T], c.[740G>T];c.[1448C>T], and c.[287dupA*];c.[1448C>T]), resulting in MADD types II/III, presented before the age of five years, depending on the time and maintenance of intervention. By contrast, the homozygous c.[1448C>T];c.[1448C>T] genotype, which causes MADD type III, presented later in life. Except for the type I, I/II and II cases, urinary metabolic markers for MADD improved/normalised following treatment with riboflavin and L-carnitine. Furthermore, genetic analyses of the most frequent variants (c.[1067G>A] and c.[1448C>T]) revealed a shared haplotype in the region of *ETFDH*, with SA population-specific allele frequencies of <0.00067–0.00084%.

Conclusions: This study reveals the first extensive genotype–phenotype profile of a MADD patient cohort from the diverse and understudied SA population. The pathogenic variants and associated variable phenotypes were characterised, which will enable early screening, genetic counselling, and patient-specific treatment of MADD in this population.

3.3 BACKGROUND

Multiple acyl-CoA dehydrogenase deficiency (MADD; ORPHAcode 26791), also known as glutaric aciduria type II (GAII), is an autosomal recessive disorder that occurs at a global and ethnically variable prevalence of approximately 1 in 200,000 births (Orphanet, 2014). It is caused by pathogenic variants (at least 590) in the *ETFA*, *ETFB* and *ETFDH* genes, which encode for the alpha and beta subunits of the electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO; EC:1.5.5.1), respectively. Collectively, ETF and ETFQO are responsible for re-oxidising reduced mitochondrial flavin adenine dinucleotide ($FADH_2$), which in turn sustains mitochondrial fatty acid β-oxidation (FAO), amino acid catabolism and choline metabolism (Ghisla & Thorpe, 2004; Mereis *et al.,* 2021).

Clinically, MADD may be divided into three phenotypes. The first and second (MADD types I and II, respectively) are characterised by the neonatal-onset of severe and often fatal symptoms with multi-system involvement (including leukodystrophy, hypotonia, cardiomyopathy, hepatomegaly, and renal abnormalities), as well as hypoglycaemia, hyperammonaemia, and metabolic acidosis (with/without ketosis) (Frerman & Goodman, 2001; Grünert, 2014; van Rijt *et al.,* 2019). MADD types I and II may be distinguished from each other by the presence (type I) or absence (type II) of congenital abnormalities and

are caused, at essentially equal frequencies, by pathogenic variants in *ETFA*, *ETFB*, and *ETFDH* (Grünert, 2014). By contrast, the third phenotype (MADD type III) has a later-onset and presents with milder/delayed symptoms which are highly heterogeneous. Such symptoms may include recurrent/intermittent episodes of lethargy and vomiting; muscle, cardiac and/or liver involvement; as well as lipid storage myopathy, hypoglycaemia, metabolic acidosis, and/or hyperammonaemia. MADD type III is reported to be caused mainly by *ETFDH* variants and is also known as riboflavin (Rb)-responsive MADD owing to its amenability to treatment (Grünert, 2014; Yıldız *et al.,* 2019). To estimate the burden of disease, a disease severity scoring system (MADD-DS3; MADD-disease severity 3) may be used (van Rijt *et al.,* 2019). Moreover, disease-severity is often linked to the patient's response to treatment, which includes a high-caloric diet with fat and protein restriction, the strict avoidance of fasting, and supplementation with L-carnitine and Rb (as first-line treatment) (Gordon, 2006; Mosegaard *et al.,* 2020).

At present, South Africa (SA) does not have a compulsory newborn screening (NBS) programme. Instead, the biochemical diagnosis of MADD follows (i) a clinical presentation in symptomatic cases, or (ii) an abnormal NBS profile in asymptomatic cases—during which MADD is defined as a secondary condition. Symptomatic patients undergo a thorough metabolic work-up including (i) urinary organic acids, (ii) plasma/serum acylcarnitines, and (iii) plasma/serum amino acids and display a unique metabolic fingerprint as described in a recent review by Mereis *et al.* (2021). As with other inherited metabolic disorders, final confirmation of the diagnosis is obtained by genetic analysis.

In SA, as with most understudied populations, pathogenic variant screening for inherited metabolic disorders is less common. Such screening is, however, offered for a small selection of disorders (including glutaric aciduria type I, isovaleric acidaemia, galactosemia, and MPV17-hepatocerebral mitochondrial DNA depletion syndrome) following the comprehensive description of cohorts in literature. Currently, substantial knowledge of the genotype–phenotype and biochemical profiles, and the subsequent response to treatment are not available for MADD in the different SA populations. Prior to this study, a novel pathogenic *ETFDH* variant (c.[1067G>A], p.[Gly356Glu]) and one previously described pathogenic variant (c.[1448C>T], p.[Pro483Leu]) were reported to result in MADD in the White SA population (van der Westhuizen *et al.,* 2018). In the homozygous state, these variants cause MADD types I and III, respectively, and it was proposed by van der Westhuizen *et al.* (2018) that the prevalence of c.[1067G>A] in this SA population could possibly be the result of a founder effect. In our study, we aimed to address these hypotheses and other limitations by investigating a cohort of 14 recruited MADD patients, under the auspices of the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD). We extensively characterised the clinical phenotype, biochemical profile, and genetics of MADD in SA, and provide knowledge that will contribute to its timely diagnosis, clinical management, and therapeutic intervention.

3.4 METHODS

3.4.1 Cohort selection and sampling

Following informed consent/assent, the patients in this study were enrolled as part of the ICGNMD study with ethical approval numbers 19/LO/1796 (HRA and HCRW), NWU-00966-19-A1 and NWU-00966-19-A1-01 (North-West University; NWU), 296/2019 (University of Pretoria; UP), B19/01/002 (Stellenbosch University; SU), and 605/2020 (University of Cape Town; UCT).

Probands and their affected/unaffected first-degree relatives were recruited either retrospectively or prospectively, based on a clinical and metabolic diagnosis of MADD, via one of three SA academic, state-funded hospitals: Steve Biko Academic Hospital, Tygerberg Hospital, and Red Cross War Memorial Children's Hospital. For (i) genetic, (ii) protein, and (iii) metabolic analyses, the following samples were obtained: (i) whole blood, saliva (living patients), or urine (deceased patients from whom no blood or saliva was available), (ii) primary skin fibroblasts (P5 and P9), as well as (iii) urine collected during the first metabolic presentation (P1, P4–P6, P8–P12 and P14)/during metabolic decompensation (P7), and after therapeutic management (P1–P11, P13 and P14).

3.4.2 Clinical and biochemical investigations

Patients were extensively evaluated by paediatric or adult neurologists according to protocols set forth by the ICGNMD. This included obtaining relevant demographic information, family history, medical history, and current treatment (Table 3.1). Moreover, deep phenotypic data (Supplementary Material 1, Appendix B.2) were collected during a comprehensive clinical re-assessment of each proband.

SDS-PAGE and Western blot analysis of primary skin fibroblasts, established from two adult patients (P5 and P9) and two healthy controls (matched in age, gender, and ethnicity to the patients), were performed to evaluate the steady state level of ETFQO. Immunoblotting was conducted using primary antibodies at 1:1,000 directed against ETFQO (ab131376; Abcam, Cambridge, UK) and β-actin (as housekeeping gene; ab6276; Abcam), and an HRP-conjugated secondary antibody at 1:10,000 (ab97023; Abcam). Findings are shown in Supplementary Material 3 (Appendix B.4).

For targeted metabolic analyses, urine organic acids and their glycine conjugates were extracted, derivatised, and analysed using the 7890A gas chromatography system coupled to the 5977A MSD mass spectrometer (Agilent Technologies, California, USA) as described by Erasmus *et al.* (1985) and refined by Reinecke *et al.* (2012). Data acquisition was facilitated with the 5977 MassHunter Data Acquisition software (B.07.04.2260; Agilent Software); Automated Mass spectral Deconvolution and Identification

System software (AMDIS v2.73; National Institute for Standards and Technology) was used to identify the component peaks and perform spectral deconvolution. Underivatised amino acids in urine were analysed using validated liquid chromatography-triple quadrupole mass spectrometry and the MassChrom® Amino Acid Analysis kit (75111; Chromsystems, Gräfelfing, DE). Samples were prepared via the Microlab STAR Liquid Handling System (Hamilton, Nevada, USA) and analysed on the Infinity 1290 II liquid chromatography system coupled to the 6470 Triple Quadrupole liquid chromatography–mass spectrometer (Agilent Technologies). For urine acylcarnitine analysis, electrospray ionisation–tandem mass spectrometry was performed using the 1290 Infinity II liquid chromatography system coupled to the 6410 Triple Quadrupole liquid chromatography–mass spectrometer (Agilent Technologies) as described by Pitt *et al.* (2002). A standard mixture of stable acylcarnitine isotopes was used for quantification (Swanepoel *et al.,* 2021). Amino acid and acylcarnitine data were acquired and quantified using MassHunter Data Acquisition software and MassHunter QQQ Quantitative software (v10.00; Agilent Software), respectively. All reported processed data are shown in Supplementary Material 2 (Appendix B.3).

3.4.3 Genetic analyses

To identify potential pathogenic variants in the proband DNA, WES was performed at Macrogen Europe (Amsterdam, NL) using the NovaSeq 6000 HiSeq platform (Illumina Inc., San Diego, USA) and the SureSelect Human All Exon V6 panel (5190-8865; Agilent Technologies). Reads (50× average depth coverage) were aligned to the Genome Reference Consortium Human Build (GRCh) 38.p13, and variants were annotated according to GRCh38 using the Ensembl Variant Effect Predictor (v108) (McLaren *et al.,* 2010). High-quality variants were filtered by applying the Genomics' England PanelApp, "Rhabdomyolysis and metabolic muscle disorders_1.57" panel (Martin *et al.,* 2019); the pathogenicity of potential disease-causing variants was evaluated and classified as such, according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards *et al.,* 2015). All potential pathogenic variants identified were confirmed and segregation analysis in family members was conducted via Sanger sequencing.

3.4.4 Allele frequency and haplotyping

To estimate the allele (carrier) frequency of the two most encountered variants in the cohort (c.[1067G>A] and c.[1448C>T]), PCR–RFLP analysis was performed as previously described (van der Westhuizen *et al.,* 2018) on newborn dried blood spot (DBS) samples of the four largest SA population groups—African, White SA, Indian, and mixed ethnicity. To this end, a total of 2,844 anonymised and randomised DBS

samples per variant per population group were used: 594 representatives of mixed ethnicity as well as 750 representatives of each of the following populations: African, White SA and Indian ethnicity. The samples represented an equal distribution of healthy males and females and were kindly provided by the NBS laboratory at the NWU Centre for Human Metabolomics (CHM).

Next, we investigated whether these two variants arose due to founder mutations by determining the haplotype(s) of the affected individuals using a GSA v3.0 array (Illumina Inc.). Sample processing was performed at UCL Genomics (UCL Great Ormond Street Institute of Child Health, London, UK), according to the manufacturer's instructions; the resulting raw IDAT files were processed using Genome Studio (v2.0.5; Illumina Inc.) and converted to PLINK (v1.9) format (PLINK, 2023). Samples and variants with a call rate below 90% were excluded from further analysis. Thereafter, samples were compared to determine familial relations and phased using Eagle (v2.4.1) (Eagle, 2023). Finally, haplotype sharing between each sample was determined with Germline2 software (v1.0) (Nait *et al.,* 2020) and shared regions were visualised using R (v4.2.2).

3.5 RESULTS AND DISCUSSION

3.5.1 Cohort

Over a period of three years and using a retrospective and prospective recruitment strategy at three different centres across SA, 12 apparently unrelated families (ten of White SA and two of mixed ethnicity) with 14 clinically affected patients (5 males and 9 females) were recruited. All patients were born to non-consanguineous parents and disease onset ranged from birth to 41 years, with four deaths recorded (at 9 days, 14 days, 3 months, and 23 years, respectively). Where possible, a consistent clinical re-evaluation was conducted according to ICGNMD guidelines; for most of the cases, biological samples acquired during acute metabolic presentation (n = 12/14) and following therapeutic management (n = 13/14) were collected/available and could be re-analysed (for the remainder, the original data were used, where available).

It should be noted that SA patients of African and Indian ethnicity have previously been diagnosed with MADD on a biochemical level at the CHM (NWU) and the NHLS Inherited Metabolic Disease Molecular Laboratory (UCT)—the two SA facilities where metabolic testing is offered as a main service (unpublished data). However, while all possible efforts were made to include patients of these population groups, they were regrettably lost to follow-up prior to the study's onset and an opportunity for subsequent genetic confirmation did not present itself. Moreover, no new cases were identified in these population groups during this study. We therefore recognise a potential referral bias within the population groups included. It is our hope that this problem can be addressed by metabolic and genetic NBS in the future.

3.5.2 Clinical, biochemical, and genetic features

Overall, the SA MADD cohort displayed heterogeneous clinical presentations [Table 3.1 and Supplementary Material 1 (Appendix B.2)], together with the characteristic diagnostic urinary metabolites associated with MADD [Table 3.2 and Supplementary Material 2 (Appendix B.3)]. While plasma/serum acylcarnitine and amino acid findings are typically used for diagnosis, this matrix was limited for the majority of the patients (sample collection and analyses were conducted over a 30-year period) and consequently, the levels of these metabolites in the urine are reported instead. The variants identified, their location in ETFQO, and their pathogenicity, according to the ACMG criteria, are described in Table 3.3 (Richards *et al.,* 2015). The elucidation of these variants in the SA populations now enable rapid screening methods which were not available before, thereby allowing the timely confirmation of the disorder (in hours) in symptomatic patients. This prompt identification of MADD-related variants can be used to aid the reproductive choices of carrier/affected patients and is especially valuable for prenatal testing and the testing of neonates who may require immediate therapy following birth (i.e., those affected with MADD type I/II). In all recruited cases, a resolved genotype–phenotype result was obtained. Three distinct groups were observed, based on disease severity and response to treatment, as discussed below.

3.5.2.1 Severe, Rb-unresponsive MADD patient group

The first group included three patients (P6, P8 and P12), who presented with the hallmark features of neonatal-onset MADD. Clinically, their symptoms were severe and progressive, and patients were metabolically and phenotypically unresponsive to treatment with Rb. The first case was a male neonate (P6), born prematurely (at 38 weeks) to parents of White SA ethnicity, who presented within the first week of life with acute metabolic decompensation. Key clinical features included congenital cardiac abnormalities (pulmonary stenosis, a patent foramen ovale with a right to left shunt, and a large atrial septum aneurysm) and convulsions with early neonatal death on day 9 of life. Metabolic profiling indicated the typical MADD biochemical fingerprint (Ruiz-Sala & Peña-Quintana, 2021), including dicarboxylic aciduria, increased 2-hydroxyglutaric acid, elevated glycine conjugates (short-, short-branched-, and medium-chain-related), increased disease-associated acylcarnitine conjugates, as well the characteristic increase in sarcosine on the amino acid profile. Both mitochondrial fatty acid β-oxidation and branched-chain amino acid catabolism were affected, correlating with previous studies on severe MADD cases (van Rijt *et al.,* 2019). The patient displayed a MADD-DS3 score of 30, further supporting a diagnosis of MADD type I. A then novel homozygous variant—c.[1067G>A] (p.[Gly356Glu])—with *in silico* and structural evidence of pathogenicity (ACMG classification: likely pathogenic) was identified in this neonate (van der Westhuizen *et al.,* 2018).

Table 3.1: Demographic, clinical, and genetic information of the SA MADD cohort

Group	**Rb-unresponsive (severe phenotype; n = 3)**			**Variably Rb-responsive (moderate phenotype; n = 8)**								**Rb-responsive (mild phenotype; n = 3)**		
Patient	**P12**	**P8**	**P6**	**P13**	**P2**	**P3**	**P9**	**P10**	**P11**	**P1**	**P7**	**P4**	**P14**	**P5**
Gender	F	F	M	F	F	M	M	F	M	M	F	F	F	F
Ethnicity	Mixed	Mixed	White	White	White	White	White	White	White	White	White	White	White	White
Consanguinity	None	None	None	None	None	None	None	None	None	None	None	None	None	None
Clinical														
Age of onset/death	Birth/D14	Birth/M3	Birth/D9	Y3	M8	Y4	M4	M9	Y3	Birth	M9/Y23	Y41	Y8	Y22
Major organ systems involved	Liver Muscle	Liver Muscle	Congenital Liver Muscle	Muscle	Muscle	Liver Muscle	CNS Liver Muscle	Liver	Muscle	CNS Liver Muscle	CNS Liver Muscle	PNS Muscle	Liver Muscle	Liver Muscle
MADD-DS3 score	**24**	**21**	**30**	**3**	**3**	**6**	**6.75**	**9**	**9**	**13.95**	**22.5**	**5.5**	**7.5**	**11.4**
Corresponding type	I/II	I/II	I	III	III	III	III	III	III	II	III	III	III	III
***ETFDH* genotype**														
c.[1067G>A];c.[1067G>A]	–	+	+	–	–	–	–	–	–	–	–	–	–	–
c.[1448C>T];c.[1448C>T]	–	–	–	–	–	–	–	–	–	–	–	+	+	+
c.[976G>C];c.[1067G>A]	+	–	–	–	–	–	–	–	–	–	–	–	–	–
c.[1067G>A];c.[1448C>T]	–	–	–	–	–	–	+	+	+	+	+	–	–	–
c.[287dupA*];c.[1448C>T]	–	–	–	–	+	+	–	–	–	–	–	–	–	–
c.[740G>T];c.[1448C>T]	–	–	–	+	–	–	–	–	–	–	–	–	–	–

The MADD-DS3 score (van Rijt *et al.*, 2019) increases with disease severity and reflects the clinical symptoms exhibited during decompensation. Consequently, it is affected by the time elapsed (and thus the progression of symptoms) from presentation to successful diagnosis. The MADD-DS3 score is used as a guide only and is not an absolute indication of the degree of pathogenicity of a variant. Red, yellow, and green: high, moderate, and low MADD-DS3 scores within the cohort, respectively. Abbreviations: +: present and validated; CNS: central nervous system; D and number: age in days; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene (NM_004453.4); F: female; M: male; M and number: age in months; MADD-DS3: multiple acyl-CoA dehydrogenase deficiency-disease severity 3; n: number of patients; PNS: peripheral nervous system; Rb: riboflavin; Y and number: age in years.

Table 3.2: Diagnostic urine MADD metabolites of the SA MADD cohort

Group	Rb-unresponsive (severe phenotype; n = 3)		Variably Rb-responsive (moderate phenotype; n = 8)		Rb-responsive (mild phenotype; n = 3)	
Treatment (sample number)	Untr (s = 3)	Tr (s = 2)	Untr (s = 5)	Tr (s = 8)	Untr (s = 3)	Tr (s = 3)
Urine Organic Acids						
Ethylmalonic acid	**1.7**	**2.5**	**1.8**	**1.1**	**2.0**	**1.0**
Glutaric acid	**3.0**	**3.0**	**2.8**	**0.6**	**2.0**	**0.0**
2-Methylbutyrylglycine	**1.7**	**1.5**	**0.0**	**0.0**	**1.0**	**0.0**
N-Hexanoylglycine	**2.0**	**1.5**	**2.4**	**1.6**	**2.0**	**0.0**
N-Isovalerylglycine	**3.0**	**3.0**	**0.0**	**0.0**	**1.0**	**0.0**
N-Isobutyrylglycine	**1.3**	**1.5**	**1.2**	**0.5**	**1.0**	**0.0**
2-Hydroxyglutaric acid	**3.0**	**3.0**	**1.4**	**1.3**	**3.0**	**1.0**
cis-4-Decenedioic acid	**2.0**	**0.5**	**0.6**	**0.4**	**0.0**	**0.0**
C6-C10 dicarboxylic acids[§]	**1.7**	**1.3**	**1.2**	**0.5**	**1.5**	**0.0**
Treatment (sample number)	Untr (s = 2)	Tr (s = 2)	Untr (s = 7)	Tr (s = 8)	Untr (s = 3)	Tr (s = 2)
Urine Acylcarnitines[†,‡]						
Free carnitine	**2.0**	**2.0**	**1.3**	**2.3**	**1.3**	**1.5**
Butyryl-/isobutyryl carnitine	**3.0**	**3.0**	**2.6**	**3.0**	**1.7**	**1.5**
Isovalerylcarnitine	**3.0**	**3.0**	**2.1**	**1.8**	**1.7**	**1.5**
Glutarylcarnitine	**3.0**	**3.0**	**3.0**	**2.9**	**3.0**	**3.0**
Hexanoylcarnitine	**2.0**	**3.0**	**1.6**	**0.6**	**0.7**	**0.5**
Octanoylcarnitine	**2.0**	**3.0**	**2.3**	**1.6**	**0.7**	**1.5**
Decanoylcarnitine	**2.5**	**2.5**	**2.6**	**0.6**	**1.0**	**0.5**
Treatment (sample number)	Untr (s = 2)	Tr (s = 2)	Untr (s = 7)	Tr (s = 8)	Untr (s = 3)	Tr (s = 2)
Urine Amino acids[†]						
Sarcosine	**3.0**	**3.0**	**2.9**	**0.9**	**2.7**	**0.0**
4-Hydroxyproline	**3.0**	**1.5**	**0.4**	**0.0**	**1.0**	**0.0**
Proline	**3.0**	**3.0**	**0.9**	**1.0**	**1.0**	**1.0**

§ The average weighted mean of adipic acid (C6), suberic acid (C8), and sebacic acid (C10) is given.

† Acylcarnitine and amino acid assessments are typically conducted on plasma/serum, but this matrix was limited due to sample stability (collection ranged over a 30-year period).

‡ Increased free carnitine is beneficial and promotes fatty acid conjugation and release. Likewise, elevated acylcarnitine conjugates indicate sufficient detoxification.

Values represent the number of times a metabolite is increased above the reference range, as a weighted mean of each group (≥3: severely elevated; ≥2: elevated; >1: slightly elevated; ≤1: normal). Metabolites due to secondary consequences are not reported. Red, yellow, and green: high, moderate, and low values, respectively. Abbreviations: n: number of patients; Rb: riboflavin; s: number of available samples; Tr: treated; Untr: untreated.

Table 3.3: *ETFDH* variants identified in the SA MADD cohort

Variant GRCh38.p13	Exon	Protein GRCh38.p13	Protein domain	rsID	ACMG classification	Allele frequencies (% x10^{-3})			
						gnomAD[†]		H3Africa	This study
c.[287dupA*]	3/13 (ENSE00003628977)	p.[Asp97Glyfs*24]	FAD-binding	–	Likely pathogenic	AF	-	-	-
						E	-	-	-
						AS + I	-	-	-
						M	-	-	-
c.[740G>T]	7/13 (ENSE00001126657)	p.[Gly247Val]	FAD-binding	rs1384574225	Likely pathogenic	AF	0	-	-
						E	0	-	-
						AS + I	0	-	-
						M	0	-	-
c.[976G>C]	9/13 (ENSE00001126640)	p.[Gly326Arg]	Ubiquinone-binding	–	Uncertain significance	AF	-	-	-
						E	-	-	-
						AS + I	-	-	-
						M	-	-	-
c.[1067G>A]	9/13 (ENSE00001126640)	p.[Gly356Glu]	Ubiquinone-binding	–	Likely pathogenic	AF	-	-	<0.67
						E	-	-	<0.67
						AS + I	-	-	<0.67
						M	-	-	<0.84
c.[1448C>T]	11/13 (ENSE00003510567)	p.[Pro483Leu]	Carboxy-terminal	rs377656387	Pathogenic	AF	0	-	<0.67
						E	0.04	-	<0.67
						AS + I	0.03	-	<0.67
						M	0	-	<0.84

[†] Ethnicities are used as reported on gnomAD: AF: African; AS + I: Asian and Indian; E: European non-Finnish; M: mixed ethnicity. Abbreviations: ACMG: American College of Medical Genetics and Genomics; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene (NM_004453.4); FAD: flavin adenine dinucleotide; GRCh: Genome Reference Consortium Human Build; H3Africa: Human Heredity and Health in Africa; rsID: reference single nucleotide polymorphism (SNP) cluster ID.

The second patient was a female infant (P8), born at term to parents of mixed ethnicity, who presented at birth with metabolic acidosis, hypotonia and feeding difficulties. Disease-specific metabolic markers were similar to those of P6, and the MADD-DS3 score of 21 was high. The patient succumbed at the age of three months. The homozygous c.[1067G>A] variant was subsequently identified; however, based on the lack of any reported congenital features (absent post-mortem examination), a diagnosis of MADD type I/II was given. The third case was a female neonate (P12), born at term to parents of mixed ethnicity. A urinary organic acid profile typical of MADD was confirmed by the hospital that made the diagnosis (no residual urine collected before or after treatment was available to re-analyse for the purpose of this study). Clinical features included metabolic acidosis, hypoglycaemia, hyperammonaemia, pancytopaenia, acute kidney injury, hyponatremia, and hypocalcaemia, and the patient succumbed at 14 days of age. Based on the absence of any reported congenital features (no post-mortem examination) and the high MADD-DS3 score of 24, a diagnosis of MADD type I/II was given. Whole exome sequencing (WES) and segregation analysis revealed that the patient was compound heterozygous for c.[976G>C];c.[1067G>A]

(p.[Gly326Arg];p.[Gly356Glu]). Variant c.[976G>C] is classified as a likely pathogenic variant of unknown significance according to the ACMG criteria and, to our knowledge, has been reported to occur in only one late-onset case of Chinese ethnicity by Xi *et al.* (2014) as compound heterozygous with the common variant, c.[250G>A] (p.[Ala84Thr]; ACMG classification: pathogenic). By contrast, the c.[1067G>A] variant has only been encountered in the SA population to date (van der Westhuizen *et al.,* 2018).

Considering the treatment-unresponsive metabolic profile (P6 and P12) and rapid clinical deterioration of patients P6, P8 and P12, it may be inferred that c.[1067G>A] is a highly pathogenic variant. Its presence on both alleles, or its bi-allelic combination with another variant affecting the same protein domain (ubiquinone-binding domain) of ETFQO, appears to lead to insufficient enzymatic compensation for adequate ETFQO activity. While it is evident that L-carnitine supplementation facilitated the formation of disease specific acylcarnitine conjugates, we hypothesise that the homozygous c.[1067G>A];c.[1067G>A] and compound heterozygous c.[976G>C];c.[1067G>A] genotypes result in an ETFQO protein of which the folding cannot be sufficiently rescued/stabilised by Rb treatment.

3.5.2.2 Moderate, variably Rb-responsive MADD patient group

The second group included eight patients who presented with moderate, heterogeneous phenotypes, all showing a varying response to treatment. The onset of symptoms was observed in the neonatal period (P1), infancy (P2, P7, P9 and P10), as well as childhood (P3, P11 and P13), and all patients displayed the characteristic clinical features of MADD. These included metabolic decompensation (n = 5), muscle weakness (n = 4), muscle pain (n = 3), hypotonia (n = 5), neck flexor weakness (n = 5), susceptibility to fatigue (n = 2), restrictive ventilatory defect (n = 1), gastrointestinal involvement (n = 6), elevated creatine kinase (CK) (n = 2), recurrent infections (n = 2), lethargy (n = 2), cognitive disability (n = 2), delayed gross motor development (n = 2), migraine/paroxysmal headache (n = 3), seizures (n = 2), coma (n = 3), skeletal involvement (n = 2), and liver dysfunction (n = 4). Uncommon symptoms included ketosis at the time of metabolic crisis (n = 5) and Beevor's sign (n = 1). Owing to the availability of data, the baseline urine organic acids of only five of the eight patients are reported, and the data of P7 represent the urinary organic acids present upon considerable decompensation near the time of demise.[10] At first presentation, patients P1, P7 and P9–P11 displayed an increase (to a variable extent) in the diagnostic urine organic acid markers

[10] P2 and P3 (siblings) were diagnosed prior to 1995 (before the storage of electronic records at the contributing diagnostic facility) and did not decompensate following the onset of treatment. Although P7 was diagnosed at the same time, the patient decompensated considerably near the time of her demise. The urinary organic acid data collected during this period of decompensation, while the patient was on Rb and L-carnitine, are reported.

associated with MADD, albeit less pronounced than that of P6, P8 and P12. Most of these patients had increased concentrations of urinary glutaric acid, ethylmalonic acid, dicarboxylic acids, and 2-hydroxyglutaric acid. The excretion of *N*-hexanoylglycine and, to a variable extent, branched-chain-related glycine conjugates were mostly observed. Moreover, all eight patients displayed increased disease-associated urinary acylcarnitines and elevated sarcosine. The biomarker assessment correlated with previous observations in moderate cases where FAO seems to be initially/mostly affected and branched-chain amino acid catabolism is influenced to a lesser extent (van Rijt *et al.,* 2019). It is important to note that the metabolic profiling was greatly dependent on the time of sample collection and that P3 and P9 had received L-carnitine treatment from an early stage in their lives due to the prior diagnosis of a sibling with MADD. Apart from P7, the clinical symptoms, together with most of the urine metabolites, improved upon dietary adjustment in combination with treatment with L-carnitine (P9 and P10), L-carnitine and Rb (P2, P3, P11 and P13), or L-carnitine, Rb, and coenzyme Q10 (P1). Carnitine conjugation indicated that accumulating acyl-CoAs were being detoxified via the carnitine transportation system, which likely explains the less prominent MADD organic acid signature observed. By contrast, the metabolic response to treatment of P7—who succumbed to a stroke at the age of 23 years—was more comparable to that of severe MADD, a finding that correlated with the severity of the clinical presentation as summarised in Table 3.1 and Supplementary Material 1 (Appendix B.2).

WES and segregation analysis by Sanger sequencing revealed four compound heterozygous variants in this group of White SA-ethnicity patients. These included: (i) c.[740G>T];c.[1448C>T] (p.[Gly247Val];p.[Pro483Leu]) in P13, (ii) c.[287dupA*];c.[1448C>T] (p.[Asp97Glyfs*24]; p.[Pro483Leu]) in siblings P2 and P3, and (iii) c.[1067G>A];c.[1448C>T] (p.[Gly356Glu];p.[Pro483Leu]) in P1, P7, P9, P10, and P11. The novel c.[287dupA*] variant affects the third exon of *ETFDH*, leading to a premature stop codon, and is classified as likely pathogenic according to the ACMG criteria. The c.[740G>T] variant shares the same classification as c.[287dupA*], and has been reported only once before as a compound heterozygous variant along with the likely pathogenic c.[389A>T] (p.[Asp130Val]) variant in a late-onset MADD case of Chinese ethnicity (Hong *et al.,* 2019). All variants identified in this group encode for highly conserved amino acids.

Once again, the MADD-DS3 scores confirmed that the disease burden increases when the c.[1067G>A] variant is present. This finding is corroborated by the level of steady state ETFQO protein in skin fibroblasts of P5 (c.[1448C>T];c.[1448C>T]) and P9 (c.[1067G>A];c.[1448C>T]), which show a 55% and 73% decrease, respectively, in comparison to fibroblasts from healthy controls (Supplementary Material 3, Appendix B.4). Similarly, van der Westhuizen *et al.* (2018) reported an 83% decrease in the steady state level of ETFQO in muscle from a patient with a compound heterozygous c.[1067G>A];c.[1448C>T] genotype, when compared to a healthy control. By contrast to the severe, Rb-unresponsive MADD patient group, four of the five patients affected by the c.[1067G>A] variant (P9, P10, P11 and P1) in this group

were found to be very amenable to treatment. It is, therefore, reasonable to conclude that when c.[1067G>A] is encountered as a compound heterozygous variant along with a variant which affects a different protein domain of ETFQO (e.g., c.[1448C>T]), sufficient enzymatic compensation occurs which may allow for adequate ETFQO activity. Based on the onset of disease, a diagnosis of either MADD type II (P1) or type III (P2, P3, P7, P9, P10, P11 and P14) was given to the patients in this group.

3.5.2.3 Mild, Rb-responsive MADD patient group

The final group included three patients (P4, P5 and P14) of White SA ethnicity, who presented later in life with mild and non-progressive (treatment-related) phenotypes. Clinically, their symptoms were heterogenous, but to some extent characteristic of MADD. The disease presentation included metabolic decompensation (n = 2), muscle weakness (n = 3), neck flexor weakness (n = 2), gastrointestinal involvement as the disease progressed (n = 1), elevated CK (n = 2), lethargy (n = 1), encephalopathy (n = 1), cerebral white matter abnormalities (n = 1), and liver dysfunction (n = 1), with two patients exhibiting ketosis. Initially, statin-induced myositis was suspected in P5 until hepatic features, including lipid deposits on the liver biopsy and raised transaminases prompted a metabolic work-up. P5 and P14 displayed urine metabolites associated with MADD, including dicarboxylic aciduria, raised 2-hydroxyglutaric acid (P5), acylglycine conjugates (with less prominent branched-chain-related conjugates), short- and medium-chain acylcarnitines as well as the presence of sarcosine on the amino acid profile. As indicated earlier in the moderate MADD group, FAO tends to be more affected compared to the branched-chain amino acid catabolism in the less severe cases (van Rijt *et al.,* 2019), which correlated with our findings. Literature shows that plasma/serum acylcarnitine profiling is typically most informative when diagnosing a late-onset MADD case, as organic acid profiling may only be remarkable at the time of a metabolic crisis or catabolic status induced by fasting. However, acylcarnitine assessments have been inconclusive in some cases, particularly if the patient has insufficient free carnitine available to promote conjugation (Grünert, 2014; Ruiz-Sala & Peña-Quintana, 2021). The latter has also led to false negative NBS results in mild MADD cases, as reported by Lin *et al.* (2021). In our study, P14 showed a free carnitine concentration below the limit of detection and normal butyryl/isobutyryl-, isovaleryl- and glutarylcarnitine levels in plasma/serum (results not shown). Interestingly, this patient presented with severe ketosis, as well as a prominent increase in 2-hydroxyglutaric acid and *cis*-4-decenedioic acid with unremarkable increases in glutaric acid and ethylmalonic acid. These inconsistent urinary organic acid findings corroborate the observations of Goodman *et al.* (1980) and support their suggestion to discontinue the use of the term "Glutaric aciduria type II," as it may be diagnostically misleading. In addition, our data indicate that mild cases of MADD may be missed or incorrectly diagnosed (e.g., as a different fatty acid oxidation disorder) on a biochemical level, depending on the metabolic (i.e., anabolic/catabolic status due to the time of sample collection) and systemic free carnitine status of the patient. Consequently, this study emphasises the metabolite variation

within this disease group and advocates for repeat testing if the clinical presentation and routine chemistry are suggestive of MADD.

Following dietary adjustment and treatment with L-carnitine, Rb and coenzyme Q10 (P5 and P14), the clinical and biochemical aberrations of P14 essentially normalised. P4, however, presented with only increased ethylmalonic acid. This patient, the mother of P2 and P3, underwent metabolic testing following the children's diagnosis and was on L-carnitine treatment at the time of sample collection. Clinical–biochemical improvements, specifically after therapeutic intervention, have been observed in several cases of late-onset MADD, and our clinical and metabolic results (although only three cases were included) strongly correlate with previous investigations (Grünert *et al., 2014;* Gempel *et al.,* 2007).

Genetic analyses revealed that all three patients had a homozygous genotype for the known pathogenic c.[1448C>T] (p.[Pro483Leu]) variant. This genotype (c.[1448C>T];c.[1448C>T]) has been reported in numerous other cases that, similarly to this group, presented with adult-onset, Rb-responsive MADD (Gempel *et al.,* 2007; van der Westhuizen *et al.,* 2018). The Rb-responsive nature of this variant is further corroborated in a study by Cornelius *et al.* (2012), in which it was shown that ETFQO activity in c.[1448C>T]-modified HEK293 cells could be restored from ~45 to ~85% (corresponding to an increase of ~50 to ~80% steady-state ETFQO) when moderately Rb-deficient cultures were treated with a saturating concentration of Rb. Therefore, considering the literature, together with the time of onset, severity and treatment response of this group, a diagnosis of MADD type III was given to P4, P5 and P14, despite moderate MADD-DS3 scores.

3.5.3 Allele frequency spectrum and haplotypes

To determine the allele frequency of the variants identified, PCR–RFLP analysis was used to screen the two most frequently occurring variants, c.[1067G>A] and c.[1448C>T], in the four largest population groups in SA. The study yielded no homozygous or heterozygous genotypes for any of the variants in any of the population groups assessed so that the allele frequencies of the population groups investigated were calculated as <0.00067% (African, White SA, and Indian ethnicity) or <0.00084% (mixed ethnicity) (Table 3.3). All five variants identified in the cohort were subsequently compared to gnomAD (v2.1.1) (gnomAD, 2023) and the Human Heredity and Health in Africa (H3Africa) project (H3Africa, 2023). Of these variants, only two were recorded on gnomAD, displaying exome allele frequencies of <0.0001% (c.[740G>T] and c.[1448C>T]); hitherto, none of the variants has been identified in the H3Africa data. The absence of the variants in the above-mentioned population databases, together with the frequency at which they were identified in the SA cohort, indicates with high probability the pathogenicity of these five variants and supports their causative role in MADD.

Haplotyping was performed on those patients from whom sufficient DNA could be obtained. Upon recruitment, patients and their families were invited to self-report their ethnicity and region of birth. Consequently, haplotyping was performed on ten patients of White SA ethnicity (P1–P6, P9–P11 and P13) and one patient of mixed ethnicity (P8) born in various geographical regions across SA (including the central, north-eastern, north-western, south-eastern, and south-western provinces).

Apart from the siblings, P2 and P3, and their mother, P4, all patients were found to be unrelated down to the second degree. DNA samples from those patients harbouring the c.[1067G>A] variant (P1, P6, P8 and P9–P11), displayed variable lengths of a shared haplotype on one allele, with a minimal overlapping region of 7.2 Mb (Figure 3.1). Of these samples, the two with a homozygous c.[1067G>A];c.[1067G>A] genotype (P6 and P8) exhibited homozygosity for the shared haplotype in the region of *ETFDH*.

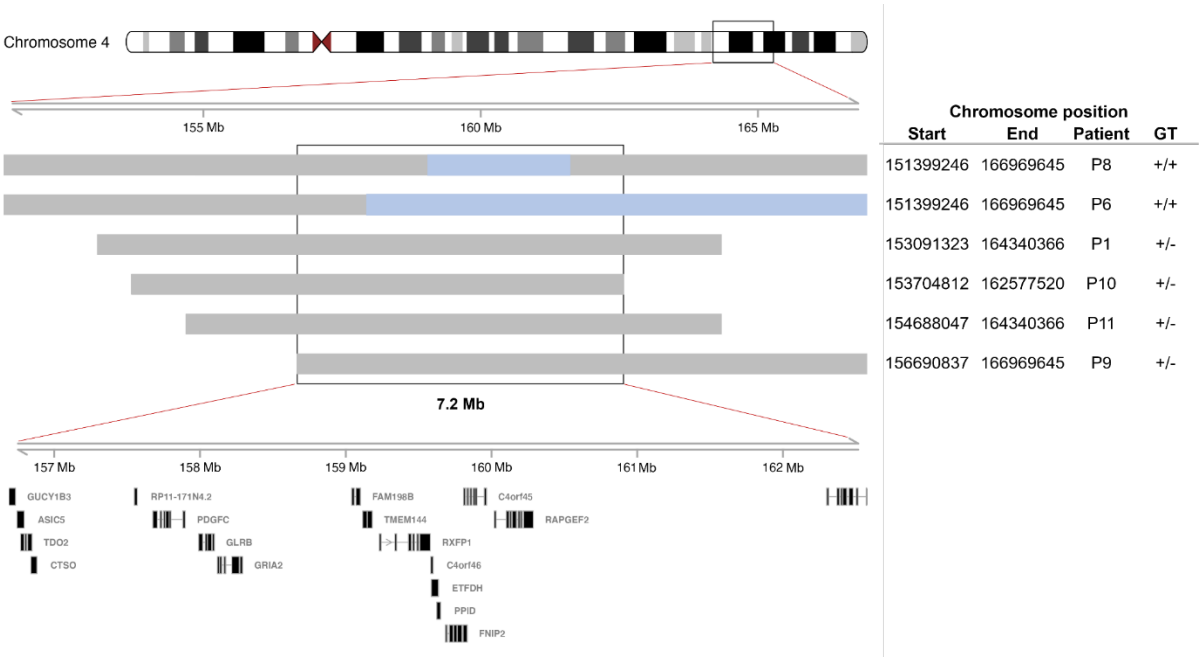


Figure 3.1: Shared haplotype of patients affected by *ETFDH* c.[1067G>A]

All samples listed show variable lengths of a shared haplotype on a single allele (grey) with a minimal overlapping region of 7.2 Mb, and samples P6 and P8 exhibit homozygosity (c.[1067G>A];c.[1067G>A]) for the shared haplotype (blue). The shared region includes the genes listed. These results indicate a founder for the c.[1067G>A] variant in the White SA population. Abbreviations: *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; GT: genotype.

Similar results were obtained for the DNA samples of those patients harbouring the c.[1448C>T] variant [P1, P4 (including P2 and P3 due to their first-degree relation), P5, P9–P11 and P13], with a minimal overlapping region of 4 Mb (Figure 3.2). Again, the two patients who had a homozygous c.[1448C>T];c.[1448C>T] genotype (P4 and P5), displayed homozygosity for the shared haplotype in the *ETFDH* region. These findings suggest the presence of two separate founder haplotypes on which the c.[1067G>A] and c.[1448C>T] variants arose, with that of the former in the White SA population. However, without access to additional control data from the same population(s), it is currently not possible to estimate the haplotype frequency with confidence.

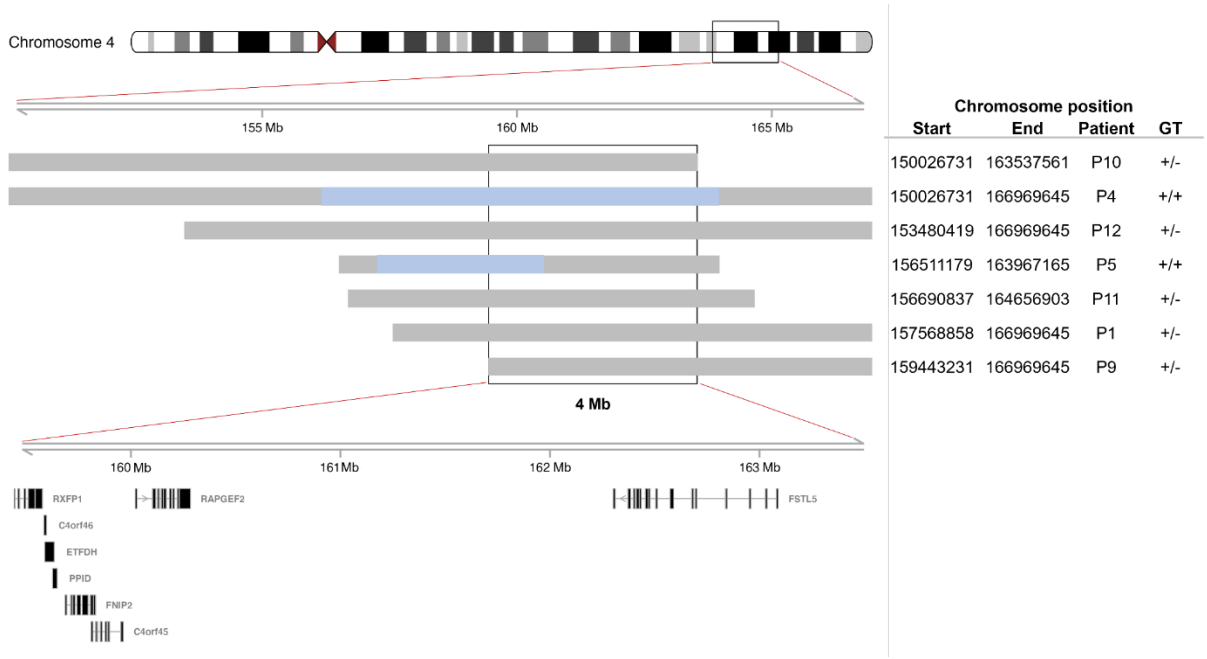


Figure 3.2: Shared haplotype of patients affected by *ETFDH* c.[1448C>T]

All samples listed show variable lengths of a shared haplotype on a single allele (grey) with a minimal overlapping region of 4 Mb, and samples P4 and P5 exhibit homozygosity (c.[1448C>T];c.[1448C>T]) for the shared haplotype (blue). The shared region includes the genes listed. These results indicate a founder for the c.[1448C>T] variant. Abbreviations: *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; GT: genotype.

3.6 CONCLUSION

This study provides the first extensive clinical and biochemical profiles, along with the genetic aetiology, of MADD in the diverse and understudied SA population. Within the cohort investigated, we describe one novel (c.[287dupA*]) and four previously identified causal variants in *ETFDH*, with an autosomal

recessive inheritance pattern. In addition, the data support the suspicion that MADD is more prevalent in the White population of SA (van der Westhuizen *et al.,* 2018). We also demonstrate a distinct shared haplotype for each of the two most common variants (c.[1067G>A] and c.[1448C>T]), suggesting the existence of a founder for each, with that of c.[1067G>A] likely having arisen in the White SA population. Furthermore, we show that, depending on the variant(s) involved, it is possible to anticipate the clinical progression and treatment response of the patient, which underscores the need for genetic confirmation in SA. It is our belief, that this genotype–phenotype correlation of MADD in the SA population will assist physicians—who rarely encounter this disorder—to recognise and treat MADD in a more efficient manner. In addition, our study provides background for the subsequent genetic counselling of families and patient-specific treatment of local MADD cases. Altogether, the data reported support a policy of including MADD in the NBS program of SA.

3.7 FINAL ALLELE FREQUENCY SPECTRUM

To improve the statistical power (see Appendix C.5) of the allele frequency estimation of the *ETFDH* variants c.[1067G>A] and c.[1448C>T], additional available samples collected from newborns born to the southern populations of SA were assessed by PCR–RFLP analysis, as described in Section 3.4.4. These samples included DNA isolated from umbilical cord DBSs of healthy individuals of African (258 samples screened for c.[1067G>A] and 729 samples screened for c.[1448C>T]) and mixed ethnicity (481 samples screened for c.[1067G>A] and 735 samples screened for c.[1448C>T]) (Meldau *et al.,* 2018). Within these groups no c.[1067G>A] or c.[1448C>T] heterozygotes or homozygotes, were detected. By combining these findings with those reported in Section 3.5.3, the final allele frequency of c.[1067G>A] was estimated as <0.00050 in the African and <0.00047 in the mixed population groups, whereas that of the c.[1448C>T] variant was estimated as <0.00034 in the African and <0.00038 in the mixed population groups (calculated as explained in Section C.5.2). Taken together, these estimations predict that the c.[1067G>A] and c.[1448C>T] variants will occur in <5 African and <1 White SA, Indian/Asian, or mixed ethnicity newborns per annum, respectively[11].

[11] Calculated from the number of births per annum (1,175,776) and percentage distribution of African (81.4%), mixed (8.2%), White (7.3%), and Indian/Asian (2.7%) population groups in SA (Statistics South Africa, 2023).

Chapter 4

Functional evaluation of MADD in patient fibroblasts

4.1 CHAPTER INTRODUCTION

This chapter addresses the second aim of this study; namely, to evaluate the functional properties of selected pathogenic variants frequently identified in the cohort via an *in vitro* approach. To achieve this, primary dermal fibroblasts, established from patient skin biopsies, were utilised. This cell type has been used for decades to evaluate and aid the diagnosis of FAO disorders, and more specifically, MADD (Frerman & Goodman, 1985; Manning *et al*., 1990). Functional studies in dermal fibroblasts, which express ETFQO, have been shown to correlate with the phenotype and disease severity of MADD in both neonatal and adult patients and can be used to study various aspects of the disorder, including protein expression (Cornelius *et al*., 2013; van Rijt *et al.,* 2019). In addition, patient fibroblasts offer a genotype-matched tool to analyse mitochondrial respiration (Zdrazilova *et al*., 2022).

In this chapter, the materials and methods required to accomplish Objectives 2.1–2.3, are first described. This entails an overview of the ethics and patient consent, the establishment and propagation of primary patient fibroblasts, and the genotype-validation thereof (Objective 2.1). This is followed by a description of the functional analyses conducted on the patient fibroblasts, including the evaluation of the steady state levels of ETFQO (Objective 2.2.a) and mitochondrial RC CI–CV (Objective 2.2.b), as well as the measurement of mitochondrial respiration (Objective 2.3). The results obtained are then reported and discussed (Objectives 2.1–2.3), after which the chapter concludes with a summary of the findings. Supplementary information may be found in Appendix C.1 and C.2.

4.2 MATERIALS AND METHODS

4.2.1 Ethics approval and patient consent/assent

The analyses described in this chapter were conducted in accordance with the approved ethical application NWU-00966-19-A1-01 [Health Research Ethics Committee (NWU-HREC) of the Faculty of Health Sciences, NWU] shown in Appendix C.1. Patient recruitment and informed consent were performed as described in Appendix C.2.

4.2.2 Primary patient fibroblasts

4.2.2.1 Sample collection

Primary dermal fibroblasts were obtained from skin biopsies, collected over a period of three months. For this study, we attempted to collect as many skin biopsies as possible from the participating patients.

However, due to the logistical challenges associated with this process, including the remote locations of the patients and the unwillingness of parents to consent to an additional invasive procedure on paediatric patients, we were only able to obtain three biopsies. Fortunately, these cases included the predominantly observed genotypes in this study. In addition, three healthy Southern African controls (who were unrelated to and not part of the study cohort, did not have a diagnosis of an IEM, did not present with any symptoms of MADD, and were matched as closely as possible to the patients in terms of age, gender, and ethnicity) were also recruited (see Table 4.1).

Table 4.1: Patients and controls from whom skin biopsies (fibroblasts) were collected

Patient/control	*ETFDH* genotype	Gender	Age at collection (Feb–May 2023)	Ethnicity
P3	c.[287dupA*];c.[1448C>T]	Male	Y28	White
P5	c.[1448C>T];c.[1448C>T]	Female	Y42	White
P9	c.[1067G>A];c.[1448C>T]	Male	Y19	White
C1	n.a	Male	Y22	White
C2	n.a	Female	Y34	White
C3	n.a	Male	Y22	White

Abbreviations: C: control; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; Feb: February; n.a: not applicable; Y: age in years.

Biopsies of 3 mm diameter × 4 mm depth were excised aseptically from the forearm of each individual by trained medical professionals and stored in 1× Dulbecco's Modified Eagle Medium with GlutaMAX™ (DMEM; 31966-021, Gibco™, Thermo Scientific™, Massachusetts, USA) containing 1% (v/v) penicillin:streptomycin (P/S; 15140-122, Gibco™, Thermo Scientific™) for the duration (1–4 h) of sample transport. The process involved numbing the excision site with lidocaine-prilocaine (EMLA® cream 5%; 826367038, Aspen, Durban, SA), disinfecting the site with 70% ethanol (prepared in Milli-Q® water[12]), allowing the site to dry, and extracting the biopsy with a disposable biopsy punch (BPP-30F, Kai Medical, Seki-shi, Gifu Prefecture, JP), surgical forceps, and -scalpel. Following sample collection, the area was disinfected and the wound was covered with Granuflex® extra thin wound dressing (S150, Convatec, Reading, UK) to heal.

[12] Milli-Q® water refers to water that has been purified by the Milli-Q® Ultrapure water purification system (ZIQ7000T0C, Merck, Darmstadt, DE).

4.2.2.2 Establishing primary fibroblasts

Skin biopsies were transported to, and established by, Lancet Laboratories (Mayo Clinic, Roodepoort, Johannesburg, SA) according to an in-house dissociated fibroblast culture method: Using a sterile scalpel, each biopsy was dissected into four 7 mm^3 pieces in a petri dish containing 1 mL of a 1:6 solution of 0.5% (v/v) trypsin-ethylenediaminetetraacetic acid (EDTA) (15400-054, Gibco™, Thermo Scientific™):Ham's F-12 culture medium (11765054, Gibco™, Thermo Scientific™). The pieces were digested by incubation at 37°C for 1 h in a polypropylene tube containing 5 mL of pre-warmed[13] 1:6 0.5% (v/v) trypsin-EDTA:Ham's F-12 medium, and pelleted by centrifugation (2000 rpm, 10 min)[14]. The pellet was then digested for an additional 30 min at 37°C in 5 mL of pre-warmed, filter-sterilised 0.027% (w/v) collagenase type II (C1764, Sigma-Aldrich®, Missouri, USA) prepared in Ham's F-12 culture medium. Next, the digested biopsy was pelleted as before, resuspended in 3.5 mL of Ham's F-12 culture medium containing 20% (v/v) foetal bovine serum (FBS; 10499-044, Gibco™, Thermo Scientific™), and allowed to proliferate undisturbed for eight days in a 25 cm^2 tissue culture flask at standard culturing conditions[15].

Following this period, 2 mL of additional fresh culture medium was added to the established dermal fibroblasts, which exhibited the characteristic fusiform morphology (Takashima, 1998). Depending on cell growth, the culture medium was replaced every 48 h or 72 h (5 mL total) and cells were subcultured 1:2 upon reaching 80% confluency, by scraping. After recovery and growth to 80% confluency, primary fibroblast samples were transported to the NWU where the culture medium was switched to 5 mL recovery medium [1× DMEM with GlutaMAX™ containing 20% (v/v) FBS and 1% P/S] per 25 cm^2 tissue culture flask. To obtain multiple aliquots of low-passage number samples (passage 2–4), cells were subcultured 1:2 every 72 h (doubling time) by trypsinisation according to an established standard operating procedure (SOP). To this end, 1× phosphate buffered saline (PBS; 18912-014, Gibco™, Thermo Scientific™) prepared in autoclaved Milli-Q® water and 1× trypsin-EDTA [0.05% (w/v)] prepared in 1× PBS, were used. Aliquots of 90% confluent cells were cryopreserved at -80°C in recovery medium containing 5% (v/v) dimethyl sulfoxide (DMSO; D2650, Sigma-Aldrich®), according to an established SOP.

[13] Throughout this study, pre-warmed solutions were heated to 37°C, unless otherwise stated.

[14] Throughout this study, all centrifugation steps were carried out at 22°C, unless otherwise stated.

[15] Incubation at 37°C in a humidified atmosphere with 5% CO_2.

4.2.2.3 Culturing primary fibroblasts for analyses

In preparation for each assay, cell lines of similar passage number were restarted, either at once (steady state protein assays), or one at a time (respirometry assays) in 25 cm^2 tissue culture flasks containing 5 mL recovery medium, according to an established SOP. Following a recovery period of 48 h, cells were trypsinised, resuspended in complete growth medium [1× DMEM with GlutaMAX™ containing 10% (v/v) FBS and 1% (v/v) P/S], and counted via the Muse® Cell Analyzer (0500-3115, Millipore™, Luminex, Texas, USA), using Guava® ICF instrument cleaning fluid (4200-0140), the Muse® System check kit (MCH100101), and Guava® ViaCount™ reagent (4000-0041, Luminex) as per the manufacturer's instructions. To avoid variability due to seeding density-related differences, equal numbers of cells were transferred to 75 cm^2 tissue culture flasks (15 mL media) and allowed to proliferate. Upon reaching a confluency of 90% (five days), cells were harvested by trypsinisation, pelleted by centrifugation (500 ×*g*, 5 min), and processed as required by each assay. For genotype-validation, cells were harvested by trypsinisation following the 48 h recovery period.

4.2.3 Genotype validation of patient fibroblasts

Patient fibroblasts were cultured to passage three to five, harvested, and pelleted as described in Section 4.2.2.3. DNA was isolated from the resulting pellet using the *Quick*-DNA™ Miniprep Plus kit (D4069, Zymo Research, Inqaba Biotec, Pretoria, SA) according to the manufacturer's instructions. To confirm the *ETFDH* genotype of each patient's fibroblasts, sequence validation was performed by Sanger sequencing. To achieve this, 25 ng of each cell line's DNA was amplified by polymerase-chain reaction (PCR) using 1× Phire HS II Master Mix (F170L, Thermo Scientific™), 0.5 µM of the corresponding forward and reverse primers (listed in Table 4.2), and nuclease-free water (total volume 25 µL). PCR amplification was performed on a T100™ thermal cycler (1861096, Bio-Rad, California, USA) as follows: Initial denaturation at 98°C for 5 min, followed by 34 cycles of denaturation at 98°C for 5 s, annealing at the respective annealing temperature (T_a) of each primer pair for 5 s and extension at 72°C for 20 s, with a final extension step at 72°C for 1 min.

Successful amplification of each sample was confirmed by electrophoresis (at 4 V/cm) of the amplicons and a molecular weight marker (GeneRuler™ 50 bp DNA ladder; SM0371, Thermo Scientific™) on a 2% (w/v) agarose gel (Agarose E; 8100; Conda, Separations, Johannesburg, SA) prepared with 1× Bionic™ Buffer (B6185, Sigma-Aldrich®) and containing 5 µg/mL ethidium bromide. The amplicons were visualised by ultraviolet (UV) detection, using the ChemiDoc™ MP system (17001402, Bio-Rad) and Image Lab software (v6.1). PCR-products were then sent to Central Analytic Facilities (CAF, Stellenbosch, SA) for post-PCR cleanup and Sanger sequencing of the forward (5'–3') and reverse (3'–5') DNA strands of the amplicons, as described in Appendix C.4.

Table 4.2: Primer pairs flanking the variants detected in the MADD cohort.

ETFDH variant(s) flanked	Primer type	Primer sequence (5'–3')	Primer size (nt)	Amplicon size (bp)	T_a (°C)
c.[287dupA*]	Forward	CCT ATA CCC TGT TGC CTA	18	731	52
	Reverse	GGG TTT CCT ATA TTC CAG T	19		
c.[740G>T]	Forward	TGC GTA AGC TGG AAA CAG TGA	21	643	49
	Reverse	CTA GCC CCT TGA AAA ATA TCG C	22		
c.[976G>C] and c.[1067G>A]	Forward	GCA CAT AGT GCT CCA AAT AC	20	580	57
	Reverse	CAT GCC TGG CTA ATC TTT CC	20		
c.[1448C>T]	Forward	CAC ACA TTT GGG CAG TTT	18	568	57
	Reverse	AAA CTG ATC TGT CCA TCG G	19		

The *ETFDH* c.[740G>T] variant was not expected to be present in the fibroblasts and was thus not screened for. In this instance, the primer pair was used for Sanger validation and segregation analysis of patient whole blood DNA (Appendix C.4). Units and abbreviations: bp: base pairs; DNA: deoxyribonucleic acid; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; nt: nucleotides; T_a: annealing temperature.

4.2.4 Evaluation of steady state ETFQO and mitochondrial RC enzyme levels

4.2.4.1 Sample preparation

Patient and control primary fibroblasts were cultured to passage eight, harvested, and pelleted as described in Section 4.2.2.3. The pellet was washed twice in 1× PBS to remove residual medium and frozen at -20°C until use. On the day of analysis, each pellet was thawed and lysed by incubation at 0°C for 20 min in radioimmunoprecipitation assay (RIPA) buffer (R0278, Sigma-Aldrich®) containing 1× cOmplete™, Mini, EDTA-free protease inhibitor cocktail (11836170001, Roche, Sigma-Aldrich®). The whole cell lysate was sonicated at 0°C via three bursts of 5 s, interspersed with 30 s resting periods, and the supernatant was collected by centrifugation (13,000 ×*g*, 20 min, 4°C). Hereafter, the protein concentration was determined via the bicinchoninic acid (BCA) method as described by Smith *et al.* (1985), using BCA (B9643, Sigma-Aldrich®), $CuSO_4 \cdot 5H_2O$ (C2284, Sigma-Aldrich®), and a standard protein curve prepared from bovine serum albumin (BSA, P0914, Sigma-Aldrich®). Absorbance was detected at 562 nm using the Synergy™ HT Multi-detection microtiter plate reader (7091000, BioTek® Instruments, Vermont, USA) and protein concentrations were calculated by the Gen5™ Data Analysis software (v1.11.5).

4.2.4.2 SDS-PAGE and Western blot analyses

The steady state levels of ETFQO and mitochondrial RC CI–CV were determined in the whole cell lysates of C1, C2, C3, P3, P5, and P9 via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses (Laemmli, 1970). All steps were carried out as per the manufacturer's

instructions: Samples containing 50 µg of protein were prepared in 1× Laemmli Sample Buffer (1610737, Bio-Rad), 355 mM β-mercaptoethanol (M-3148, Sigma-Aldrich®) and Milli-Q® water, and denatured at 100°C for 5 min[16]. The protein bands and molecular weight marker (Precision Plus Protein™ Dual Xtra Prestained Protein Standards; 1610377, Bio-Rad) were loaded on a 1 mm 10% TGX Stain-Free™ FastCast™ acrylamide gel[17] (1610183, Bio-Rad), and separated by electrophoresis in 1× Tris/glycine/sodium dodecyl sulfate (SDS) running buffer (1610732, Bio-Rad). Electrophoresis proceeded at 40 V/cm for 22 min using the Mini-PROTEAN® Tetra Cell (1658004; Bio-Rad).

Next, the protein bands of each gel were transferred, using a semi-dry method, to a 0.2 µm polyvinylidene difluoride membrane (Trans-Blot® Turbo™ Mini PVDF Transfer Pack; 1704156, Bio-Rad) via the Trans-Blot® Turbo™ Blotting System (1704155, Bio-Rad), using the Standard SD protocol (1.0 A and 25 V). The total protein content of each blot was then visualised by 1 min of UV light exposure, using the ChemiDoc™ MP system and Stain Free Blot protocol (Image Lab software), and both blots were incubated[18] in blocking buffer [1× tris buffered saline (TBS) with 1% (w/v) casein; 1610782, Bio-Rad)] for 1.5 h. To detect the proteins of interest, the blots were incubated overnight at 4°C in immunodetection solvent [1× TBS with 1% (w/v) casein] containing the following primary antibodies: Mouse monoclonal anti-ETFQO antibody (1:1,000; ab131376, Abcam, Cambridge, UK) or mouse monoclonal Total OXPHOS rodent WB antibody cocktail (1:250; ab110413, Abcam) reactive against human. The ETFQO-immunoblotted membrane was further incubated overnight at 4°C in immunodetection solvent containing the mouse monoclonal β-actin primary antibody (1:1,000; ab6276, Abcam) reactive against human, in order to visualise the housekeeping protein, β-actin[19].

On the following day, each blot was washed three times for 5 min in 1× TBS (1706435, Bio-Rad) with 0.001% (v/v) Tween®20 (1610781, Bio-Rad). Hereafter, both blots were incubated for 1.5 h in immunodetection solvent containing goat polyclonal horseradish peroxidase (HRP)-conjugated secondary antibody, reactive against mouse (1:10,000; ab97023, Abcam). Both blots were washed as before, followed by a final wash step of 5 min in 1× TBS. To detect the proteins of interest, each blot was incubated in Clarity™ Western Enhanced Chemiluminescence (ECL) Substrate (1705060, Bio-Rad) for 5 min and visualised using the ChemiDoc™ MP system and Chemi Hi Resolution protocol (Image Lab software). In

[16] In an attempt to detect the CIV subunit, MTCO1, repeat analyses were performed with samples denatured at 50°C for 5 min (results not shown) (Mitomap, 2023).

[17] Each gel was prepared by the addition of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; T7024, Sigma-Aldrich®) and ammonium persulfate (APS; A3678, Sigma-Aldrich®) as recommended by the manufacturer.

[18] All incubation steps for blocking, immunoblotting, and washing were performed by gentle agitation on a waving platform shaker at 26 rpm.

[19] This step was performed following visualisation of ETFQO and all following steps were repeated.

addition, the colorimetric protocol was used to image the molecular weight marker. Data processing entailed subtracting the background noise from the signal of the total protein content and bands of interest. The volume intensity of each band of interest was then normalised to that of the corresponding lane's total protein content and expressed as a percentage relative to the mean volume intensity of C1 and C2 (ETFQO) or C1 and C3 (CI–CV). The size of each band was determined relative to the molecular weight marker, using the Image Lab software. For each assay, one replicate per sample was analysed and consequently, no statistical analyses could be performed. The trends observed are given and discussed in Section 4.3.2 (ETFQO) and 4.3.3 (CI–CV).

4.2.5 Evaluation of respiration in permeabilised fibroblasts

4.2.5.1 Sample preparation

The experimental approach for this method, which allows two samples to be analysed per run, was to analyse one control- (chamber A) and one patient cell line (chamber B) per day. Given that short-term differences in cell passage are reported not to affect OXPHOS in dermal fibroblasts, cells were cultured to passage two, three, or four on the respective day of analysis, and harvested and pelleted as described in Section 4.2.2.3 (Ye & Hoppel, 2013). The pellet was immediately resuspended in preheated substrate-limited mitochondrial respiration (MiR05) medium, prepared[20] as described in Gnaiger *et al.* (2000). Hereafter, cells were counted via the Muse® Cell Analyzer according to the manufacturer's instructions, and 1×10^6 cells/mL were prepared in preheated MiR05 medium.

4.2.5.2 Analysis of the respiration in permeabilised cells

Respiration was measured using the Oroboros Oxygraph-2k (O2k) system (10022-02) and DatLab (v7.4.0.4) software (Oroboros, Innsbruck, AT). To allow specific compounds to access the mitochondrion, it was necessary to selectively permeabilise the plasma membrane by the addition of digitonin. To ensure that the outer mitochondrial membrane was not damaged by this action, the concentration of digitonin was

[20] MiR05 medium consists of 5 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N,N*-tetraacetic acid (EGTA; E4378), 3 mM $MgCl_2 \cdot 6H_2O$ (M2670), 60 mM lactobionic acid (153516), 20 mM taurine (T0625), 10 mM potassium phosphate monobasic (P5655), 20 mM HEPES (H7523), 100 mM D-sucrose (84097), and 1 g/L (w/v) BSA (fatty acid-free; A6003, Sigma-Aldrich®) dissolved in sterile Milli-Q® water (Gnaiger *et al.,* 2000).

first optimised[21] in preparation for the specific Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol (results not shown).

On the day of each assay, both chambers of the Oroboros O2k were cleaned and the polarographic oxygen sensors were air-calibrated as specified by the manufacturer[22]. The respiration assay (as described below) was then performed, after which the chambers were once again cleaned[23] and incubated with 70% ethanol prepared in Milli-Q® water. The respiration of each cell line was assessed using the SUIT-002 O2 ce-pce D007 protocol. This protocol provides a means to measure respiratory control in permeabilised cells by specifically targeting the F-pathway in the OXPHOS state[24], during which FAO (in the presence of anaplerotic pathways) is not overestimated (Bioblast, 2023). In addition, this protocol provides information on the FN-, FNS-, and FNSGp-pathways[25] in the OXPHOS state, as well as the FNSGp-pathway in the electron transfer state[26]. A description and visualisation of each pathway may be found in Table 4.3 and Figure 4.1, respectively (Bioblast, 2023).

Table 4.3: Oroboros O2k terminology

Pathway	Junction†	Substrates	Description	SUIT-relevant inhibitor
F	Q, (N)	M.1, Oct	Oct supplies electrons, via the generation of $FADH_2$ by FAO dehydrogenases, to ETF, and subsequently, to ETFQO, CoQ10, CIII, and CIV. FAO simultaneously generates NADH and also depends on CI's electron throughput. Consequently, a non-saturating concentration of malate is required as co-substrate to prevent acetyl-CoA accumulation in the TCA cycle.	Rot, Ama
N	N, Q	M.2, P, G	Substrate(s) supplies electrons, via the generation of NADH by dehydrogenases, to CI, and subsequently, to CoQ10, CIII and CIV.	Rot, Ama
S	S, Q	S	Substrate supplies electrons, via the generation of $FADH_2$ by succinate dehydrogenase, to CII, and subsequently, to the CoQ10, CIII, and CIV.	Ama
Gp	Q	Gp	Substrate supplies electrons, via the generation of $FADH_2$ by glycerophosphate dehydrogenase, to CoQ10, and subsequently, to CIII and CIV.	Ama

See with Figure 4.1. † The N-, S- and Q-junctions refer to junctions for the convergent electron flow through the ETC as a result of substrates that fuel the N-pathway, S-pathway, and N-, S-, F- and Gp-pathways, respectively. Abbreviations: Ama: antimycin A; CoA: coenzyme A; CI–CIV: complex I–IV; CoQ10: coenzyme Q10; ETF: electron

[21] Protocol used: SUIT-010 O2 ce-pce D008 protocol (Bioblast, 2023).

[22] Protocols used: O2k-cleaning_BeforeUse and O2 calibration_air (Bioblast, 2023).

[23] Protocol used: O2k-cleaning_AfterUse (Bioblast, 2023).

[24] The OXPHOS state measures the respiratory capacity of mitochondria following activation by saturating concentrations of ADP and inorganic phosphate.

[25] FN-, FNS-, and FNSGp-pathways signify the effect of the sum of the individual pathways shown in Table 4.3.

[26] The electron transfer state measures the respiratory capacity of the ETC in uncoupled mitochondria.

transfer flavoprotein; ETFQO: ETF-ubiquinone oxidoreductase; $FADH_2$: reduced flavin adenine dinucleotide; FAO: mitochondrial fatty acid β-oxidation; G: glutamate; Gp: glycerophosphate; M.1: malate (non-saturating concentration); M.2: malate (saturating concentration); NADH: reduced nicotinamide adenine dinucleotide; Oct: octanoylcarnitine; P: pyruvate; RC: respiratory chain; Rot: rotenone; S: succinate; SUIT: Substrate-Uncoupler-Inhibitor Titration; TCA: tricarboxylic acid.

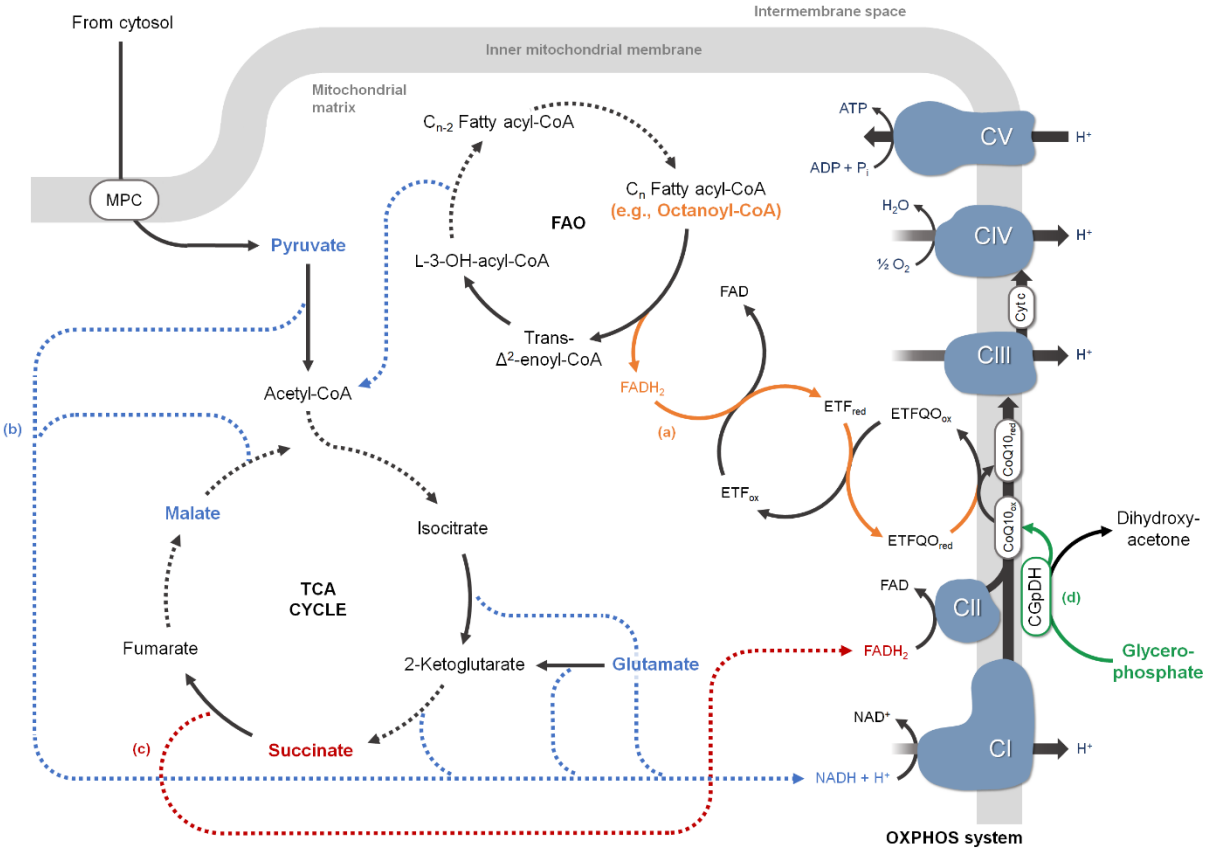


Figure 4.1: Respiration stimulated during the SUIT-002 O2 ce-pce D007 protocol

See with description of each pathway in Table 4.3. (a) F-pathway, (b) N-pathway, (c) S-pathway, and (d) Gp-pathway. Dashed lines indicate omitted metabolic steps. Abbreviations: ADP and ATP: adenosine diphosphate and -triphosphate (respectively); CGpDH: inner mitochondrial membrane flavoprotein-linked L-glycerol 3-phosphate dehydrogenase complex; CI–CV: mitochondrial respiratory chain complexes I–V; CoA: coenzyme A; CoQ10: coenzyme Q10; Cyt c: cytochrome c; ETF: electron transfer flavoprotein; ETFQO: ETF-ubiquinone oxidoreductase; FAD and $FADH_2$: flavin adenine dinucleotide (oxidised and reduced, respectively); FAO: mitochondrial fatty acid β-oxidation; MPC: mitochondrial pyruvate carrier; NAD^+ and NADH: nicotinamide adenine dinucleotide (oxidised and reduced, respectively); $_{ox}$: oxidised; OXPHOS: oxidative phosphorylation; P_i: inorganic phosphate; $_{red}$: reduced; SUIT: Substrate-Uncoupler-Inhibitor Titration; TCA: tricarboxylic acid.

To each chamber of the Oroboros O2k, 2 mL of the prewarmed cell suspension (1×10^6 cells/mL) was added. Compounds were injected in the order shown in Table 4.4 and the O_2 flux per volume [pmol/(s·mL)] was recorded. Each compound was injected following the stabilisation of O_2 flux per volume brought on by the previous injection. The O_2 flux per volume readings were processed by selecting a subset of data points (called a mark) within the stabilised region of the trace, obtained after each injection. The median of the mark was calculated and the baseline flux (i.e., non-mitochondrial respiration; the O_2 flux per volume obtained following the injection of antimycin A) was subtracted from the reading to obtain background-corrected (bc) specific flux. To normalise mitochondrial respiration, the specific flux (bc) values obtained before and after the injection of selected compounds were used to calculate the flux control efficiency (FCE) according to Equation 4.1:

$$\mathrm{FCE} = (Z_X\text{-}Y_X)/Z_X = 1\text{-}(Y_X/Z_X) \tag{4.1}$$

Table 4.4: Compounds injected during the SUIT-002 O2 ce-pce D007 protocol

Step	Compound	Cat. #†	Final conc	Purpose of injection
1.1	Dig	D5628	10 µg/mL	Selective permeabilisation of plasma membrane to allow entry of compounds to cell.
1.2	D	A5285	2.5 mM	Stimulates catabolism of endogenous substrates.
2	M.1	M1000	0.1 mM	Added to aid catabolism of Oct. Does not saturate N-pathway.
3.1	Oct	9358	0.5 mM	Stimulation of F-pathway in the presence of malate.
3.2	c	C7752	10 µM	Added to determine mitochondrial outer membrane damage. Damage leads to an increase in respiration. Slight increase in respiration can be inherent to cell line.
4	M.2	M1000	2 mM	Simultaneous stimulation of N-pathway (due to M.2) and F-pathway (due to Oct and M.1).
5	P	P2256	5 mM	Simultaneous stimulation of N-pathway (due to P and M.2) and F-pathway (due to Oct and M.1).
6	G	G1626	10 mM	Simultaneous stimulation of N-pathway (due to G, P, and M.2) and F-pathway (due to Oct and M.1).
7	S	S2378	10 mM	Simultaneous stimulation of S-pathway (due to S), N-pathway (due to G, P, and M.2) and F-pathway (due to Oct and M.1).
8	Gp	G7886	10 mM	Simultaneous stimulation of Gp-pathway (due to Gp), S-pathway (due to S), N-pathway (due to G, P, and M.2) and F-pathway (due to Oct and M.1).
9	U*	C2759	0.5 µM	Added in increments until maximum flux is obtained. Stimulation of N-, F-, S-, and Gp-pathway in uncoupled state. Used to test limitation of OXPHOS capacity relative to electron transport capacity.
10	Rot	R8875	0.5 µM	Inhibits CI and, thus, N- and F-pathways. S- and Gp-pathways in uncoupled state are still stimulated.
11	Ama	A8674	2.5 µM	Inhibits CIII and, thus, N-, F-, S-, and Gp-pathways. Used to obtain baseline flux.

† All products were purchased from Sigma-Aldrich®, except Oct (VUmc, Amsterdam, NL). * Denotes addition of multiple titrations. Abbreviations: Ama: antimycin A; c: cytochrome c; Cat. #: catalogue number; conc: concentration; D: adenosine triphosphate; Dig: digitonin; G: glutamate; Gp: glycerophosphate; M.1: malate (non-saturating concentration); M.2: malate (saturation concentration); Oct: octanoylcarnitine; OXPHOS: oxidative phosphorylation; P: pyruvate; Rot: rotenone; S: succinate, SUIT: Substrate-Uncoupler-Inhibitor Titration; U: carbonyl cyanide 3-chlorophenylhydrazone (CCCP).

FCE describes the change of respiration resulting from the injection of a metabolic control variable, X, and is calculated as the change in flux between Y_X and Z_X (i.e., before and after the injection of X, with Y_X denoting the smaller flux, and Z_X the larger), normalised against a reference flux, Z_X. To determine the effect of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on respiration, as well as potential damage to the outer mitochondrial membrane caused by the addition of digitonin, the coupling control efficiency (CCE) and cytochrome c control efficiency (Cyt c CE) were calculated, respectively. These calculations proceeded according to Equation 4.1, where Y_X is the specific flux (bc) value prior to the addition of CCCP or cytochrome c (Cyt c), and Z_X is the specific flux (bc) value after the addition. In this instance, it was possible to analyse two replicates of each cell line to get an estimation of repeatability. As only two replicates were analysed per cell line, statistical analyses could not be performed. Instead, the trends observed are given and discussed in Section 4.3.4.

4.3 RESULTS AND DISCUSSION

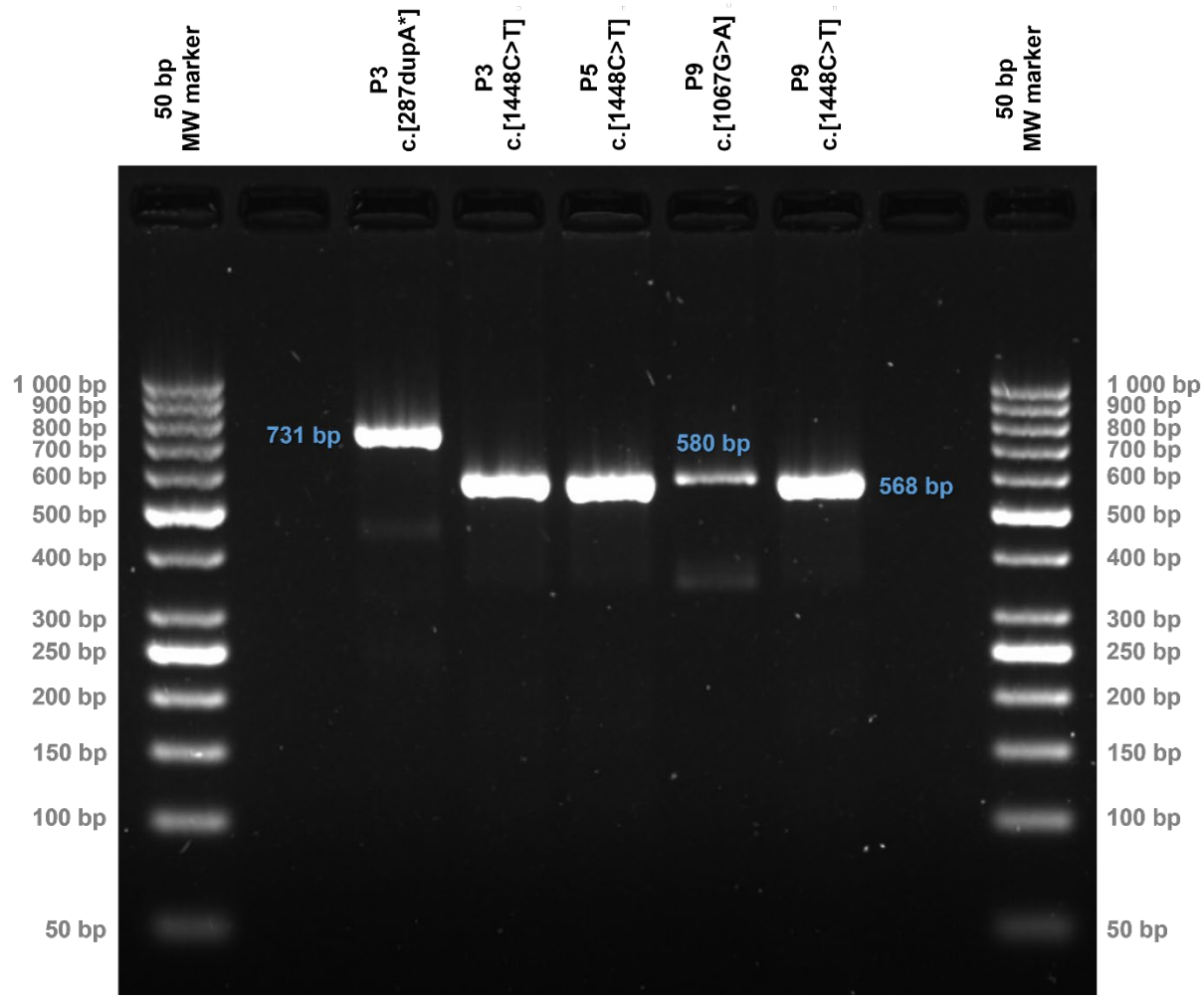
To evaluate the effect of the *ETFDH* genotype on the functional level *in vitro*, primary dermal fibroblast cultures were successfully established from skin biopsies of patients (P3, P5, and P9) and healthy controls (C1, C2, and C3). The following section describes and discusses the results obtained following genotype validation, the evaluation of steady state ETFQO and mitochondrial RC CI–CV, and the analysis of respiration in permeabilised cells.

4.3.1 Genotype validation of patient fibroblasts

4.3.1.1 PCR amplification

Prior to performing functional analyses, it was necessary to validate the genotype of each patient cell line. This was achieved by PCR amplification and Sanger sequencing, as explained in Section 4.2.3. Following PCR analysis, the successful amplification of cellular DNA from P3, P5, and P9 was verified by agarose gel electrophoresis. The results obtained after visualising the PCR amplicons, are shown in Figure 4.2.

Considering the *ETFDH* variant(s) of (i) P3, (ii) P5, and (iii) P9 (Table 4.1), bands corresponding to (i) 731 bp (c.[287dupA*]) and 568 bp [c.[1448C>T]), (ii) 568 bp (c.[1448C>T]), as well as (iii) 580 bp (c.[1067G>A]) and 568 bp [c.[1448C>T]), were expected for each patient, respectively. As presented in Figure 4.2, each amplified product yielded bands of the anticipated size, indicating successful amplification of the relevant DNA sequences. While the lanes containing the amplified product of P3 c.[287dupA*] and P9 c.[1067G>A] displayed additional faint bands of 480 bp and 360 bp, respectively, these bands



corresponded to consistently-observed, non-specific PCR products and were not the result of sample contamination. Consequently, Sanger validation was performed on each PCR product.

Figure 4.2: Agarose gel electrophoresis depicting the successful amplification of DNA sequences containing the *ETFDH* variant(s), isolated from patient skin fibroblasts

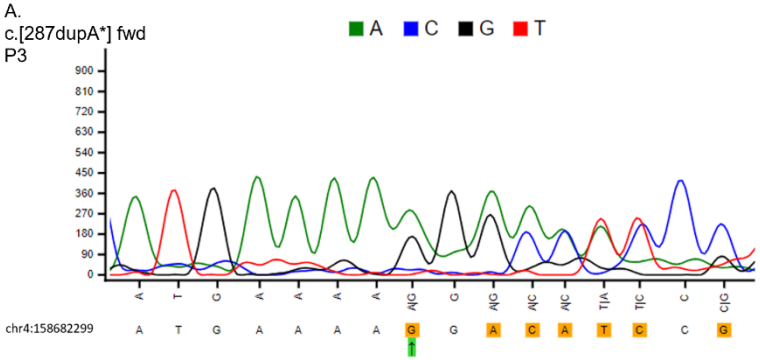
The theoretical size (in bp) of the PCR-amplified products are shown for patient P3, P5 and P9, together with a 50 bp molecular weight marker on either side of the gel. Units and abbreviations: bp: base pairs; MW: molecular weight.

4.3.1.2 Sanger validation

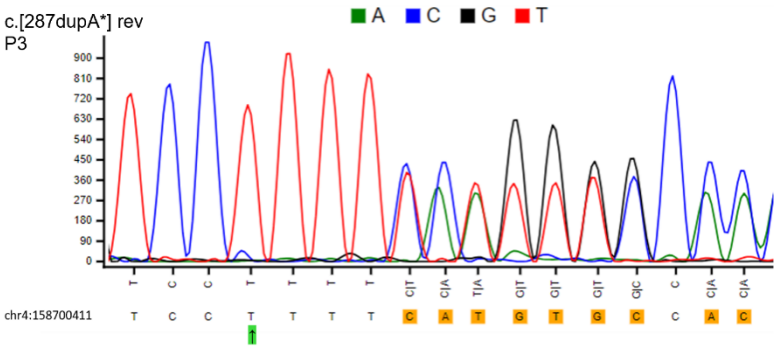
The data obtained following Sanger sequencing of the forward (5'–3') and reverse (3'–5') DNA strands of each PCR product were visualised by aligning the .abi files to the *ETFDH* gene sequence of the corresponding strand of the human reference genome (Build GRCh38.p13), using the SAGE alignment tool (Tracy v0.7.5; GEAR-Genomics, 2023; Rausch *et al.,* 2020). Results are depicted in Figure 4.3a–c.

A.

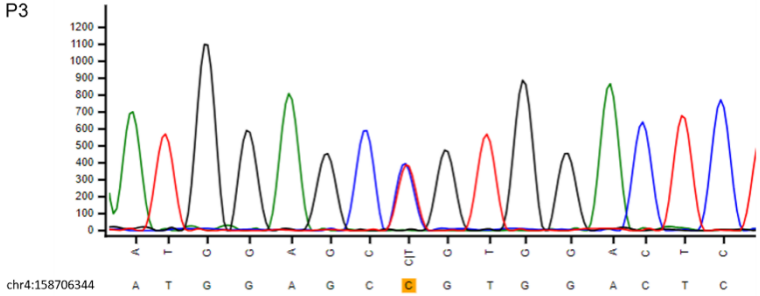
c.[287dupA*] fwd
P3



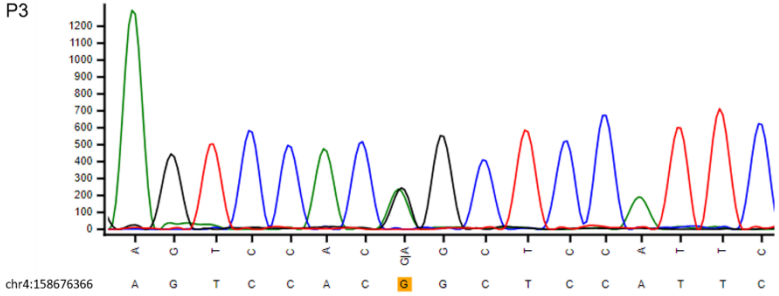
c.[287dupA*] rev
P3



c.[1448C>T] fwd
P3

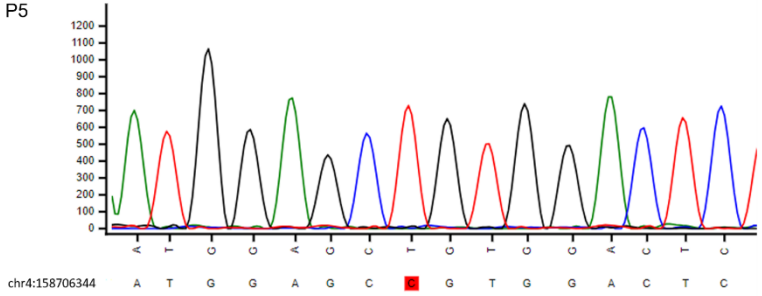


c.[1448C>T] rev
P3

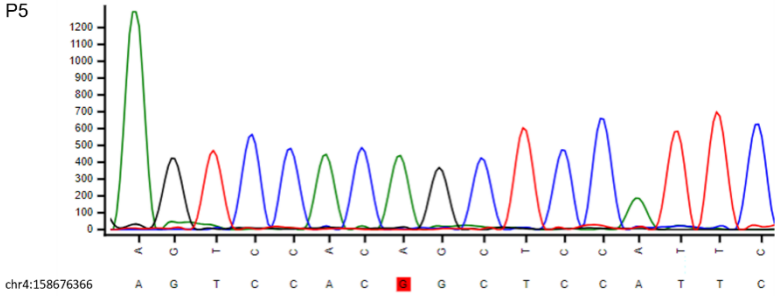


B.

c.[1448C>T] fwd
P5



c.[1448C>T] rev
P5



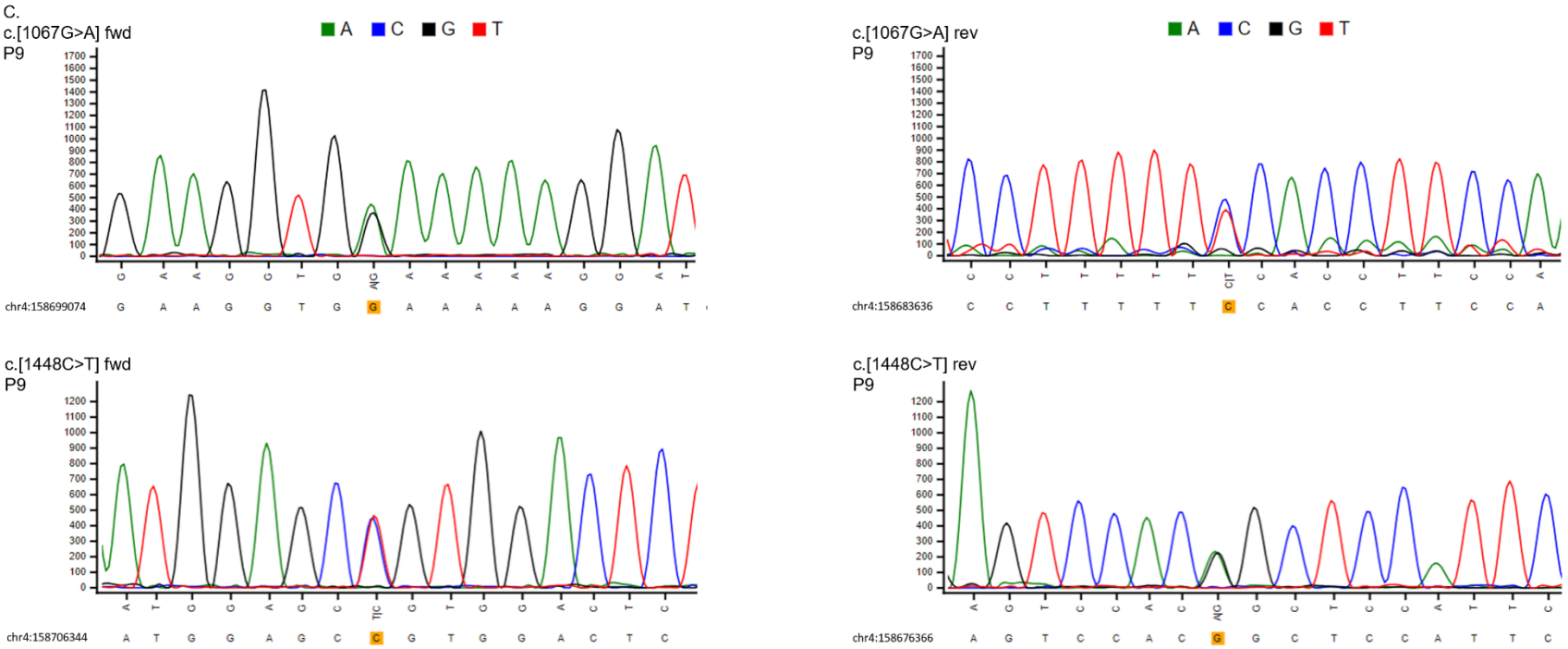


Figure 4.3.a–c: Electropherograms confirming the presence of the relevant *ETFDH* variant(s) in skin fibroblasts of patients P3, P5, and P9

The Sanger data from the forward and reverse DNA strands are depicted. Altered nucleotides are highlighted in yellow (heterozygous variant), red (homozygous variant), or green (duplication). The (a) *ETFDH* c.[287dupA*];c.[1448C>T] genotype of P3, (b) *ETFDH* c.[1448C>T];c.[1448C>T] genotype of P5, and (c) *ETFDH* c.[1067G>A];c.[1448C>T] genotype of P9. Abbreviations: chr: chromosome; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; fwd: forward strand; rev: reverse strand.

Given that each variant is located >200 bp from the 5' and 3'-ends of the PCR product, confident base calls could be made, since any erroneous sequencing of the 5' and 3'-ends—due to instrumental error—was avoided[27]. Each electropherogram yielded large, individual, and evenly-spaced peaks, with a low level of baseline noise. Moreover, no ambiguous or incorrect base calls were encountered in at least 100 bp of sequence up- and downstream to the variant of interest. Taken together, the results indicated high-quality sequencing data.

As expected, the c.[1448C>T] variant was present as a heterozygous or homozygous variant in (i) P3 and P9 or (ii) P5, respectively. This was indicated as (i) overlapping C and T base calls, or (ii) the presence of T instead of C, at position chr4:158706351 of the forward strand. Likewise, c.[287dupA*] and c.[1067G>A] were present as heterozygous variants in (iii) P3 and (iv) P9, respectively, as was evident from (iii) the duplication of a single A at position chr4:158682306 of the forward strand, followed by a frameshift of the open reading frame, and (iv) overlapping G and A base calls at position chr4:158699081 of the forward strand. For each patient, complimentary results were observed on the reverse strand at positions chr4:158676359 (i and ii), chr4:158700408 (iii), and chr4:158683629 (iv). Consequently, it was confirmed that each cell line contained cells from the respective patient and could, therefore, be used for functional analyses.

4.3.2 Evaluation of steady state ETFQO levels

To evaluate the effect of the *ETFDH* genotype on the structural stability of ETFQO, the relative quantity of ETFQO was determined in whole cell lysates of P3, P5, P9, and two healthy controls (C1 and C2) via SDS-PAGE and Western blot analyses, as described in Section 4.2.4. The detected protein bands were normalised against the total protein content of the corresponding lane—a method that has been shown to be more sensitive, reliable, and robust, by comparison to housekeeping protein normalisation (Gürtler *et al*., 2013). The normalised values were then expressed as a percentage of the mean quantity of ETFQO in C1 and C2. The results obtained are presented in Figure 4.4.a–b.

[27] During Sanger sequencing, sequencing errors can occur at the 5' and 3'-ends of the sequenced amplicon, due to inherent instrumental error.

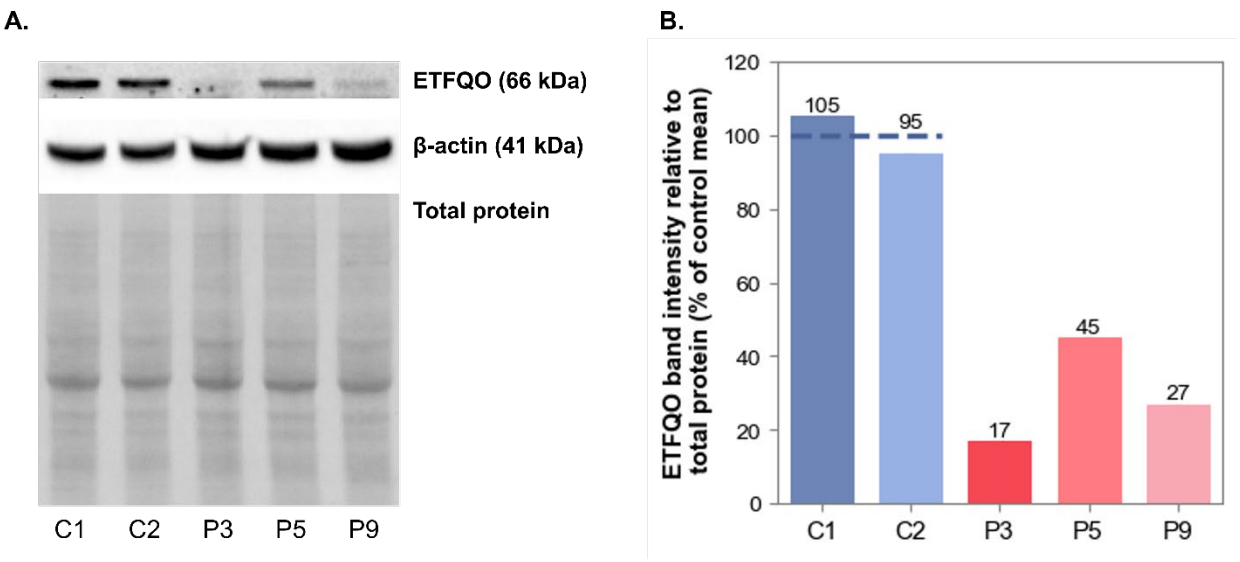


Figure 4.4.a–b: Structural instability of ETFQO in skin fibroblasts of P3, P5, and P9

(a) Cell lysate, prepared from primary skin fibroblasts of P3, P5, and P9 as well as two healthy controls, C1 and C2, were separated on a 10% SDS-PAGE gel and immunoblotted with primary antibodies against ETFQO (reported weight of the monomeric protein: 64 kDa) and β-actin (42 kDa). (b) Band volume intensities were normalised to the total protein content of each corresponding lane and quantified as a percentage, relative to the mean (100%) of both controls (n = 1). Abbreviations: ETFQO: electron transfer flavoprotein-ubiquinone oxidoreductase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis .

By comparison to healthy controls, ETFQO (observed here as a band of 66 kDa) was found to be decreased in all three patients with the largest reduction observed for P3 (-83%; c.[287dupA*];c.[1448C>T]), followed by P9 (-73%; c.[1067G>A];c.[1448C>T]), and P5 (-55%; c.[1448C>T];c.[1448C>T]). While this finding does not correspond to the MADD-DS3 scores of the patients relative to each other (Table 3.1; P3 < P9 < P5), it should be noted that the reported disease severity was influenced by numerous external factors in this instance (e.g., the delayed diagnosis of P5, the early treatment intervention of P3 and P9 due to affected older siblings and the treatment strategy of P9, which, up until his genetic diagnosis of MADD, included only L-carnitine). As mentioned in Chapter 3, this thesis marks the first report of the c.[287dupA*] variant. Likewise, to our knowledge, no comparative data exists for patients affected by the c.[1448C>T];c.[1448C>T] genotype. The severe decrease in ETFQO stability observed for P3 may be explained by the effect of the c.[287dupA*] variant on protein maturation; i.e., the introduction of a premature termination codon in exon three of *ETFDH*—a thirteen-exon gene. Within the cell, C-terminally truncated messenger ribonucleic acid (mRNA) or proteins can be detected and degraded via nonsense-mediated mRNA decay or protein degradation pathways (Shi *et al.,* 2015). Therefore, two possibilities exist: Either the severe decrease in ETFQO of the c.[287dupA*];c.[1448C>T] genotype is

because the remaining allele is the only protein-producing allele, or the protein, were it to be produced, is severely truncated and likely unstable (molecular weight of the variant precursor protein: ~13.12 kDa, and native precursor protein: 67 kDa) (Cornelius *et al.,* 2012; Expasy, 2023). Moreover, studies by Westover *et al.* (2003) and Cornelius *et al.* (2012), respectively, report a decrease in the stability of ETFQO caused by variants affecting the carboxy-terminal (i.e., c.[1448C>T]), as well as the c.[1448C>T];c.[1448C>T] genotype (in HEK293 cells), specifically.

Considering the c.[1067G>A];c.[1448C>T] genotype, steady state levels of ETFQO have been reported once before in patient skeletal muscle (van der Westhuizen *et al.,* 2018). These studies demonstrated a similar, albeit slightly more prominent, decrease (-83%) in the stability of ETFQO. While this may be due to biological variation, it may also be attributed to the increased energy demand of muscle tissue compared to fibroblasts. By comparing the decrease in steady state ETFQO observed for P9 and P5, it is reasonable to conclude that the presence of the c.[1067G>A] variant contributes to a larger decrease in the stability of ETFQO.

4.3.3 Evaluation of steady state mitochondrial RC enzyme levels

The effect of the *ETFDH* genotype on the expression of mitochondrial RC CI–CV was analysed by SDS-PAGE and Western blot analyses of whole cell lysates from P3, P5, and P9, as well as two healthy controls (C1 and C3), as described in Section 4.2.4. Mitochondrial RC CI–CV were probed using an antibody cocktail of mouse monoclonal antibodies, reactive against human and directed against the following subunits of each complex: NDUFB8 (~20 kDa; CI subunit), SDHB (~30 kDa; CII subunit), UQCRC2 (~48 kDa; CIII subunit), MTCO1 (40 kDa; CIV subunit), and ATP5A (55 kDa; CV subunit; also known as ATP5F1A). The band intensity of each subunit was normalised to the total protein content of the respective lane, and expressed as a percentage of the mean quantity of the corresponding subunit in C1 and C3. Results are reported in Figure 4.5.a–f. Changes in band intensity larger than 10% of the mean of C1 and C3, are discussed below.

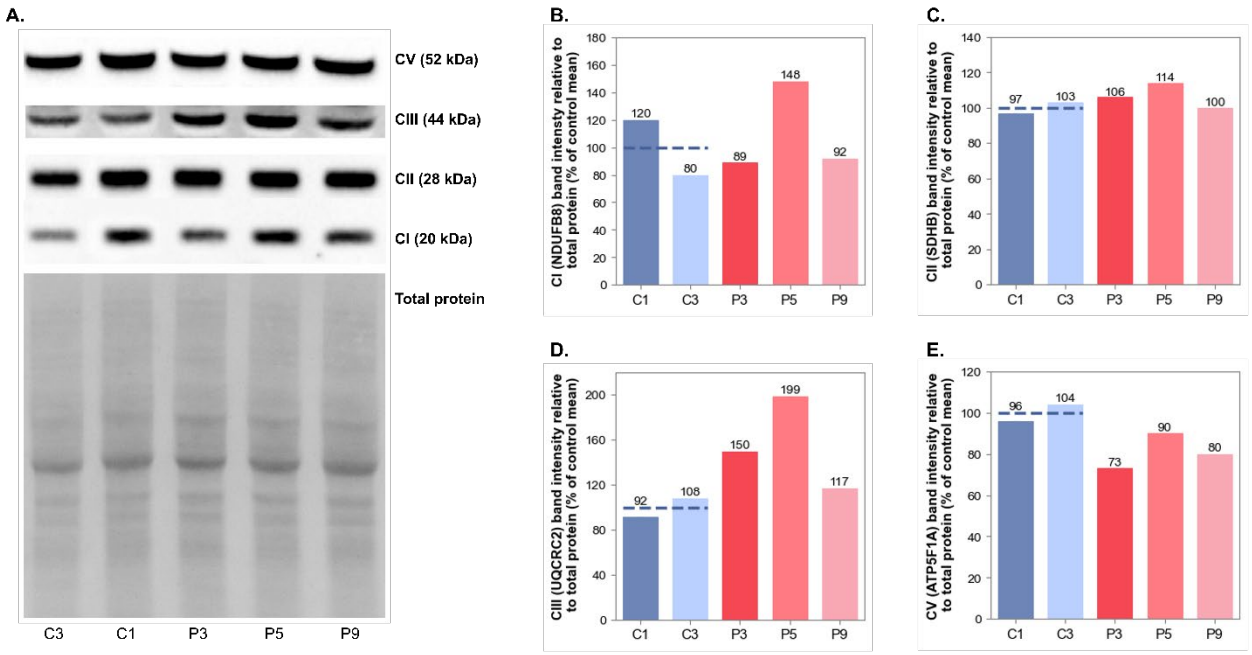


Figure 4.5.a–e: Changes in the expression of mitochondrial RC CI–CIII and CV in skin fibroblasts of P3, P5, and P9

(a) Cell lysate, prepared from primary skin fibroblasts of P3, P5, and P9 as well as two healthy controls, C1 and C3, were separated on a 10% SDS-PAGE gel and immunoblotted with primary antibodies against subunits of CI, CII, CIII, and CV. (b) Band volume intensities were normalised to the total protein content of each corresponding lane and quantified as a percentage, relative to the mean (100%) of both controls (n = 1), for (b) CI, (c) CII, (d) CIII, and (e) CV. Note the difference in sample order between (a) and (b–e). Abbreviations: CI–CIII and CV: complex I to III and complex V; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

As shown in Figure 4.5.a, bands corresponding to the anticipated size of the subunits of CI, CII, CIII, and CV, were obtained. The CIV subunit, MTCO1, however, could not be visualised despite repeating the assay at different denaturing temperatures (50°C and 100°C; results not shown). P5 displayed increased expression of CI (+48%), CII (+14%), and CIII (+99%), which may be indicative of increased mitochondrial biogenesis—a compensatory mechanism defined as the upregulated expression of mitochondrial components in response to augmented energy demands/stress (Jones *et al.,* 2012). This finding is corroborated by studies by Cornelius *et al*. (2014) and Wen *et al*. (2013), who demonstrated an increase in the mitochondrial mass of Rb-responsive MADD patient fibroblasts by comparison to healthy controls. Cornelius *et al.* (2014), who also reported an increase in reactive oxygen species (ROS) production and mitophagy for these cells, argued that mitochondrial biogenesis may be upregulated as a way to sustain energy production in the presence of the increased removal of mitochondria damaged by ROS.

By contrast, CI was found to be decreased in P3 (-11%), and both P3 and P9 displayed reduced levels of CV (-27% and -20%, respectively). Considering the greater decrease in ETFQO stability observed for these two genotypes, with that of P3 being the most extreme (Figure 4.4.b), it could be argued that CI and CV are decreased as a result of secondary mitochondrial derangement. Indeed, MADD has been associated with the secondary dysfunction of ETC components (Gempel *et al*., 2007; Gregersen & Olsen, 2010; Henriques *et al.*, 2010; Olsen *et al*., 2007). Moreover, a shift in the metabolism, supported by the secondary decrease of FAO enzymes and ETC components, has also been demonstrated for MADD patient fibroblasts as a potential adaptive response to increased levels of ROS brought on by electron leakage from the damaged ETC and variant ETFQO (Cornelius *et al.,* 2014; Song *et al.,* 2009). Therefore, it is suggested that the c.[287dupA*];c.[1448C>T] and (to a lower extent) the c.[1067G>A];c.[1448C>T] genotypes are less reliant on mitochondrial OXPHOS for the production of ATP when compared to healthy controls or the milder c.[1448C>T];c.[1448C>T] genotype. As fibroblasts are considered a more glycolytic cell type, it is suspected that this finding will be even more pronounced in oxidative tissues, e.g., muscle (van den Heuvel *et al.*, 2004).

Finally, as shown for P5, an increase in CIII was also observed for P3 (+50%) and P9 (+17%). In this instance, it is important to note that the subunit targeted to assess CIII, was UQCRC2 (cytochrome *b*-c_1 complex subunit 2). In the mitochondrion, CI, CIII, and CIV are organised into supercomplex structures (I/IIIn, I/IIIn/IVn, and III/IVn) to allow for substrate channelling and catalytic efficiency (Dudkina *et al.*, 2011; Guo *et al.*, 2017; Stroh *et al.*, 2004; Wang *et al.,* 2019). Following fatty acid oxidation, electrons from $FADH_2$ are donated by ETFQO to CoQ10 near the CoQ10 reduction site of CIII. By conducting extensive investigations via multiple modalities, Wang *et al.* (2019) were able to definitively demonstrate the close association and physical interaction between ETFQO and CIII via UQCRC2. This protein subunit is located at the CoQ10 reduction site of CIII on the matrix side of the inner mitochondrial membrane. Therefore, it is hypothesised that the observed increase in UQCRC2 could be a cellular response to repair the defective interaction between CIII and ETFQO, resulting from the structurally unstable ETFQO. In support hereof, the degree to which CIII (i.e., UQCRC2) is increased in P3 and P9 is inversely proportional to the relative quantity of structurally stable ETFQO (Figure 4.5.b), whereas the large increase observed for P5, may be explained as the compounded effect of the hypothesised cellular repair response, together with the upregulation of mitochondrial biogenesis.

4.3.4 Evaluation of respiration in permeabilised fibroblasts

The effect of the *ETFDH* genotype on respiration was evaluated using high-resolution respirometry on permeabilised fibroblasts of patients P3, P5 and P9, and controls C1–C3 via the Oroboros O2k, as described in Section 4.2.5 and shown in Figure 4.6.a–g.

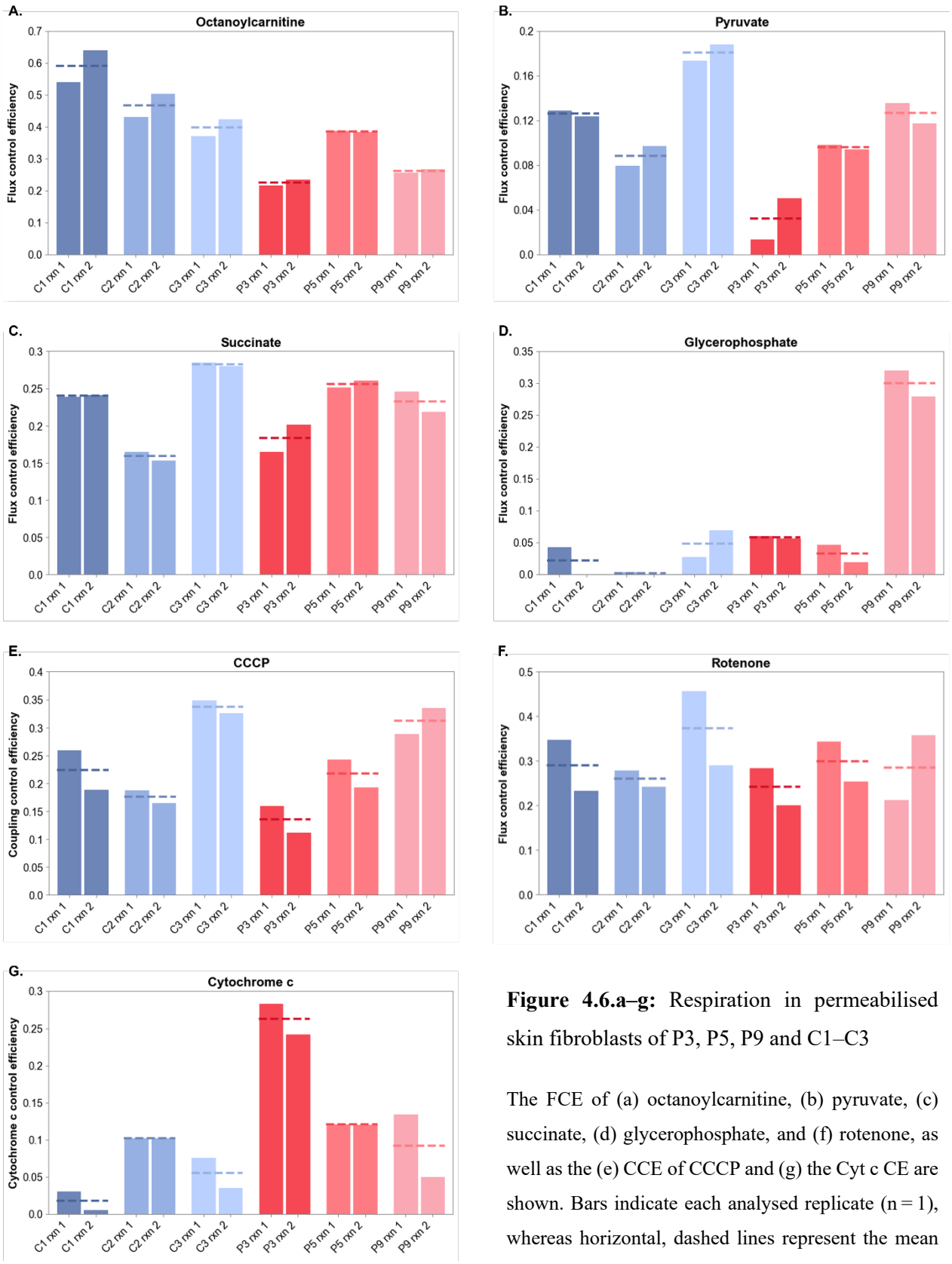


Figure 4.6.a–g: Respiration in permeabilised skin fibroblasts of P3, P5, P9 and C1–C3

The FCE of (a) octanoylcarnitine, (b) pyruvate, (c) succinate, (d) glycerophosphate, and (f) rotenone, as well as the (e) CCE of CCCP and (g) the Cyt c CE are shown. Bars indicate each analysed replicate (n = 1), whereas horizontal, dashed lines represent the mean of both replicates. Abbreviations: CCCP: carbonyl cyanide 3-chlorophenylhydrazone; CCE: coupling control efficiency; Cyt c CE: cytochrome c control efficiency; FCE: flux control efficiency; rxn: reaction (replicate).

During each analysis, substrates of the F-, N-, S- and Gp-pathways were sequentially added, followed by CCCP and rotenone (see Tables 4.3 and 4.4 for more detail). To determine the effect of the injected compounds on cellular respiration, the FCE and CCE were calculated (Equation 4.1). Furthermore, any potential damage to the outer mitochondrial membrane owing to the addition of digitonin, was evaluated by calculating the Cyt c CE. Respiration through the F-, FN-, FNS, and FNSGp-pathways is discussed below by interpretation of the FCE of one representative substrate of each pathway. In addition, the effect of CCCP, rotenone, and digitonin is deliberated.

To account for variability produced by external factors, the polarographic oxygen sensors were calibrated prior to each assay, during which signal stability (i.e., noise and drift), sensor response time, and instrumental background O_2 flux were evaluated (Bioblast, 2023). Moreover, potential variability due to differences in sample concentration was excluded during data processing by the calculation of control efficiency values. Nevertheless, some degree of variation was still observed between replicates of the same sample (inter-day variation). Since replicates were analysed on different days, this variability was attributed to human error and is most likely the result of subtle differences encountered during cell culture (e.g., the effect of cell culture density or time on metabolism). By contrast, the differences observed between C1, C2, and C3, were ascribed to biological variation. As each control and patient could only be analysed in duplicate, it was not possible to calculate the statistical significance between the respiration of each patient and that of the controls; consequently, the trends observed for each patient are described by comparison to the lowest or highest control value recorded. While publications of Oroboros assays that report three or less replicates per sample (cells) or per individual (tissues) exist (Doerrier *et al.,* 2018; Oemer *et al.,* 2021; Reda *et al.,* 2019; Zuccarelli *et al.,* 2021), it is recommended that future analyses include more replicates to increase the statistical power of the assay.

Figure 4.6.a displays the change in respiration due to the addition of octanoylcarnitine. This substrate stimulates the F-pathway in the presence of a low (non-saturating) concentration of malate, which in turn stimulates the tricarboxylic acid (TCA) cycle to allow the incorporation of FAO-produced acetyl-CoA. By comparison to C3 (yielding the lowest control FCE_{oct}), P3 and P9 displayed decreased FCE_{oct}, with that of P3 being the lowest. This indicates a decrease in respiration through the F-pathway and corresponds to the relative quantity of ETFQO observed for P3 and P9 (Figure 4.4.b). By contrast, the FCE_{oct} of P5 was comparable to that of C3, despite a decrease in steady state ETFQO. This implies that the c.[1448C>T];c.[1448C>T] genotype has a negative effect on the stability of ETFQO, without considerably decreasing the activity of the enzyme. The same finding was observed by Cornelius *et al.* (2012), who reported minor to no alterations in the ETFQO activity of c.[1448C>T];c.[1448C>T] HEK293 cells.

The change in respiration due to the addition of pyruvate is shown in Figure 4.6.b. While pyruvate stimulates the N-pathway, FCE_{pyr} represents the FN-pathway due to the prior addition of octanoylcarnitine (and malate, now at a saturating concentration). P3 once again displayed a decrease in respiration by

comparison to C2 (yielding the lowest control FCE_{pyr}). This finding corresponds to P3's slight decrease in CI expression, as shown in Figure 4.5.b, and indicates that cumulative respiration through the F- and N-pathways are decreased for the c.[287dupA*];c.[1448C>T] genotype. P5, however, showed respiration comparable to that of C2. Since P5 displayed normal respiration via the F-pathway, as well as increased CI expression (Figure 4.5.b), this finding was as expected and implies that the c.[1448C>T];c.[1448C>T] genotype is capable of compensating for decreased energy production via upregulated mitochondrial biogenesis of CI. Interestingly, P9 displayed the highest FCE_{pyr}—comparable to that of C1. As respiration through the F-pathway was decreased in P9 and a normal level of CI was observed (Figure 4.5.b), this observation might be the result of the more effective catabolism of pyruvate by P9, rather than mitochondrial biogenesis of CI, allowing for normal respiration through the FN-pathway.

The addition of succinate stimulates the S-pathway. With the prior addition of octanoylcarnitine, malate (saturating concentration), pyruvate and glutamate, Figure 4.6.c represents the change in respiration through the FNS-pathway. As shown, the FCE_{suc} of P3, P5, and P9 were comparable to that of the healthy controls. Considering the expression of CII (Figure 4.5.c) as well as the FCE_{oct} and FCE_{pyr} displayed by P3, FCE_{suc} indicates an increased efficiency of succinate catabolism in this sample in the absence of increased mitochondrial biogenesis of CII, resulting in normal respiration through the FNS-pathway. Among the patients, the highest FCE_{suc} was observed for P5. Considering that P5 also exhibited increased expression of CII (Figure 4.5.c), the c.[1448C>T];c.[1448C>T] genotype also appears to compensate for decreased energy production via increased mitochondrial biogenesis of CII. The FCE_{suc} of P9, was as expected considering the unchanged levels of CII (Figure 4.5.c). Overall, the results observed for FCE_{suc} of the patients demonstrate, at the level of the OXPHOS system, how the use of alternative substrates can allow patients with MADD to restore energy production.

An interesting finding was the increase in respiration displayed by P9 as a result of glycerophosphate. Figure 4.6.d depicts the change in respiration due to glycerophosphate (which stimulates the Gp-pathway) and represents respiration through the FNSGp-pathway [due to the prior addition of octanoylcarnitine, malate (saturating concentration), pyruvate, glutamate, and succinate]. Whereas C1–C3, P3, and P5 yield values close to zero, indicating that the contribution of glycerophosphate to the cumulative respiration converging at the Q-junction (see Table 4.3 for the explanation of junctions) is low or absent, P9 (c.[1067G>A];c.[1448C>T]) appears to use glycerophosphate much more readily.

Figure 4.6.e displays the change in respiration due to CCCP, also known as the CCE. Upon addition of CCCP, the ETC is uncoupled and respiration is stimulated maximally. This allows the measure of uncoupled respiration through the FNSGp-pathway (in this instance) and determines the E-P excess capacity (Doerrier *et al.,* 2018). The latter indicates the difference between uncoupled respiration (E) and coupled respiration in the presence of adenosine diphosphate (ADP), inorganic phosphate and specific OXPHOS substrates (P) (Bioblast, 2023). If $E - P > 0$, the ETC operates at sub-maximal rate when coupled

and consequently, E-P excess is a measure of the coupling efficiency of the ETC. Similar to the previous FCE values, P3 displayed a decrease in CCE by comparison to C2 (yielding the lowest control CCE). This suggests that either (i) the ETC of P3 is less coupled, or (ii) that P3 has lower excess respiratory capacity. Considering the effect of digitonin on the outer mitochondrial membrane (discussed later), it is possible that the former could have contributed to this finding. In addition, a lower excess capacity of c.[287dupA*];c.[1448C>T] is also likely and indicates that the ETC of P3 reacts poorly to metabolic stress. By contrast, the CCE of P5 and P9 were comparable to C1 and C3, respectively. This may be explained by the upregulated mitochondrial biogenesis observed for P5 (Figure 4.5.b–d). For P9, this finding correlates with the more effective use of N- and Gp-substrates in the absence of increased mitochondrial biogenesis instead.

Next, the effect of rotenone was investigated. Following the addition of rotenone, respiration via the N- and F-pathways cease (Doerrier *et al.,* 2018). Figure 4.6.f, therefore, depicts respiration as a result of the SGp-pathway in uncoupled mitochondria. Upon inspection of the mean FCE_{rot} of each patient, that of P5 and P9 were found to be comparable to C1, whereas that of P3 showed a slight decrease in comparison to C2 (yielding the lowest control FCE_{rot}). However, considering the variation observed between the replicates, the latter finding is most likely not statistically significant.

To assess potential damage to the outer mitochondrial membrane, Cyt c CE was determined, as shown in Figure 4.6.g. Since excess digitonin is capable of damaging the outer mitochondrial membrane, it is recommended to optimise the concentration prior to experimentation. A study by Ye & Hoppel (2013) showed that the optimal concentration of digitonin remains relatively constant when assessed in different patients' primary fibroblast cell lines, and reported an optimal concentration of 2–3 µg/mL per 0.6×10^6 cells/mL (i.e., 3.33–5 µg/mL per 1×10^6 cells/mL). Likewise, Bioblast (2023) describes a typical optimal digitonin concentration of 5 µg/mL per 1×10^6 cells/mL. Following optimisation on the fibroblasts of C2, an optimal digitonin concentration of 5 µg/mL per 1×10^6 cells/mL was obtained (results not shown). After the final analyses, the calculation of Cyt c CE showed a slight increase in respiration. According to Bioblast (2023), stimulation of respiration following the addition of Cyt c could indicate damage to the outer mitochondrial membrane. However, a small increase is expected for some cell types if observed consistently. For C1–C3, P5, and P9, the Cyt c CE was consistent and fairly low (0.02–0.12), indicating an intact outer mitochondrial membrane. P3, however, yielded a mean Cyt c CE of 0.26. During analyses, exceptional care was taken to assess the same number of cells in the presence of the same concentration of digitonin for all samples. Considering the clinical phenotype (Table 3.1), it is suggested that the pathology associated with the c.[287dupA*];c.[1448C>T] genotype alters the properties of the outer mitochondrial membrane, resulting in mitochondria that are more vulnerable to experimental damage, as reported by Bioblast (2023).

4.4 CHAPTER CONCLUSION

In conclusion, the results reported in this chapter confirm, via the *in vitro* analyses of primary patient dermal fibroblasts, the pathogenicity of the c.[287dupA*];c.[1448C>T], c.[1448C>T];c.[1448C>T], and c.[1067G>A];c,[1448C>T] genotypes. By investigating the relative quantity of ETFQO, it is evident that the structural stability of ETFQO is decreased most by c.[287dupA*];c.[1448C>T], followed by c.[1067G>A];c.[1448C>T], and the homozygous c.[1448C>T];c.[1448C>T] genotype. Moreover, the analysis of the relative quantity of mitochondrial RC CI–CIII and CV, as well as the mitochondrial respiration, indicate two putative adaptive responses to MADD, based on the severity of the variant: namely, that milder variants (like c.[1448C>T]) result in an upregulation of mitochondrial biogenesis, whereas more severe variants (like c.[287dupA*] and c.[1067G>A]) redirect metabolism away from the OXPHOS system and towards alternative metabolic pathways. Further studies (which were beyond the scope of this thesis) are, however, required to confirm these hypotheses and elucidate the specific mechanisms involved.

Chapter 5

Summary and Conclusions

5.1 INTRODUCTION

Multiple acyl-CoA dehydrogenase deficiency (MADD; ORPHAcode 26791), also known as glutaric aciduria type II (GAII), is an IEM affecting FAO, BCAA catabolism, amino acid catabolism, and choline metabolism (Mosegaard *et al.,* 2020; Orphanet, 2014). The disorder is caused by autosomal recessive variants in the *ETFDH*, *ETFA*, and *ETFB* genes. Additionally, MADD-like phenotypes have been described resulting from variants in *SLC52A1* (autosomal dominant), *SLC52A2–3*, *SLC25A32,* and *FLAD1* (autosomal recessive) (Green *et al.*, 2010; Haack *et al.*, 2012; Ho *et al.*, 2011; Johnson *et al.*, 2012; Olsen *et al.*, 2016; Schiff *et al.*, 2016; Taylor *et al.*, 2014). However, the largest contribution to disease by far, stems from pathogenic variants in *ETFDH* (59%) (Mereis *et al.,* 2021). This gene encodes for ETFQO, the protein responsible for the sequential transfer of electrons from different substrates, including fatty acyl-CoA moieties, via ETF, to CoQ10 and then CIII of the OXPHOS system; thereby replenishing FAD (required by up to 14 mitochondrial flavoproteins) and sustaining mitochondrial metabolism. Clinically, *ETFDH* deficiency may present with a heterogeneous, late-onset phenotype (MADD type III) accompanied by muscle, hepatic and/or cardiac involvement, recurrent/intermittent episodes of vomiting and lethargy, as well as hypoglycaemia and metabolic acidosis (Frerman & Goodman, 2001; Grünert, 2014; van Rijt *et al.*, 2019). Moreover, albeit to a lesser extent, *ETFDH* deficiency may also manifest as more severe, and mostly fatal, neonatal-onset phenotypes of MADD. These subtypes are distinguished from each other by the presence (type I) or absence (type II) of congenital anomalies and are characterised by multi-system involvement (neurological, muscle, cardiac, hepatic, and/or renal abnormalities), as well as hyperammonaemia, hypoglycaemia and metabolic acidosis (with or without ketosis). Of the three clinical phenotypes, MADD type III is exceptionally amenable to treatment, whereas the treatment responsiveness of MADD types I and II is absent or variable, respectively (Gordon 2006; Mosegaard *et al.*, 2020; van Rijt *et al.,* 2020). Consequently, early diagnosis and treatment are imperative to reduce the morbidity and mortality of MADD patients, especially those affected by MADD types II and III.

In understudied populations, including Southern Africa, MADD is still under-recognised and under-diagnosed, due in part to socio-economic obstacles and competing burdens of disease. However, it is the limited expertise in specialised clinical and diagnostic fields, due to the paucity of research, that constitutes the largest limitation (Raga *et al.*, 2022). In addition, Southern Africa does not have a compulsory NBS program as of yet, and the diagnosis of IEMs is performed on an elective basis instead, following the onset of symptoms or upon parental request (Conradie *et al.,* 2021). Therefore, considering the number of live births per annum as well as the global prevalence of MADD, it is hypothesised that the current referral rate (2–3 cases annually) is significantly lower than the statistically estimated rate of incidence (5–6 cases annually) (Orphanet, 2014; Statistics South Africa, 2023; Vorster & Meldau, 2023, personal communication). In Southern Africa, diagnosis proceeds mainly by metabolic testing, with little to no genetic confirmation, as opposed to well-studied populations. A key problem with this approach is

that metabolic testing is only informative when conducted during the catabolic state or metabolic crisis and may result in numerous missed cases (Chaya *et al.*, 2018). Moreover, variants leading to rare disease tend to be population-specific (Bustamante *et al.,* 2011). Therefore, the absence of genetic screening precludes the discovery of population-specific variants, which could otherwise contribute to the development of more rapid and inexpensive screening procedures. This lack of genetic information further hampers research into the genotype–phenotype correlation of, and the carrier frequency and mechanisms involved in, population-specific variants of MADD, which may offer a more guided approach to treatment. Consequently, a comprehensive description of a disease cohort is required to characterise the clinical phenotype, metabolic presentation, genetic aetiology, and functional aspects of MADD in Southern Africa.

Previous research of MADD in Southern Africa includes a single study performed at this institution of three patients of White SA ethnicity in which one novel (c.[1067G>A]) and one known (c.[1448C>T]) pathogenic *ETFDH* variant were identified (van der Westhuizen *et al.*, 2018). Based on these and other anecdotal findings, it was suggested that, in Southern Africa, the prevalence of MADD may be more similar to organic acidaemias—a group of more prevalent IEMs. In addition, it was hypothesised that the c.[1067G>A] variant could be a founder mutation and that MADD may be more frequently encountered in the White SA population. To address these questions and to comprehensively investigate MADD in all of the understudied populations of Southern Africa, this study was launched in collaboration with the ICGNMD—an international, multi-continental partnership with an overall aim to elucidate the genetic aetiology of NMDs (including MADD) in understudied populations (Wilson *et al.*, 2023). The first aim of this thesis was to characterise the clinical and metabolic properties, as well as the genetics, of a Southern African cohort diagnosed with MADD. Following the identification of potential pathogenic variants, primary dermal fibroblasts were cultured from consenting study participants and used to fulfil the second aim: namely, to evaluate the functional properties of selected pathogenic variants frequently identified in the cohort *in vitro.* To self-evaluate the scientific contributions of this study, this chapter presents a summary of the key findings and conclusions drawn from this thesis, structured according to the study objectives. For the sake of readability, the data is presented in the following order: Objective 1.1, 1.3, 1.2 and 1.4, 1.5, 2.1, 2.2, 2.3 and 2.4. This is followed by a discussion of the strengths, limitations, and future prospects of the study.

5.2 SUMMARY OF THE FINDINGS

The objectives set for each aim of this study were successfully achieved. The following sections describe the main results observed, additional points of discussion, and the ensuing conclusions.

5.2.1 Objective 1.1: Recruitment of the Southern African MADD cohort

Objective 1.1: "Select and recruit a cohort of Southern Africa patients that had been diagnosed with MADD at four diagnostic centres in SA."

For the purpose of this study, it was possible to recruit individuals from 12 unrelated[28] pedigrees, either retrospectively or prospectively, over a three-year period (June 2020–June 2023). Patient referral and recruitment proceeded via the NWU (referrals only)[29] and four ICGNMD-affiliated academic state hospitals [including Steve Biko Academic Hospital (UP), Tygerberg Hospital (SU), Red Cross War Memorial Children's Hospital (UCT), and Groote Schuur Hospital (referrals only; UCT)] to which individuals of all socio-economic backgrounds have access. The final study cohort consisted of 14 patients, including five males (one deceased) and nine females (three deceased), diagnosed clinically and metabolically with MADD between 1992 and 2021. In addition, 21 unaffected first-degree relatives were recruited for segregation analyses. The size of the study cohort was comparable to that of similar studies (Olsen *et al*., 2007; van Rijt *et al*., 2019; Yotsumoto *et al.*, 2008; Zhu *et al.*, 2014) and included individuals across the central, north-eastern, north-western, south-eastern, and south-western provinces of SA, as well as Namibia. Recruitment via academic state hospitals, which, along with the NHLS, serve the wider population of SA, presented the best opportunity for individuals of all socio-economic backgrounds to partake in this research study. While this study only included individuals who self-identified as having White SA (12 patients) and mixed (two patients) ethnicities, it should be noted that MADD has previously been diagnosed[30], albeit less frequently, in Southern African patients of both African and Indian ethnicity via the CHM (NWU) and NHLS IMD molecular laboratory (UCT) (NMP, 2023; UCT, 2023; Vorster & Meldau, 2023, personal communication). However, despite making all possible efforts to include patients of these population groups (including project campaigning), these individuals were regrettably lost to follow-up prior to the onset of this study and no additional cases were encountered. Therefore, it is not possible to draw any conclusions regarding the clinical, metabolic, genetic, and functional properties of MADD in the African and Indian populations of Southern Africa at this time. Based on the frequency of the recruitment of patients of White SA ethnicity, it is highly likely that MADD is more prevalent in this population group, by comparison to the other populations of Southern Africa.

[28] Individuals were unrelated to the second degree.

[29] Patients were only traced by these institutions, whereas study enrolment and clinical re-evaluation were conducted by healthcare professionals of the remaining three academic state hospitals.

[30] Diagnoses were made based on metabolic testing only.

5.2.2 Objective 1.3: Variants associated with MADD in Southern Africa

Objective 1.3: "Investigate genes known to be affected in MADD, using whole exome sequencing (WES)."

WES analysis of all patients belonging to the Southern African MADD cohort (performed on DNA isolated from whole blood, saliva, or urine, as available) resulted in a genetic diagnosis of MADD, due to five causal variants in the *ETFDH* gene (NM_004453.4). These included the novel duplication variant, c.[287dupA*] (p.[Asp97Glyfs*24]), as well as four known non-synonymous single nucleotide variants {c.[740G>T] (p.[Gly247Val]) (rs1384574225), c.[976G>C] (p.[Gly326Arg]), c.[1067G>A] (p.[Gly356Glu]), and c.[1448C>T] (p.[Pro483Leu]) (rs377656387)}. The five variants encode for highly conserved amino acids and were encountered as homozygous or compound heterozygous genotypes: c.[1067G>A];c.[1067G>A] (P6 and P8), c.[1448C>T];c.[1448C>T] (P4, P5, and P14), c.[287dupA*];c.[1448C>T] (P2 and P3), c.[740G>T];c.[1448C>T] (P13), c.[1067G>A];c.[1448C>T] (P1, P7, and P9–P11), and c.[976G>C];c.[1067G>A] (P12). Moreover, segregation analyses of the biological parents (and siblings, where possible) revealed an autosomal recessive pattern of inheritance, as is expected for MADD caused by *ETFDH* variants, and confirmed the patient variants to be *in trans* (Orphanet, 2014).

To date, the c.[1067G>A] variant has not yet been reported in any population group other than the White and mixed ethnicity populations of Southern Africa (van der Westhuizen *et al.,* 2018). Of the five variants reported in this study, only the c.[1448C>T] variant has previously been described in the well-studied European populations from which the White SA population descended since the mid-17th century. To our knowledge, this study further presents the second report of the c.[740G>T] and c.[976G>C] variants, which have previously only been identified in two single cases of Chinese ethnicity (Hong *et al.,* 2019; Xi *et al.,* 2014). Considering the population distribution of the known variants, the number of variants identified, as well as the discovery of a novel variant, the genetic cause of MADD in Southern Africa appears to be population-specific and to some extent, unique. This underscores the importance of genetic confirmation of MADD in Southern Africa and how it can enable the development of more rapid screening methods [e.g., the PCR–RFLP method described in Objective 1.5 and the future use of single nucleotide polymorphism (SNP) panels], which will aid the timeous diagnosis of future patients.

5.2.3 Objectives 1.2 and 1.4: Clinical and metabolic profiles of the Southern African MADD cohort and the evaluation of variant pathogenicity

Objective 1.2: "Collect relevant pre-existing or new samples and generate clinical and biochemical (metabolic) data of the study cohort."

Objective 1.4: "Identify and confirm existing and/or novel putative pathogenic genetic variants from the study cohort and compare the results to the data obtained in Objective 1.2."

To investigate potential genotype–phenotype associations in the cohort, consistent and extensive clinical re-evaluations were conducted by the contributing paediatric or adult neurologists, where possible, according to standardised protocols set forth by the ICGNMD (Wilson *et al.,* 2023). To estimate the burden of disease, the MADD-DS3 disease severity system was used (van Rijt *et al.,* 2019). In addition, urine metabolic profiles were re-analysed on available samples collected during acute metabolic presentation and post-treatment. Patients could be categorised into three distinct groups by comparing the disease severity and treatment response:

5.2.3.1 Severe, Rb-unresponsive MADD patient group

The first group included three infants, one male of White SA ethnicity (P6) and two females of mixed ethnicity (P8 and P12), who presented at birth with severe and progressive clinical symptoms of the liver and muscle that were characteristic of neonatal-onset MADD. All patients displayed elevated or severely elevated diagnostic MADD metabolites and were metabolically and phenotypically unresponsive to treatment with Rb. While L-carnitine supplementation did promote the formation of disease-specific acylcarnitine conjugates, all patients succumbed between the second week and third month of life. Congenital cardiac aberrations were present in one patient (P6) and, considering his high MADD-DS3 score (30), a diagnosis of MADD type I was given. By contrast, no congenital features were reported for the remaining two patients (P8 and P12). However, in the absence of a post-mortem examination, congenital features could not be ruled out and, therefore, a diagnosis of MADD type I/II was given, corresponding to the high MADD-DS3 scores observed (21 and 24, respectively).

Genetically, WES revealed two genotypes in this group, the homozygous c.[1067G>A];c.[1067G>A] (P6 and P8) and compound heterozygous c.[976G>C];c.[1067G>A] (P12). Upon evaluation of the pathogenicity, both the c.[1067G>A] and c.[976G>C] variants were found to be extremely rare, having been reported only twice or once before, respectively (van der Westhuizen *et al.,* 2018; Xi *et al.,* 2014). In addition, both are located in the ubiquinone-binding domain of *ETFDH* and are classified, by the ACMG, as either likely pathogenic with *in silico* and structural evidence (c.[1067G>A]), or a variant of unknown significance with moderately pathogenic *in silico* predictions (c.[976G>C]). Furthermore,

van der Westhuizen *et al.* (2018) (and Section 4.3.2 of this thesis) demonstrated a decrease in the stability of ETFQO when c.[1067G>A] forms part of the genotype. Considering the patients' lack of response to treatment and their rapid clinical deterioration, together with the low allele frequencies reported for this variant (Sections 3.5.3 and 3.7), it can thus be concluded that c.[1067G>A] is a highly pathogenic variant. By comparing the disease severity observed in this group to that of the other groups (Sections 5.2.3.2 and 5.2.3.3), it is further hypothesised that the presence of c.[1067G>A] on both alleles (P6 and P8), or on one allele *in trans* with another variant affecting the same protein domain (i.e., c.[976G>C]), results in an enzyme of which the stability, and thus activity, is diminished to such an extent that it cannot be sufficiently rescued by Rb treatment. Neither can adequate enzyme activity be provided by proteins translated from the less severely affected allele.

5.2.3.2 Moderate, variably Rb-responsive MADD patient group

The second group included eight individuals of White SA ethnicity (four males and four females). Symptom-onset spanned the neonatal period (P1), infancy (P2, P7, P9, P10) and childhood (P3, P11, P13), and included moderate features of MADD [with muscle, hepatic, muscle and hepatic, or central nervous system (CNS), muscle, and hepatic involvement], as well as uncommon reports of ketosis (P2, P3, P7, P9, and P10) and Beevor's sign (P13). All patients displayed a variable increase in the diagnostic urine markers of MADD, which was less pronounced when compared to that of the first group. Following treatment[31], the clinical symptoms and majority of the metabolites normalised for seven of the eight patients (excluding P7), with those on L-carnitine displaying successful fatty-acyl conjugation. It should be noted that therapeutic intervention of P3 and P9 was started earlier in life, due to the diagnosis of an older sibling with MADD, which likely attenuated disease progression. By contrast, the metabolic profile of P7 resembled that of the first group near the time of her demise (at 23 years, following a stroke with evidence of emboli in the right hemisphere) and her MADD-DS3 score was high (22.5). Notably, however, this patient had a family history of thrombotic/embolic episodes, which may have contributed to her clinical outcome. Based on the time of disease-onset and their disease severity scores (ranging from 3–13.95; P13 < P2 < P3 < P9 < P10 < P11 < P1), patients were given a diagnosis of either MADD type II (P1) or type III (P1, P2, P3, P7, and P9–P11).

In this group, three compound heterozygous genotypes, including c.[287dupA*];c.[1448C>T], c.[740G>T];c.[1448C>T], and c.[1067G>A];c.[1448C>T] were identified by WES. When evaluating the

[31] Treatment varied and included dietary adjustment with treatment by L-carnitine (P9 and P10), L-carnitine and Rb (P2, P3, P7, P11, P13), or L-carnitine, Rb, and CoQ10 (P1). All patients not on Rb therapy at the time of clinical re-evaluation were advised by the contributing neurologist to include Rb in their treatment regime.

pathogenicity, the novel c.[287dupA*] variant was found to produce a premature stop-codon in exon three of *ETFDH* (a 13-exon gene). Moreover, the c.[287dupA*] and c.[740G>T] variants are located in the FAD-binding domain of ETFQO and are rare, with the c.[740G>T] variant having only been reported in one other case of late-onset MADD (Hong *et al.,* 2019). Both variants are also classified as likely pathogenic according to the ACMG criteria. The c.[1448C>T] variant, on the other hand, is classified as pathogenic, which is supported by the low allele frequencies identified in this thesis (described in Sections 3.5.3 and 3.7). It is well-studied and is known to be associated with late-onset MADD, as observed in seven of the eight patients described in this group (Gempel *et al.,* 2007; Grünert, 2014; van der Westhuizen *et al.,* 2018). In addition, the variant has been shown to be Rb-responsive, a finding which corresponds to the therapeutic response of six of the eight patients (Cornelius *et al.,* 2012). Considering these factors, together with the pathogenic nature of c.[1067G>A] (see Section 5.2.3.1), the genotypes identified in this group, therefore, explain the clinical and metabolic presentations observed. Notably, the presence of the c.[1067G>A] variant appeared to increase the burden of disease, as reflected by the presence of CNS symptoms (in three of the five affected cases), higher MADD-DS3 scores, and one fatality. However, when encountered as a compound heterozygous variant along with the milder c.[1448C>T] variant (which affects the carboxy-terminal protein domain of ETFQO), we conclude that sufficient enzymatic compensation is provided by the less severely affected allele to allow for adequate ETFQO activity (described in more detail in Sections 5.2.6 and 5.2.7).

5.2.3.3 Mild, Rb-responsive MADD patient group

The final group included three female patients of White SA ethnicity (P4, P5, and P14), who presented later in life with mild and non-progressive clinical symptoms. While their phenotypes were heterogeneous [involving hepatic and muscle, as well as hepatic, muscle, and peripheral nervous system (PNS) features], they were characteristic of late-onset MADD, with uncommon findings of ketosis in P5 (mild) and P14 (severe). The urinary metabolic profiles of P4 and P5 indicated slightly elevated to elevated metabolic markers of MADD, which were lower than those of the group discussed previously (Section 5.2.3.2). Interestingly, however, P14 displayed severely elevated 2-hydroxyglutaric acid and *cis*-4-decenedioic acid, with unremarkable increases in the more typical MADD metabolites (glutaric acid and ethylmalonic acid), and normal acylcarnitines (specifically C4 and C5DC). The latter is likely due to free carnitine depletion at the time of metabolic decompensation, whereas the variation observed for the other metabolic findings underscore the importance of genetic confirmation and advocate for a more holistic interpretation of the phenotypic and metabolic presentation of MADD in adults.

Following therapeutic intervention with L-carnitine, Rb, and CoQ10, the clinical and metabolic abnormalities essentially normalised. Moreover, the patients in this group displayed moderate MADD-DS3 scores (ranging from 5.5–11.4; P4 < P14 < P5) and were given a diagnosis of MADD type III. It should,

however, be noted that the diagnosis of P5 was especially delayed, which contributed to a higher final MADD-DS3 score as a result of severe hepatic decompensation and hospitalisation. Following WES, the homozygous c.[1448C>T];c.[1448C>T] genotype was identified in each patient. As mentioned in Section 5.2.3.2, the pathogenic c.[1448C>T] variant is well-known to cause late-onset, Rb-responsive MADD (i.e., type III) and consequently, corroborated the clinical and metabolic findings in this group (Cornelius *et al.,* 2012; Gempel *et al.,* 2007; Grünert, 2014).

5.2.3.4 Conclusion

In summary, the Southern African MADD cohort could be classified into three groups based on disease severity and treatment response (Table 5.1). Within each group, the severity of the clinical presentation, metabolic profile, and variants (severity; i > ii > iii) were found to correspond, together with the time of onset (age; i < ii < iii), clinical and metabolic response to treatment (response; i < ii < iii), and the diagnostic phenotype (MADD type I or I/II, type II or III, type III; i, ii, iii). The data obtained from Objectives 1.2 and 1.4 indicate that it is possible to anticipate the clinical progression and response to treatment when the variant(s) involved are known, thereby highlighting the importance of genetic confirmation in Southern Africa.

Table 5.1: Grouping of the Southern African MADD cohort

Group	(i) Severe, Rb-unresponsive	(ii) Moderate, Rb-responsive	(iii) Mild, Rb-responsive
Ethnicity	White SA (n = 1) and mixed (n =2)	White SA (n = 8)	White SA (n = 3)
Onset	Birth	Birth to early childhood	Late childhood to adulthood
Clinical symptoms	Severe (characteristic)	Moderate (characteristic)	Mild (heterogeneous)
Metabolic profile	Severe (characteristic)	Moderate (characteristic)	Mild (heterogeneous)
Genotypes	• c.[1067G>A];c.[1067G>A] • c.[976G>C];c.[1067G>A]	• c.[287dupA*];c.[1448C>T] • c.[740G>T];c.[1448C>T] • c.[1067G>A];c.[1448C>T]	• c.[1448C>T];c.[1448C>T]
Treatment response	None	Variable	Good
MADD phenotype	I (n = 1) or I/II (n = 2)	II (n = 1) or III (n = 7)	III (n= 3)

Decreasing disease severity

Abbreviations: MADD: multiple acyl-CoA dehydrogenase deficiency; Rb: riboflavin; SA: South African.

5.2.4 Objective 1.5: Allele frequencies and haplotypes of *ETFDH* c.[1067G>A] and c.[1448C>T] in Southern Africa

Objective 1.5: "Determine the allele frequency and haplotype of selected prevalent variants in the four largest population groups of SA."

Next, the allele frequencies of c.[1067G>A] and c.[1448C>T] (the two variants most regularly encountered in the study cohort) were estimated in the four largest population groups of SA. Accordingly, DBS and DNA samples of equal distributions of healthy male and female African (1,008–1,479), mixed (1,231–1,485), White (750), and Indian (750) newborns were assessed in Southern Africa's northern and southern populations, as available. From this, the estimated allele frequency of c.[1067G>A] was calculated as <0.00050% (African ethnicity), 0.00067% (White SA or Indian ethnicity), and <0.00047% (mixed ethnicity), whereas that of c.[1448C>T] was determined to be <0.00034% (African ethnicity), 0.00067 (White SA or Indian ethnicity), and <0.00038% (mixed ethnicity). This provides an estimate of the carrier frequency, suggesting that, per annum, <5 African- and <1 White, Indian, or mixed ethnicity newborns will carry the c.[1067G>A] or c.[1448C>T] variant in SA. By further investigating the five variants identified in the cohort on population databases, all were found to be absent from H3Africa and only two (c.[1448C>T] and c.[740G>T]) were recorded on gnomAD (with exome allele frequencies <0.0001%) (gnomAD, 2023; H3Africa, 2023). This, together with the low estimated allele frequencies {(<0.0001%[32]; less than that (1.35%) of the most commonly occurring pathogenic MADD variant, *ETFDH* c.[250G>A]}, indicate with high probability, the pathogenicity of the five variants and validate their aetiological role in MADD (Prasun, 2020; Richards *et al.,* 2015). It should be noted that the PCR–RFLP approach employed to achieve this objective was also adjusted to assess the c.[740G>T] variant (results not shown) and is available as a rapid and inexpensive screening method to test for the presence of these three variants in future MADD patients.

In addition, haplotype analyses were performed to determine whether the c.[1067G>A] [as hypothesised by van der Westhuizen *et al.* (2018)] and c.[1448C>T] variants arose as a result of founder effects. In both instances, DNA samples from patients harbouring one or both of the variants yielded a shared haplotype on one allele with a minimal overlapping region of 7.2 Mb (c.[1067G>A]) and 4 Mb (c.[1448C>T]). Moreover, patients who were homozygous for each variant exhibited homozygosity for the shared haplotype in the region of *ETFDH*, and familial relations among patients (excluding the family of P4, P2 and P3) were absent down to the second degree. These findings support the existence of a founder effect for both these variants. However, it is likely that only the c.[1067G>A] variant originated in the White SA

[32] A variant absent from 1,000 analysed individuals is considered moderately pathogenic according to the ACMG criteria (Richards *et al.,* 2015).

population, as c.[1448C>T] is also widely reported in other population groups (Gempel *et al.,* 2007; Grünert, 2014). Due to the lack of publicly available control genetic data from the same population groups, it was not possible to estimate the haplotype frequency with confidence. Should control genetic data become available for Southern African populations in the future, this research question may be revisited.

5.2.5 Objective 2.1: Patient fibroblast establishment and genotype validation

Objective 2.1: "Acquire primary fibroblasts from patient- (where available) and control skin biopsies."

To assess the structural and functional properties of MADD *in vitro*, primary dermal fibroblasts were established from skin biopsies of three MADD patients (P3, P5, and P9) and three healthy controls (C1–C3; age-, gender-, and ethnicity-matched). While logistical challenges limited the number of donating patients, it was possible to obtain fibroblasts of the predominantly observed genotypes (including c.[287dupA*];c.[1448C>T], c.[1448C>T];c.[1448C>T], and c.[1067G>A];c.[1448C>T]). Visually, all cell cultures exhibited an approximately equal growth rate (doubling time: ~72 h) and displayed the expected characteristic fusiform morphology of fibroblasts (Takashima, 1998). Moreover, experimental variability (due to differences in sample storage, confluency, and age) was avoided by collecting skin biopsies and establishing cultures over a short period of time (three months). Additionally, equal numbers of cells were seeded at similar passages per flask, five days prior to each assay. Cells were harvested for analysis while in the exponential growth phase (i.e., at 90% confluency) to ensure a constant and maximal growth rate, with regular cell population doubling. Genotyping of each patient cell line via PCR and Sanger sequencing, yielded unambiguous and high-quality sequencing data, which confirmed the expected genotype of each cell line and ruled out any possible sample mix-ups.

5.2.6 Objective 2.2: Evaluation of steady state ETFQO and mitochondrial RC enzyme levels

Objective 2.2: "Characterise selected relevant molecular properties of these in vitro models, including (a) the steady state protein level of the affected enzyme(s) and (b) mitochondrial RC enzymes CI–CV."

Upon investigating the steady state level of ETFQO as a result of the *ETFDH* genotype, it was found that the largest reduction occurred in P3 (c.[287dupA*];c.[1448C>T], -83%), followed by P9 (c.[1067G>A];c.[1448C>T], -73%) and P5 (c.[1448C>T];c.[1448C>T], -55%). Firstly, as the c.[287dupA*] variant introduces a premature termination codon in exon three of *ETFDH* (a thirteen-exon gene), it is possible that the resulting mRNA or protein is degraded more significantly compared to the other two genotypes (Shi *et al.*, 2015). In this case, the only protein present would be that encoded by the remaining allele, which carries the pathogenic c.[1448C>T] variant. Alternatively, if the c.[287dupA*]-carrying allele were to produce a protein product, the latter would be severely truncated and

likely unstable. Secondly, the reduction observed for c.[1448C>T];c.[1448C>T] genotype, is corroborated by studies of Westover *et al.* (2003) and Cornelius *et al.* (2012). These authors demonstrated a decrease in ETFQO stability, due to variants affecting the carboxy-terminal domain of the protein (like c.[1448C>T]), and the c.[1448C>T];c.[1448C>T] genotype itself, respectively (Gempel *et al.,* 2007). Finally, when comparing the results of the c.[1067G>A];c.[1448C>T] genotype to that of c.[1448C>T];c.[1448C>T], it becomes clear that the c.[1067G>A] variant produces a larger decrease in ETFQO. This is corroborated by similar results demonstrated by van der Westhuizen *et al.* (2018) in c.[1067G>A];c.[1448C>T] patient muscle. In addition, the more severely compromised ETFQO stability of P9, by comparison to P5, further supports the increased burden of disease experienced by individuals with the c.[1067G>A];c.[1448C>T] genotype by comparison to the c.[1448C>T];c.[1448C>T] genotype [MADD-DS3: 6.72, 9, 9, 13.95, and 22.5 versus 5.5, 7.5, and 11.4[33]]. In conclusion, the c.[287dupA*];c.[1448C>T], c.[1067G>A];c.[1448C>T], and c.[1448C>T];c.[1448C>T] genotypes have an increasingly detrimental effect on the stability of ETFQO, which likely affects the protein's function.

Next, the effect of the *ETFDH* genotype on the steady state level of mitochondrial RC enzymes CI–CV was assessed. While it was not possible to visualise MTCO1 (the probed subunit of CIV), the subunits of CI–CIII and CV were detected. For the c.[1448C>T];c.[1448C>T] genotype (P5), an increase in the relative quantity of CI (+48%), CII (+14%) and CIII (+99%) was observed. A decrease in ETFQO stability caused by the c.[1448C>T];c.[1448C>T] genotype has been linked to an increase in ROS and mitophagy (Cornelius *et al.,* 2014). Therefore, the augmented expression of CI–CIII was attributed to an upregulation of mitochondrial biogenesis as a potential compensatory mechanism to sustain energy production under these conditions (Cornelius *et al.,* 2014; Wen *et al.*, 2013). By contrast, the c.[287dupA*];c.[1448C>T] genotype (P3) displayed a decrease in CI (-11%) and CV (-27%), while the c.[1067G>A];c.[1448C>T] genotype (P9) exhibited a decrease in CV (-20%). These findings are proposed to be the result of secondary mitochondrial derangement (Gempel *et al.*, 2007; Gregersen & Olsen, 2010; Henriques *et al.*, 2010; Olsen *et al.*, 2007) caused by a larger decrease in ETFQO stability. This is supported by the work of Cornelius *et al*. (2014) who demonstrated a secondary decrease in FAO enzymes and ETC components in MADD patient fibroblasts. As a possible explanation, they hypothesised a shift in metabolism as an adaptive response to increased ROS (caused by electron-leakage from defective ETFQO and ETC components). Therefore, it can be concluded that, depending on the severity of the variant, the cell engages different rescue mechanisms to increase its energy production and limit cellular damage. In cases where the variant is mild and leads to a smaller decrease in enzyme activity, the cell responds by upregulating mitochondrial

[33] Note that the MADD-DS3 score of P5 (11.4) was likely compounded by the considerable delay in diagnosis, as explained in Section 5.2.3.3.

biogenesis and increases the use of the OXPHOS system. However, when the variant is more severe, cellular metabolism putatively shifts away from FAO and OXPHOS to alternative metabolic pathways.

Lastly, the relative quantity of CIII was found to be increased for each genotype {c.[287dupA*];c.[1448C>T] (P3; +50%), c.[1067G>A];c.[1448C>T] (P9; +17%), and c.[1448C>T];c.[1448C>T] (P5; +99%)}. The CIII subunit probed in this study was UQCRC2, which is located at CIII's CoQ10 reduction site. Considering the study by Wang *et al.* (2019), which reports that ETFQO physically interacts with CIII via UQCRC2, it is hypothesised that the upregulation of this subunit may be a cellular response to repair the defective connection between CIII and ETFQO, which is caused by structurally unstable ETFQO. This is further supported by the inverse proportional relationship between the relative quantities of ETFQO and UQCRC2, as observed for P3 and P9. By contrast, the increase in UQCRC2 of P5 is proposed to be the combined effect of this, together with upregulated mitochondrial biogenesis.

5.2.7 Objective 2.3: Evaluation of respiration in permeabilised fibroblasts

Objective 2.3: "Investigate the oxidation of relevant substrates in permeabilised primary fibroblasts."

As a final analysis, the effect of the *ETFDH* genotype on respiration through the OXPHOS system was assessed. Despite accounting for variability caused by external factors and differences in sample concentration, inter-day variation between each replicate, as well as variation between C1–C3, were observed. The former was attributed to the effect of subtle cell culture differences, while the latter was ascribed to biological variation. In spite of this, the results were analysed, and control and patient samples were compared.

Of the patients analysed, cells with the c.[287dupA*];c.[1448C>T] genotype (P3) yielded the largest decrease in FCE through the F-pathway (which is fed by FAO in the presence of a non-saturating concentration of malate). This cell line further displayed decreased respiration through the FN-pathway. On both accounts, the findings corresponded to the decreased steady state levels of ETFQO (most decreased) and CI, respectively. Upon addition of an S-pathway substrate, respiration through the FNS-pathway could be restored to levels comparable to those of a healthy control. Considering the normal steady state levels of CII and the decreased FCE values preceding this addition, this may imply an increased efficiency in this patient's fibroblasts to catabolise S-pathway substrates. As explained in Section 4.3.4, an increase in respiration following the addition of Cyt c indicates potential damage to the outer mitochondrial membrane but may be inherent to some cell types if observed consistently (Bioblast, 2023). This was the case for the dermal fibroblasts analysed in this study (C1–C3, P5, and P9), which displayed a small, but consistent increase in Cyt c CE following optimisation of the digitonin concentration and consequently, indicated an intact outer mitochondrial membrane. By comparison, however, P3 exhibited a larger Cyt c

CE. As this finding was observed in both replicates, it is hypothesised that the pathology associated with the c.[287dupA*];c.[1448C>T] genotype results in an outer mitochondrial membrane that is more vulnerable to experimental damage (Bioblast, 2023). Uncoupling of the mitochondria of P3 further resulted in a decreased CCE, when compared to the healthy controls. One possible explanation is the increase in Cyt c CE, which may contribute to a less coupled ETC. However, when taking into account the overall lower respiration displayed by P3 when compared to C1–C3, P5, and P9, a decrease in CCE may also indicate a lower excess respiratory capacity of the cell line.

As observed for P3, cells with the c.[1067G>A];c.[1448C>T] genotype (P9) also exhibited decreased respiration through the F-pathway, which once again corresponded (proportionally by comparison to P3) to the decreased steady state level of ETFQO. Interestingly, however, respiration through the FN-pathway was increased, despite no increase in the relative quantity of CI. It is, therefore, hypothesised that P9's fibroblasts are capable of more effectively catabolising N-pathway substrates. Moreover, P9 was the only cell line to display increased respiration through the FNSGp-pathway following the addition of glycerophosphate, which implies that this cell line is also more capable of utilising glycerophosphate. Once uncoupled, the fibroblasts of P9 exhibited normal CCE. Considering their more efficient use of N- and Gp-pathway substrates, it may be concluded that cells with the c.[1067G>A];c.[1448C>T] genotype maintain sufficient excess respiratory capacity. When inspecting the respiration of c.[1448C>T];c.[1448C>T] cells, it was concluded that the F-pathway was unaffected in this patient (P5), despite a decrease in the structural stability of ETFQO. This finding was corroborated by Cornelius *et al.* (2012), who reported little to no changes in the ETFQO activity of c.[1448C>T];c.[1448C>T] HEK293 cells. Furthermore, upon evaluating the FN- and FNS-pathways, normal respiration was observed. These results were supported by the upregulation of CI and CII expression, and offered additional evidence that this genotype leads to the upregulation of mitochondrial biogenesis (see Section 5.2.6). Consequently, the normal CCE observed for P5 was as expected and indicated sufficient excess respiratory capacity in cells affected by the c.[1448C>T];c.[1448C>T] genotype.

In conclusion, the findings of this objective show that the c.[287dupA*];c.[1067G>A] genotype decreases respiration through the F- and FN-pathways in fibroblasts and may compromise the stability of the mitochondrial outer membrane, thereby contributing to a less coupled ETC or a lower excess respiratory capacity. The c.[1448C>T];c.[1448C>T] genotype, on the other hand, displays sufficient respiration through the F-pathway with increased flux through the FN- and FNS-pathways. These results are attributed to the genotype, which does not considerably affect the activity of ETFQO and leads to an upregulation of mitochondrial biogenesis (Cornelius *et al.*, 2012; Cornelius *et al.*, 2014; Wen *et al.*, 2013). Finally, respiration through the F-pathway is also decreased by the c.[1067G>A];c.[1448C>T] genotype. However, in this instance, fibroblasts are capable of amending respiration via the more effective use of N- and Gp-pathway substrates. In addition, the use of the S-pathway substrate, succinate, appears to restore respiration in c.[287dupA*];c.[1067G>A] fibroblasts. These findings, demonstrate the benefit of alternative

energy substrates. By considering the different responses to N-, S-, and Gp-pathway substrates, the value of patient-specific treatment is evident.

5.2.8 Objective 2.4: Description of MADD in Southern Africa

Objective 2.4: "Propose a list of pathogenic variants and their clinical and biochemical characteristics in the Southern African population that can be translated to national diagnostic services."

The following provides a summary for multidisciplinary teams (including clinicians, pathologists, medical scientists, and dieticians) involved in the diagnosis of MADD in the Southern African population and gives a brief description of the clinical, biochemical (metabolic), genetic, and functional findings of this study:

This study investigated a cohort of 14 Southern African patients (12 of White SA and two of mixed ethnicity) diagnosed clinically and metabolically with MADD. All patients were born to non-consanguineous parents. WES analysis of the cohort revealed five autosomal recessive variants in the *ETFDH* (NM_004453.4) gene including, four non-synonymous single nucleotide variants {c.[740G>T] (p.[Gly247Val]) (rs1384574225), c.[976G>C] (p.[Gly326Arg]), c.[1067G>A] (p.[Gly356Glu]), and c.[1448C>T] (p.[Pro483Leu]) (rs377656387)}, and one duplication variant {c.[287dupA*] (p.[Asp97Glyfs*24])}. All variants were further classified as likely pathogenic or pathogenic according to the ACMG criteria and were encountered as two homozygous (c.[1067G>A];c.[1067G>A] and c.[1448C>T];c.[1448C>T]) and four compound heterozygous genotypes (c.[287dupA*];c.[1448C>T], c.[740G>T];c.[1448C>T], c.[1067G>A];c.[1448C>T], and c.[976G>C];c.[1067G>A]) (Richards *et al.,* 2015). Each patient presented with hallmark clinical features and urinary metabolic markers of MADD and could be grouped based on disease severity and treatment response, as summarised in Table 5.2.

The clinical and metabolic profiles of mild and/or late-onset MADD patients were heterogeneous, and it is advised to take special care when diagnosing patients of this kind. Time of sampling is also of particular importance when investigating the metabolic profile of MADD. To be most informative, it is recommended that sampling be done during the catabolic state or metabolic crisis, or before the next feeding (for neonates), following minimal medication intake and prior to the administration of glucose infusion. Notably, our data showed that, depending on the time of sample collection or systemic free carnitine status, a diagnosis of mild and/or late-onset MADD may be missed metabolically. In such cases, repeat metabolic testing is advised (including the assessment of plasma/serum free carnitine and acylcarnitines)[34] along with

[34] Diagnostic metabolic markers of MADD include urinary organic acids, plasma/serum acylcarnitines and plasma/serum amino acids. This study reports urinary metabolites only, due to the unavailability of historic blood plasma/serum sample data.

a more holistic interpretation of the findings. As reported, FAO is initially and primarily affected, followed by BCAA catabolism in a clinically mild or moderate presentation of MADD (van Rijt *et al.,* 2019).

Moreover, the use of disease severity scores, such as MADD-DS3 was found to be helpful when diagnosing the specific phenotype. However, it is important to note that the duration of symptom-onset to diagnosis is particularly significant and, if delayed, may result in skewed data. Apart from MADD types I, I/II, or II, the clinical phenotype and urinary metabolic profile typically improves following dietary adjustment[35] together with Rb, L-carnitine, or Rb and L-carnitine therapy. While L-carnitine supplementation does remove toxic accumulating metabolites, Rb should always be included as a first-line treatment to stabilise ETFQO and to limit the strain on the detoxification system (Cornelius *et al.,* 2012; Gordon 2006; Mels *et al.,* 2011; Sim *et al.,* 2002).

Genetically, this study marks the first report of the c.[287dupA*] variant, which has been identified in a single Southern African family in this instance. The c.[1067G>A] variant is highly pathogenic and is suspected to lead to a terminal phenotype when encountered as a homozygous genotype or compound heterozygous genotype along with a variant affecting the same protein domain (e.g., c.[976G>C]). Based on *in vitro* findings, we hypothesise that c.[287dupA*] is also highly pathogenic, whereas the c.[1448C>T] variant contributes to a milder presentation. In addition, the c.[1067G>A] and c.[287dupA*] variants appear to significantly increase the pathogenicity when encountered as part of a compound heterozygous genotype with another variant. Considering the allele (carrier) frequency, the c.[1067G>A] and c.[1448C>T] variants are expected to occur in <5 African- and <1 White SA, Indian, or mixed ethnicity newborns per annum, with the c.[1067G>A] variant likely being the result of a founder effect introduced to the White SA population. Finally, it should be noted that, in Southern Africa, rapid and inexpensive methods are available to screen for genetic variants following a primary diagnosis by metabolic testing. Since the data in this study indicate it is possible to anticipate the clinical progression and treatment response of a patient when the variant(s) involved are known, clinicians are urged to genetically confirm a diagnosis of MADD. Importantly, while not detected in this instance, it should be kept in mind that MADD can also be caused by variants in the *ETFA* and *ETFB* genes, whereas variants in the *SLC52A1−3, SLC25A32,* and *FLAD1* genes result in MADD-like phenotypes.

[35] Dietary adjustments include a high-caloric diet with fat and protein restriction, and the strict avoidance of fasting.

Table 5.2: Summary of the clinical, biochemical (metabolic) and genetic features of MADD in Southern Africa

Genotypes		c.[1067G>A];c.[1067G>A]	c.[976G>C];c.[1067G>A]	c.[740G>T];c.[1448C>T]	c.[287dupA*];c.[1448C>T]	c.[1067G>A];c.[1448C>T]	c.[1448C>T];c.[1448C>T]
Clinical	*Onset*	Birth	Birth	Early childhood	Infant-early childhood	Birth-early childhood	Late childhood-adulthood
	Major organ systems involved[†]	• Liver • Muscle • Congenital	• Liver • Muscle • Congenital?	• Muscle	• Liver • Muscle	• Liver • Muscle • CNS	• Liver • Muscle • PNS
	Unusual symptoms	-	-	Beevor's sign	Ketosis	Ketosis	Ketosis
	Fatal	Yes (<M3)	Yes (<14D)	No	No	Observed	No
	Severity	Severe	Severe	Moderate	Moderate	Moderate	Mild
	Phenotype	Type I/II	Type I/II	Type III	Type III	Type II/III	Type III
Treatment response		Unresponsive	Unresponsive	Variably responsive	Variably responsive	Variably responsive	Responsive
Urinary metabolic markers[‡]							
	Organic acids	↑↑↑ EMA, GA, iVGly, 2HGA, DC6 ↑↑-↑↑↑ 2-MBGly ↑-↑↑↑ iBGly N-↑↑↑ c4DC8 ↑-↑↑ HGly, iBGly N DC8, DC10	-	-	-	↑↑↑ HGly ↑↑-↑↑↑ GA N-↑↑↑ EMA, iBGly, 2-HGA, c4DC8, DC6, DC8, DC10 N 2-MBGly, iVGly	↑↑↑ 2HGA, c4DC8 N-↑↑↑ EMA, GA, 2-MBGly, HGly, iVGly, iBGly, DC6, DC8
	Acylcarnitines	↑↑↑ C4, C5, C5DC ↑↑-↑↑↑ C10 ↑-↑↑↑ C6, C8	-	-	↑↑↑ C5DC, C10 ↑↑-↑↑↑ C4 ↑-↑↑↑ C5, C8 ↑-↑↑ C6	↑↑↑ C5DC ↑↑-↑↑↑ C10 ↑-↑↑↑ C4, C6 N-↑↑↑ C5, C8	↑↑↑ C5DC ↑-↑↑↑ C4 N-↑↑↑ C5DC N-↑↑ C6 ↑ C10 N-↑ C8
	Amino acids	↑↑↑ Sarcosine, 4-OH-Pro, Pro	-	-	↑↑↑ Sarcosine N-↑↑ Pro N-↑ 4-OH-Pro	↑↑-↑↑↑ Sarcosine N-↑↑↑ Pro N-↑↑ 4-OH-Pro	↑↑-↑↑↑ Sarcosine N-↑↑↑ 4-OH-Pro, Pro

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† For details, see Appendix B.2.

‡ Acylcarnitine and amino acid assessments are typically conducted on plasma/serum. In this study, this matrix was limited due to sample stability.

Arrows represent the number of times a metabolite is increased above the reference range, as a weighted mean of each group (↑↑↑: substantial increase; ↑↑: moderate increase; ↑: slight increase; N: normal). Metabolites due to secondary consequences are not reported.

Abbreviations: 2-HGA: 2-hydroxyglutaric acid; 2-MBGly: 2-methylbutyrylglycine; 4-OH-Pro: 4-hydroxyproline; C4: butyryl-/isobutyrylcarnitine; c4DC8: *cis*-4-decenedioic acid; C5: isovalerylcarnitine; C5DC: glutarylcarnitine; C6: hexanoylcarnitine; C8: octanoylcarnitine; C10: decanoylcarnitine; D and number: age in days; DC6: adipic acid; DC8: suberic acid; DC10: sebacic acid; EMA: ethylmalonic acid; GA: glutaric acid; HGly: *N*-hexanoylglycine; iBGly: *N*-isobutyrylglycine; iVGly: *N*-isovalerylglycine; M and number: age in months; Pro: proline

5.3 STRENGTHS, LIMITATIONS, AND FUTURE PROSPECTS

5.3.1 Strengths

In this study, recruitment of the cohort was performed by four academic public hospitals, which serve the wider population and collaborate with the two primary diagnostic centres for IEMs in SA (including the CHM and NHLS IMD molecular laboratory) (NMP, 2023; UCT, 2023). According to the most recent national census, 84.2% of the SA population is not covered by a private medical aid scheme (Statistics South Africa, 2023). Consequently, 90.3% of the African, 81.8% of the mixed, 51.1% of the Indian/Asian, and 28.1% of the White population are dependent on the services of public hospitals for medical care. Participant recruitment was further aided by a project awareness campaign, which proceeded via academic, clinical, and non-profit organisations (RDSA, 2023; SASHG, 2022). By employing these recruitment strategies, we believe that this study was inclusive and accessible to all, and allowed individuals of diverse ethnic and socio-economic backgrounds, residing in different regions of Southern Africa (including SA and Namibia), an equal opportunity to participate. Moreover, standardised protocols developed by the ICGNMD were used by adult and paediatric neurologists to comprehensively re-assess the clinical presentation of the recruited study cohort (Wilson *et al.,* 2023). These protocols included multiple accredited neuromuscular assessments, and to ensure comparability across different centres, all clinical presentations were recorded according to human phenotype ontology (HPO) terminology (HPO, 2023).

Another strength of this study was the ability to perform targeted metabolic profiling of newly collected samples via validated diagnostic methods at the CHM. In addition, historical clinical and metabolic data collected at the initial presentation of disease and following the implementation of treatment were available

for the majority of study participants, dating as far back as 1992. In this manner, the study was able to compare diagnostic metabolic markers of MADD recorded during states of decompensation and treatment to provide information on the therapeutic response of the participants and/or genotypes. To determine the allele frequency of the two variants that were most regularly observed in the Southern African MADD cohort, this study included well-powered sample sizes of 3,583 (c.[1067G>A]) and 4,308 (c.[1448C>T]) individuals. The PCR–RFLP methods employed to achieve this, which have since been extended to assess the c.[740G>T] variant (results not shown)[36], now provide clinicians with a rapid and inexpensive genetic screening test that can be used, following the metabolic diagnosis of future cases of MADD. This study further describes an updated (up until October 2023) list of all variants reported on PubMed to lead to MADD and MADD-like phenotypes (Mereis *et al.,* 2021). This knowledge is of particular importance to the global scientific community and will facilitate comparative research studies. In the updated list, 154 new variants leading to MADD and MADD-like phenotypes are shown. It is important to note that these variants were identified in the mere three years spanning the initial (October 2020) and updated literature search (up until October 2023) of Mereis *et al.* (2021). This considerable increase [35.3% of the total number of variants originally reported in Mereis *et al.* (2021)] highlights the number of variants that are yet to be discovered as genomic technologies advance and emphasises the impact of genetic screening. Finally, the investigation of patient dermal fibroblasts allowed for the confirmation of a genotype–phenotype correlation and offered insights that may contribute to patient-tailored treatment. Taken together, this study provides the first extensive evaluation of the clinical, biochemical (metabolic), genetic, and functional aspects of MADD in the understudied populations of Southern Africa.

5.3.2 Limitations

This study was limited in the sense that the cohort investigated only included individuals of two ethnic backgrounds (White SA and mixed). However, it is worth noting that MADD has previously been diagnosed in single cases in Southern African patients of African and Indian ethnicity by metabolic testing at the CHM and NHLS IMD molecular laboratory (Vorster & Meldau, 2023, personal communication). It is regrettable that, despite making every effort to include participants of these population groups (as explained in Section 5.3.1), these few patients were lost to follow-up prior to the onset of this study and that new cases did not present throughout the study's duration. It is our hope that this problem can be addressed by metabolic and genetic NBS in the future. Considering the metabolic analyses performed, another limitation was the unavailability of historic plasma/serum acylcarnitine and amino acid data. As

[36] The c.[287dupA*] and c.[976G>C] variants do not form part of homopolymeric regions and cannot be detected by PCR–RFLP analysis.

the majority of participants in this study were metabolically diagnosed during a period in which plasma/serum samples were not frequently requested or analysed due to logistical challenges, acylcarnitine and amino acid data from viable plasma/serum samples collected upon first presentation of MADD were not available and consequently, urinary data was evaluated instead.

One of the major aspects limiting genetic analyses in Southern Africa, and which also limited this study, is the paucity of publicly available control genetic data from the Southern African populations (including individuals of White SA ethnicity). Without this data, it was not possible to estimate, with confidence, the haplotype frequencies in this study and, as a result, we cannot definitively confirm the founder effects identified for the c.[1067G>A] and c.[1448C>T] variants in the White SA population at this time. The use of patient-derived dermal fibroblasts presents another potential limitation. At the onset of this thesis, one of its objectives was to develop disease models of the identified genotypes using CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein) in a commercial cell line. However, it was not possible to produce the desired disease models in this instance, due to the delayed provision of the reagents required for this process; thus, patient fibroblasts were obtained instead. While patient fibroblasts are a well-studied sample source and frequently used in the diagnosis of IEMs, their metabolism is more glycolytic (Cornelius *et al*., 2013; Leipnitz *et al.,* 2018; Olsen *et al.,* 2007; van den Heuvel *et al.*, 2004; van Rijt *et al.,* 2020). In addition, the genetic background of every patient is unique and will, therefore, contribute to biological variation and the results obtained. In SA, tissue biopsies (including skin biopsies) are also not generally requested as part of the IEM diagnostic process; consequently, no samples were available for the deceased individuals in this study (e.g., P6, P8, P12). It is clear that this, together with the logistical challenges faced to obtain patient skin biopsies, limited the number and replicates of genotypes studied *in vitro*. Finally, while it did not subtract from the study, another limitation was the inability to definitively identify the MTCO1 subunit (CIV) during steady state protein analyses. This protein is highly hydrophobic and known to exhibit variable migration in different SDS-PAGE gel systems (Mitomap, 2023). Although the analysis was repeated with samples denatured at different temperatures (results not shown), it was not possible to identify the subunit with confidence, and therefore, the relative quantity of CIV could not be studied.

5.3.3 Future prospects

The observations made in this study warrant the development of *in vitro* MADD disease models via gene editing technology (e.g., CRISPR/Cas), to study, in more detail, the genotypes identified in an energetically more relevant cell line(s) with a consistent genetic background. Such a cell line(s) may include any of the affected tissues and will allow the evaluation of the underlying mechanisms involved in the pathogenicity and treatment-mediated rescue of MADD, in the absence of patient-derived biological variation. An

interesting finding of this study was the increased expression (observed in all patient fibroblasts) of UQCRC2, the CIII subunit with which ETFQO has been shown to associate (Wang *et al.,* 2019). As the quantity of UQCRC2 was further found to be inversely proportional to that of ETFQO in two instances, future studies examining the hypothesis that UQCRC2 is upregulated as a cellular response to overcome the physical disconnect between FAO and the ETC may offer interesting new insights into the structural assembly of these metabolic pathways. In addition, this study demonstrates two metabolic adaptive responses to decreased energy production caused by pathogenic *ETFDH* variants. From the results, it is suggested that less severe genotypes (e.g., c.[1448C>T];c.[1448C>T]), in which the function of ETFQO is less affected, result in the upregulation of mitochondrial biogenesis (Cornelius *et al.,* 2012). By contrast, it is proposed that those genotypes caused by more severe variants (e.g., c.[287dupA*];c.[1448C>T] and c.[1067G>A];c.[1448C>T]), in which function is clearly affected, produce a shift in the metabolism from OXPHOS to alternative metabolic pathways. While the cause of such adaptive responses (e.g., ROS-production or mitophagy) was beyond the scope of this thesis, future studies regarding specific adaptive responses to MADD are warranted and may offer therapeutic benefit.

Considering the dramatic decrease in steady state ETFQO quantity, as well as the altered expression (and respiration) of the OXPHOS complexes, future investigations of the enzymatic activity of these protein and protein-complexes (which were beyond the scope of this thesis) are justified to evaluate potential diagnostic targets of MADD as well as the contribution of secondary mitochondrial dysfunction to the observed phenotype. Studies (e.g., respirometry or enzymatic activity) regarding the response of MADD to different therapeutic interventions (e.g., Rb, ketone bodies, and bezafibrate) are also required to determine whether there is merit to genotype-specific (i.e., patient-specific) treatment strategies for this IEM (Gautschi *et al.,* 2015; Shioya *et al.*, 2014, Yamada *et al.*, 2017; van Rijt *et al.,* 2020). Finally, one of the late objectives of the ICGNMD, to which this thesis has contributed, was to build trial-ready cohorts in understudied populations with the aim to increase the potential for clinical trial involvement in future therapeutic studies.

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Appendix A

Peer-reviewed article i and
Supplementary Material:
Chapter 2

A.1 PEER-REVIEWED ARTICLE I

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Review article

Disorders of flavin adenine dinucleotide metabolism: MADD and related deficiencies

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ABSTRACT

Multiple acyl-coenzyme A dehydrogenase deficiency (MADD), or glutaric aciduria type II (GAII), is a group of clinically heterogeneous disorders caused by mutations in electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO) – the two enzymes responsible for the re-oxidation of enzyme-bound flavin adenine dinucleotide ($FADH_2$) via electron transfer to the respiratory chain at the level of coenzyme Q10. Over the past decade, an increasing body of evidence has further coupled mutations in FAD metabolism (including intercellular riboflavin transport, FAD biosynthesis and FAD transport) to MADD-like phenotypes. In this review we provide a detailed description of the overarching and specific metabolic pathways involved in MADD. We examine the eight associated genes (*ETFA, ETFB, ETFDH, FLAD1, SLC25A32* and *SLC52A1–3*) and clinical phenotypes, and report ~436 causative mutations following a systematic literature review. Finally, we focus attention on the value and shortcomings of current diagnostic approaches, as well as current and future therapeutic options for MADD and its phenotypic disorders.

FAD metabolism and MADD facts

- Flavoproteins are intricately involved in the mitochondrial metabolism and require oxidised FAD as cofactor.
- The re-oxidation of $FADH_2$ is mediated by the ETF-ETFQO system, while the synthesis and transport of FAD depends on the combined action of RFVT1–3, FADS and MFT.
- Genetic defects in *ETFA*, *ETFB* and *ETFDH* lead to a potentially fatal mitochondrial myopathy called multiple acyl-CoA dehydrogenase deficiency (MADD).
- Disorders associated with mutations in *FLAD1*, *SLC25A32* and *SLC52A1–3* also frequently present with MADD-like phenotypes and -biochemistry.
- The effective diagnosis of MADD, resulting from >436 mutations, is challenging and involves a combination of biochemical investigations, enzyme/flux assays and next generation sequencing.
- Apart from MADD Types I and II and severe FADS dysfunction, the majority of MADD(-like) phenotypes are very amendable to treatment by riboflavin.

1. Introduction

Acyl-coenzyme A (CoA) dehydrogenases (ACDHs), a family of homologous mitochondrial flavoproteins that contain flavin adenine dinucleotide (FAD) as a non-covalently-bound prosthetic group, are key players in mitochondrial metabolism (Battaile et al., 2002; Henriques et al., 2010). Of the 11 ACDHs currently identified, a total of nine partake in: (i) mitochondrial fatty acid β-oxidation (FAO), (ii) branched chain amino acid (BCAA) catabolism and (iii) amino acid catabolism (Ghisla and Thorpe, 2004; Zhang et al., 2019). These include: (i) acyl-CoA dehydrogenase 9 (ACAD9), short-, medium-, long-, and very-long-chain acyl-CoA dehydrogenase (SCAD, MCAD, LCAD and VLCAD), (ii) isovaleryl-CoA-, isobutyryl-CoA-, and short branched-chain acyl-CoA dehydrogenase (IVD, IBD and SBCAD) and (iii) glutaryl-CoA

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dehydrogenase (GCDH). To enable the sustained operation of these enzymes, their reduced FAD groups require continuous re-oxidation by the concerted action of electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO) (Ghisla and Thorpe, 2004). In addition, three other mitochondrial dehydrogenases rely on ETF and ETFQO as redox partners, including 2-hydroxyglutarate- (2HGDH), dimethylglycine- (DMGDH; involved in amino acid metabolism), and sarcosine dehydrogenase (SDH; involved in choline metabolism), while FAD is also required for the function of hydroxyproline- (HYPDH) and proline dehydrogenase (PRODH) (Ostrander et al., 2009; Summitt et al., 2015; Toplak et al., 2019; Zhang et al., 2019).

The importance of the ETF-ETFQO system is exemplified by the existence of a variety of different inborn errors of metabolism, collectively called multiple acyl-CoA dehydrogenase deficiency (MADD) (Goodman and Frerman, 1984; Grünert, 2014). MADD, also known as glutaric aciduria type II (GAII), may be classified as a combined FAO- and protein

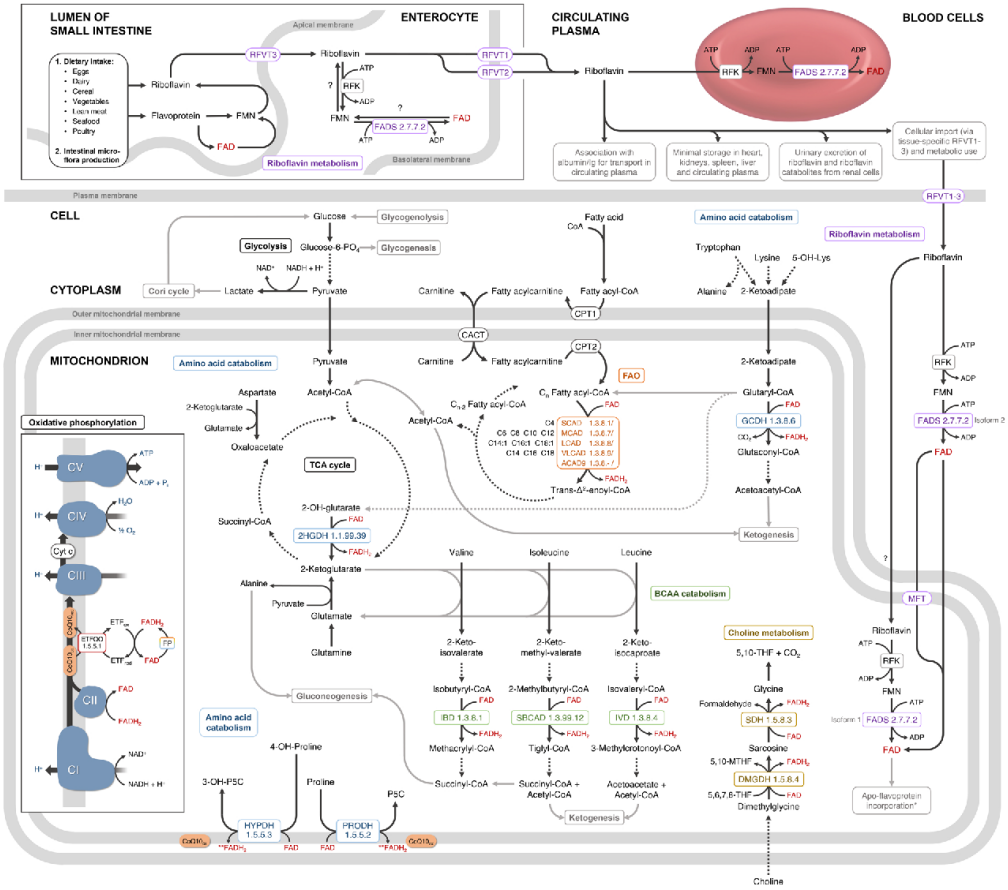

Fig. 1. Riboflavin metabolism and flavoproteins affected in MADD(-like disorders) In the small intestine, flavoproteins are denatured, thereby releasing FMN and FAD which can be converted into riboflavin via non-specific hydrolases. Riboflavin is then absorbed into the blood and subsequently taken up by different blood cells (including erythrocytes and leukocytes) or bound to albumin/immunoglobulins to ensure transport through the circulation and tissue-uptake via cell-type specific RFVTs for cellular use or urinary excretion. Once inside the cell, riboflavin can be converted to the different enzymatically active cofactors (FMN and FAD) within the cytoplasm and mitochondrion. A deficiency of FAD, caused by a genetic defect in ETF, ETFQO, RFVT1 3, FADS or MFT may affect at least 14 flavoproteins, resulting in the accumulation of their associated metabolites. Dashed lines indicate omitted metabolic steps. Question marks refer to presently undefined hydrolases and mitochondrial import-export mechanisms. * Hypothesized to occur in the mitochondrion and cytoplasm via protein-protein interactions between FADS and apo-flavoproteins; ** Hypothesized to donate electrons directly to $CoQ10_{ox}$. Abbreviations: 2HGDH, 2-hydroxyglutarate dehydrogenase; ACAD9, acyl-CoA dehydrogenase 9; ADP and ATP, adenosine diphosphate and -triphosphate (respectively); CACT, carnitine-acylcarnitine translocase; CI CV, mitochondrial respiratory chain complexes I V; CoA, coenzyme A; CPT1 and CPT2, carnitine palmitoyltransferase 1 and 2; CoQ10, coenzyme Q10; Cyt c, cytochrome c; DMGDH, dimethylglycine dehydrogenase; ETF, electron transfer flavoprotein; ETFQO, ETF-ubiquinone oxidoreductase; FAD and $FADH_2$, flavin adenine dinucleotide (oxidised and reduced, respectively); FADS, FAD synthase; FAO, mitochondrial fatty acid β-oxidation; FMN, flavin mononucleotide; FP, flavoprotein; GCDH, glutaryl-CoA dehydrogenase; HYPDH, hydroxyproline dehydrogenase; IBD, isobutyryl-CoA dehydrogenase; Ig, immunoglobulin; IVD, isovaleryl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; MFT, mitochondrial FAD transporter; MTHF, methyl-tetrahydrofolate; NAD^+ and NADH, nicotinamide adenine dinucleotide (oxidised and reduced, respectively); OH, hydroxy; ox, oxidised; P5C, Δ^1-pyrroline-5-carboxylate; P_i, inorganic phosphate; PO_4, phosphate; PRODH, proline dehydrogenase; red, reduced; RFK, riboflavin kinase; RFVT1 3, riboflavin transporter 1 3; SBCAD, short branched-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; SDH, sarcosine dehydrogenase; TCA, tricarboxylic acid; THF, tetrahydrofolate; VLCAD, very-long-chain acyl-CoA dehydrogenase (For interpretation of the metabolic pathways to colour in this figure, the reader is referred to the web version of this article).

metabolism disorder resulting in lipid storage myopathy and is historically caused by mutations in the genes coding for ETF and ETFQO (Vasiljevski et al., 2018; Wanders et al., 2010). More recently, mutations in the genes coding for enzymes involved in the metabolism of riboflavin – the precursor of FAD – have also been shown to present as MADD-like phenotypes (Bosch et al., 2012; Green et al., 2010; Haack et al., 2012; Ho et al., 2011; Johnson et al., 2012, 2010; Mosegaard et al., 2020; Olsen et al., 2016; Schiff et al., 2016; Taylor et al., 2014). In this review, we present the current state of knowledge on the metabolic pathways which are affected in MADD and the genes involved in the disorders of FAD metabolism from the perspective of both the ETF-ETFQO system as well as riboflavin transport and metabolism. We provide an overview of the phenotypes and diagnostic procedures used to identify these disorders and discuss available and future therapeutic avenues.

2. Pathways

In its fully oxidised state, FAD functions by accepting hydrogen ions and electrons from the substrates of mitochondrial flavoproteins. In order to maintain mitochondrial flavoproteome homeostasis, the resulting $FADH_2$ needs to be re-oxidised by the action of ETF, which is a heterodimer comprised of a 30 kDa ETFα and 28 kDa ETFβ subunit located in the mitochondrial matrix (Frerman and Goodman, 2001; Ruzicka and Beinert, 1975). This protein in turn, is re-oxidised by ETFQO, a 64 kDa monomer embedded in the inner mitochondrial membrane, which feeds the transferred electrons into the respiratory chain at the level of coenzyme Q10 (CoQ10) (Frerman, 1987). Together, ETF and ETFQO couple various metabolic pathways to the oxidative phosphorylation (OXPHOS) system for the production of adenosine triphosphate (ATP) (Fig. 1).

In humans, FAD and its precursor flavin mononucleotide (FMN) are produced from riboflavin, a water-soluble vitamin (B2) which itself cannot be synthesized in humans, but instead is obtained from dietary sources and in part from intestinal microflora (Fig. 1) (Barile et al., 2016). From the small (and less so, the large) intestine, free riboflavin is transported into the enterocyte via the action of riboflavin transporter (RFVT) 3, and released into the blood by RFVT1 and 2. From here, riboflavin (bound to albumin/immunoglobulin) can be transferred to various tissues and transported into cells by RFVT1−3, which are expressed in a tissue-specific manner (see Barile et al., 2016 for review). Once inside the cytoplasm, riboflavin is phosphorylated to FMN by riboflavin kinase (RFK), and subsequently adenylated to FAD by FAD synthase (FADS) (Brizio et al., 2006; Henriques et al., 2010; Leone et al., 2018). A similar process takes place in the mitochondrion following the import of riboflavin. The enzymatically active cofactor (FAD) can then be transported to and from the mitochondrion via the mitochondrial folate/FAD transporter (MFT), and incorporated into apo-flavoproteins. Moreover, MFT is involved in the mitochondrial export of FAD for the maintenance of flavin homeostasis and FAD-dependant cell signalling processes (Giancaspero et al., 2015; Vergani et al., 1999). Since humans have a very low storage capacity for riboflavin, any surplus will be excreted in urine, a factor which contributes to riboflavin's low toxicity, even at pharmacological doses.

Due to the central catalytic function of FAD in ETF, ETFQO, RFVT1−3, FADS and MFT, as well as its role in CoQ10 reduction, at least 14 flavoproteins (nine ACDHs, 2HGDH, DMGDH, SDH, HYPDH and PRODH), distributed across a multitude of interconnected secondary metabolic pathways, may be affected in MADD(-like disorders) (Frerman and Goodman, 2001; Henriques et al., 2010). Under normal physiological conditions, ATP is produced via glycolysis and FAO, of which the latter is the main energy pathway in the heart, skeletal muscle and kidney. However, in MADD the ACDH-catalysed reactions within the FAO and BCAA catabolic pathways are deficient and metabolism shifts to glycolysis, followed by glycogenolysis in order to cope with the energy demand. Accordingly, pyruvate (and subsequently lactate) increases, while acetyl-CoA production declines and various fatty acyl-CoA species accumulate, resulting in the secondary accumulation of the corresponding acylglycine and -carnitine conjugates. The decrease in acetyl-CoA limits hepatic ketogenesis and results in the proteolytic degradation of muscle tissue to supply glucogenic and ketogenic substrates for energy production. The decreased availability of FAD further reduces the catabolism of BCAAs and other amino acids (lysine, 5-hydroxylysine, tryptophan, alanine, glutamate, aspartate, sarcosine, proline and 4-hydroxyproline) in the muscle, leading to the accumulation of their related intermediates and to a decrease in the ketogenic substrate, acetoacetate. While MADD generally presents without ketonuria, it should be noted that exceptions have been reported, owing to variants that result in adequate residual levels of the affected ACDHs in FAO and BCAA catabolism (Cairns and Connell., 2018; Chandra et al., 2018). MADD caused by mutations in *ETFDH* may also be associated with secondary CoQ10 deficiency, which, together with the metabolic stress, will lead to a decreased activity of the respiratory chain complexes (Gempel et al., 2007). Consequently, depending on the severity of the genetic variant, MADD may give rise to varying degrees of disturbed energy homeostasis, as well as the accumulation of toxic metabolites and cellular starvation.

3. Clinical and genetic features

Deficiencies of those flavoproteins that rely on ETF and ETFQO and their cofactor (FAD and thus riboflavin) lead to a number of disease phenotypes that are generally recognized as *metabolic myopathies* (Gaspar et al., 2019). These disorders have overlapping clinical features and are biochemically characterized by a set of metabolite abnormalities typical for MADD, as described in more detail in the diagnosis section. The deficiencies and resulting phenotypes associated with the genes involved in this metabolism are summarized in Table 1. Table S1 provides a list of 436 published pathogenic genetic variants (mutations) that have been associated with these phenotypes, as well as 158 additional potential pathogenic variants from the Ensembl Genome Browser (https://www.ensembl.org/). Table S2 provides an overview of the distribution of the variants in the gene structures. It should be noted that most of these 594 variants where not scrutinized using current classification guidelines to determine pathogenicity (Richards et al., 2015).

3.1. *Multiple acyl-CoA dehydrogenase deficiency (MADD/GAII)*

The first case of MADD/GAII was described over 40 years ago (Przyrembel et al., 1976). The disorder presents with a heterogeneous and multi-system phenotype which may be divided into three clinical classes including Types I and II – two ultimately fatal, neonatal-onset subtypes presenting with or without congenital features [MADD-severe (S)], and Type III – a mild and/or later-onset form [MADD-mild (M)]. As the disease severity correlates well with MADD's response to high-dose riboflavin treatment, it has been suggested to classify these phenotypes as riboflavin-unresponsive (RU-MADD) or -responsive (RR-MADD) (Yıldız et al., 2019).

Together, MADD has a general prevalence of 1 to 9:1,000,000 and an estimated birth prevalence of 1:200,000 (https://www.orpha.net/; ORPHA:26791), with wide variation reported between different countries and ethnicities. Historically, MADD is caused by autosomal recessive mutations in the *ETFA* (36), *ETFB* (19) and *ETFDH* (280) genes, which encode the alpha and beta subunits of ETF, and ETFQO, respectively (Table S1). The majority of these reported mutations are private and more or less equally distributed in both MADD Types I and II (Table S1), while Type III is most frequently caused by mutations in *ETFDH* (Grünert, 2014). In addition, MADD Types I and II display a strong genotype-phenotype correlation, with the former most often being associated with homozygous null mutations and the latter resulting from mutations often leading to amino acid substitutions which allow some level of residual enzyme activity. Conversely, while individuals with MADD Type III have at least one missense mutation and do not display a

Table 1
Clinical phenotypes of genetic disorders associated with ETF, ETFQO and FAD metabolism.

Deficiency	**ETF and ETFQO**			**Riboflavin transporters**			**FAD synthase**	**MFT**
OMIM	#231680/608053/130410/#231675			607883/#615026	607882	613350/#211500/ #211530	610595/#255100	610815
Gene*	*ETFA*, *ETFB*, *ETFDH* for classification as glutaric aciduria II (GA2)A, GA2B, GA2C, respectively			*SLC52A1*	*SLC52A2*	*SLC52A3*	*FLAD1*	*SLC25A32*
Protein	ETFα, ETFβ, ETFQO			RFVT1	RFVT2	RFVT3	FADS	MFT
Clinical phenotypes**	MADD Type I (MADD-S, RU-MADD)	MADD Type II (MADD-S, RU-MADD)	MADD Type III (MADD-M, RR-MADD)	Riboflavin deficiency	BVVLS-2	Fazio-Londe disease/ BVVLS-1	Lipid storage myopathy	Riboflavin-responsive exercise intolerance
Inheritance	AR	AR	AR	AD, possibly haploinsufficiency, maternal	AR	AR	AR	AR
Onset	neonatal, severe	neonatal	mild and/or late-onset	neonatal / pregnancy	all ages	all ages	all ages	early age
Clinical and biochemical features	• with congenital anomalies • multi-system involvement • hypotonia • cardiomyopathy • hepatomegaly • nonketotic hypoglycemia • metabolic acidosis • hyperammonemia (see text for detailed metabolic profile)	• without congenital anomalies	• high variability • recurrent episodes of lethargy, vomiting • muscle, cardiac, liver involvement • hypoglycemia • metabolic acidosis (see text for detailed metabolic profile)	• transient neonatal riboflavin deficiency • lethargy • hypotonia • poor peripheral circulation • hypothermia • lactic acidosis • MADD-type metabolites	• ponto-bulbar palsy • sensory ataxia • optic atrophy • early-onset muscle weakness of upper limbs and axial muscles • hearing loss • cranial nerve deficits • respiratory symptoms • feeding difficulties • MADD-type • metabolites are possible	• ponto-bulbar palsy • muscle weakness in upper limbs and axial muscles • hearing loss (BVVLS-1 only) • cranial nerve deficits • respiratory symptoms • feeding difficulties • sensory symptoms • MADD-type metabolites are possible	• hypotonia • muscle weakness • swallowing and speech difficulties • respiratory symptoms • lipid storage myopathy • acylcarnitine abnormalities or ethylmalonic-and/or adipic aciduria	• recurrent exercise intolerance • acylcarnitine abnormalities
Effective Treatment***	diet (low fat and protein)	diet (low fat and protein)	riboflavin, carnitine, glycine, CoQ10	riboflavin	riboflavin	riboflavin	riboflavin	riboflavin
Prognosis (with treatment)	fatal	fatal	good	good	good	good	mostly fatal	good
References	Frerman and Goodman, 2001; Grünert, 2014; van Rijt et al., 2019			Chiong et al., 2007; Mosegaard et al., 2017	Bosch, 2020; Foley et al., 2014; Jaeger and Bosch, 2016	Bosch, 2020; Bosch et al., 2011; Jaeger and Bosch, 2016	Bosch, 2020; Olsen et al., 2016	Bosch, 2020; Schiff et al., 2016

*See Table S1 for variants; ** Recognized by OMIM; *** Variability should be recognized. Abbreviations: AD, autosomal dominant; AR, autosomal recessive; BVVLS-1 and -2, Brown-Vialetto-Van Laere syndrome 1 and -2; MADD-M and -S, MADD-mild and -severe; RR- and RU-MADD, riboflavin-responsive and -unresponsive MADD.

significantly reduced activity of the affected enzyme(s), the genotype-phenotype correlation is poor (Grünert, 2014).

3.2. Riboflavin transporter deficiencies

Although dietary deficiency of riboflavin has been well-documented (Powers, 2003), genetic disorders of the three riboflavin transporters (RFTV1–3) have only been recognized in the past two decades. Of these, the least frequently reported is a deficiency of RFVT1, which has only been described in three cases of dominantly inherited, adult-onset or neonatal (transiently expressed) riboflavin deficiency (Chiong et al., 2007; Ho et al., 2011; Mosegaard et al., 2017). More frequently, two related autosomal recessive disorders of RFVT2 [Brown-Vialetto-Van Laere syndrome (BVVLS)-2] and RFVT3 (Fazio-Londe disease/BVVLS-1) have been described, with BVVLS-2 showing distinguishing features of ponto-bulbar palsy, ataxia and optic atrophy (Bosch et al., 2011; Cornelius et al., 2012; Foley et al., 2014; Ho et al., 2011; Olsen et al., 2016; Schiff et al., 2016). At present, only two mutations have been published for *SLC52A1* (coding for RFVT1), whereas 31 and 52 mutations have been published for *SLC52A2* and *3* (coding for RFVT2 and 3), respectively (see Table S1).

3.3. FAD synthase and transporter deficiencies

Two additional, mostly riboflavin-responsive phenotypes, resulting from dysfunction in the synthesis or transport of FAD, have been identified. A lipid storage myopathy with a variable age of onset and a mostly fatal outcome, is associated with FADS deficiency, resulting from mutations in *FLAD1*, of which 13 have been reported (Table S1). Furthermore, three mutations have hitherto been identified in *SLC25A32*, which cause a deficiency of the inner mitochondrial membrane carrier MFT and result in a milder, recurrent exercise intolerance phenotype. To date, however, no mutations have been identified in human *RFK* (involved in FAD biosynthesis) (Ryder et al., 2019). Owing to the many unsolved cases of riboflavin deficiency, it is very likely that the number of affected genes will increase in the future as research advances (Olsen et al., 2016).

4. Diagnosis

Dysfunction of ETF, ETFQO and the riboflavin metabolism, together with the associated secondary flavoprotein deficiencies, affects a myriad of metabolic pathways – as summarized in Fig. 1. These deficiencies are expressed as phenotypes that are recognized in a clinical setting through their varied neonatal- or late-onset MADD clinical presentations, and via newborn screening (NBS) programmes in which they are included (Frerman and Goodman, 2001; Houten et al., 2016; Sahai et al., 2014).

4.1. Conventional diagnostic approach

In the event of a MADD-related clinical presentation (symptomatic patients) or abnormal NBS profile (asymptomatic patients), the differential diagnosis of MADD(-like disorders), as illustrated in Fig. 2, typically proceeds via broad-spectrum metabolite analyses. This may be

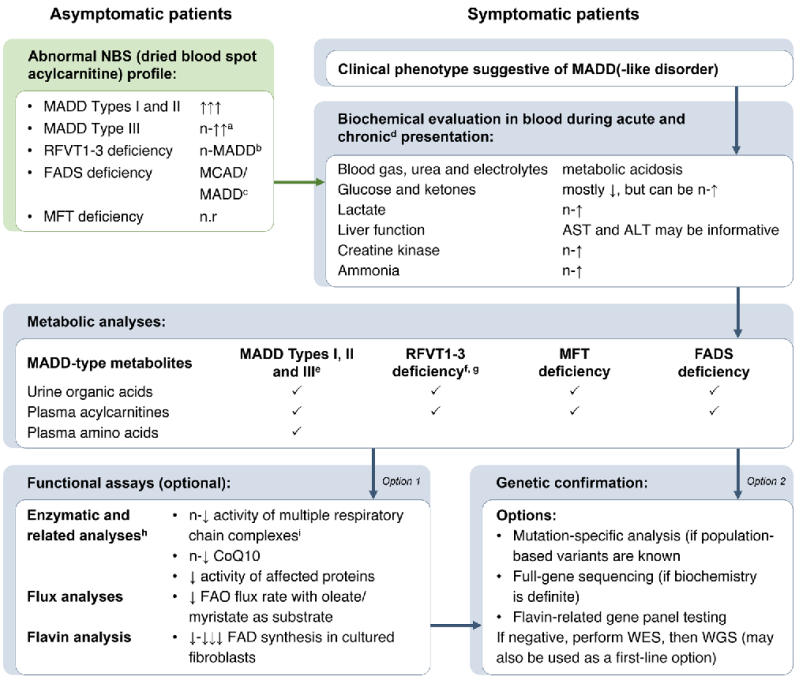

Fig. 2. Overview of the differential diagnosis for MADD(-like disorders) [a] Low birth weight and/or total parenteral nutrition may result in a MADD-like acylcarnitine profile (Shai et al., 2008); [b] NBS is not feasible for the diagnosis of RFVT2 and 3 deficiencies (due to suspected compensation by maternal riboflavin), whereas RFVT1 deficiency has displayed normal or abnormal (MADD) NBS profiles in the two reported cases (Bosch et al., 2011; Ho et al., 2011; Mosegaard et al., 2017); [c] NBS has only been reported in three cases (Muru et al., 2019; Ryder et al., 2019; Yamada et al., 2019); [d] Biochemical parameters may normalize and abnormal liver function is more to likely to occur later in the disease progression; [e] Metabolic analysis is most indicative during catabolic status/metabolic crisis, as the profile may be normal otherwise; [f] Some genotypes may have normal metabolic profiling; [g] Metabolite analysis can be indicative, but may be normal in some patients (molecular analysis of RFTV1-3 is required for definite diagnosis) (Jaeger and Bosch, 2016); [h] Should enzymatic assays be unavailable, diagnosis may proceed directly to genetic analyses or a combination of flux- and genetic assays; [i] Only CII activity has been analysed in MFT deficiency (Hellebrekers et al., 2017). ✓, can be indicative; ↓, ↓↓↓, slight and substantial decrease; ↑, ↑↑ and ↑↑↑, slight, moderate and substantial increase. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CoQ10, coenzyme Q10; FAD, flavin adenine dinucleotide; FADS, FAD synthase; FAO, mitochondrial fatty acid β-oxidation; MADD, multiple acyl-CoA dehydrogenase deficiency; MFT, mitochondrial folate/FAD transporter; n, normal; n.r, not reported; NBS, newborn screening; RFVT, riboflavin transporter; WES, whole exome sequencing; WGS, whole genome sequencing.

followed by functional (enzymatic/flux) assays, with a range of options for genetic analyses essential to confirm the diagnosis.

At present, metabolic investigations remain the cornerstone of diagnosis and involve the analysis of urinary organic acids (GC–MS) along with plasma amino acids (GC-/LC–MS/MS) and acylcarnitines (LC–MS/MS). Urinary organic acids suggestive of MADD include a combination of elevated glutaric-, ethylmalonic-, adipic-, suberic-, sebacic-, dodecanedioic-, 2-hydroxyglutaric-, 3-hydroxyisovaleric-, and 5-hydroxyhexanoic acid, in conjunction with C4 and C5 glycine conjugates. Moreover, elevated concentrations of proline, 4-hydroxyproline (neonatal-onset) and sarcosine (late-onset) are common biochemical features (Frerman and Goodman, 2001; Grünert, 2014). Acylcarnitine profiling, which is also used in NBS, is highly predictive and reflects the intra-mitochondrial accumulation of various acyl-CoAs as well as the corresponding acylcarnitines generated by carnitine palmitoyltransferase 2 (CPT2) and carnitine acetyltransferase (CrAT). A typical MADD-specific acylcarnitine profile would include C4, C5, C5-DC, C6, C8, C10, C12, C14:1, C16 and C18:1 acylcarnitine species (Grünert, 2014; Houten et al., 2016; Violante et al., 2013; Wanders et al., 2010). Important, however, is that abnormal metabolite profiles may, in some instances, only be observed during symptomatic events and may improve or even normalize when clinical symptoms are absent (Chaya et al., 2018). For metabolites to be most indicative, samples should be obtained during the catabolic state or metabolic crisis, or before the next feeding (for neonates), following minimal medication intake.

Although metabolic profiling can be highly predictive for a particular inborn error of metabolism, enzymatic and/or flux analyses have, in general, been shown to be paramount in pinpointing the enzyme defect with certainty and disclosing the level of the deficiency. However, in the case of MADD, direct measurement of ETFα, ETFβ and ETFQO activity has been notoriously difficult to perform routinely, mainly due to the requirement of purified ETF, which is not commercially available. As reviewed by Wanders et al. (2010), radio- and stable isotope-labelled FAO flux analyses, which are based on methods developed in the 1990s in fibroblasts and lymphocytes, have proven to be of great value to distinguish MADD from other FAO disorders (Manning et al., 1990; Niezen-Koning et al., 1992; Olpin et al., 1992). In addition, FAO flux analysis using oleate as substrate in patients' fibroblasts has been found to correlate with the clinical phenotype as determined using a recently proposed MADD-disease severity scoring system (MADD-DS3) (van Rijt et al., 2019). Considering the clinical and biochemical variability that makes MADD prone to misdiagnosis when using a differential diagnosis with limited assays (Frerman and Goodman, 2001; Vasiljevski et al., 2018; Yıldız et al., 2018), the study of van Rijt et al. (2019) illustrated the extensive nature of the investigations required to obtain markers for early prediction of disease severity that would allow preventative and follow-up treatment.

4.2. Postgenomic era diagnostic approach

With the expanding ability, lowered cost and advantages of whole exome- (WES) and whole genome sequencing (WGS) to study complex inherited diseases, the application of these genomic tools have become an increasingly popular option to include earlier in the diagnostic process at several laboratories (Haack et al., 2012; Lieber et al., 2013; Neveling et al., 2013; Wortmann et al., 2017, 2015). Altogether, genetic approaches have resulted in the rapid expansion of gene and mutation identification, as summarized in Table S1. Of the eight genes and ~436 reported mutations, as well as other potential variants involved in MADD(-like disorders), five genes and the vast majority of mutations have been identified using next generation sequencing (NGS). With a limited number of genes involved in this group of disorders, gene panels have proven to be exceptionally useful, particularly when run in parallel with functional (flux) assays. Nevertheless, the value of biochemical and enzymatic/flux analyses, as well as the use of disease expression systems in unraveling the impact of genetic variants, diagnostic variability and the pathophysiology and treatment of MADD, should not be underestimated (Henriques et al., 2009; Tolomeo et al., 2020).

5. Treatment and prognosis

As a first-line treatment, early oral supplementation of riboflavin at a high dose (100−400 mg/day) is advised for all MADD(-like) phenotypes (Mosegaard et al., 2020; Ryder et al., 2019). This vitamin has been shown to dramatically improve the clinical and biochemical abnormalities associated with MADD Type III (especially in cases of *ETFDH* deficiency), RFVT1−3 defects, MFT deficiency and milder cases of FADS dysfunction without major side effects (Auranen et al., 2017; Jaeger and Bosch, 2016; Ryder et al., 2019; Schiff et al., 2016). Studies on *ETFDH* deficiency have attributed the molecular mechanism of riboflavin's response to the chaperone function of FAD, which promotes folding of the variant protein or otherwise stabilizes its folding intermediates or mature form (Cornelius et al., 2012; Henriques et al., 2009). However, this improvement, the extent of which depends on the nature of the variant, usually does not completely correct for the variant-induced structural defect(s). Other possible explanations for the beneficial effect of riboflavin include the increase of flavin content to compensate for the loss of FAD, and the induction of flavoprotein-transcription and -translation by FAD (Henriques et al., 2010; Nagao and Tanaka, 1992).

In MADD(-like disorders), riboflavin supplementation is often combined with a high-caloric diet reduced in fat and protein, the strict avoidance of fasting, and/or oral supplementation with glycine and L-carnitine to promote the export of acylglycine and -carnitine conjugates (Gordon, 2006). The latter is especially important since acyl-CoA esters cannot traverse the mitochondrial- or plasma membrane, but should be approached with caution as L-carnitine may worsen symptoms, at least in some cases (Gempel et al., 2007). Riboflavin may further be combined with CoQ10 to decrease the oxidative stress and treat MADD Type III with secondary CoQ10 deficiency, whereas bezafibrate has been shown to improve MADD in the absence of riboflavin, although its *in vivo* application requires further research (Cornelius et al., 2014; Gempel et al., 2007; Xi et al., 2014; Yamada et al., 2017; Yamaguchi et al., 2012). Moreover, the efficacy and safety of D,L-3-hydroxybutyric acid (100−2,600 mg/kg/day, administered orally or via a nasogastric/gastrostomy tube) to treat MADD by bypassing the disturbed ketogenesis was recently demonstrated, with the authors reporting a longer survival rate (notably in MADD Type II) and clinical improvement in 70 % of the patients (including MADD Types II and III and RFVT3 deficiency) (van Rijt et al., 2020).

Despite these treatment options, mortality remains high in severe MADD(-like) phenotypes (most notably MADD Types I and II) (van Rijt et al., 2020). More effective treatment strategies are thus still desperately needed and, consequently, future research in enzyme replacement-, gene- and gene expression therapies hold great promise in MADD as the phenotype can often be rescued by even a slight increase in the activity of the variant protein (Ørngreen and Vissing, 2017). Likewise, disease expression models are of immeasurable value in the study of therapeutic options (Tolomeo et al., 2020).

6. Conclusion

FAD and the flavoproteins it supports, participate in a number of catabolic pathways which all converge on the mitochondrion. Deficiencies of FAD's synthesis, transport and dedicated redox machinery are thus expected to have a severe impact on a wide range of metabolic functions. In this article, we have provided a succinct overview of these processes and the clinical phenotypes resulting from genetic deficiencies of the FAD metabolism, which, at present, include eight genes with at least 436 reported mutations that lead to a range of different MADD (-like) phenotypes. *ETFDH* remains the gene with the most (64 %) reported mutations followed by *SLC52A3* (12 %), while the other genes each exhibit less than 9 % of the reported mutations. With the exception

of MADD Types I and II, an important feature of these disorders is the possibility of effective treatment. At present, these disorders are included in few NBS programmes and an effective early metabolic diagnosis relies predominantly on disease expression (catabolic state) (Austin and Dawkins, 2017; Baynam et al., 2020). Together, this warrants the development of novel diagnostic approaches – notably with the focus on finding more successful and direct avenues for diagnostics in a more inclusive global population. Over the past decade genomic approaches have, alongside biochemical investigations, proven their value in the diagnosis of MADD(-like disorders). Considering the limited number of genes involved, the earlier inclusion of mutational analysis in diagnosis, and the development of targeted gene therapy should therefore be considered in earnest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2020.105899.

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A.2 SUPPLEMENTARY MATERIAL 1

Method

In order to identify reported pathogenic variants resulting in MADD and MADD-like phenotypes, a systematic literature search was performed in PubMed (https://pubmed.ncbi.nlm.nih.gov/, October 2023) using the following terms (without quotation marks): “Multiple acyl CoA dehydrogenase deficiency”, “Glutaric aciduria type 2”, “Glutaric acidemia type 2”, “ETFDH”, “ETFA”, “ETFB”, “Flavin adenine dinucleotide synthase deficiency”, “FAD synthase deficiency”, “FLAD1”, “SLC25A32”, “Riboflavin transporter deficiency”, “Brown-Vialetto-Van Laere”, “Fazio-Londe”, “SLC52A1”, “SLC52A2” and “SLC52A3”. The search yielded 1192 articles (published between 1962 and November 2023), of which 253 described pathogenic variants in MADD-related genes. The remainder did not report any applicable variants (871 articles), could not be reviewed due to language constraints (61 articles), or were not available to the authors (7 articles). An additional 41 articles, which were not identified by the search, but contained MADD-related variants (cited in the identified literature), were also included. Finally, the *ETFDH* (ENST00000511912.6), *ETFA* (ENST00000557943.6), *ETFB* (ENST00000309244.9), *FLAD1* (ENST00000292180.8), *SLC25A32* (ENST00000297578.9), *SLC52A1* (ENST00000254853.10), *SLC52A2* (ENST00000643944.2) and *SLC52A3* (ENST00000645534.1) genes were searched against the Genome Reference Consortium Human Build (GRCh) 38.p13 via Ensembl (https://www.ensembl.org/, October 2020) and gnomAD v2.1.1 (Genome Aggregation Database; https://gnomad.broadinstitute.org/, October 2020) to supplement any missing information of the variants that were reported in literature, and to identify all current additional variants with a CADD (Combined Annotation Dependent Depletion) score of ≥30 (likely pathogenic/pathogenic) (Rentzsch *et al.*, 2019). Synonymous variants were excluded from both searches. The results are displayed in Table A.1.

Table A.1: Reported (literature and Ensembl and gnomAD v2.1.1 databases) pathogenic variants in MADD-related genes (up until October 2023)

#	Affected gene	Exon	Intron/ non-coding	Nucleotide change (position)	Nucleotide change (allele call)	Amino acid change (predicted from cDNA)	rsID	Reference*	CADD score
1	*ETFA*		5'-UTR	chr15:76311473 C>T	c.-85G>A	n.r.	rs982154601	[291]	n.a
2	*ETFA*	1		n.a	c.1-40G>A	n.r	n.a	[79] [193] [225]	n.a
3	*ETFA*	1		chr15:76311382C>T	c.7C>T	p.Arg3*	n.a	[10] [23] [60] [134] [275]	0
4	*ETFA*	1		chr15:76311363 T>TGCC CCGGAGCC	c.15_25dup11	p.Gln9Argfs*20	rs1384386872	[18]	n.a
5	*ETFA*	2		chr15:76295725G>A	c.52C>T	p.Arg18*	rs754202690	[28] [18]	0
6	*ETFA*	2		chr15:76295706A>T	c.71T>A	p.Val24Glu	rs979879737	-	31
7	*ETFA*	2		chr15:76295703delA	c.72delA	p.Ile25*	n.a	[18] [72]	n.a
8	*ETFA*	2		chr15:76295702delA	c.73delA	p.Ile25*	n.a	[18] [193]	n.a
9	*ETFA*	3		chr15:76292699A>C	c.188T>G	p.Val63Gly	rs1231262303	-	33
10	*ETFA*	3		chr15:76292686A>G	c.200T>C	p.Leu67Pro	n.a	[155] [225]	n.a
11	*ETFA*	3		chr15:76292645T>G	c.242A>C	p.His81Pro	rs972293793	[193]	25
12	*ETFA*	4		chr15:76292499A>C	c.283T>G	p.Leu95Val	n.a	[23] [72] [275]	n.a
13	*ETFA*	4		chr15:76286463A>C	c.284T>G	p.Leu95Trp	rs119458969	[27] [72]	32
14	*ETFA*	4		chr15:76292436C>T	c.346G>A	p.Gly116Arg	rs119458971	[2] [7]	27
15	*ETFA*	4		chr15:76292435 C>A	c.347G>T	p.Gly116Val	n.a	[194]	29
16	*ETFA*	5		chr15:76287943G>T	c.354C>A	p.Asn118Lys	n.a	[69] [203]	n.a
17	*ETFA*	5		chr15:76287944ins	c.355_356insC	p.Leu119Profs*10	n.a	[18] [72]	n.a
18	*ETFA*	5		chr15:76287932C>T	c.365G>A	p.Arg122Lys	rs773865838	[18] [72] [225]	27
19	*ETFA*	5		chr15:76283808C>T	c.370G>A	p.Ala124Thr	rs1390694827	[82]	24
20	*ETFA*	5		chr15:76287866A>G	c.431T>C	p.Phe144Ser	rs755055339	[18]	32
21	*ETFA*	6		chr15:76286480del	c.453_470del	p.152_157del	n.a	[2]	n.a
22	*ETFA*	6		chr15:76286463A>C	c.470T>G	p.Val157Gly	rs119458969	[1] [2] [7]	32
23	*ETFA*	6		chr15:76286455del	c.478delG	p.Asp160Metfs*4	n.a	[10] [23] [72] [275]	n.a
24	*ETFA*	6		chr15:76286439A>G	c.494T>C	p.Val165Ala	rs749455172	[18] [69] [72]	28
25	*ETFA*	6		chr15:76286431C>A	c.502G>T	p.Val168Phe	rs779987487	[34] [72]	24
26	*ETFA*	6		chr15:76286421G>A	c.512C>T	p.Thr171Ile	rs1801591	[2] [42] [174]	28
27	*ETFA*		6	chr:15:76286380C>G	c.553-1G>C	exon skipping or frameshift	n.a	[23] [134] [275]	n.a
28	*ETFA*	7		chr15:76285665A>G	c.635T>C	p.Leu212Pro	n.a	[18] [72]	n.a

29	*ETFA*	7		chr15:76285649C>T	c.652G>A	p.Val218Met	rs753461278	[201]	26
30	*ETFA*	7		chr15:76285645-7628653del	c.656_664del	p.Val219_Gly221del	n.a	[134]	n.a
31	*ETFA*		7	chr15:76285635-76285636del	c.664+1_664+2del	n.r	rs1567215572	[193]	n.a
32	*ETFA*	8		chr15:76283823 G>A	c.667C>T	p.Arg223fs	rs769976586	[158] [234]	40
33	*ETFA*	8		chr15:76283788 TAAC>T	c.699_701delGTT	p.Leu234del	n.a	[293]	n.a
34	*ETFA*	8		chr15:76283774T>A	c.716A>T	p.Asp239Val	rs1260250211	-	31
35	*ETFA*	9		chr15:76274491 C>A	c.737G>T	p.Gly246Val	n.a	[250]	33
36	*ETFA*	9		chr15:76274488G>A	c.740C>T	p.Ala247Val	rs1466909697	-	32
37	*ETFA*	9		chr15:76274485G>A	c.743C>T	p.Ser248Phe	rs1384518039	-	33
38	*ETFA*	9		chr15:76274483G>C	c.745C>G	p.Arg249Gly	rs1353938548	-	32
39	*ETFA*	9		chr15:76274483 G>A	c.745C>T	p.Arg249Cys	n.a	[18]	32
40	*ETFA*	9		chr15:76274477 C>T	c.751G>A	p.Ala251Thr	n.a	[186]	29
41	*ETFA*	9		chr15:76274464A>C	c.764G>T	p.Gly255Val	n.a	[10] [23] [72] [275]	n.a
42	*ETFA*	9		chr15:76274442T>C	c.786A>G	p.Gln262Arg	rs373871967	[34] [72]	0
43	*ETFA*	9		chr15:76274432 T>C	c.796A>G	p.Tyr266Ala	n.a	[277]	40
44	*ETFA*	9		chr15:76274431G>A	c.797C>T	p.Thr266Met	rs119458970	[2] [7] [12] [18] [72] [106] [193] [197] [225] [234]	32
45	*ETFA*	9		chr15:76274428C>T	c.799G>A	p.Gly267Arg	n.a	[10] [23] [60] [134] [275]	n.a
46	*ETFA*	9		chr15:76274417-76274422CTACTA>CTA	c.809_811del	p.Val270del	rs779140971	[2] [225]	0
47	*ETFA*	9		chr15:76274416G>A	c.812C>T	p.Ala271Val	rs1567211944	-	32
48	*ETFA*	10		chr15:76232280del	c.817_882del	p.Glu273_Lys294del	n.a	[2]	n.a
49	*ETFA*	10		chr15:76231361A>T	c.854A>T	p.Gln285Leu	n.a	[155] [225]	n.a
50	*ETFA*	10		chr15:76231336-76231344del	c.875_878delACAG	p.Asn292Alafs*23	n.a	[18] [72]	n.a
51	*ETFA*	10		chr15:76231335T>C	c.880A>G	p.Lys294Glu	rs774043017	-	34
52	*ETFA*	10		chr15:76231333C>G	c.882G>C	p.Lys294Asn	rs905969804	-	34
53	*ETFA*	11		n.a	c.(882+1_883-1)_(963+1_964-1)del	exon deletion	n.a	[158]	n.a
54	*ETFA*	11		chr15:76226892-76226812del	c.883_963del	p.Thr295_Lys321del	n.a	[12]	n.a
55	*ETFA*	11		chr15:76225895G>A	c.917C>T	p.Pro306Leu	rs768924673	-	31
56	*ETFA*	11		chr15:76225875A>T	c.937T>A	p.Tyr313Asn	rs1482839883	[36] [72]	27

57	*ETFA*	11		chr15:76225848-76225849delC	c.963+1delG	splice variant	rs746488455	[18] [72]	n.a
58	*ETFB*	1		chr19:51366314G>A	c.13C>T	p.Arg5Cys	rs1422832903	-	33
59	*ETFB*	1		chr19:51366283T>G	c.44A>C	p.Asp15Ala	rs981006954	-	31
60	*ETFB*	1		chr19:51366277C>G	c.49G>C	p.Ala17Pro	n.a	[21]	n.a
61	*ETFB*	1		chr19:51366271T>A	c.55A>T	p.Lys19*	n.a	[23] [91] [275]	n.a
62	*ETFB*	1		chr19:51366271T>C	c.56A>G	p.Lys19Arg	rs767751467	-	31
63	*ETFB*	1		n.a	exon 1 skipping?	exon 1 skipping?	n.a	[275]	n.a
64	*ETFB*	2		chr19:51354289del	c.77delG	p.Thr27Profs*8	n.a	[23] [275]	n.a
65	*ETFB*	2		chr19:51354288del	c.78delG	p.Thr27Profs*8	n.a	[91]	n.a
66	*ETFB*	2		chr19:51857540delC	c.80delC	p.Gly28Valfs*7	n.a	[23] [275]	n.a
67	*ETFB*	2		chr19:51354244A>G	c.122T>C	p.Phe41Ser	rs746082442	-	33
68	*ETFB*	2		chr19:51354242A>G	c.124T>C	p.Cys42Arg	rs774387920	[11] [72]	25
69	*ETFB*	2		chr19:51857622delGGA	c.138-140delGGA	p.Glu47del	n.a	[275]	n.a
70	*ETFB*	2		chr19:5134223-51354225del	c.143_145delAGG	p.Glu48del	n.a	[91]	n.a
71	*ETFB*	2		chr19:51857475G>C	c.145G>C	p.Ala49Pro	n.a	[186] [265]	29
72	*ETFB*	2		chr19:51354179C>T	c.187G>A	p.Ala63Thr	rs142966954	[193]	26
73	*ETFB*		2	chr19:51353294C>A	c.217-4G>T	n.r	rs149557388	[225]	0
74	*ETFB*	3		chr19:51353663-51353665del	c.217_375del	p.Glu73_Gln125del	n.a	[12]	n.a
75	*ETFB*	3		chr19:51856487 G>A	c.274C>T	p.Pro92Ser	rs758509148	[112]	13
76	*ETFB*	3		chr19:51856483dup	c.278dupC	p.Ala94Serfs*36	n.a	[254]	24
77	*ETFB*	3		chr19:51353205G>A	c.302C>T	p.Pro101Leu	rs1249159210	-	32
78	*ETFB*	3		chr19:51353191G>A	c.316C>T	p.Arg106Trp	rs748730384	-	33
79	*ETFB*	3		chr19:51856418delGAG	c.343_345delGAG	p.Glu115del	rs777551226	[186] [265]	22
80	*ETFB*	3		chr19:51353134G>C	c.373C>G	p.Gln125Glu	rs1568467526	-	32
81	*ETFB*	4		chr19:51353132C>G	c.375G>C	p.Gln125His	rs1173733121	[12]	31
82	*ETFB*	4		chr19:51353132-	c.375G>C + c.375_376ins(9)	p.Gln125His + p.His125_Ala126ins(3)	n.a	[12]	n.a
83	*ETFB*	4		chr19:51350385C>T	c.382G>A	p.Asp128Asn	rs104894678	[12] [24] [72]	25
84	*ETFB*		4	chr19:51350309G>A	c.438+20C>T	n.r	rs114985874	[225]	0
85	*ETFB*	5		chr19:51347045G>A	c.452C>T	p.Ser151Phe	rs74735908	-	33
86	*ETFB*	5		chr19:51347036C>T	c.461C>T	p.Thr154Met	rs1130426	[4] [182]	25

87	*ETFB*	5		chr19:51856410-51857427	c.466_624del	p.Glu156_Val208del	n.a	[4]	n.a
88	*ETFB*	5		chr19:51347007G>A	c.490C>T	p.Arg164Trp	rs148045813	[23] [91] [275]	29
89	*ETFB*	5		chr19:51347006C>T	c.491G>A	p.Arg164Gln	rs104894677	[4] [7] [23] [72] [91] [134] [275]	28
90	*ETFB*	5		chr19:51346926G>A	c.571C>T	p.Arg191Cys	rs772976948	[18] [72] [179]	34
91	*ETFB*	5		chr19:51346925C>T	c.572G>A	p.Arg191His	rs769951998	-	32
92	*ETFB*	5		chr19:51346916G>A	c.581C>T	p.Thr194Met	rs369138107	-	33
93	*ETFB*	5		chr19:51346908T>C	c.589A>G	p.Asn197Asp	rs747411704	-	32
94	*ETFB*	5		chr19:51346902T>G	c.595A>C	p.Met199Leu	rs975423352	-	34
95	*ETFB*		5	chr19:51346900del	c.597+1G>C	p.Gly147_Met199del	n.a	[91]	n.a
96	*ETFB*		5	chr19:51346900del	c.597+1G>C	p.Gly148_Met200del	n.a	[4] [23] [72] [134] [275]	n.a
97	*ETFB*	6		chr19:51345381T>C	c.598A>G	p.Lys200Glu	rs920672041	-	33
98	*ETFB*	6		chr19:51345375-19:51345377del	c.604_606delAAG	p.Lys202del	n.a	[11] [72]	n.a
99	*ETFB*	6		chr19:51848616 ATCT>A	c.614_616delAGA	p.Lys205del	rs767519060	[11]	22
100	*ETFDH*		5'UTR	chr4:158672382A>G	c.-75A>G	transcript expression decreased	rs1001221143	[66] [72] [80] [274]	0
101	*ETFDH*	1		chr4:158672458T>C	c.2T>C	p.Met1Thr	rs121964953	[3] [7] [8] [275]	21
102	*ETFDH*	1		chr4:158672459G>C	c.3G>C	p.Met1Ile	rs1340862175	[48] [47] [72] [80] [82] [88] [167] [201] [274] [277] [279]	22
103	*ETFDH*	1		chr4:159593628 A>G	c.20A>G	p.Lys7Arg	rs780104191	[288] [294]	15
104	*ETFDH*	1		chr4:158672490G>C	c.34G>C	p.Ala12Pro	rs1172887273	[149] [201] [180] [225]	22
105	*ETFDH*	1		chr4:158,682,236-158,695,602	c.34_607del	p.Ala12Serfs*9	n.a	[247]	n.a
106	*ETFDH*		1	chr4:158679459 T>G	c.35-1008T>G	splice variant (pseudoexon)	n.a	[247]	7.277
107	*ETFDH*		1	chr4:158679699 A>G	c.35-768A>G	splice variant (pseudoexon)	n.a	[247]	21
108	*ETFDH*		1	chr4:158680443 ATAAT>A	c.35-23_35-20delTAAT	n.r.	n.a	[274]	13
109	*ETFDH*	1-5		n.a	exon 1-5del	deletion of exon 1-5	n.a	[178] [277]	n.a
110	*ETFDH*	1-6		n.a	exon 1-6del	deletion of exon1-6	n.a	[275] [277]	n.a
111	*ETFDH*	1-7		n.a	exon 1-7del	multiple deletions in exon 1-7	n.a	[171]	n.a
112	*ETFDH*		1	chr4:158672495G>C	c.34+5G>C	splice variant	rs1373597092	[106] [179] [182] [224]	0
113	*ETFDH*	2		n.a	n.r	p.Ala12fs	n.a	[20] [72] [201]	n.a
114	*ETFDH*		1	chr4:158680465del	c.35-2A>C	p.Ala12_59delinsGly	n.a	[180]	n.a
115	*ETFDH*	2		chr4:158680468del	c.36delA	p.Tyr13Ilefs*7	n.a	[7] [12] [59]	n.a

116	*ETFDH*	2		chr4: between 158680476 & 158680477	c.45_46insC	p.Phe16*22	n.a	[12]	n.a
117	*ETFDH*	2		n.a	n.r	p.Phe16Cys	n.a	[7]	n.a
118	*ETFDH*	2		chr4:158680483dup	c.50dupA	p.His17Glnfs*6	n.a	[106]	n.a
119	*ETFDH*	2		chr4:158680483dup	c.51dupT	p.Ala18Cysfs*5	n.a	[7] [8] [12] [15] [20] [59] [64] [72] [201] [193] [225] [292]	n.a
120	*ETFDH*	2		chr4:158680485C>G	c.53C>G	p.Ala18Gly	n.a	[167] [201]	n.a
121	*ETFDH*	2		chr4:158680493T>C	c.61T>C	p.Ser21Pro	n.a	[167] [201]	n.a
122	*ETFDH*	2		chr4:158680497A>G	c.65A>G	p.Lys22Arg	n.a	[57] [110] [201] [277]	n.a
123	*ETFDH*	2		chr4:158680499G>A	c.67G>A	p.Ala23Thr	n.a	[178]	n.a
124	*ETFDH*	2		chr4:158680511C>T	c.79C>T	p.Pro27Ser	rs537038850	[36] [72] [96] [201] [193] [216]	15
125	*ETFDH*	2		chr4:158680524C>T	c.92C>T	p.Thr31Ile	rs11559290	[141] [167] [201] [173] [216]	10
126	*ETFDH*	2		chr4:158680524T>C	c.92T>C	p.Ile31Thr	n.a	[25] [29] [59]	n.a
127	*ETFDH*	2		chr4:158680553C>T	c.121C>T	p.Arg41*	rs773668457	[7] [8] [128] [252] [247]	0
128	*ETFDH*	2		chr4:158680554G>T	c.122G>T	p.Arg41Leu	n.a	[95] [254]	n.a
129	*ETFDH*	2		chr4:158680578A>G	c.146A>G	p.Tyr49Cys	n.a	[8]	n.a
130	*ETFDH*	2		chr4:158680584G>A	c.152G>A	p.Arg51Gln	rs534388496	[47] [72] [80] [136] [167] [201] [245] [274] [273]	26
131	*ETFDH*	2		chr4:158680587A>T	c.155A>T	p.Asp52Val	n.a	[259]	25
132	*ETFDH*	2		chr4:158680589A>G	c.158A>G	p.Lys53Arg	n.a	[74]	n.a
133	*ETFDH*	2		chr4:158680603G>A	c.171G>A	p.Trp57*	rs949249162	[164]	0
134	*ETFDH*	2		chr4:158680604G>A	c.172G>A	p.Glu58Lys	rs1473188524	[72] [80] [201]	20
135	*ETFDH*	2		chr4:158680604G>T	c.172G>T	p.Glu58*	n.a	[47]	n.a
136	*ETFDH*		2	chr4:158680609T>C	c.175+2T>C	truncated	n.a	[66] [72] [80] [274]	n.a
137	*ETFDH*		2	chr4:158682002del	c.176-194_176-193del	n.r.	n.a	[270]	n.a
138	*ETFDH*		2	chr4:158682194 G>A	c.176-1G>A	splice variant	n.a	[277]	35
139	*ETFDH*		2	chr4:158682194G>T	c.176-1G>T	p.Gly58fs 3	n.a	[180]	n.a
140	*ETFDH*	3		chr4:158682193A>G	c.176-2A>G	p.Ile72Thr	n.a	[167] [201]	n.a
141	*ETFDH*	3		chr4: between 158682194 & 158682195	c.177insT	p.Asp59fs	n.a	[117] [201]	n.a
142	*ETFDH*	3		chr4:158682210G>A	c.191G>A	p.Arg64Lys	n.a	[47] [72] [80] [201]	n.a
143	*ETFDH*	3		chr4:158682231del	c.210_212del	p.Val71del	n.a	[66] [72] [274]	n.a

144	*ETFDH*	3		chr4:158682229-158682234TGTTGT>TGT	c.213_215del	p.Val72del	rs796051966	[72] [80]	0
145	*ETFDH*	3		chr4:158682245G>A	c.226G>A	p.Ala76Lys	n.a	[72] [80]	n.a
146	*ETFDH*	3		chr4:158682245G>A + 4:158682247C>A	c.226G>A+ c.227C>A	p.Ala76Lys	n.a	[72] [80] [201]	n.a
147	*ETFDH*	3		chr4:158682248G>A	c.229G>A	p.Gly77Ser	n.a	[143] [205] [274]	n.a
148	*ETFDH*	3		chr4:158682255C>G	c.236C>G	p.Ala79Gly	n.a	[66] [72] [80] [164] [274]	n.a
149	*ETFDH*	3		chr4:158682402T>C	c.242T>C	p.Leu81Pro	rs1554031490	[38] [57] [59] [66] [72] [80] [110] [167] [201] [274]	31
150	*ETFDH*	3		chr4:158682263T>C	c.244T>C	p.Ser82Pro	n.a	[6] [8] [20] [59] [72] [201] [224]	n.a
151	*ETFDH*	3		chr4:158682264C>T	c.245C>T	p.Ser82Phe	rs887871605	[8] [93] [96] [201] [269]	27
152	*ETFDH*	3		chr4:158682269G>A	c.250G>A	p.Ala84Thr	rs121964954	[23] [25] [26] [29] [33] [35] [37] [41] [49] [48] [47] [59] [62] [66] [68] [72] [73] [80] [88] [96] [97] [99] [103] [119] [133] [141] [147] [167] [199] [201] [173] [178] [180] [183] [198] [218] [222] [240] [274] [277] [281]	28
153	*ETFDH*	3		chr4:158682269C>T	c.251C>T	p.Ala84Val	n.a	[48] [62] [66] [72] [80] [201] [274] [275]	n.a
154	*ETFDH*	3		chr4:158682275C>T	c.256C>T	p.Arg86Cys	rs1242099513	[66] [72] [80] [274]	27
155	*ETFDH*	3		chr4:158682284-4:1586822875del	c.265_266delCA	p.Gln89Valfs*5	n.a	[149] [201] [180]	n.a
156	*ETFDH*	3		chr4:158682276G>A	c.257G>A	p.Arg86His	rs777655131	[251]	25
157	*ETFDH*	3		chr4:159603464T>A	c.293T>A	p.Ile98Asn	n.a	[264]	26
158	*ETFDH*	3		chr4:158682314C>G	c.295C>G	p.Arg99Gly	n.a	[62] [72] [201]	n.a
159	*ETFDH*	3		chr4:158682314C>T	c.295C>T	p.Arg99Cys	rs371493232	[62] [82] [88] [110] [157] [167] [201] [180] [274]	31
160	*ETFDH*	3		chr4:158682315G>A	c.296G>A	p.Arg99His	rs376630579	-	32
161	*ETFDH*	3		chr4:158682318T>A	c.299T>A	truncated	n.a	[15]	n.a
162	*ETFDH*	3		chr4:158682321G>A	c.302G>A	p.Cys101Tyr	rs1436363240	-	30
163	*ETFDH*	3		chr4:158682322T>A	c.303T>A	p.Cys101*	n.a	[82] [88] [201] [180]	n.a
164	*ETFDH*	3		chr4:158695620A>G	c.313A>G	p.Lys105Glu	rs398124154	[106]	23

165	*ETFDH*	3		chr4:158684642G>A	c.315G>A	p.Met105Ile	rs1311814302	[167] [201]	0
166	*ETFDH*	3		chr4:158682344A>G	c.325A>G	p.Ile109Val	rs1448698249	[289]	21
167	*ETFDH*	3		chr4:158682345T>C	c.326T>C	p.Ile109Thr	n.a	[294]	27
168	*ETFDH*	3		chr4:158682353C>T	c.334C>T	p.His112Tyr	n.a	[16] [20] [59] [72] [201]	n.a
169	*ETFDH*	3		chr4:158682365G>C	c.346G>C	p.Gly116Arg	rs1458858994	-	31
170	*ETFDH*	3		chr4:158682368G>C	c.349G>C	p.Ala117Pro	n.a	[47] [72] [80] [201]	n.a
171	*ETFDH*	3		chr4:158682372G>T	c.353G>T	p.Cys118Phe	rs1467428857	[72] [80] [97] [201] [208] [274]	29
172	*ETFDH*	3		chr4: between 158682378 & 158682379	c.360_361insT	p.Pro121Serfs*5	n.a	[66]	n.a
173	*ETFDH*	3		chr4: between 158682380 & 158682381	c.361_362insT	p.Pro121Leufs*5	n.a	[72] [80] [274]	n.a
174	*ETFDH*	3		chr4:158682345G>A	c.365G>A	p.Gly122Asp	rs760629096	[270]	6
175	*ETFDH*	3		chr4:158682399T>A	c.380T>A	p.Leu127His	rs121964956	[26] [29] [59] [62] [72] [103] [201] [183] [240] [263]	27
176	*ETFDH*	3		chr4:158682399T>C	c.380T>C	p.Leu127Pro	rs1244950045	[73] [201]	27
177	*ETFDH*	3		chr4:158682399T>G	c.380T>G	p.Leu127Arg	n.a	[38] [59] [72] [80] [274] [277]	n.a
178	*ETFDH*	3		n.a	n.r	p.Leu127Cys	n.a	[164]	n.a
179	*ETFDH*	3		chr4:158682402T>C	c.383T>C	p.Phe128Ser	rs1554031490	[41] [62] [72] [201]	31
180	*ETFDH*	3		chr4:158682408A>T	c.389A>T	p.Asp130Val	rs199899494	[38] [48] [59] [66] [72] [80] [82] [88] [94] [129] [168] [164] [167] [201] [178] [180] [208] [227] [229] [274] [275] [277]	29
181	*ETFDH*	3		chr4:158682410T>G	c.391T>G	p.Trp131Gly	rs758081107	-	33
182	*ETFDH*	3		chr4:158682412G>C	c.393G>C	p.Trp131Cys	rs1292908960	[38] [59] [72] [274]	34
183	*ETFDH*		3	chr4:158682425G>A	c.405+1G>A	truncated	n.a	[38] [59] [72] [80] [274]	n.a
184	*ETFDH*		3	chr4:158682425G>T	c.405+1G>T	truncated	n.a	[47] [72] [80] [201]	n.a
185	*ETFDH*		3	chr4:158682427A>T	c.405+3A>T	splice variant or truncated	rs796051965	[14] [72] [179] [182]	n.a
186	*ETFDH*		3	chr4:158684590A>G	c.406-2A>G	splice variant	n.a	[96] [201]	n.a
187	*ETFDH*	4		chr4:158684593C>T	c.407C>T	p.Ala136Val	rs758242572	-	33
188	*ETFDH*	4		chr4:158684595C>T	c.409C>T	p.Pro137Ser	n.a	[25] [59] [72] [80] [103] [201]	n.a
189	*ETFDH*	4		chr4:158684599C>T	c.412C>T	p.Leu138Phe	n.a	[136] [201]	n.a
190	*ETFDH*	4		chr4:158684599T>G	c.413T>G	p.Leu138Arg	rs779896449	[8] [14] [52] [72] [201] [205] [235]	28

191	*ETFDH*	4		chr4:159605757C>T	c.419C>T	p.Thr140Ile	rs754506098	[240]	26
192	*ETFDH*	4		chr4:158684613-158684619del	c.427_433del	truncated	n.a	[5] [7] [8]	n.a
193	*ETFDH*	4		chr4:158684619G>C	c.433G>C	p.Asp145His	n.a	[47] [72] [80] [201]	n.a
194	*ETFDH*	4		chr4:158684635-15868439	c.449_453delTAACA	p.Leu150*	n.a	[99] [201]	n.a
195	*ETFDH*	4		chr4:1586846375A>G	c.451A>G	p.Thr151Ala	n.a	[136] [201]	n.a
196	*ETFDH*	4		chr4:158684639del	c.453delA	p.Glu152Argfs*15	n.a	[99] [201]	n.a
197	*ETFDH*	4		chr4:158684649A>G	c.463A>G	p.Arg155Gly	rs549150456	[179] [225] [247]	21
198	*ETFDH*	4		chr4:158684656C>T	c.470C>T	p.Pro157Leu	n.a	[277]	27
199	*ETFDH*	4		chr4:158684659T>G	c.473T>G	p.Val158Gly	n.a	[159]	n.a
200	*ETFDH*		4	chr4:158684664T>C	c.487-9T>C	truncated	n.a	[80] [274]	n.a
201	*ETFDH*		4	chr4:158685092T>C	c.488-9T>C	truncated	rs1554031705	[38] [59] [72]	n.a
202	*ETFDH*	5		chr4:158685111G>A	c.498G>A	p.Met166Ile	n.a	[201] [198]	n.a
203	*ETFDH*	5		chr4:158685116A>G	c.503A>G	p.Asn168Ser	n.a	[66] [72] [80] [274]	n.a
204	*ETFDH*	5		chr4:158685121G>T	c.508G>T	p.Gly170Cys	rs775548362	-	32
205	*ETFDH*	5		chr4:158685124A>G	c.511A>G	p.Asn171Asp	n.a	[178] [277]	n.a
206	*ETFDH*	5		chr4:158685131T>G	c.518T>G	p.Ile173Met	n.a	[82] [88] [201]	n.a
207	*ETFDH*	5		chr4:158685134T>C	c.521T>C	p.Val174Ala	rs1244950045	[211] [264]	27
208	*ETFDH*	5		chr4:159606288C>T	c.523C>T	p.Arg175Cys	rs762928354	[264]	33
209	*ETFDH*	5		chr4:158685137G>A	c.524G>A	p.Arg175His	rs121964955	[20] [23] [27] [31] [33] [35] [49] [59] [66] [68] [72] [80] [93] [96] [103] [134] [143] [201] [186] [205] [211] [222] [249] [263] [272] [274] [275] [277] [281] [282]	33
210	*ETFDH*	5		chr4:158685137G>C	c.524G>C	p.Arg175Pro	rs121964955	-	33
211	*ETFDH*	5		chr4:158685137G>T	c.524G>T	p.Arg175Leu	rs121964955	[26] [29] [59] [72] [80] [82] [88] [201] [180] [274] [281]	33
212	*ETFDH*	5		chr4:158685141G>C	c.528G>C	p.Leu176Phe	n.a	[66] [80] [72] [180] [274]	n.a
213	*ETFDH*	5		chr4:158685142G>A	c.529G>A	p.Gly177Arg	rs1463795907	-	32
214	*ETFDH*	5		chr4:158685148A>G	c.535A>G	p.Lys179Glu	n.a	[167] [201]	n.a
215	*ETFDH*	5		chr4:158685149T>C	c.536T>C	p.Leu179Ser	n.a	[221]	n.a
216	*ETFDH*	5		chr4:158685155G>A	c.542G>A	p.Ser181Asn	n.a	[180]	n.a

217	*ETFDH*	5		chr4:158685158G>A	c.545G>A	p.Trp182*	rs767249944	[8]	0
218	*ETFDH*	5		chr4:158685173C>T	c.560C>T	p.Ala187Val	rs369912835	[136] [201] [210] [211] [214] [264] [290]	26
219	*ETFDH*	5		chr4:158685181C>G	c.568C>G	p.Leu190Val	n.a	[274]	17
220	*ETFDH*	5		chr4:158685184G>A	c.571G>A	p.Gly191Ser	n.a	[96] [201]	n.a
221	*ETFDH*	5		chr4:158685190G>A	c.577G>A	p.Glu193Lys	n.a	[275]	32
222	*ETFDH*	5		chr4:158685191A>G	c.578A>G	p.Glu193Gly	rs1287661192	-	32
223	*ETFDH*	5		chr4:158685193 G>GTA	c.582_583insTA	p.Pro196Thrfs*17	n.a	[251]	n.a
224	*ETFDH*	5		chr4:158685200A>G	c.587A>G	p.Tyr196Cys	n.a	[167] [201] [178]	n.a
225	*ETFDH*	5		chr4:158685200C>A	c.587C>A	p.Pro196His	n.a	[273]	27
226	*ETFDH*	5		chr4:158685214G>C	c.601G>C	p.Ala201Pro	n.a	[274]	25
227	*ETFDH*		5	chr4:158685224insT	c.606+5insT	n.r	n.a	[136] [201] [245]	n.a
228	*ETFDH*		5	chr4:158685220G>A	c.606+1G>A	n.r	rs1241072742	[193]	0
229	*ETFDH*		5	chr4:158685220insT	c.606+1_606+2insT	splice variant	n.a	[258]	n.a
230	*ETFDH*		5	chr4:159606372insT	c.606+4insT	n.r.	n.a	[264]	n.a
231	*ETFDH*		5	chr4:158685226A>G	c.606+7A>G	truncated	rs1561240026	[66] [72] [80] [274]	0
232	*ETFDH*	6		chr4:158690358A>T	c.617A>T	p.His206Leu	n.a	[167] [201]	n.a
233	*ETFDH*	6		chr4:158690363G>C	c.622G>C	p.Asp208His	n.a	[96] [201] [184]	n.a
234	*ETFDH*	6		chr4:158690367del	c.623_626delATGG	p.Asp208Valfs*3	n.a	[217]	n.a
235	*ETFDH*	6		chr4:158690384G>A	c.643G>A	p.Ala215Thr	rs755214552	[49] [48] [72] [103] [201] [281]	28
236	*ETFDH*	6		chr4:158690393G>A	c.652G>A	p.Asp218Asn	rs748289922	[8] [21] [72] [201]	25
237	*ETFDH*	6		chr4:158690406A>C	c.665A>C	p.Gln222Pro	rs1482632936	[8] [225] [228]	23
238	*ETFDH*	6		chr4:159611572C>A	c.679C>A	p.Pro227Thr	rs141407224	[255]	26
239	*ETFDH*	6		chr4:158690420C>T	c.679C>T	p.Pro227Ser	rs141407224	[216]	25
240	*ETFDH*	6		n.a	exon 6del	deletion of exon 6	n.a	[274]	n.a
241	*ETFDH*		6	chr4:158690426G>A	c.684+1G>A	n.r	n.a	[48] [201]	n.a
242	*ETFDH*		6	chr4:158690426G>T	c.684+1G>T	splice variant	n.a	[201] [195]	n.a
243	*ETFDH*	7		chr4:158695503T>A	c.691T>A	p.Phe231Ile	n.a	[167] [201]	n.a
244	*ETFDH*	7		chr4:158695504T>C	c.692T>C	p.Phe231Ser	n.a	[72] [80] [274]	n.a
245	*ETFDH*	7		chr4:158695512G>A	c.700G>A	p.Gly234Arg	rs1032041993	-	32
246	*ETFDH*	7		chr4:158695518dupG	c.706dupG	p.Glu236Glyfs*5	n.a	[213]	n.a
247	*ETFDH*	7		chr4:158695527G>A	c.715G>A	p.Ala239Thr	n.a	[38] [59] [72] [80] [82] [88] [201] [274]	n.a

248	*ETFDH*	7		chr4:158695537C>T	c.725C>T	p.Thr242Ile	rs777646291	[279]	25
249	*ETFDH*	7		chr4:158695540T>C	c.728T>C	p.Ile243Thr	rs1397900640	[17] [72] [96] [201]	24
250	*ETFDH*	7		chr4:158697599T>C	c.731T>C	p.Phe244Ser	rs755907131	[21] [72] [201]	24
251	*ETFDH*	7		chr4:158695548G>A	c.736G>A	p.Glu246Lys	n.a	[94] [149] [201] [180] [281]	n.a
252	*ETFDH*	7		chr4:158695551G>C	c.739G>C	p.Gly247Arg	rs758218892	-	32
253	*ETFDH*	7		chr4:158695552G>A	c.740G>A	p.Gly247Asp	n.a	[274]	28
254	*ETFDH*	7		chr4:158695552G>T	c.740G>T	p.Gly247Val	rs1384574225	[180]	29
255	*ETFDH*	7		chr4:158697637T>C	c.769T>C	p.Tyr257His	rs1376613646	[96] [201]	28
256	*ETFDH*	7		chr4:158697638A>G	c.770A>G	p.Tyr257Cys	rs776108871	[38] [49] [48] [47] [57] [59] [66] [72] [80] [81] [82] [88] [89] [103] [110] [170] [147] [150] [152] [164] [167] [201] [178] [180] [181] [183] [216] [251] [274] [277] [281]	29
257	*ETFDH*	7		chr4:158695587A>T	c.775A>T	p.Lys259fs	n.a	[274]	37
258	*ETFDH*	7		chr4:158695598G>T	c.786G>T	p.Leu262Phe	n.a	[8]	n.a
259	*ETFDH*	7		chr4:1586956175C>G	c.805C>G	p.Gln269Glu	rs1580411897	[274]	24
260	*ETFDH*	7		chr4:158695618A>T	c.806A>T	p.Gln269Leu	rs776320810	[12] [15] [20] [59] [72] [201]	26
261	*ETFDH*	7		chr4:158695619A>C	c.807A>C	p.Gly269His	rs1265381182	[95] [254] [242]	23
262	*ETFDH*	7		chr4:158695624A>G	c.812A>G	p.Tyr271Cys	n.a	[159]	n.a
263	*ETFDH*	7		chr4:158695626G>A	c.814G>A	p.Gly272Arg	rs763541530	[21] [72] [168] [201] [176] [211] [216]	32
264	*ETFDH*	7		chr4:1586956308T>C	c.818T>C	p.Ile273Thr	rs766845829	[260] [273]	24
265	*ETFDH*	7		chr4:158695632G>T	c.820G>T	p.Gly274*	n.a	[39] [59] [72] [201] [225]	n.a
266	*ETFDH*	7		chr4:158695633G>A	c.821G>A	p.Gly274Glu	n.a	[48] [82] [88] [201]	n.a
267	*ETFDH*	7		chr4:158695641G>A	c.829G>A	p.Glu277Lys	rs1554032712	-	34
268	*ETFDH*	7		chr4:158695641insG	c.829dupG	p.Glu277Glyfs*6	n.a	[273] [274]	n.a
269	*ETFDH*	7		n.a	exon 7del	deletion of exon7	n.a	[277]	n.a
270	*ETFDH*	7-8		n.a	exon 7-8del	deletion of exon 7-8	n.a	[171]	n.a
271	*ETFDH*		7	chr4:158695645T>C	c.831+2T>C	n.r	rs150832878	[48] [201]	n.a
272	*ETFDH*		7	chr4:158695646A>C	c.831+3A>C	n.r	n.a	[171] [201]	n.a
273	*ETFDH*		7	chr4:158697558G>A	c.832-1G>A	exon 8 skipping	n.a	[37] [72] [201]	n.a
274	*ETFDH*		7	chr4:158697243C>T	c.832-316C>T	n.r.	rs145207902	[270]	344

275	*ETFDH*	8		chr4:158697562T>C	c.835T>C	p.Trp279Arg	rs754082348	[66] [72] [80] [274]	33
276	*ETFDH*	8		chr4:158697563G>T	c.836G>T	p.Trp279Leu	rs1377478090	-	33
277	*ETFDH*	8		chr4:158697564G>T	c.837G>T	p.Trp279Cys	rs1230343117	-	34
278	*ETFDH*	8		chr4:158697580A>G	c.853A>G	p.Asn285Asp	n.a	[273]	23
279	*ETFDH*	8		chr4:158697585G>A	c.858G>A	p.Trp286*	rs1235904433	[225]	0
280	*ETFDH*	8		chr4:158697599T>G	c.872T>G	p.Val291Gly	n.a	[38] [59] [66] [72] [80] [164] [167] [201] [218] [274]	n.a
281	*ETFDH*	8		chr4:158697604C>G	c.877C>G	p.His293Asp	n.a	[8] [35] [45] [72] [96] [201]	n.a
282	*ETFDH*	8		chr4:158697608C>T	c.881C>T	p.Thr294Ile	n.a	[17] [72] [201] [193]	n.a
283	*ETFDH*	8		chr4:158697617G>T	c.890G>T	p.Trp297Leu	n.a	[76] [111] [134] [201] [275]	n.a
284	*ETFDH*	8		chr4:158697618G>A	c.891G>A	p.Trp297fs	n.a	[274]	39
285	*ETFDH*	8		chr4:158697619C>T	c.892C>T	p.Pro298Ser	n.a	[99] [201]	n.a
286	*ETFDH*	8		chr4:158697623T>C	c.896T>C	p.Leu299Ser	n.a	[225]	n.a
287	*ETFDH*	8		chr4:158697643G>A	c.916G>A	p.Gly306Arg	rs761008371	-	32
288	*ETFDH*	8		chr4:158697644G>A	c.917G>A	p.Gly306Glu	n.a	[294]	29
289	*ETFDH*	8		chr4:158697647C>G	c.920C>G	p.Ser307Cys	n.a	[89] [110] [133] [162] [201] [180] [274]	n.a
290	*ETFDH*	8		chr4:158697647C>G	c.920C>T	p.Ser307Phe	n.a	[251]	27
291	*ETFDH*	8		chr4:158697649T>G	c.922T>G	p.Phe308Val	rs1218671512	[23] [59] [72] [201] [275]	29
292	*ETFDH*	8		chr4:158697653T>C	c.926T>C	p.Leu309Pro	rs1276785426	-	32
293	*ETFDH*	8		chr4:158697653T>G	c.926T>G	p.Leu309Arg	rs1276785426	-	31
294	*ETFDH*	8		chr4:158697656A>G	c.929A>G	p.Tyr310Cys	rs796051958	[263]	32
295	*ETFDH*	8		chr4:158697667G>A	c.940G>A	p.Glu314Lys	n.a	[211] [225]	n.a
296	*ETFDH*	8		chr4:158697673G>A	c.946G>A	p.Glu316Lys	n.a	[275]	26
297	*ETFDH*	8		chr4:158697676C>A	c.949C>A	p.Pro317Thr	n.a	[47] [72] [80] [201]	n.a
298	*ETFDH*	8		chr4:158697677C>G	c.950C>G	p.Pro317Arg	n.a	[76] [111] [134] [201] [275]	n.a
299	*ETFDH*	8		chr4:158697680T>C	c.953T>C	p.Leu318Pro	n.a	[159]	n.a
300	*ETFDH*	8		chr4:158697685G>C	c.958G>C	p.Ala320Pro	rs752675985	-	33
301	*ETFDH*	8		chr4:158697686C>T	c.959C>T	p.Ala320Val	rs751725601	[199] [201]	25
302	*ETFDH*	8		chr4:158697691G>A	c.964G>A	p.Gly322Ser	rs867300806	-	32
303	*ETFDH*	8		chr4:158697691G>A	c.964G>T	p.Gly322Cys	rs867300806	[228]	32
304	*ETFDH*	8		chr4:158703459 A>G	c.970G>A	p.Val324Met	n.a	[247]	n.a

305	*ETFDH*	8		chr4:158697697G>T	c.970G>T	p.Val324Leu	n.a	[229]	n.a
306	*ETFDH*		8	chr4:158697701 T>G	c.972+2A>G	splice variant	n.a	[275]	n.a
307	*ETFDH*	9		chr4:158698987G>T	c.973G>T	p.Val325Phe	rs549454407	-	34
308	*ETFDH*	9		chr4:158698987del	c.973del144	p.Val325_Gln372del	n.a	[274]	n.a
309	*ETFDH*	9		chr4:158698675del	c.973del312	p.Val325del48	n.a	[38] [59] [72] [80]	n.a
310	*ETFDH*	9		chr4:158698990G>C	c.976G>C	p.Gly326Arg	n.a	[72] [80] [201]	n.a
311	*ETFDH*	9		chr4:158698999T>C	c.985T>C	p.Tyr329His	rs750084469	-	31
312	*ETFDH*	9		chr4:158699000A>G	c.986A>G	p.Tyr329Cys	rs1561247687	-	32
313	*ETFDH*	9		chr4:158699007T>G	c.993T>G	p.Asn331Lys	n.a	[38] [48] [59] [80] [201] [181]	n.a
314	*ETFDH*	9		chr4:158699012A>G	c.998A>G	p.Tyr333Cys	rs781531865	[49] [48] [72] [80] [103] [201] [183] [263] [274] [281]	23
315	*ETFDH*	9		chr4:158699013C>G	c.999C>G	p.Tyr333fs	n.a	[275]	35
316	*ETFDH*	9		chr4:158699015T>C	c.1001T>C	p.Leu334Pro	rs377686388	[7] [8] [20] [59] [72] [201] [225]	30
317	*ETFDH*	9		chr4:159620170G>C	c.1004G>C	p.Ser335Thr	rs770140708	[264]	24
318	*ETFDH*	9		chr4:158699040G>T	c.1026G>T	p.Arg342Ser	n.a	[72] [80] [274]	n.a
319	*ETFDH*	9		chr4:158699041T>C	c.1027T>C	p.Trp343Arg	n.a	[66] [72] [80] [136] [201] [180] [214] [264] [274]	n.a
320	*ETFDH*	9		chr4:158699042T>C	c.1028T>C	p.Met343Thr	n.a	[167] [201]	n.a
321	*ETFDH*	9		chr4:158699048A>G	c.1034A>G	p.His345Arg	n.a	[295]	22
322	*ETFDH*	9		chr4:158699051A>G	c.1037A>G	p.His346Arg	rs1358691961	[8]	27
323	*ETFDH*	9		chr4:158699058A>C	c.1044A>C	p.Leu348Phe	n.a	[167] [201]	n.a
324	*ETFDH*	9		chr4:158699063G>A	c.1049G>A	p.Arg350Gln	rs139306043	[29] [59]	8
325	*ETFDH*	9		chr4:158699072T>C	c.1058T>C	p.Leu353Ser	rs1286771338	-	32
326	*ETFDH*	9		chr4:158699074G>T	c.1060G>T	p.Glu354*	n.a	[20] [59] [72] [201]	n.a
327	*ETFDH*	9		chr4:158699077G>A	c.1063G>A	p.Gly355Ser	rs1361421769	-	31
328	*ETFDH*	9		chr4:158699081G>A	c.1067G>A	p.Gly356Glu	n.a	[161] [201] [189] [205]	n.a
329	*ETFDH*	9		chr4:158699087G>A	c.1073G>A	p.Arg358Lys	rs796051959	[142]	26
330	*ETFDH*	9		chr4:158699088G>C	c.1074G>C	p.Arg358Ser	n.a	[12] [15] [59] [72] [142] [201] [223] [225]	n.a
331	*ETFDH*	9		chr4:158699092G>C	c.1078G>C	p.Ala360Pro	n.a	[134] [275]	n.a
332	*ETFDH*	9		chr4:158699097C>G	c.1083C>G	p.Tyr361fs	n.a	[273]	25

333	*ETFDH*	9		chr4:158699098G>A	c.1084G>A	p.Gly362Arg	rs369711837	[23] [38] [59] [72] [80] [82] [88] [134] [201] [274] [275]	32
334	*ETFDH*	9		chr4:158699121del	c.1085_1107del	p. Ala363Leufs*18	n.a	[277]	n.a
335	*ETFDH*	9		chr4:158699110C>T	c.1096C>T	p.Leu366Phe	n.a	[23] [59] [72] [201] [275]	n.a
336	*ETFDH*	9		chr4:158699113A>G	c.1099A>G	p.Asn367Asp	n.a	[72] [80] [225] [274]	n.a
337	*ETFDH*	9		chr4:158699120G>A	c.1106G>A	p.Gly369Asp	rs754418186	[182]	32
338	*ETFDH*	9		chr4:158699120G>C	c.1106G>C	p.Gly369Ala	rs754418186	[85] [225]	31
339	*ETFDH*		9	chr4:158703421A>G	c.1117-2A>G	splice variant	rs764060500	[120] [201]	0
340	*ETFDH*	10		chr4:158703423T>C	c.1117T>C	p.Ser373Pro	rs1774518301	[252]	32
341	*ETFDH*	10		chr4:158703422C>T	c.1118C>T	p.Ser373Phe	n.a	[193]	n.a
342	*ETFDH*	10		chr4:158703429C>A	c.1123C>A	p.Pro375Thr	n.a	[167] [201] [227] [277]	n.a
343	*ETFDH*	10		chr4:158703436T>C	c.1130T>C	p.Leu377Pro	rs387907170	[19] [59] [72] [123] [166] [201] [193] [197] [216] [261]	27
344	*ETFDH*	10		chr4:158703447G>C	c.1141G>C	p.Gly381Arg	rs1466787789	[13] [72] [132] [201] [200] [202] [197] [225]	33
345	*ETFDH*	10		chr4:158703448 G>C	c.1142G>C	p.Gly381Ala	n.a	[289]	28
346	*ETFDH*	10		n.a	n.r	p.Leu384Ile	n.a	[164]	n.a
347	*ETFDH*	10		chr4:158703465G>A	c.1157G>A	p.Gly386Asp	n.a	[143] [201] [205]	n.a
348	*ETFDH*	10		chr4:158703463 G>T	c.1157G>T	p.Gly386Val	n.a	[274]	28
349	*ETFDH*	10		chr4:158703471C>A	c.1165C>A	p.Pro389Thr	n.a	[201] [197]	n.a
350	*ETFDH*	10		chr4:158703507del	c.1198_1201delACTC	p.His401Glnfs*3	n.a	[123] [201] [197]	n.a
351	*ETFDH*	10		chr4:158703508A>G	c.1202A>G	p.His401Arg	n.a	[206]	n.a
352	*ETFDH*	10		chr4:158703510A>G	c.1204A>G	p.Thr402Ala	rs754240915	[176]	23
353	*ETFDH*	10		chr4:158703510A>T	c.1204A>T	p.Thr402Ser	n.a	[201] [195]	n.a
354	*ETFDH*	10		chr4:158703511C>T	c.1205C>T	p.Thr402Ile	n.a	[72] [80] [274] [289] [294]	n.a
355	*ETFDH*	10		chr4:158703514C>T	c.1208C>T	p.Ala403Val	n.a	[23] [27] [59] [72] [201] [275]	n.a
356	*ETFDH*	10		chr4:158703517T>C	c.1211T>C	p.Met404Thr	rs779253471	[38] [44] [59] [72] [80] [82] [88] [92] [149] [164] [167] [201] [178] [180] [274] [277]	27
357	*ETFDH*	10		chr4:158703523G>A	c.1217G>A	p.Ser406Asn	n.a	[60] [134] [275]	n.a

358	*ETFDH*	10		chr4:158703533A>C	c.1227A>C	p.Leu409Phe	rs1200031596	[38] [48] [59] [66] [72] [80] [82] [88] [110] [167] [201] [178] [180] [216] [274] [277] [281]	24
359	*ETFDH*	10		chr4:158703534G>A	c.1228G>A	p.Ala410Thr	rs927133458	-	31
360	*ETFDH*	10		chr4:158703540G>T	c.1234G>T	p.Glu412*	rs398124151	[225]	0
361	*ETFDH*	10		chr4:158703552del	c.1241_1246del	p.Ile414_Phe415del	n.a	[96] [201] [184]	n.a
362	*ETFDH*	10		chr4:158703550T>C	c.1244T>C	p.Phe415Ser	rs1050622882	-	33
363	*ETFDH*	10		chr4:158703563del	c.1254_1257delAACT	p.Thr419Valfs*9	n.a	[49] [72] [201]	n.a
364	*ETFDH*	10		chr4:158703579del	c.1270_1273del	n.r	n.a	[152] [201]	n.a
365	*ETFDH*	10		chr4:158703577C>G	c.1271C>G	p.Thr424Ser	n.a	[167] [201]	n.a
366	*ETFDH*	10		chr4:158703587-158703588AA>-	c.1281_1282delAA	p.Ile428Argfs*6	rs1233725939	[66] [72] [80] [150] [167] [201] [178] [181] [274]	0
367	*ETFDH*	10		chr4:158703587-158703588delAT	c.1282_1283delAT	p.Ile428Argfs*6	rs1233725939	[47]	n.a
368	*ETFDH*	10		chr4:158703591G>C	c.1285G>C	p.Gly429Arg	rs759044284	[20] [52] [72] [201]	28
369	*ETFDH*		10	chr4:158703592G>A	c.1285+1G>A	p.Gly429Aspfs21*	rs767046886	[153] [210] [214] [277]	0
370	*ETFDH*		10	chr4:158703593T>C	c.1285+2T>C	splice variant	n.a	[178] [277]	n.a
371	*ETFDH*	11		chr4:158703594G>A	c.1286G>A	p.Gly429Glu	n.a	[169]	n.a
372	*ETFDH*	11		chr4:158706198T>A	c.1295T>A	p.Val432Gly	n.a	[66] [72] [80] [274]	n.a
373	*ETFDH*	11		chr4:158706206T>C	c.1303T>C	p.Tyr435His	rs1057519158	-	32
374	*ETFDH*	11		chr4:158706215A>G	c.1312A>G	p.Asn438Asp	rs1485959982	[294]	19
375	*ETFDH*	11		chr4:158706228C>T	c.1325C>T	p.Ser442Leu	rs1442766122	[254] [235]	26
376	*ETFDH*	11		chr4:158706230T>C	c.1327T>C	p.Trp443Arg	n.a	[274]	28
377	*ETFDH*	11		chr4:158706234T>C	c.1331T>C	p.Val444Ala	rs760234838	[96] [117] [201] [264]	23
378	*ETFDH*	11		chr4:158706236T>C	c.1333T>C	p.Trp445Arg	rs1156517377	-	32
379	*ETFDH*	11		chr4:158706254G>A	c.1351G>A	p.Gly451Arg	n.a	[178]	n.a
380	*ETFDH*	11		chr4:158706254G>C	c.1351G>C	p.Val451Leu	rs558005496	[20] [59] [72] [201] [193]	24
381	*ETFDH*	11		chr4:158706257A>G	c.1354A>G	p.Arg452Gly	n.a	[171] [201]	n.a
382	*ETFDH*	11		chr4:158706258del	c.1355delG	p.Arg452Lysfs*3	n.a	[25] [59] [72] [201]	n.a
383	*ETFDH*	11		chr4:158706258G>A	c.1355G>A	p.Arg452Lys	n.a	[8]	n.a
384	*ETFDH*	11		chr4:158706269C>A	c.1366C>A	p.Pro456Thr	n.a	[16] [20] [59] [72] [201]	n.a
385	*ETFDH*	11		chr4:158706269C>T	c.1366C>T	p.Pro456Ser	rs751821289	[96] [160] [201] [221] [264]	25

386	*ETFDH*	11		chr4:158706270C>A	c.1367C>A	p.Pro456Gln	n.a	[216]	n.a
387	*ETFDH*	11		chr4:158706270C>T	c.1367C>T	p.Pro456Leu	rs398124152	[8] [9] [19] [20] [22] [23] [52] [59] [64] [72] [111] [201] [206] [225] [275]	25
388	*ETFDH*	11		chr4:158706271C>T	c.1368C>T	p.Pro456Leu	n.a	[8] [23]	n.a
389	*ETFDH*	11		chr4:159627424T>C	c.1369T>C	p.Ser457Pro	rs1404615636	[262]	25
390	*ETFDH*	11		chr4:158706275-158706278del	c.1372_1375delTGCC	p.Cys458Thrfs*10	n.a	[66] [72] [80] [274]	n.a
391	*ETFDH*	11		chr4:158706281G>T	c.1378G>T	p.Gly460*	n.a	[72] [80] [201] [274]	n.a
392	*ETFDH*	11		chr4:158706287C>G	c.1384C>G	p.Leu462Val	rs1438551187	[86] [201]	19
393	*ETFDH*	11		chr4:158706290G>A	c.1387G>A	p.Gly463Ser	rs1561251385	-	32
394	*ETFDH*	11		chr4:158706290G>C	c.1387G>C	p.Gly463Arg	rs1561251385	[211]	32
395	*ETFDH*	11		chr4:158706291G>A	c.1388G>A	p.Gly463Asp	rs1561251388	-	31
396	*ETFDH*	11		chr4:158706295-158706296del	c.1392_1393del	truncated	n.a	[7] [8]	n.a
397	*ETFDH*	11		chr4:158706298T>G	c.1395T>G	p.Tyr465*	rs745714733	[38] [49] [59] [66] [72] [80] [82] [88] [103] [167] [201] [178] [180] [208] [274] [277] [281]	0
398	*ETFDH*	11		chr4:158706298dup	c.1395dupT	p.Gly466Trpfs*24	n.a	[170] [201] [274]	n.a
399	*ETFDH*	11		chr4:158706302G>A	c.1399G>A	p.Gly467Arg	n.a	[82] [88] [201]	n.a
400	*ETFDH*	11		chr4:158706302G>C	c.1399G>C	p.Gly467Arg	rs1174882036	[38] [59] [72] [80] [180] [183] [217] [263]	33
401	*ETFDH*	11		chr4:158706303G>C	c.1400G>C	p.Gly467Arg	n.a	[25] [59] [72] [201]	n.a
402	*ETFDH*	11		chr4:158706314A>G	c.1411A>G	p.Thr471Ala	rs1247869602	[274]	23
403	*ETFDH*	11		chr4:158706317G>A	c.1414G>A	p.Gly472Arg	rs746598421	[12] [52] [59] [95] [193] [254]	33
404	*ETFDH*	11		chr4:158706318G>T	c.1415G>T	p.Gly472Val	rs768181815	-	32
405	*ETFDH*	11		chr4:158706328C>A	c.1425C>A	p.Tyr475*	n.a	[15]	n.a
406	*ETFDH*	11		chr4:158706339G>C	c.1436G>C	p.Arg479Thr	n.a	[38] [59] [72] [80] [274]	n.a
407	*ETFDH*	11		chr4:158706348A>T	c.1445A>T	p.Glu482Val	n.a	[20] [59] [72] [201]	n.a
408	*ETFDH*	11		chr4:158706351C>T	c.1448C>T	p.Pro483Leu	rs377656387	[19] [20] [22] [52] [59] [72] [80] [161] [201] [175] [189] [197] [211] [230] [274]	27

409	*ETFDH*	11		chr4:158706353T>C	c.1450T>C	p.Trp484Arg	n.a	[48] [57] [92] [110] [143] [164] [201] [180] [205] [274] [277]	n.a
410	*ETFDH*	11		chr4:158706357C>G	c.1454C>G	p.Thr485Ser	n.a	[47] [72] [80] [167] [201] [180] [208] [274]	n.a
411	*ETFDH*	11		chr4:158706360T>C	c.1457T>C	p.Leu486Pro	rs751839607	-	33
412	*ETFDH*		11	chr4:158706372del	c.1468+1delG	n.r	n.a	[86] [201]	n.a
413	*ETFDH*		11	chr4:158706373T>G	c.1468+2T>G	p.Gly490fs 15	n.a	[180]	n.a
414	*ETFDH*	12		chr4:158706630-158706632	c.1471_1473delins(8)	p.Ser491Glnfs*3	n.a	[96] [201]	n.a
415	*ETFDH*	12		chr4:158706635A>C	c.1475A>C	p.Asp492Ala	rs763487621	-	32
416	*ETFDH*	12		chr4:158706647T>A	c.1487T>A	p.Leu496His	n.a	[275]	31
417	*ETFDH*	12		chr4:158706647T>C	c.1487T>C	p.Leu496Pro	rs863224869	-	33
418	*ETFDH*	12		chr4:158706666del	c.1506delC	p.Cys502*	n.a	[169]	n.a
419	*ETFDH*	12		chr4:158706674T>C	c.1514T>C	p.Ile505Thr	rs764164607	[226]	28
420	*ETFDH*	12		chr4:158706679T>C	c.1519T>C	p.Tyr507His	rs779944719	-	31
421	*ETFDH*	12		chr4:158706679T>G	c.1519T>G	p.Tyr507Asp	rs779944719	[27] [59] [72] [134] [201] [275]	32
422	*ETFDH*	12		chr4:158706682C>A	c.1522C>A	p.Pro508Thr	rs751394068	[61] [72] [201]	26
423	*ETFDH*	12		chr4:158706684del	c.1524delA	p.Lys509Asnfs*16	n.a	[201] [197]	n.a
424	*ETFDH*	12		chr4:158706688C>T	c.1528C>T	p.Pro510Ser	n.a	[66] [72] [80] [274]	n.a
425	*ETFDH*	12		chr4:158706691G>A	c.1531G>A	p.Asp511Asn	rs780768015	[38] [58] [59] [66] [72] [80] [136] [201] [178] [210] [211] [264] [274] [277]	33
426	*ETFDH*	12		chr4:158706694G>A	c.1534G>A	p.Gly512Arg	n.a	[178] [277]	n.a
427	*ETFDH*	12		chr4:158706704G>T	c.1544G>T	p.Ser515Ile	n.a	[72] [75] [201]	n.a
428	*ETFDH*	12		chr4:158706708T>C	c.1547T>C	p.Phe516Ser	n.a	[157]	n.a
429	*ETFDH*	12		chr4:158706710A>C	c.1550A>C	p.Asp517Ala	rs777302338	-	31
430	*ETFDH*	12		chr4:158706712C>G	c.1552C>G	p.Leu518Val	n.a	[178] [277]	n.a
431	*ETFDH*	12		chr4:158706716T>G	c.1556T>G	p.Leu519Trp	rs1270988246	-	31
432	*ETFDH*	12		chr4:159627872del	c.1560delA	p.Ser521Leufs*4	n.a	[239]	n.a
433	*ETFDH*	12		chr4:158706724delG	c.1564delG	p.Val522Trpfs*3	n.a	[294]	n.a
434	*ETFDH*	12		chr4: between 158706743 & 158706744	c.1583_1584insA	p.Asn528Lysfs*3	n.a	[61] [72] [201]	n.a
435	*ETFDH*	12		chr4:158706748A>G	c.1586A>G	p.His529Arg	n.a	[82] [88] [201] [180]	n.a

436	*ETFDH*	12		chr4:158706755A>C	c.1595A>C	p.Asp532Ala	rs766367908	-	31
437	*ETFDH*	12		chr4:158706757C>T	c.1597C>T	p.Gln533*	n.a	[167] [201]	n.a
438	*ETFDH*	12		chr4:158706759G>C	c.1599G>C	p.Gln533His	n.a	[259]	25
439	*ETFDH*	12		chr4:158706761C>T	c.1601C>T	p.Pro534Leu	rs200920510	[23] [39] [59] [72] [103] [131] [134] [159] [201] [175] [179] [182] [225] [269] [275]	27
440	*ETFDH*	12		chr4:158706783del	c.1623delT	truncated	n.a	[7] [8]	n.a
441	*ETFDH*	12		chr4:158706806del	c.1647_1648delCT	n.r	n.a	[128]	n.a
442	*ETFDH*	12		chr4:158706809delCT	c.1648_1649delCT	p.Leu550Valfs*4	rs1469053638	[247]	n.a
443	*ETFDH*	12		chr4:158706809T>G	c.1649T>G	p.Leu550Pro	n.a	[83] [136] [201]	n.a
444	*ETFDH*	12		chr4:158706817T>C	c.1657T>C	p.Tyr553His	rs182913453	[72] [80] [183] [263] [274]	27
445	*ETFDH*	12		chr4:158706818A>G	c.1658A>G	p.Tyr553Cys	rs1561251884	[248]	24
446	*ETFDH*	12		chr4:158706827C>G	c.1667C>G	p.Pro556Arg	n.a	[205]	n.a
447	*ETFDH*	12		chr4:158706829G>A	c.1669G>A	p.Glu557Lys	rs1279728239	[81] [201] [290]	33
448	*ETFDH*	12		chr4:158708489C>T	c.1675C>T	p.Arg559*	rs1292587000	[23] [48] [59] [60] [72] [134] [201] [230] [275]	26
449	*ETFDH*	12		chr4:158706836G>A	c.1676G>A	p.Arg559Gln	rs768961719	-	34
450	*ETFDH*	12		chr4:158706836G>C	c.1676G>C	p.Arg559Pro	rs768961719	-	34
451	*ETFDH*	12		chr4:158706841T>C	c.1681T>C	p.Cys561Arg	rs1561251922	-	32
452	*ETFDH*	12		chr4:158706842G>T	c.1682G>T	p.Cys561Phe	rs774278013	-	33
453	*ETFDH*	12		chr4:158706845C>T	c.1685C>T	p.Pro562Leu	rs993314323	[7] [8]	27
454	*ETFDH*		12	chr4:158706851G>T	c.1690+1G>T	p.Arg559*	rs917285990	[5] [7] [8]	n.a
455	*ETFDH*		12	chr4:159628004T>G	c.1690+2T>G	splice variant	rs767238539	[235]	33
456	*ETFDH*		12	chr4:158708361C>G	c.1691-3C>G	splice variant or absence of transcript	rs776853600	[35] [37] [38] [47] [59] [66] [72] [80] [96] [119] [201] [180] [263]	n.a
457	*ETFDH*	13		chr4:158708366G>C	c.1693G>C	p.Val565Leu	rs769893690	[225]	28
458	*ETFDH*	13		chr4:158708372G>A	c.1699G>A	p.Glu567Lys	n.a	[216]	n.a
459	*ETFDH*	13		chr4:158708376T>G	c.1703T>G	p.Phe568Ser	n.a	[48] [201]	n.a
460	*ETFDH*	13		chr4:158708388A>T	c.1715A>T	p.Glu572Val	rs1169887640	-	32
461	*ETFDH*	13		chr4:158708390C>T	c.1717C>T	p.Gln573*	n.a	[48] [201]	n.a
462	*ETFDH*	13		chr4:158708399G>A	c.1726G>A	p.Gly576Arg	rs760578708	-	32
463	*ETFDH*	13		chr4:158708405C>T	c.1732C>T	p.Arg578Trp	rs763912783	[96] [201]	24

464	*ETFDH*	13		chr4:158708417A>T	c.1744A>T	p.Asn582Tyr	n.a	[47] [72] [80] [201]	n.a
465	*ETFDH*	13		chr4:158708420G>A	c.1747G>A	p.Ala583Thr	rs780666554	-	32
466	*ETFDH*	13		chr4:158708436A>G	c.1763A>G	p.His588Arg	rs781498366	[47] [72] [80] [201]	26
467	*ETFDH*	13		chr4:158708436A>T	c.1763A>T	p.His588Leu	n.a	[48] [201]	n.a
468	*ETFDH*	13		chr4:158708441A>G	c.1768A>G	p.Lys590Glu	n.a	[19] [72] [59] [201]	n.a
469	*ETFDH*	13		chr4:158708441A>T	c.1768A>T	p.Lys590*	n.a	[193]	n.a
470	*ETFDH*	13		chr4:158708446-158708447	c.1773_1774delA	p.Cys592*	rs767795266	[47] [72] [80] [129] [201]	n.a
471	*ETFDH*	13		chr4:158708446-158708447	c.1773_1774delAT	p.Cys592*	n.a	[82] [88] [201] [264]	n.a
472	*ETFDH*	13		chr4:158708447T>C	c.1774T>C	p.Cys592Arg	n.a	[23] [59] [72] [134] [201] [225] [275]	n.a
473	*ETFDH*	13		chr4:158708447T>G	c.1774T>G	p.Cys592Arg	n.a	[27] [31] [201]	n.a
474	*ETFDH*	13		chr4:158708456A>G	c.1783A>G	p.Lys595Glu	rs1422165935	-	31
475	*ETFDH*	13		chr4:158708459G>A	c.1786G>A	p.Asp596Asn	rs1165102742	[44] [72] [201]	33
476	*ETFDH*	13		chr4:158708460A>G	c.1787A>G	p.Asp596Gly	n.a	[131] [134] [201] [275]	n.a
477	*ETFDH*	13		chr4:158708463C>T	c.1790C>T	p.Pro597Leu	n.a	[201] [197]	n.a
478	*ETFDH*	13		chr4:158708480T>C	c.1807T>C	p.Trp603Arg	rs1561252786	-	33
479	*ETFDH*	13		chr4:158708482G>A	c.1809G>A	p.Trp603*	rs796051960	[58] [72] [201]	0
480	*ETFDH*	13		chr4:158708483G>T	c.1810G>T	p.Val604Leu	rs1436514087	[82]	28
481	*ETFDH*	13		chr4:158708485del	c.1812delG	p.Val605Tyrfs 34	n.a	[207]	n.a
482	*ETFDH*	13		chr4:158708492dupG	c.1819dupG	p.Glu607Glyfs*16	n.a	[277]	n.a
483	*ETFDH*	13		chr4:158708500-158709501	c.1827_1828insCAC	p.610insHis	n.a	[180]	n.a
484	*ETFDH*	13		chr4:158708501G>A	c.1828G>A	p.Gly610Arg	n.a	[82] [88] [201] [180] [264]	n.a
485	*ETFDH*	13		chr4:158708504G>A	c.1831G>A	p.Gly611Arg	n.a	[29] [59] [72] [201]	n.a
486	*ETFDH*	13		chr4:158708505G>A	c.1832G>A	p.Gly611Glu	rs761669036	[8]	31
487	*ETFDH*	13		chr4:158708515C>A	c.1842C>A	p.Tyr614*	rs765049360	[193] [225]	0
488	*ETFDH*	13		chr4:158708518dup	c.1842_1845dupCAAT	p.Gly616Glnfs*8	n.a	[276]	n.a
489	*ETFDH*	13		chr4:159629670insCAAT	c.1845_1846insCAAT	p.Gly616Glnfs*8	n.a	[263]	n.a
490	*ETFDH*	13		chr4:159629676G>A	c.1851G>A	p.Met617Ile	rs762536343	[262]	24
491	*ETFDH*	13		chr4:158708525T>C	c.1852T>C	p.*618Gln	rs765742496	[21] [72] [201] [179]	0
492	*ETFDH*	13		chr4:158708525T>C	c.1852T>C	p.*618Glnext*14	rs765742496	[46] [179] [264]	18
493	*ETFDH*		3'UTR	chr4:158708662A>C	c.1989A>C	n.r	n.a	[72]	n.a

494	*FLAD1*	1		chr1:154984015-154984018GGGG>GGG	c.324delG	p.Arg109Alafs*3	rs876661314	[105] [172]	n.a
495	*FLAD1*	1		chr1:154984044T>AC	c.350T>A	p.Ile117Asn	rs751960963	-	32
496	*FLAD1*	1		chr1:154984066G>T	c.372G>T	p.Lys124Asn	rs1338709810	-	35
497	*FLAD1*	1		chr1:154988105G>A	c.373G>A	p.Gly125Arg	rs747895040	-	31
498	*FLAD1*	2		chr1:154988129-154988136TTCTTTCT>TTCT	c.401_404delTTCT	p.Phe134Cysfs*8	rs876661313	[78] [105] [165] [172]	n.a
499	*FLAD1*	2		chr1:154988148T>C	c.416T>C	p.Leu139Pro	rs775833539	-	30
500	*FLAD1*	2		chr1:154988174C>T	c.442C>T	p.Arg148*	rs1426328465	[185]	0
501	*FLAD1*	2		chr1:154988229T>C	c.497T>C	p.Phe166Ser	rs1203866786	-	31
502	*FLAD1*	2		chr1:154988230del	c.498delC	p.Ser167Profs*20	rs876661315	[105] [172]	n.a
503	*FLAD1*	2		chr1:154988240T>C	c.508T>C	p.Phe170Leu	rs761928040	[105] [172]	23
504	*FLAD1*	2		chr1:154988258-154988269GCAGGGGGCATC>CA	c.526_537delinsCA	p.Ala176Glnfs*8	rs876661312	[105] [172]	0
505	*FLAD1*	2		chr1:154988264G>A	c.532G>A	p.Gly178Ser	rs920886455	-	31
506	*FLAD1*	2		chr1:154988270G>A	c.538G>A	p.Gly180Ser	rs376637647	-	31
507	*FLAD1*	2		chr1:154988270G>C	c.538G>C	p.Gly180Arg	rs376637647	-	32
508	*FLAD1*	2		chr1:154988286 A>T	c.554A>T	p.Asp185Val	rs1261326694	[287]	29
509	*FLAD1*	2		chr1:154988300-154988301dupGC	c.568_569dupGC	p.Val191Glnfs*10	rs876661310	[105] [114] [172]	n.a
510	*FLAD1*	2		chr1:154988477C>T	c.745C>T	p.Arg249*	rs199979286	[172] [188] [196] [241] [271] [275]	0
511	*FLAD1*	2		chr1:154988529del	c.797_798delAGinsT	p.Glu266Valfs*3	n.a	[145]	n.a
512	*FLAD1*	2		chr1:154988567-154988568TT>T	c.836delT	p.Phe279Serfs*45	rs876661311	[105] [172]	n.a
513	*FLAD1*	2		chr1:154988663G>C	c.931G>C	p.Gly311Arg	rs764740796	-	31
514	*FLAD1*	3		chr1:154989605A>G	c.1163A>G	p.Glu388Gly	rs1043646797	-	31
515	*FLAD1*	3		chr1:154989655G>A	c.1213G>A	p.Gly405Arg	rs754788619	-	31
516	*FLAD1*	3		chr1:154989658G>T	c.1216G>T	p.Gly406Cys	rs1404487345	-	33
517	*FLAD1*	3		chr1:154989662A>T	c.1220A>T	p.Lys407Ile	rs1266041411	-	31
518	*FLAD1*	3		chr1:154989707G>C	c.1265G>C	p.Arg422Thr	rs748771067	-	33
519	*FLAD1*	5		chr1:154990341A>G	c.1367A>G	p.Tyr456Cys	rs1275925705	-	32

520	*FLAD1*	5		chr1:154990367G>A	c.1393G>A	p.Gly465Ser	rs773436746	-	32
521	*FLAD1*	5		chr1:154990382G>A	c.1408G>A	p.Ala470Thr	rs775463868	-	32
522	*FLAD1*	5		chr1:154990440G>A	c.1466G>A	p.Arg489His	rs1447858195	-	33
523	*FLAD1*	5		chr1:154990456-154990460delCCT	c.1484_1486delCCT	p.Ser495del	rs876661309	[105] [172]	n.a
524	*FLAD1*	5		chr1:154990498G>T	c.1524G>T	p.Trp508Cys	rs1031039007	-	32
525	*FLAD1*		5	chr1:154992708C>G	c.1555-3C>G	splice variant	n.a	[145]	n.a
526	*FLAD1*	6		chr1:154992746C>T	c.1588C>T	p.Arg530Cys	rs771466122	[105] [114] [172] [185] [273] [274]	26
527	*FLAD1*	6		chr1:154992747G>A	c.1589G>A	p.Arg530His	rs146007674	-	33
528	*FLAD1*	6		chr1:154992747 G>C	c.1589G>C	p.Arg530Pro	n.a	[274]	32
529	*FLAD1*	6		chr1:154992768G>C	c.1610G>C	p.Cys537Ser	rs143499209	-	32
530	*FLAD1*	6		chr1:154992777A>G	c.1619A>G	p.Tyr540Cys	rs1299471459	-	32
531	*FLAD1*	7		chr1:154993030G>A	c.1757G>A	p.Arg586His	rs766627769	-	31
532	*SLC25A32*		5' UTR	chr8:12:98593715del	c.-264_31delins(14)	deletion of Met start codon	n.a	[121]	n.a
533	*SLC25A32*	1		chr8:103414868C>T	c.70G>A	p.Glu24Lys	rs932761592	-	32
534	*SLC25A32*	1		chr8:103414816T>C	c.122A>G	p.His41Arg	rs1472757179	-	30
535	*SLC25A32*	1		chr8:103414810A>G	c.128T>C	p.Leu43Pro	rs149273860	-	34
536	*SLC25A32*	1		chr8:103414808C>G	c.130G>C	p.Asp44His	rs766681889	-	33
537	*SLC25A32*	1		chr8:103414795A>C	c.143T>G	p.Ile48Ser	rs567748395	-	34
538	*SLC25A32*	2		chr8:103407760G>A	c.179C>T	p.Pro60Leu	rs747038599	-	31
539	*SLC25A32*	2		chr8:103407754T>C	c.185A>G	p.Tyr62Cys	rs772386221	-	32
540	*SLC25A32*	2		chr8:104419895G>T	c.272G>T	p.Gly91Val	n.a	[256]	26
541	*SLC25A32*	2		chr8:103407644A>G	c.295T>C	p.Tyr99His	rs760697865	-	32
542	*SLC25A32*	3		chr8:103404859T>C	c.308A>G	p.Tyr103Cys	rs764848283	-	31
543	*SLC25A32*	3		chr8:103404778G>C	c.389C>G	p.Ala130Gly	rs973996007	-	34
544	*SLC25A32*	4		chr8:103404776C>T	c.391G>A	p.Gly131Arg	rs770178649	-	34
545	*SLC25A32*	4		chr8:103403291C>T	c.425G>A	p.Trp142*	rs147014855	[107]	n.a
546	*SLC25A32*	4		chr8:103403276C>T	c.440G>A	p.Arg147His	rs142329098	[107]	27
547	*SLC25A32*	4		chr8:103403195T>C	c.521A>G	p.Tyr174Cys	rs1563717284	[267]	30
548	*SLC25A32*	4		chr8:103403186T>C	c.530A>G	p.Glu177Gly	rs772497373	-	31
549	*SLC25A32*	4		chr8:103403178G>A	c.538C>T	p.Arg180Cys	rs374025789	-	33

550	*SLC25A32*	4		chr8:103403168T>C	c.548A>G	p.Tyr183Cys	rs864622028	-	32
551	*SLC25A32*	5		chr8:103402014A>G	c.593T>C	p.Leu198Pro	rs757326114	-	32
552	*SLC25A32*	6		chr8:103401648T>C	c.680A>G	p.Tyr227Cys	rs1202272743	-	33
553	*SLC25A32*	6		chr8:103401636G>A	c.692C>T	p.Ala231Val	rs1238398531	-	31
554	*SLC25A32*	6		chr8:103401633G>A	c.695C>T	p.Ala232Val	rs759232426	-	31
555	*SLC25A32*	6		chr8:103401630A>G	c.698T>C	p.Leu233Pro	rs145785157	-	32
556	*SLC25A32*	6		chr8:103401628A>G	c.700T>C	p.Ser234Pro	rs770819305	-	31
557	*SLC25A32*	6		chr8:104413852A>G	c.704A>G	p.Lys235Arg	n.a	[267]	29
558	*SLC25A32*	6		chr8:103401600T>C	c.728A>G	p.Tyr243Cys	rs971518583	-	32
559	*SLC25A32*	6		chr8:103401597G>A	c.731C>T	p.Pro244Leu	rs1262662383	-	31
560	*SLC25A32*	6		chr8:103401594T>C	c.734A>G	p.Tyr245Cys	rs983458477	-	32
561	*SLC25A32*	6		chr8:103401520A>T	c.808T>A	p.Trp270Arg	rs1209946662	-	32
562	*SLC25A32*	7		chr8:103400468A>C	c.891T>G	p.Phe297Leu	rs1270709178	-	31
563	*SLC25A32*	7		chr8:103400460T>C	c.899A>G	p.Tyr300Cys	rs141856398	-	32
564	*SLC25A32*	7		chr8:103400439A>G	c.920T>C	p.Leu307Ser	rs1401357629	-	31
565	*SLC52A1*	1		chr17:5034604G>A	c.3G>A	p.Met1Ile	n.a	[286]	22
566	*SLC52A1*	1		chr17:5034539C>A	c.68C>A	p.Ser22Tyr	rs560354267	[286]	22
567	*SLC52A1*	1-5		n.a	exon 1-2, 4-5	multiple deletions in exon 1-2, 4-5	n.a	[209] [286]	n.a
568	*SLC52A1*	1-5		n.a	exon 1-5del	multiple deletions in exon 1-5	n.a	[209] [286]	n.a
569	*SLC52A1*	2		chr17:5034280A>G	c.209A>G	p.Gln70Arg	rs346822	[43]	4
570	*SLC52A1*	2-3		n.a	exon 2-3del	deletion across exon 2-3	n.a	[286]	n.a
571	*SLC52A1*	2-3		n.r	1.9kb del	n.r	n.a	[43]	n.a
572	*SLC52A1*	3		chr17:5033603G>A	c.886G>A	p.Val296Met	rs2304445	[43]	22
573	*SLC52A1*	3		n.a	exon 3del	deletion of exon 3	n.a	[209] [286]	n.a
574	*SLC52A1*		4	chr17:5033250C>T	c.1134+11G>A	exon 4 skipping	rs141935493	[127] [286]	n.a
575	*SLC52A2*		1	chr8:144359183G>A	c.-110-1G>A	splice variant	rs1554853682	[118] [151] [280] [285]	n.a
576	*SLC52A2*	1		chr8:144359368 T>TCCTGG	c.75_76insCCTGG	p.Ala26Profs*42	n.a	[244]	n.a
577	*SLC52A2*	1		chr8:144359384T>G	c.91T>G	p.Trp31Gly	rs1554853760	[280]	33
578	*SLC52A2*	1		chr8:144359385G>C	c.92G>C	p.Trp31Ser	rs797045199	[71]	26
579	*SLC52A2*	1		chr8:8:144359396C>T	c.103C>T	p.Pro35Ser	rs1554853770	[285]	25
580	*SLC52A2*	1		chr8:144359423G>A	c.130G>A	p.Gly44Ser	rs782089755	-	33

581	*SLC52A2*		2	chr8:144359622G>C	c.131-1G>C	splice variant	rs782305211	[285]	33
582	*SLC52A2*	2		chr8:144359627G>T	c.135G>T	p.Trp45Cys	rs1064795599	[285]	33
583	*SLC52A2*	2		chr8:144359641dup	c.149dupA	p.Tyr50*	rs782095095	[283]	n.a
584	*SLC52A2*	2		chr8:144359647C>T	c.155C>T	p.Ser52Phe	rs397514657	[63] [204] [212] [246] [280] [285]	21
585	*SLC52A2*	2		chr8:144359659C>T	c.167C>T	p.Ala56Val	rs781884010	[192] [280]	24
586	*SLC52A2*	2		chr8:144359674G>A	c.182G>A	p.Gly61Asp	rs1554853862	-	32
587	*SLC52A2*	2		chr8:144359677T>G	c.185T>G	p.Leu62Arg	rs782192446	[278] [285]	25
588	*SLC52A2*	2		chr8:144359721G>A	c.229G>A	p.Glu77Lys	rs782089251	[126]	24
589	*SLC52A2*	2		chr8:144359737G>C	c.245G>C	p.Arg82Pro	rs377110942	[138] [285]	12
590	*SLC52A2*	2		chr8:144359779C>T	c.287C>T	p.Ala96Val	rs1554853898	[285]	24
591	*SLC52A2*	2		chr8:144359779 CCT>C	c.292_293delCT	p.Leu98Valfs*91	n.a	[285]	n.a
592	*SLC52A2*	2		chr8:144359789G>C	c.297G> C	p.Trp99Cys	rs782591841	[118] [151] [280] [285]	27
593	*SLC52A2*	2		chr4:158682341C>T	c.322C>T	p.Gln108*	n.a	[231] [285]	n.a
594	*SLC52A2*	2		chr8:144359842T>C	c.350T>C	p.Leu117Pro	n.a	[243] [257] [284]	24
595	*SLC52A2*	2		chr8:144359845C>A	c.353C>A	p.Ala118Asp	rs117500243	[113] [231] [285]	15
596	*SLC52A2*	3		chr8:144359860T>C	c.368T>C	p.Leu123Pro	rs397514538	[50] [54] [156] [280] [285]	22
597	*SLC52A2*	3		chr8:144359869G>A	c.377G>A	p.Cys126Tyr	rs998301770	[285]	26
598	*SLC52A2*	3		chr8:144359875C>T	c.383C>T	p.Ser128Leu	rs374071862	[126] [280] [285]	21
599	*SLC52A2*	3		chr8:144359893C>T	c.401C>T	p.Pro134Leu	rs1447838904	[101] [280] [285]	23
600	*SLC52A2*	3		chr4:158682424-158682426delCTT	c.405_407delCTT	p.Phe135del	n.a	[219] [280] [285]	20
601	*SLC52A2*	3		chr8:144359910C>T	c.419C>T	p.Pro140Leu	n.a	[50] [55] [156]	n.a
602	*SLC52A2*	3		chr8:144359913C>A	c.421C>A	p.Pro141Thr	rs377740960	[108] [280] [285]	22
603	*SLC52A2*	3		chr8:144359941G>A	c.449G>A	p.Gly150Asp	rs1365149837	-	32
604	*SLC52A2*	3		chr8:144359969C>G	c.477C>G	p.Cys159Trp	n.a	[285]	13
605	*SLC52A2*	3		chr8:144359988G>A	c.496G>A	p.Gly166Ser	rs1554854006	-	32
606	*SLC52A2*	3		chr8:144359989G>A	c.497G>A	p.Gly166Asp	rs782636375	-	31
607	*SLC52A2*	3		chr8:144359997C>T	c.505C>T	p.Arg169Cys	rs782345472	[113] [163] [231] [191] [280] [285]	21
608	*SLC52A2*	3		chr8:144360085G>A	c.593G>A	p.Trp198*	n.a	[192] [280]	39
609	*SLC52A2*	3		chr8:144360076 CCTT>C	c.589_591delTTC	p.Phe197del	rs781879748	[285]	n.a
610	*SLC52A2*	3		chr8:144360109C>T	c.617C>T	p.Ala206Val	n.a	[283]	3
611	*SLC52A2*	3		chr8:144360187del	c.696_703del	p.Leu233Glyfs*32	n.a	[215] [280]	n.a

612	*SLC52A2*	3		chr8:144360192C>T	c.700C>T	p.Gln234*	rs797045200	[71] [280] [285]	0
613	*SLC52A2*	3		chr8:144360241 TGC>T	c.750_751delGC	p.Gln251Argfs*16	n.a	[285]	n.a
614	*SLC52A2*	3		chr8:144360243C>T	c.751C>T	p.Gln251*	rs1312209529	[285]	37
615	*SLC52A2*	3		chr8:144360300C>T	c.808C>T	p.Gln270*	rs375088539	[90] [280] [283] [285]	0
616	*SLC52A2*	3		chr4:158695636G>T	c.824G>T	p.Arg275Leu	n.a	[231] [285]	n.a
617	*SLC52A2*	3		chr8:144359368insGC CTGCTGG	c.832_c.833insGCCT GCTGG	p.Cys278delinsCysLeu LeuGly	n.a	[244]	n.a
618	*SLC52A2*	3		chr8:144360343C>A	c.851C>A	p.Ala284Asp	rs398123067	[71] [280] [285]	19
619	*SLC52A2*	3		chr8:144360353C>A	c.861C>A	p.Asn287Lys	n.a	[285]	167
620	*SLC52A2*	3		chr8:144360355C>T	c.863C>T	p.Ala288Val	rs764545993	[126] [231] [285]	23
621	*SLC52A2*	3		chr8:144360403C>T	c.911C>T	p.Pro304Leu	n.a	[285]	25
622	*SLC52A2*	3		chr8:144360406A>G	c.914A>G	p.Tyr305Cys	rs398123068	[71] [280] [285]	23
623	*SLC52A2*	3		chr8:144360408G>A >T	c.916G>A	p.Gly306Arg	rs398124641	[50] [55] [71] [70] [77] [101] [104] [113] [115] [144] [156] [231] [280] [285]	26
624	*SLC52A2*	3		chr8:144360409G>A	c.917G>A	p.Gly306Glu	rs781923855	[154] [190] [280] [285]	23
625	*SLC52A2*	3		chr8:144360427T>C	c.935T>C	p.Leu312Pro	rs754320812	[71] [113] [126] [204] [212] [246] [280] [285]	24
626	*SLC52A2*	3		chr8:144360460T>C	c.968T>C	p.Leu323Pro	rs781842708	[177] [280] [285]	24
627	*SLC52A2*	3		chr8:144360465T>G	c.973T>G	p.Cys325Gly	n.a	[137] [280] [285]	27
628	*SLC52A2*	3		chr8:144360604T>C	c.1016T>C	p.Leu339Pro	rs148234606	[50] [54] [71] [87] [90] [104] [115] [126] [156] [219] [268] [280] [285]	26
629	*SLC52A2*	3		chr8:144360610	c.1022_1023insC	p.Leu342Profs*103	n.a	[190] [280] [285]	n.a
630	*SLC52A2*	3		chr8:144360611 CCT>C	c.1030_1031delCT	p.Leu344Alafs*100	rs1328651461	[285]	n.a
631	*SLC52A2*	3		chr8:8:144360640G> A	c.1052G>A	p.Gly351Asp	rs782728994	[285]	22
632	*SLC52A2*	3		chr8:8:144360675C> G	c.1087C>G	p.Pro363Ala	rs1131691969	[285]	25
633	*SLC52A2*	3		chr8:144360676C>T	c.1088C>T	p.Pro363Leu	rs797045202	[126] [231] [253] [280] [285]	28
634	*SLC52A2*	3		chr8:144360814delTG G	c.1135_1137delTGG	p.Trp379del	n.a	[243] [257] [284]	n.a
635	*SLC52A2*	3		chr8:144360814G>C	c.1137G>C	p.Trp379Cys	rs782764685	-	34
636	*SLC52A2*	3		chr8:144360817del	c.1140delG	p.Leu381Cysfs*9	rs879254305	[138] [285]	n.a
637	*SLC52A2*	3		chr8:144360819T>C	c.1142T>C	p.Leu381Pro	rs1554854563	-	33

638	*SLC52A2*	4		chr8:144360866del21 chr8:144360836 TCCTACGTGAAGG TGGCAGCCAGCTC CCTG>T	c.1161_1189del29	p.Tyr388Alafs*47	n.a	[285]	n.a
639	*SLC52A2*	4		chr8:144360920G>A	c.1243G>A	p.Gly415Ser	n.a	[285]	31
640	*SLC52A2*	4		chr8:144360921G>A	c.1244G>A	p.Gly415Asp	rs782245545	-	32
641	*SLC52A2*	4		chr8:144360921G>C	c.1244G>C	p.Gly415Ala	rs782245545	[285]	27
642	*SLC52A2*	4		chr8:144360921G>T	c.1244G>T	p.Gly415Val	rs782245545	-	32
643	*SLC52A2*	4		chr8:144360927T>C	c.1250T>C	p.Leu417Pro	rs782065920	[215] [280] [285]	28
644	*SLC52A2*	4		chr8:144360932G>A	c.1255G>A	p.Gly419Ser	rs397514658	[63] [204] [212] [280] [285]	26
645	*SLC52A2*	4		chr8:144360935G>A	c.1258G>A	p.Ala420Thr	rs368924997	[71] [278] [280] [283] [285]	23
646	*SLC52A2*	4		chr8:144360936C>T	c.1259C>T	p.Ala420Val	rs1554854637	[285]	24
647	*SLC52A2*	4		chr8:144360944A>G	c.1267A>G	p.Met423Val	rs560659799	[285]	24
648	*SLC52A2*	4		chr8:144360945T>C	c.1268T>C	p.Met423Thr	n.a	[285]	24
649	*SLC52A2*	4		chr8:144360966A>G	c.1289A>G	p.Tyr430Cys	rs781873022	[285]	23
650	*SLC52A2*	4		chr8:144361004T>C	c.1327T>C	p.Cys443Arg	n.a	[100] [126] [156] [280] [285]	n.a
651	*SLC52A2*	4		chr8:144361005G>A	c.1328G>A	p.Cys443Tyr	n.a	[190] [280] [285]	n.a
652	*SLC52A3*	2		chr20:765738 C>T	c.37G>A	p.Gly13Arg	rs146302587	[126] [238]	24
653	*SLC52A3*	2		chr20:765731C>A	c.44G>T	p.Gly15Val	n.a	[102] [285]	n.a
654	*SLC52A3*	2		chr20:765726A>G	c.49T>C	p.Trp17Arg	rs797045190	[40] [50] [225] [285]	29
655	*SLC52A3*	2		chr20:765717T>G	c.58A>C	p.Ile20Leu	rs148387972	[126]	25
656	*SLC52A3*	2		chr20:765713T>C	c.62A>G	p.Asn21Ser	rs199588390	[50] [53] [108] [148] [231] [237] [238] [285]	26
657	*SLC52A3*	2		chr20:765705A>G	c.70T>C	p.Trp24Arg	rs1288873761	-	32
658	*SLC52A3*	2		chr20:765704C>T	c.71G>A	p.Trp24*	n.a	[122] [285]	n.a
659	*SLC52A3*	2		chr20:765693G>T	c.82C>A	p.Pro28Thr	rs267606688	[32] [50] [231] [285]	27
660	*SLC52A3*	2		chr20:765669C>T	c.106G>A	p.Glu36Lys	rs267606686	[30] [50] [65] [113] [126] [135] [231] [285]	27
661	*SLC52A3*	2		chr20:765662C>G	c.113G>C	p.Trp38Ser	n.a	[231] [232]	n.a
662	*SLC52A3*	2		chr20:765615C>T	c.160G>A	p.Gly54Arg	rs797045191	[50] [55]	25
663	*SLC52A3*	2		chr20:765602A>T	c.173T>A	p.Val58Asp	rs797045192	[50] [51] [231] [285]	29

664	*SLC52A3*	2		chr20:765582G>A	c.193C>T	p.Arg65Trp	rs537244674	[98] [231] [285]	20
665	*SLC52A3*	2		chr20:765564C>T	c.211G>A	p.Glu71Lys	rs267606683	[32] [50] [285]	26
666	*SLC52A3*	2		chr20:765564C>A	c.211G>T	p.Glu71*	rs267606683	[30] [50] [285]	0
667	*SLC52A3*	2		chr20:765551A>G	c.224T>C	p.Ile75Thr	rs797045193	[50] [55] [285]	26
668	*SLC52A3*	2		chr20:765423G>A	c.352G>A	p.Val118Met	rs1481863428	[126]	26
669	*SLC52A3*	2		chr20:765401G>T	c.374C>A	p.Thr125Asn	rs767263985	[126] [140] [231] [236]	27
670	*SLC52A3*	2		chr20:765392G>A	c.383C>T	p.Pro128Leu	rs890766612	[84] [231] [285]	31
671	*SLC52A3*	2		chr20:765381G>A	c.394C>T	p.Arg132Trp	rs267606684	[30] [50] [231] [220] [285]	26
672	*SLC52A3*	2		chr20:765372T>C	c.403A>G	p.Thr135Ala	rs527853872	[126] [285]	5
673	*SLC52A3*	2		chr20:765339 C>T	c.436G>A	p.Gly146Arg	rs1461455465	[285]	27
674	*SLC52A3*	2		chr20:765329 C>T	c.446G>A	p.Gly149Asp	rs749668781	[285]	25
675	*SLC52A3*	2		chr20:765320G>A	c.455C>T	p.Pro152Leu	rs1255062029	-	32
676	*SLC52A3*	2		chr20:765308G>A	c.467C>T	p.Ala156Val	rs1318600253	-	31
677	*SLC52A3*	2		chr20:765278 C>G	c.497G>C	p.Cys166Ser	n.a	[125]	25
678	*SLC52A3*	2		chr20:765273T>G	c.502A>C	p.Asn168His	rs1337277627	[220]	23
679	*SLC52A3*	2		chr20: between 764021 & 764022	c.568-19_-18insCTGATTGAC	insertion	rs11273404	[50] [51]	n.a
680	*SLC52A3*	3		chr20:763937G>A	c.634C>T	p.Arg212Cys	rs778479139	[126] [231] [285]	22
681	*SLC52A3*	3		chr20:763932G>C	c.639C>G	p.Tyr213*	rs778363575	[30] [32] [40] [50] [65] [140] [225] [285]	0
682	*SLC52A3*	3		chr20:763912G>T	c.659C>A	p.Pro220His	rs797045194	[50] [53] [231] [285]	26
683	*SLC52A3*	3		chr4:158690401delT	c.662delT	p.Leu221Argfs*10	n.a	[231]	n.a
684	*SLC52A3*	3		chr20:763901A>G	c.670T>C	p.Phe224Leu	rs267606685	[30] [50] [285]	27
685	*SLC52A3*	3		chr20:763900A>C	c.671T>G	p.Phe224Cys	rs797045197	[285]	32
686	*SLC52A3*	3		chr20:763893del	c.678_680del	p.Leu227del	n.a	[225]	n.a
687	*SLC52A3*	3		chr20:763861 G>A	c.710C>T	p.Ala237Val	rs773916730	[237]	26
688	*SLC52A3*	3		chr20:763850 G>A	c.721C>T	p.Leu241Phe	n.a	[236]	26
689	*SLC52A3*	3		chr4:158695540G>A	c.728G>A	p.Arg243His	n.a	[231]	n.a
690	*SLC52A3*	3		chr20:763775G>A	c.796C>T	p.Arg266Trp	rs370499474	[50] [51] [285]	25
691	*SLC52A3*	3		chr20:763636G>A	c.935C>T	p.Ala312Val	rs752218005	[50] [53] [231] [124] [238] [285]	24
692	*SLC52A3*	3		chr20:763616G>A	c.955C>T	p.Pro319Ser	rs797045195	[50] [51] [285]	29
693	*SLC52A3*	3		chr20:763615G>A	c.956C>T	p.Pro319Leu	rs760533974	-	33
694	*SLC52A3*	3		chr20:763612G>C	c.959C>G	p.Ser320Cys	rs535368648	-	33

695	*SLC52A3*	3		chr20:763612G>A	c.959C>T	p.Ser320Phe	rs535368648	-	33
696	*SLC52A3*	3		chr20:763598A>G	c.973T>C	p.Ser325Pro	rs1246925461	-	32
697	*SLC52A3*	3		chr20:763597G>A	c.974C>T	p.Ser325Phe	rs748083229	-	33
698	*SLC52A3*	3		chr20:763585 T>C	c.986A>G	p.Tyr329Cys	n.a	[238]	27
699	*SLC52A3*	3		chr20:763582C>A	c.989G>T	p.Gly330Val	rs797045196	[50] [56] [285]	25
700	*SLC52A3*	3		chr20:763525 G>A	c.1046C>T	p.Ser349Leu	rs1439187603	[233]	23
701	*SLC52A3*	3		chr20:763523A>T	c.1048T>A	p.Leu350Met	rs76947760	[30] [50] [285]	18
702	*SLC52A3*	3		chr20:763498C>G	c.1073G>C	p.Arg358Thr	rs892890925	-	33
703	*SLC52A3*	4		chr20:761824C>T	c.1074G>A	splice variant	rs1202138005	[126]	0
704	*SLC52A3*	4		chr 20:761817G>C	c.1081C>G	p.Leu361Val	n.a	[67]	n.a
705	*SLC52A3*	4		n.a	n.r	p.Leu369Pro	n.a	[146]	n.a
706	*SLC52A3*	4		chr20:761777 C>T	c.1121G>A	p.Gly374Glu	rs780735213	[285]	25
707	*SLC52A3*	4		chr20:761774C>T	c.1124G>A	p.Gly375Asp	rs1219868273	[53] [231] [285]	23
708	*SLC52A3*	4		chr20:761771T>C	c.1127A>G	p.Tyr376Cys	rs749962422	[67] [231]	32
709	*SLC52A3*	4		chr20:761770 G>C	c.1128C>G	p.Tyr376*	n.a	[109]	46
710	*SLC52A3*	4		chr20:761770	c.1128_1129insT	p.Tyr376*	n.a	[126] [285]	n.a
711	*SLC52A3*	4		chr20:761742A>G	c.1156T>C	p.Cys386Arg	rs1555783543	[130] [285]	33
712	*SLC52A3*	4		chr20:761738G>A	c.1160C>T	p.Pro387Leu	rs1164187566	-	34
713	*SLC52A3*	4		chr20:761735 A>C	c.1163T>G	p.Leu388Arg	n.a	[233]	25
714	*SLC52A3*		4	chr20:761240T>G	c.1198-2A>C	splice variant	rs754753126	[40] [50] [285]	0
715	*SLC52A3*	5		chr20:761233 G>GA	c.1203insT	p.Ser402Leufs*105	n.a	[67]	n.a
716	*SLC52A3*	5		chr20:761213C>T	c.1223G>A	p.Gly408Asp	rs1057524605	[125] [154] [285]	25
717	*SLC52A3*	5		chr20:761204-761201	c.1232_1233ins(60)	p.Ser411_Tyr412ins(20)	n.a	[139] [231] [285]	n.a
718	*SLC52A3*	5		chr20:761201T>C	c.1235A>G	p.Tyr412Cys	rs1359318796	-	33
719	*SLC52A3*	5		chr20:761198A>G	c.1238T>C	p.Val413Ala	rs267606687	[30] [50] [51] [98] [116] [126] [231] [285]	32
720	*SLC52A3*	5		chr20:761195T>C	c.1241A>G	p.Lys414Arg	rs772753947	-	32
721	*SLC52A3*	5		chr20:761183 C>T	c.1253G>A	p.Gly418Asp	rs797045198	[126] [285]	18
722	*SLC52A3*	5		chr20:761171C>G	c.1265G>C	p.Arg422Pro	rs200665228	-	31
723	*SLC52A3*	5		chr20:761160G>A	c.1276C>T	p.Arg426Cys	rs780025944	-	34
724	*SLC52A3*	5		chr20:761151 G>A	c.1285C>T	p.Leu429Phe	n.a	[238]	32
725	*SLC52A3*	5		chr20:761144C>T	c.1292G>A	p.Trp431*	rs1379613754	[84] [126] [231] [285]	0
726	*SLC52A3*	5		chr20:761143C>G	c.1293G>C	p.Trp431Cys	rs1060499531	-	33

727	*SLC52A3*	5		chr20:761140G>T	c.1296C>A	p.Cys432*	rs758570021	[50] [51] [231] [285]	0
728	*SLC52A3*	5		chr20:761123A>G	c.1313T>C	p.Leu438Pro	rs1167512470	[285]	33
729	*SLC52A3*	5		chr20:761120C>T	c.1316G>A	p.Gly439Asp	rs1555783467	[163] [285]	31
730	*SLC52A3*	5		chr20:761114A>G	c.1322T>C	p.Leu441Pro	rs1441733847	-	33
731	*SLC52A3*	5		chr20:761110-761112GAG>G	c.1325_1326delTC	p.Leu442Argfs*64	rs794728004	[30] [50] [266] [285]	n.a
732	*SLC52A3*	5		chr20:761109 CG>C	c.1326del12	p.Gly443Metfs*24	n.a	[125]	n.a
733	*SLC52A3*	5		chr20:761105G>A	c.1331C>T	p.Ala444Val	rs766882276	-	33
734	*SLC52A3*	5		chr20:761102A>C	c.1334T>G	p.Leu445Arg	rs761224042	-	33
735	*SLC52A3*	5		chr20:761090G>C	c.1346C>G	p.Pro449Arg	rs1322154756	-	33
736	*SLC52A3*	5		chr20:761087A>G	c.1349T>C	p.Leu450Pro	rs1237553628	-	34
737	*SLC52A3*	5		chr20:761067A>G	c.1369T>C	p.Phe457Leu	rs779750163	-	32
738	*SLC52A3*	5		chr20:761065G>C	c.1371C>G	p.Phe457Leu	rs145431028	[30] [50] [55] [115] [126] [285]	32
739	*SLC52A3*	5		chr20:761055A>C	c.1381T>G	p.Asp461Tyr	n.a	[116] [231] [285]	n.a
740	*SLC52A3*	4		chr20:761745 G>C	c.1153C>G	p.Pro385Ala	n.a	[238]	27

Appropriate adjustments were made in those instances where the nucleotide change/position and amino acid change/position did not correspond. All articles not available in English/Dutch/German could not be followed-up by the authors. * No value refers to a likely pathogenic variant identified from Ensembl and gnomAD v2.1.1 databases. Abbreviations: n.a, not applicable; n.r, not reported.

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A.3 SUPPLEMENTARY MATERIAL 2

Table A.2: Number and distribution of pathogenic variants (reported in literature, Ensembl and gnomAD databases, and in total) per exon or intron of each MADD-related gene

Gene	Status	Number of pathogenic variants																							
		Exon													Intron										
		1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7	8	9	10	11	12	13
ETFA	**Literature**[†]	3	3	2	4	5	6	3	2	**9**	3	4			0	0	0	0	0	**1**	**1**	0	0	0	0	0	
	Databases	0	1	1	0	0	0	0	1	**4**	2	1			0	0	0	0	0	0	0	0	0	0	0	0	
	Total	3	4	3	4	5	6	3	3	**13**	5	5			0	0	0	0	0	**1**	**1**	0	0	0	0	0	
ETFB	**Literature**	3	**8**	4	3	5	2								0	1	0	1	**2**	0							
	Databases	3	1	3	0	**5**	1								0	0	0	0	0	0							
	Total	6	9	7	3	**10**	3								0	1	0	1	**2**	0							
ETFDH	**Literature**[†]	5	22	**39**	12	21	9	24	24	27	27	35	30	31	**5**	4	4	2	**5**	2	4	1	1	2	2	3	0
	Databases	0	0	4	1	4	0	3	7	5	2	6	**10**	5	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	5	22	**43**	13	25	9	27	31	32	29	41	40	36	**5**	4	4	2	**5**	2	4	1	1	2	2	3	0
FLAD1	**Literature**	1	**10**	0	0	1	2	0							0	0	0	0	**1**	0	0						
	Databases	3	**6**	5	0	5	3	1							0	0	0	0	0	0	0						
	Total	4	**16**	5	0	6	5	1							0	0	0	0	**1**	0	0						
SLC25A32	**Literature**[†]	0	1	0	**3**	0	1	0							0	0	0	0	0	0	0						
	Databases	5	3	2	4	1	**9**	3							0	0	0	0	0	0	0						
	Total	5	4	2	7	1	**10**	3							0	0	0	0	0	0	0						
SLC52A1	**Literature**[†]	**2**	1	**2**	0	0									0	0	0	**1**	0								
	Databases	0	0	0	0	0									0	0	0	0	0								
	Total	**2**	1	**2**	0	0									0	0	0	**1**	0								
SLC52A2	**Literature**	4	13	**37**	12	0									**1**	**1**	0	0	0								
	Databases	1	1	**5**	2	0									0	0	0	0	0								
	Total	5	14	**42**	14	0									**1**	**1**	0	0	0								
SLC52A3	**Literature**	0	**25**	17	11	14									0	0	0	**1**	0								
	Databases	0	3	6	1	**11**									0	0	0	0	0								
	Total	0	**28**	23	12	25									0	0	0	**1**	0								

† Variants that are located in the 5'UTR and 3'UTR or not restricted to a single exon/intron have been omitted from Table A.2. This includes c.-85G>A in *ETFA*; c.-75A>G, exon 1–5del, exon 1–6del, exon 1–7del, exon 7–8del, and c.1989A>C in *ETFDH*; c.-264_31delins(14) in *SLC25A32*; and multiple deletions in exon 1–2 and 4–5, exon 1–5del, exon 2–3del, and the 1.9 kb deletion spanning exons 2 and 3 of *SLC52A1*; Red, bold values signify the exon and intron containing the largest number of variants per gene. Abbreviations: UTR: untranslated region.

Appendix B

Peer-reviewed article ii and
Supplementary Material:
Chapter 3

B.1 PEER-REVIEWED ARTICLE II

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Orphanet Journal of Rare Diseases

RESEARCH Open Access

Clinical, biochemical, and genetic spectrum of MADD in a South African cohort: an ICGNMD study

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Abstract

Background Multiple acyl-CoA dehydrogenase deficiency (MADD) is an autosomal recessive disorder resulting from pathogenic variants in three distinct genes, with most of the variants occurring in the electron transfer flavoprotein-ubiquinone oxidoreductase gene (*ETFDH*). Recent evidence of potential founder variants for MADD in the South African (SA) population, initiated this extensive investigation. As part of the International Centre for Genomic Medicine in Neuromuscular Diseases study, we recruited a cohort of patients diagnosed with MADD from academic medical centres across SA over a three-year period. The aim was to extensively profile the clinical, biochemical, and genomic characteristics of MADD in this understudied population.

Methods Clinical evaluations and whole exome sequencing were conducted on each patient. Metabolic profiling was performed before and after treatment, where possible. The recessive inheritance and phase of the variants were established via segregation analyses using Sanger sequencing. Lastly, the haplotype and allele frequencies were determined for the two main variants in the four largest SA populations.

Results Twelve unrelated families (ten of White SA and two of mixed ethnicity) with clinically heterogeneous presentations in 14 affected individuals were observed, and five pathogenic *ETFDH* variants were identified. Based on disease severity and treatment response, three distinct groups emerged. The most severe and fatal presentations were associated with the homozygous c.[1067G > A];c.[1067G > A] and compound heterozygous c.[976G > C];c.[1067G > A] genotypes, causing MADD types I and I/II, respectively. These, along with three less severe compound heterozygous genotypes (c.[1067G > A];c.[1448C > T], c.[740G > T];c.[1448C > T], and c.[287dupA*];c.[1448C > T]), resulting in MADD types II/III, presented before the age of five years, depending on the time and maintenance of intervention. By contrast, the homozygous c.[1448C > T];c.[1448C > T] genotype, which causes MADD type III, presented later in life. Except for the type I, I/II and II cases, urinary metabolic markers for MADD improved/normalised following treatment with riboflavin and L-carnitine. Furthermore, genetic analyses of the most frequent variants (c.[1067G > A]

For The ICGNMD Consortium in author group, see list of Consortium Members at https://www.ucl.ac.uk/genomic-medicine-neuromuscular-diseases/global-contributor-list.

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and c.[1448C > T]) revealed a shared haplotype in the region of *ETFDH*, with SA population-specific allele frequencies of < 0.00067–0.00084%.

Conclusions This study reveals the first extensive genotype–phenotype profile of a MADD patient cohort from the diverse and understudied SA population. The pathogenic variants and associated variable phenotypes were characterised, which will enable early screening, genetic counselling, and patient-specific treatment of MADD in this population.

Keywords Multiple acyl-CoA dehydrogenase deficiency, MADD, Glutaric aciduria type II, *ETFDH*, Riboflavin, South Africa, International Centre for Genomic Medicine in Neuromuscular Diseases, ICGNMD

Background

Multiple acyl-CoA dehydrogenase deficiency (MADD; ORPHAcode 26791), also known as glutaric aciduria type II (GAII), is an autosomal recessive disorder that occurs at a global and ethnically variable prevalence of approximately 1 in 200,000 births [1]. It is caused by pathogenic variants (at least 400) in the *ETFA*, *ETFB* and *ETFDH* genes, which encode for the alpha and beta subunits of the electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETFQO; EC:1.5.5.1), respectively. Collectively, ETF and ETFQO are responsible for re-oxidising reduced mitochondrial flavin adenine dinucleotide ($FADH_2$), which in turn sustains mitochondrial fatty acid β-oxidation (FAO), amino acid catabolism and choline metabolism [2, 3].

Clinically, MADD may be divided into three phenotypes. The first and second (MADD types I and II, respectively) are characterised by the neonatal-onset of severe and often fatal symptoms with multi-system involvement (including leukodystrophy, hypotonia, cardiomyopathy, hepatomegaly, and renal abnormalities), as well as hypoglycaemia, hyperammonaemia, and metabolic acidosis (with/without ketosis) [4–6]. MADD types I and II may be distinguished from each other by the presence (type I) or absence (type II) of congenital abnormalities and are caused, at essentially equal frequencies, by pathogenic variants in *ETFA*, *ETFB*, and *ETFDH* [5]. By contrast, the third phenotype (MADD type III) has a later-onset and presents with milder/delayed symptoms which are highly heterogeneous. Such symptoms may include recurrent/intermittent episodes of lethargy and vomiting; muscle, cardiac and/or liver involvement; as well as lipid storage myopathy, hypoglycaemia, metabolic acidosis, and/or hyperammonaemia. MADD type III is reported to be caused mainly by *ETFDH* variants and is also known as riboflavin (Rb)-responsive MADD owing to its amenability to treatment [5, 7]. To estimate the burden of disease, a disease severity scoring system (MADD-DS3; MADD-disease severity 3) may be used [6]. Moreover, disease-severity is often linked to the patient's response to treatment, which includes a high-caloric diet with fat and protein restriction, the strict avoidance of fasting, and supplementation with L-carnitine and Rb (as first-line treatment) [8, 9].

At present, South Africa (SA) does not have a compulsory newborn screening (NBS) programme. Instead, the biochemical diagnosis of MADD follows (i) a clinical presentation in symptomatic cases, or (ii) an abnormal NBS profile in asymptomatic cases—during which MADD is defined as a secondary condition. Symptomatic patients undergo a thorough metabolic work-up including (i) urinary organic acids, (ii) plasma/serum acylcarnitines, and (iii) plasma/serum amino acids and display a unique metabolic fingerprint as described in a recent review by Mereis et al. [3]. As with other inherited metabolic disorders, final confirmation of the diagnosis is obtained by genetic analysis.

In SA, as with most understudied populations, pathogenic variant screening for inherited metabolic disorders is less common. Such screening is, however, offered for a small selection of disorders (including glutaric aciduria type I, isovaleric acidaemia, galactosemia, and MPV17-hepatocerebral mitochondrial DNA depletion syndrome) following the comprehensive description of cohorts in literature. Currently, substantial knowledge of the genotype–phenotype and biochemical profiles, and the subsequent response to treatment are not available for MADD in the different SA populations. Prior to this study, a novel pathogenic *ETFDH* variant (c.[1067G > A], p.[Gly356Glu]) and one previously described pathogenic variant (c.[1448C > T], p.[Pro483Leu]) were reported to result in MADD in the White SA population [10]. In the homozygous state, these variants cause MADD types I and III, respectively, and it was proposed by van der Westhuizen et al. [10] that the prevalence of c.[1067G > A] in this SA population could possibly be the result of a founder effect. In our study, we aimed to address these hypotheses and other limitations by investigating a cohort of 14 recruited MADD patients, under the auspices of the International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD). We extensively characterised the clinical phenotype, biochemical profile, and genetics of MADD in SA, and

provide knowledge that will contribute to its timely diagnosis, clinical management, and therapeutic intervention.

Results and discussion

Cohort

Over a period of three years and using a retrospective and prospective recruitment strategy at three different centres across SA, 12 apparently unrelated families (ten of White SA and two of mixed ethnicity) with 14 clinically affected patients (five males and nine females) were recruited. All patients were born to non-consanguineous parents and disease onset ranged from birth to 41 years, with four deaths recorded (at 9 days, 14 days, 3 months, and 23 years, respectively). Where possible, a consistent clinical re-evaluation was conducted according to ICGNMD guidelines; for most of the cases, biological samples acquired during acute metabolic presentation (n = 12/14) and following therapeutic management (n = 13/14) were collected/available and could be re-analysed (for the remainder, the original data were used, where available).

It should be noted that SA patients of African and Indian ethnicity have previously been diagnosed with MADD on a biochemical level at the Centre for Human Metabolomics [North-West University (NWU)] and the NHLS Inherited Metabolic Disease Molecular Laboratory [University of Cape Town (UCT)]—the two SA facilities where metabolic testing is offered as a main service (unpublished data). However, while all possible efforts were made to include patients of these population groups, they were regrettably lost to follow-up prior to the study's onset and an opportunity for subsequent genetic confirmation did not present itself. Moreover, no new cases were identified in these population groups during this study. We therefore recognise a potential referral bias within the population groups included. It is our hope that this problem can be addressed by metabolic and genetic NBS in the future.

Clinical, biochemical, and genetic features

Overall, the SA MADD cohort displayed heterogeneous clinical presentations (Table 1 and Additional file 1), together with the characteristic diagnostic urinary metabolites associated with MADD (Table 2 and Additional file 2). While plasma/serum acylcarnitine and amino acid findings are typically used for diagnosis, this matrix was limited for the majority of the patients (sample collection and analyses were conducted over a 30-year period) and consequently, the levels of these metabolites in the urine are reported instead. The variants identified, their location in ETFQO, and their pathogenicity, according to the American College of Medical Genetics and Genomics (ACMG) criteria, are described in Table 3 [11]. The elucidation of these variants in the SA populations now enable rapid screening methods which were not available before, thereby allowing the timely confirmation of the disorder (in hours) in symptomatic patients. This prompt identification of MADD-related variants can be used to aid the reproductive choices of carrier/affected patients and is especially valuable for prenatal testing and the testing of neonates who may require immediate therapy following birth (i.e., those affected with MADD type I/II). In all recruited cases, a resolved genotype–phenotype result was obtained. Three distinct groups were observed, based on disease severity and response to treatment, as discussed below.

Severe, Rb-unresponsive MADD patient group

The first group included three patients (P6, P8 and P12), who presented with the hallmark features of neonatal-onset MADD. Clinically, their symptoms were severe and progressive, and patients were metabolically and phenotypically unresponsive to treatment with Rb. The first case was a male neonate (P6), born prematurely (at 38 weeks) to parents of White SA ethnicity, who presented within the first week of life with acute metabolic decompensation. Key clinical features included congenital cardiac abnormalities (pulmonary stenosis, a patent foramen ovale with a right to left shunt, and a large atrial septum aneurysm) and convulsions with early neonatal death on day 9 of life. Metabolic profiling indicated the typical MADD biochemical fingerprint [12], including dicarboxylic aciduria, increased 2-hydroxyglutaric acid, elevated glycine conjugates (short-, short-branched-, and medium-chain-related), increased disease-associated acylcarnitine conjugates, as well the characteristic increase in sarcosine on the amino acid profile. Both FAO and branched-chain amino acid catabolism were affected, correlating with previous studies on severe MADD cases [6]. The patient displayed a MADD-DS3 score of 30, further supporting a diagnosis of MADD type I. A then novel homozygous variant—c.[1067G > A] (p.[Gly356Glu])—with in silico and structural evidence of pathogenicity (ACMG classification: likely pathogenic) was identified in this neonate [10].

The second patient was a female infant (P8), born at term to parents of mixed ethnicity, who presented at birth with metabolic acidosis, hypotonia and feeding difficulties. Disease-specific metabolic markers were similar to those of P6, and the MADD-DS3 score of 21 was high. The patient succumbed at the age of three months. The homozygous c.[1067G > A] variant was subsequently identified; however, based on the lack of any reported congenital features (absent post-mortem examination), a diagnosis of MADD type I/II was given. The third case was a female neonate (P12), born at term to parents of

Table 1 Demographic, clinical, and genetic information of the SA MADD cohort

Group	Rb-unresponsive (severe phenotype; n = 3)			Variably Rb-responsive (moderate phenotype; n = 8)							
Patient	**P12**	**P8**	**P6**	**P13**	**P2**	**P3**	**P9**	**P10**	**P11**	**P1**	**P7**
Gender	F	F	M	F	F	M	M	F	M	M	F
Ethnicity	Mixed	Mixed	White	White	White	White	White	White	White	White	White
Consanguinity	None	None	None	None	None	None	None	None	None	None	None
Clinical											
Age of onset/death	Birth/D14	Birth/M3	Birth/D9	Y3	M8	Y4	M4	M9	Y3	Birth	M9/Y23
Major organ systems involved	Liver Muscle	Liver Muscle	Congenital Liver Muscle	Muscle	Muscle	Liver Muscle	CNS Liver Muscle	Liver	Muscle	CNS Liver Muscle	CNS Liver Muscle
MADD-DS3 score [6]	24	21	30	3	3	6	6.75	9	9	13.95	22.5
Corresponding type	I/II	I/II	I	III	III	III	III	III	III	II	III
***ETFDH* genotype**											
c.[1067G>A];c.[1067G>A]	–	+	+	–	–	–	–	–	–	–	–
c.[1448C>T];c.[1448C>T]	–	–	–	–	–	–	–	–	–	–	–
c.[976G>C];c.[1067G>A]	+	–	–	–	–	–	–	–	–	–	–
c.[1067G>A];c.[1448C>T]	–	–	–	–	–	–	+	+	+	+	+
c.[287dupA*];c.[1448C>T]	–	–	–	–	+	+	–	–	–	–	–
c.[740G>T];c.[1448C>T]	–	–	–	+	–	–	–	–	–	–	–

Group	Rb-responsive (mild phenotype; n = 3)		
Patient	**P4**	**P14**	**P5**
Gender	F	F	F
Ethnicity	White	White	White
Consanguinity	None	None	None
Clinical			
Age of onset/death	Y41	Y8	Y22
Major organ systems involved	PNS Muscle	Liver Muscle	Liver Muscle
MADD-DS3 score [6]	5.5	7.5	11.4
Corresponding type	III	III	III
***ETFDH* genotype**			
c.[1067G>A];c.[1067G>A]	–	–	–
c.[1448C>T];c.[1448C>T]	+	+	+
c.[976G>C];c.[1067G>A]	–	–	–
c.[1067G>A];c.[1448C>T]	–	–	–
c.[287dupA*];c.[1448C>T]	–	–	–
c.[740G>T];c.[1448C>T]	–	–	–

The MADD-DS3 score [6] increases with disease severity and reflects the clinical symptoms exhibited during decompensation. Consequently, it is affected by the time elapsed (and thus the progression of symptoms) from presentation to successful diagnosis. The MADD-DS3 score is used as a guide only and is not an absolute indication of the degree of pathogenicity of a variant. Red, yellow, and green: high, moderate, and low MADD-DS3 scores within the cohort, respectively

Abbreviations: + Present and validated, *CNS* Central nervous system, *D and number* Age in days, *ETFDH* Electron transfer flavoprotein-ubiquinone oxidoreductase gene (NM_004453.4), *F* Female, *M* Male, *M and number* Age in months, *MADD-DS3* Multiple acyl-CoA dehydrogenase deficiency-disease severity 3, *n* Number of patients, *PNS* Peripheral nervous system, *Rb* Riboflavin, *Y and number* Age in years

mixed ethnicity. A urinary organic acid profile typical of MADD was confirmed by the hospital that made the diagnosis (no residual urine collected before or after treatment was available to re-analyse for the purpose of this study). Clinical features included metabolic acidosis, hypoglycaemia, hyperammonaemia, pancytopaenia, acute kidney injury, hyponatremia, and hypocalcaemia, and the patient succumbed at 14 days of age. Based on

Table 2 Diagnostic urine MADD metabolites of the SA MADD cohort

Group	Rb-unresponsive (severe phenotype; n = 3)		Variably Rb-responsive (moderate phenotype; n = 8)		Rb-responsive (mild phenotype; n = 3)	
Treatment (sample number)	Untr (s = 3)	Tr (s = 2)	Untr (s = 5)	Tr (s = 8)	Untr (s = 3)	Tr (s = 3)
Urine Organic acids[1]						
Ethylmalonic acid	1.7	2.5	1.8	1.1	2.0	1.0
Glutaric acid	3.0	3.0	2.8	0.6	2.0	0.0
2-Methylbutyrylglycine	1.7	1.5	0.0	0.0	1.0	0.0
N-Hexanoylglycine	2.0	1.5	2.4	1.6	2.0	0.0
N-Isovalerylglycine	3.0	3.0	0.0	0.0	1.0	0.0
N-Isobutyrylglycine	1.3	1.5	1.2	0.5	1.0	0.0
2-Hydroxyglutaric acid	3.0	3.0	1.4	1.3	3.0	1.0
cis-4-Decenedioic acid	2.0	0.5	0.6	0.4	0.0	0.0
C6-C10 dicarboxylic acids[§]	1.7	1.3	1.2	0.5	1.5	0.0
Treatment (sample number)	Untr (s = 2)	Tr (s = 2)	Untr (s = 7)	Tr (s = 8)	Untr (s = 3)	Tr (s = 2)
Urine Acylcarnitines[†,‡]						
Free carnitine	2.0	2.0	1.3	2.3	1.3	1.5
Butyryl-/isobutyryl carnitine	3.0	3.0	2.6	3.0	1.7	1.5
Isovalerylcarnitine	3.0	3.0	2.1	1.8	1.7	1.5
Glutarylcarnitine	3.0	3.0	3.0	2.9	3.0	3.0
Hexanoylcarnitine	2.0	3.0	1.6	0.6	0.7	0.5
Octanoylcarnitine	2.0	3.0	2.3	1.6	0.7	1.5
Decanoylcarnitine	2.5	2.5	2.6	0.6	1.0	0.5
Treatment (sample number)	Untr (s = 2)	Tr (s = 2)	Untr (s = 7)	Tr (s = 8)	Untr (s = 3)	Tr (s = 2)
Urine Amino acids[†]						
Sarcosine	3.0	3.0	2.9	0.9	2.7	0.0
4-Hydroxyproline	3.0	1.5	0.4	0.0	1.0	0.0
Proline	3.0	3.0	0.9	1.0	1.0	1.0

[§] The average weighted mean of adipic acid (C6), suberic acid (C8), and sebacic acid (C10) is given

[†] Acylcarnitine and amino acid assessments are typically conducted on plasma/serum, but this matrix was limited due to sample stability (collection ranged over a 30-year period)

[‡] Increased free carnitine is beneficial and promotes fatty acid conjugation and release. Likewise, elevated acylcarnitine conjugates indicate sufficient detoxification

Values represent the number of times a metabolite is increased above the reference range, as a weighted mean of each group (≥ 3: severely elevated; ≥ 2: elevated; > 1: slightly elevated; ≤ 1: normal). Metabolites due to secondary consequences are not reported. Red, yellow, and green: high, moderate, and low values, respectively

Abbreviations: *n* Number of patients, *Rb* riboflavin, *s* Number of available samples, *Tr* Treated; *Untr* Untreated

the absence of any reported congenital features (no post-mortem examination) and the high MADD-DS3 score of 24, a diagnosis of MADD type I/II was given. Whole exome sequencing (WES) and segregation analysis revealed that the patient was compound heterozygous for c.[976G > C];c.[1067G > A] (p.[Gly326Arg];p.[Gly356Glu]). Variant c.[976G > C] is classified as a likely pathogenic variant of unknown significance according to the ACMG criteria and, to our knowledge, has been reported to occur in only one late-onset case of Chinese ethnicity by Xi et al. [13] as compound heterozygous with the common variant, c.[250G > A] (p.[Ala84Thr]; ACMG classification: pathogenic). By contrast, the c.[1067G > A] variant has only been encountered in the SA population to date [10].

Considering the treatment-unresponsive metabolic profile (P6 and P12) and rapid clinical deterioration of patients P6, P8 and P12, it may be inferred that c.[1067G > A] is a highly pathogenic variant. Its presence on both alleles, or its bi-allelic combination with another variant affecting the same protein domain (ubiquinone-binding domain) of ETFQO, appears to lead to insufficient enzymatic compensation for adequate ETFQO activity. While it is evident that L-carnitine supplementation facilitated the formation of disease specific acylcarnitine conjugates, we hypothesise that the homozygous c.[1067G > A];c.[1067G > A] and compound heterozygous c.[976G > C];c.[1067G > A] genotypes result in an ETFQO protein of which the folding cannot be sufficiently rescued/stabilised by Rb treatment.

Moderate, variably Rb-responsive MADD patient group

The second group included eight patients who presented with moderate, heterogeneous phenotypes, all showing a varying response to treatment. The onset of symptoms was observed in the neonatal period (P1),

Table 3 *ETFDH* variants identified in the SA MADD cohort

Variant GRCh38.p13	Exon	Protein GRCh38.p13	Protein domain	rsID	ACMG classification	Allele frequencies (%)			
						gnomAD[†]		H3Africa	This study
c.[287dupA*]	3/13 (ENSE00003628977)	p.[Asp97Glyfs*24]	FAD-binding	–	Likely pathogenic	AF	–	–	–
						E	–	–	–
						AS + I	–	–	–
						M	–	–	–
c.[740G > T]	7/13 (ENSE00001126657)	p.[Gly247Val]	FAD-binding	rs1384574225	Likely pathogenic	AF	0	–	–
						E	0	–	–
						AS + I	0	–	–
						M	0	–	–
c.[976G > C]	9/13 (ENSE00001126640)	p.[Gly326Arg]	Ubiquinone-binding	–	Uncertain significance	AF	–	–	–
						E	–	–	–
						AS + I	–	–	–
						M	–	–	–
c.[1067G > A]	9/13 (ENSE00001126640)	p.[Gly356Glu]	Ubiquinone-binding	–	Likely pathogenic	AF	–	–	< 0.00067
						E	–	–	< 0.00067
						AS + I	–	–	< 0.00067
						M	–	–	< 0.00084
c.[1448C > T]	11/13 (ENSE00003510567)	p.[Pro483Leu]	Carboxy-terminal	rs377656387	Pathogenic	AF	0	–	< 0.00067
						E	0.00004	–	< 0.00067
						AS + I	0.00003	–	< 0.00067
						M	0	–	< 0.00084

[†] Ethnicities are used as reported on gnomAD: AF: African; AS + I: Asian and Indian; E: European non-Finnish; M: mixed ethnicity

Abbreviations: *ACMG* American College of Medical Genetics and Genomics, *ETFDH* electron transfer flavoprotein-ubiquinone oxidoreductase gene (NM_004453.4), *FAD* flavin adenine dinucleotide, *GRCh* Genome Reference Consortium Human Build, *H3Africa* Human Heredity and Health in Africa, *rsID* reference SNP cluster ID

infancy (P2, P7, P9 and P10), as well as childhood (P3, P11 and P13), and all patients displayed the characteristic clinical features of MADD. These included metabolic decompensation (n = 5), muscle weakness (n = 4), muscle pain (n = 3), hypotonia (n = 5), neck flexor weakness (n = 5), susceptibility to fatigue (n = 2), restrictive ventilatory defect (n = 1), gastrointestinal involvement (n = 6), elevated creatine kinase (CK) (n = 2), recurrent infections (n = 2), lethargy (n = 2), cognitive disability (n = 2), delayed gross motor development (n = 2), migraine/paroxysmal headache (n = 3), seizures (n = 2), coma (n = 3), skeletal involvement (n = 2), and liver dysfunction (n = 4). Uncommon symptoms included ketosis at the time of metabolic crisis (n = 5) and Beevor's sign (n = 1). Owing to the availability of data, the baseline urine organic acids of only five of the eight patients are reported, and the data of P7 represent the urinary organic acids present upon considerable decompensation near the time of demise.[1] At first presentation, patients P1, P7 and P9–P11 displayed an increase (to a variable extent) in the diagnostic urine organic acid markers associated with MADD, albeit less pronounced than that of P6, P8 and P12. Most of these patients had increased concentrations of urinary glutaric acid, ethylmalonic acid, dicarboxylic acids, and 2-hydroxyglutaric acid. The excretion of *N*-hexanoylglycine and, to a variable extent, branched-chain-related glycine conjugates were mostly observed. Moreover, all eight patients displayed increased disease-associated urinary acylcarnitines and elevated sarcosine. The biomarker assessment correlated with previous observations in moderate cases where FAO seems to be initially/mostly affected and branched-chain amino acid catabolism is influenced to a lesser extent [6]. It is important to note that the metabolic profiling was greatly dependent on the time of sample collection and that P3 and P9 had received L-carnitine treatment from an early stage in their lives due to the prior diagnosis of a sibling with MADD. Apart from P7, the clinical symptoms, together with most of the urine metabolites, improved upon dietary adjustment in combination with treatment with L-carnitine (P9 and P10), L-carnitine and Rb (P2, P3,

[1] P2 and P3 (siblings) were diagnosed prior to 1995 (before the storage of electronic records at the contributing diagnostic facility) and did not decompensate following the onset of treatment. Although P7 was diagnosed at the same time, the patient decompensated considerably near the time of her demise. The urinary organic acid data collected during this period of decompensation, while the patient was on Rb and L-carnitine, are reported.

P11 and P13), or L-carnitine, Rb, and coenzyme Q10 (P1). Carnitine conjugation indicated that accumulating acyl-CoAs were being detoxified via the carnitine transportation system, which likely explains the less prominent MADD organic acid signature observed. By contrast, the metabolic response to treatment of P7—who succumbed to a stroke at the age of 23 years—was more comparable to that of severe MADD, a finding that correlated with the severity of the clinical presentation as summarised in Table 1 and Additional file 1.

WES and segregation analysis by Sanger sequencing revealed four compound heterozygous variants in this group of White SA-ethnicity patients. These included: (i) c.[740G > T];c.[1448C > T] (p.[Gly247Val];p.[Pro483Leu]) in P13, (ii) c.[287dupA*];c.[1448C > T] (p.[Asp97Glyfs*24];p.[Pro483Leu]) in siblings P2 and P3, and (iii) c.[1067G > A];c.[1448C > T] (p.[Gly356Glu];p.[Pro483Leu]) in P1, P7, P9, P10, and P11. The novel c.[287dupA*] variant affects the third exon of *ETFDH*, leading to a premature stop codon, and is classified as likely pathogenic according to the ACMG criteria. The c.[740G > T] variant shares the same classification as c.[287dupA*], and has been reported only once before as a compound heterozygous variant along with the likely pathogenic c.[389A > T] (p.[Asp130Val]) variant in a late-onset MADD case of Chinese ethnicity [14]. All variants identified in this group encode for highly conserved amino acids.

Once again, the MADD-DS3 scores confirmed that the disease burden increases when the c.[1067G > A] variant is present. This finding is corroborated by the level of steady state ETFQO protein in skin fibroblasts of P5 (c.[1448C > T];c.[1448C > T]) and P9 (c.[1067G > A];c.[1448C > T]), which show a 55% and 73% decrease, respectively, in comparison to fibroblasts from healthy controls (Additional file 3). Similarly, van der Westhuizen et al. [10] reported an 83% decrease in the steady state level of ETFQO in muscle from a patient with a compound heterozygous c.[1067G > A];c.[1448C > T] genotype, when compared to a healthy control. By contrast to the severe, Rb-unresponsive MADD patient group, four of the five patients affected by the c.[1067G > A] variant (P9, P10, P11 and P1) in this group were found to be very amenable to treatment. It is, therefore, reasonable to conclude that when c.[1067G > A] is encountered as a compound heterozygous variant along with a variant which affects a different protein domain of ETFQO (e.g., c.[1448C > T]), sufficient enzymatic compensation occurs which may allow for adequate ETFQO activity. Based on the onset of disease, a diagnosis of either MADD type II (P1) or type III (P2, P3, P7, P9, P10, P11 and P14) was given to the patients in this group.

Mild, Rb-responsive MADD patient group

The final group included three patients (P4, P5 and P14) of White SA ethnicity, who presented later in life with mild and non-progressive (treatment-related) phenotypes. Clinically, their symptoms were heterogenous, but to some extent characteristic of MADD. The disease presentation included metabolic decompensation (n = 2), muscle weakness (n = 3), neck flexor weakness (n = 2), gastrointestinal involvement as the disease progressed (n = 1), elevated CK (n = 2), lethargy (n = 1), encephalopathy (n = 1), cerebral white matter abnormalities (n = 1), and liver dysfunction (n = 1), with two patients exhibiting ketosis. Initially, statin-induced myositis was suspected in P5 until hepatic features, including lipid deposits on the liver biopsy and raised transaminases prompted a metabolic work-up. P5 and P14 displayed urine metabolites associated with MADD, including dicarboxylic aciduria, raised 2-hydroxyglutaric acid (P5), acylglycine conjugates (with less prominent branched-chain-related conjugates), short- and medium-chain acylcarnitines as well as the presence of sarcosine on the amino acid profile. As indicated earlier in the moderate MADD group, FAO tends to be more affected compared to the branched-chain amino acid catabolism in the less severe cases [6], which correlated with our findings. Literature shows that plasma/serum acylcarnitine profiling is typically most informative when diagnosing a late-onset MADD case, as organic acid profiling may only be remarkable at the time of a metabolic crisis or catabolic status induced by fasting. However, acylcarnitine assessments have been inconclusive in some cases, particularly if the patient has insufficient free carnitine available to promote conjugation [5, 12]. The latter has also led to false negative NBS results in mild MADD cases, as reported by Lin et al. [15]. In our study, P14 showed a free carnitine concentration below the limit of detection and normal butyryl/isobutyryl-, isovaleryl- and glutarylcarnitine levels in plasma/serum (results not shown). Interestingly, this patient presented with severe ketosis, as well as a prominent increase in 2-hydroxyglutaric acid and *cis*-4-decenedioic acid with unremarkable increases in glutaric acid and ethylmalonic acid. These inconsistent urinary organic acid findings corroborate the observations of Goodman et al. [16] and support their suggestion to discontinue the use of the term "Glutaric aciduria type II," as it may be diagnostically misleading. In addition, our data indicate that mild cases of MADD may be missed or incorrectly diagnosed (e.g., as a different fatty acid oxidation disorder) on a biochemical level, depending on the metabolic (i.e., anabolic/catabolic status due to the time of sample collection) and systemic free carnitine status of the patient. Consequently, this study emphasises the metabolite variation within this disease group and

advocates for repeat testing if the clinical presentation and routine chemistry are suggestive of MADD.

Following dietary adjustment and treatment with L-carnitine, Rb and coenzyme Q10 (P5 and P14), the clinical and biochemical aberrations of P14 essentially normalised. P4, however, presented with only increased ethylmalonic acid. This patient, the mother of P2 and P3, underwent metabolic testing following the children's diagnosis and was on L-carnitine treatment at the time of sample collection. Clinical-biochemical improvements, specifically after therapeutic intervention, have been observed in several cases of late-onset MADD, and our clinical and metabolic results (although only three cases were included) strongly correlate with previous investigations [5, 17].

Genetic analyses revealed that all three patients had a homozygous genotype for the known pathogenic c.[1448C>T] (p.[Pro483Leu]) variant. This genotype (c.[1448C>T]; c.[1448C>T]) has been reported in numerous other cases that, similarly to this group, presented with adult-onset, Rb-responsive MADD [10, 17]. The Rb-responsive nature of this variant is further corroborated in a study by Cornelius et al. [18], in which it was shown that ETFQO activity in c.[1448C>T]-modified HEK293 cells could be restored from ~45 to ~85% (corresponding to an increase of ~50 to ~80% steady-state ETFQO) when moderately Rb-deficient cultures were treated with a saturating concentration of Rb. Therefore, considering the literature, together with the time of onset, severity and treatment response of this group, a diagnosis of MADD type III was given to P4, P5 and P14, despite moderate MADD-DS3 scores.

Allele frequency spectrum and haplotypes

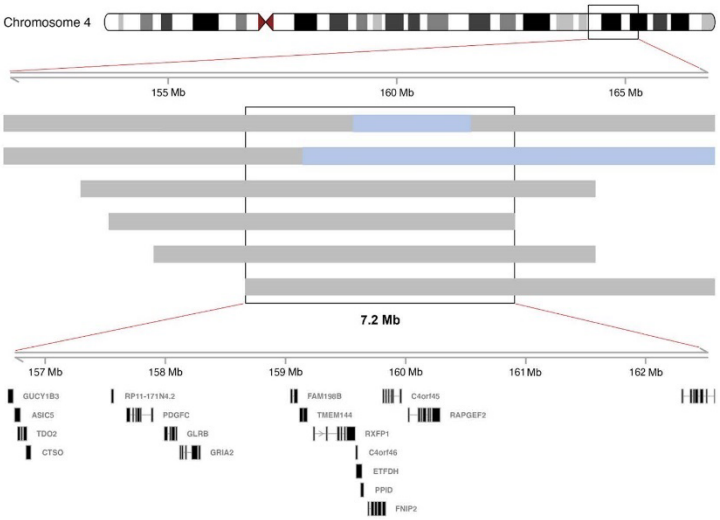
To determine the allele frequency of the variants identified, PCR–RFLP analysis was used to screen the two most frequently occurring variants, c.[1067G>A] and c.[1448C>T], in the four largest population groups in SA. The study yielded no homozygous or heterozygous genotypes for any of the variants in any of the population groups assessed so that the allele frequencies of the population groups investigated were calculated as < 0.00067% (African, White SA, and Indian ethnicity) or < 0.00084% (mixed ethnicity) (Table 3). All five variants identified in the cohort were subsequently compared to gnomAD (v2.1.1) [19] and the Human Heredity and Health in Africa (H3Africa) project [20]. Of these variants, only two were recorded on gnomAD, displaying exome allele frequencies of < 0.0001% (c.[740G>T] and c.[1448C>T]); hitherto, none of the variants has been identified in the H3Africa data. The absence of the variants in the above-mentioned population databases, together with the frequency at which they were identified in the SA cohort, indicates with high probability the pathogenicity of these five variants and supports their causative role in MADD.

Haplotyping was performed on those patients from whom sufficient DNA could be obtained. Upon recruitment, patients and their families were invited to self-report their ethnicity and region of birth. Consequently, haplotyping was performed on ten patients of White SA ethnicity (P1–P6, P9–P11 and P13) and one patient of mixed ethnicity (P8) born in various geographical regions across SA (including the central, north-eastern, north-western, south-eastern, and south-western provinces).

Apart from the siblings, P2 and P3, and their mother, P4, all patients were found to be unrelated down to the second degree. DNA samples from those patients harbouring the c.[1067G>A] variant (P1, P6, P8 and P9–P11), displayed variable lengths of a shared haplotype on one allele, with a minimal overlapping region of 7.2 Mb (Fig. 1). Of these samples, the two with a homozygous c.[1067G>A];c.[1067G>A] genotype (P6 and P8) exhibited homozygosity for the shared haplotype in the region of *ETFDH*. Similar results were obtained for the DNA samples of those patients harbouring the c.[1448C>T] variant [P1, P4 (including P2 and P3 due to their first-degree relation), P5, P9–P11 and P13], with a minimal overlapping region of 4 Mb (Fig. 2). Again, the two patients who had a homozygous c.[1448C>T];c.[1448C>T] genotype (P4 and P5), displayed homozygosity for the shared haplotype in the *ETFDH* region. These findings suggest the presence of two separate founder haplotypes on which the c.[1067G>A] and c.[1448C>T] variants arose, with that of the former in the White SA population. However, without access to additional control data from the same population(s), it is currently not possible to estimate the haplotype frequency with confidence.

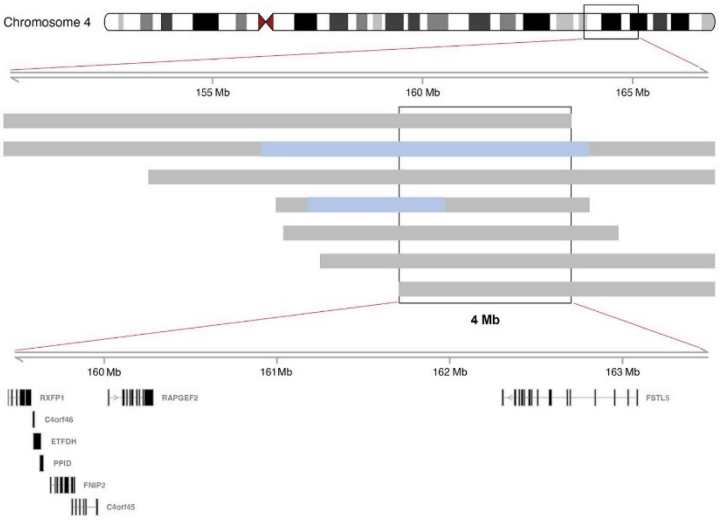
Conclusion

This study provides the first extensive clinical and biochemical profiles, along with the genetic aetiology, of MADD in the diverse and understudied SA population. Within the cohort investigated, we describe one novel (c.[287dupA*]) and four previously identified causal variants in *ETFDH*, with an autosomal recessive inheritance pattern. In addition, the data support the suspicion that MADD is more prevalent in the White population of SA [10]. We also demonstrate a distinct shared haplotype for each of the two most common variants (c.[1067G>A] and c.[1448C>T]), suggesting the existence of a founder for each, with that of c.[1067G>A] likely having arisen in the White SA population. Furthermore, we show that, depending on the variant(s) involved, it is possible to anticipate the clinical progression and treatment response of the patient, which underscores the need



Chromosome position			
Start	End	Patient	GT
151399246	166969645	P8	+/+
151399246	166969645	P6	+/+
153091323	164340366	P1	+/-
153704812	162577520	P10	+/-
154688047	164340366	P11	+/-
156690837	166969645	P9	+/-

Fig. 1 Shared haplotype of patients affected by *ETFDH* c.[1067G > A]. All samples listed show variable lengths of a shared haplotype on a single allele (grey) with a minimal overlapping region of 7.2 Mb, and samples P6 and P8 exhibit homozygosity (c.[1067G > A];c.[1067G > A]) for the shared haplotype (blue). The shared region includes the genes listed. These results indicate a founder for the c.[1067G > A] variant in the White SA population. Abbreviations: *ETFDH* electron transfer flavoprotein-ubiquinone oxidoreductase gene, *GT* genotype



Chromosome position			
Start	End	Patient	GT
150026731	163537561	P10	+/-
150026731	166969645	P4	+/+
153480419	166969645	P12	+/-
156511179	163967165	P5	+/+
156690837	164656903	P11	+/-
157568858	166969645	P1	+/-
159443231	166969645	P9	+/-

Fig. 2 Shared haplotype of patients affected by *ETFDH* c.[1448C > T]. All samples listed show variable lengths of a shared haplotype on a single allele (grey) with a minimal overlapping region of 4 Mb, and samples P4 and P5 exhibit homozygosity (c.[1448C > T];c.[1448C > T]) for the shared haplotype (blue). The shared region includes the genes listed. These results indicate a founder for the c.[1448C > T] variant. Abbreviations: *ETFDH* electron transfer flavoprotein-ubiquinone oxidoreductase gene, *GT* genotype

for genetic confirmation in SA. It is our belief, that this genotype–phenotype correlation of MADD in the SA population will assist physicians—who rarely encounter this disorder—to recognise and treat MADD in a more efficient manner. In addition, our study provides background for the subsequent genetic counselling of families and patient-specific treatment of local MADD cases. Altogether, the data reported support a policy of including MADD in the NBS program of SA.

Methods

Cohort selection and sampling

Following informed consent/assent, the patients in this study were enrolled as part of the ICGNMD study with ethical approval numbers 19/LO/1796 (HRA and HCRW), NWU-00966-19-A1 and NWU-00966-19-A1-01 (NWU), 296/2019 (University of Pretoria; UP), B19/01/002 (Stellenbosch University; SU), and 605/2020 (UCT).

Probands and their affected/unaffected first-degree relatives were recruited either retrospectively or prospectively, based on a clinical and metabolic diagnosis of MADD, via one of three SA academic, state-funded hospitals: Steve Biko Academic Hospital, Tygerberg Hospital, and Red Cross War Memorial Children's Hospital. For (i) genetic, (ii) protein, and (iii) metabolic analyses, the following samples were obtained: (i) whole blood, saliva (living patients), or urine (deceased patients from whom no blood or saliva was available), (ii) primary skin fibroblasts (P5 and P9), as well as (iii) urine collected during the first metabolic presentation (P1, P4–P6, P8–P12 and P14)/during metabolic decompensation (P7), and after therapeutic management (P1–P11, P13 and P14).

Clinical and biochemical investigations

Patients were extensively evaluated by paediatric or adult neurologists according to protocols set forth by the ICGNMD. This included obtaining relevant demographic information, family history, medical history, and current treatment (Table 1). Moreover, deep phenotypic data (Additional file 1) were collected during a comprehensive clinical re-assessment of each proband.

SDS-PAGE and Western blot analysis of primary skin fibroblasts, established from two adult patients (P5 and P9) and two healthy controls (matched in age, gender, and ethnicity to the patients), were performed to evaluate the steady state level of ETFQO. Immunoblotting was conducted using primary antibodies at 1:1,000 directed against ETFQO (ab131376; Abcam, Cambridge, UK) and β-actin (as housekeeping gene; ab6276; Abcam), and an HRP-conjugated secondary antibody at 1:10,000 (ab97023; Abcam). Findings are shown in Additional file 3.

For targeted metabolic analyses, urine organic acids and their glycine conjugates were extracted, derivatised, and analysed using the 7890A gas chromatography system coupled to the 5977A MSD mass spectrometer (Agilent Technologies, California, USA) as described by Erasmus et al. [21] and refined by Reinecke et al. [22]. Data acquisition was facilitated with the 5977 MassHunter Data Acquisition software (B.07.04.2260; Agilent Software); Automated Mass spectral Deconvolution and Identification System software (AMDIS v2.73; National Institute for Standards and Technology) was used to identify the component peaks and perform spectral deconvolution. Underivatised amino acids in urine were analysed using validated liquid chromatography-triple quadrupole mass spectrometry and the MassChrom® Amino Acid Analysis kit (75111; Chromsystems, Gräfelfing, Germany). Samples were prepared via the Microlab STAR Liquid Handling System (Hamilton, Nevada, USA) and analysed on the Infinity 1290 II liquid chromatography system coupled to the 6470 Triple Quadrupole liquid chromatography-mass spectrometer (Agilent Technologies). For urine acylcarnitine analysis, electrospray ionisation-tandem mass spectrometry was performed using the 1290 Infinity II liquid chromatography system coupled to the 6410 Triple Quadrupole liquid chromatography-mass spectrometer (Agilent Technologies) as described by Pitt et al. [23]. A standard mixture of stable acylcarnitine isotopes was used for quantification [24]. Amino acid and acylcarnitine data were acquired and quantified using MassHunter Data Acquisition software and MassHunter QQQ Quantitative software (v10.00; Agilent Software), respectively. All reported processed data are shown in Additional file 2.

Genetic analyses

To identify potential pathogenic variants in the proband DNA, WES was performed at Macrogen Europe (Amsterdam, the Netherlands) using the NovaSeq 6000 HiSeq platform (Illumina Inc., San Diego, USA) and the SureSelect Human All Exon V6 panel (5190-8865; Agilent Technologies). Reads (50×average depth coverage) were aligned to the Genome Reference Consortium Human Build (GRCh) 38.p13, and variants were annotated according to GRCh38 using the Ensembl Variant Effect Predictor (v108) [25]. High-quality variants were filtered by applying the Genomics' England PanelApp, "Rhabdomyolysis and metabolic muscle disorders_1.57" panel [26]; the pathogenicity of potential disease-causing variants was evaluated and classified as such, according to the ACMG guidelines [11]. All potential pathogenic variants identified were confirmed and segregation analysis in family members was conducted via Sanger sequencing.

Allele frequency and haplotyping

To estimate the allele (carrier) frequency of the two most encountered variants in the cohort (c.[1067G>A] and c.[1448C>T]), PCR–RFLP analysis was performed as previously described [10] on newborn dried blood spot (DBS) samples of the four largest SA population groups—African, White SA, Indian, and mixed ethnicity. To this end, a total of 2,844 anonymised and randomised DBS samples per variant per population group were used: 594 representatives of mixed ethnicity as well as 750 representatives of each of the following populations: African, White SA and Indian ethnicity. The samples represented an equal distribution of healthy males and females and were kindly provided by the NBS laboratory at the NWU Centre for Human Metabolomics.

Next, we investigated whether these two variants arose due to founder mutations by determining the haplotype(s) of the affected individuals using a GSA v3.0 array (Illumina Inc.). Sample processing was performed at UCL Genomics (UCL Great Ormond Street Institute of Child Health, London, UK), according to the manufacturer's instructions; the resulting raw IDAT files were processed using Genome Studio (v2.0.5; Illumina Inc.) and converted to PLINK (v1.9) format [27]. Samples and variants with a call rate below 90% were excluded from further analysis. Thereafter, samples were compared to determine familial relations and phased using Eagle (v2.4.1) [28]. Finally, haplotype sharing between each sample was determined with Germline2 software (v1.0) [29] and shared regions were visualised using R (v4.2.2).

Abbreviations

ACMG	American College of Medical Genetics and Genomics
AMDIS	Automated Mass spectral Deconvolution and Identification System
CK	Creatine kinase
CoA	Coenzyme A
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
EC	Enzyme Commission number
ETF	Electron transfer flavoprotein
ETFA	Electron transfer flavoprotein subunit alpha (gene)
ETFB	Electron transfer flavoprotein subunit beta (gene)
ETFDH	Electron transfer flavoprotein-ubiquinone oxidoreductase (gene)
ETFQO	Electron transfer flavoprotein-ubiquinone oxidoreductase
$FADH_2$	Flavin adenine dinucleotide (reduced)
FAO	Mitochondrial fatty acid β-oxidation
GAII	Glutaric aciduria type II
gnomAD	Genome Aggregation Database
GRCh	Genome Reference Consortium Human Build
H3Africa	Human Heredity & Health in Africa
HCRW	Health and Care Research Wales
HEK293	Human embryonic kidney 293 cell line
HRA	Health Research Authority
HRP	Horse radish peroxidase
ICGNMD	International Centre for Genomic Medicine in Neuromuscular Diseases
MADD	Multiple acyl-CoA dehydrogenase deficiency
MADD-DS3	Multiple acyl-CoA dehydrogenase deficiency-disease severity 3
Mb	Megabase(s)
MPV17	Mitochondrial inner membrane protein MPV17
MRC	Medical Research Council
n	Number of patients
NBS	Newborn screening
NHLS	National Health Laboratory Service
NHS	National Health Service
NIHR	National Institute for Health and Care Research
NRF	National Research Foundation
NWU	North-West University
NWU-HREC	North-West University-Health Research Ethics Committee
P and number	Patient
PCR-RFLP	Polymerase chain reaction-restriction fragment length
Rb	Riboflavin
SA	South Africa(n)
SAMRC	South African Medical Research Council
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SU	Stellenbosch University
UCL	University College London
UCT	University of Cape Town
UK	United Kingdom
UP	University of Pretoria
USA	United States of America
WES	Whole exome sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13023-023-03014-8.

Additional file 1. Additional Clinical Information.

Additional file 2. Additional Metabolic Information.

Additional file 3. Additional Structural Information.

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Author contributions

MB: Conceptualisation, Design, Data Acquisition, Data Analysis, Data Interpretation, Article Drafting, Article Revision; IS: Conceptualisation, Design, Data Acquisition, Data Analysis, Data Interpretation, Article Drafting, Article Revision; MD: Data Acquisition, Data Analysis, Data Interpretation, Article Drafting, Article Revision; MS: Data Acquisition, Data Analysis, Data Interpretation, Article Drafting, Article Revision; BCV: Data Acquisition, Data Analysis; GvdW: Data Acquisition, Data Analysis, Data Interpretation; CS: Data Acquisition, Data Analysis, Data Interpretation; KN: Data Acquisition, Data Analysis, Data Interpretation; FH: Data Acquisition, Data Analysis, Data Interpretation; SM: Data Acquisition, Data Analysis, Data Interpretation; RM: Data Interpretation; RWT: Data Interpretation; KP: Data Acquisition, Data Analysis, Data Interpretation; MRF: Data Acquisition, Data Analysis, Data Interpretation; JV: Data Acquisition, Data Analysis, Data Interpretation, Article Drafting;The ICGNMD Consortium: Data Acquisition, Data Analysis, Data Interpretation, Article Revision; RJAW: Design, Data Interpretation, Article Drafting, Article Revision; FHvdW: Conceptualisation, Design, Data Acquisition, Data Analysis, Data Interpretation, Article Drafting, Article Revision. The submitted work has been approved by all contributing authors.

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Availability of data and materials
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Declarations

Ethics approval and consent to participate
The patients in this study were enrolled as part of the ICGNMD study with ethical approval numbers 19/LO/1796 [Health Research Authority (HRA) and Health and Care Research Wales (HCRW)], NWU-00966-19-A1 and NWU-00966-19-A1-01 [Health Research Ethics Committee (NWU-HREC) of the Faculty of Health Sciences, North-West University], 296/2019 (Faculty of Health Sciences Research Ethics Committee, University of Pretoria), B19/01/002 (Health Research Ethics Committee, Stellenbosch University), and 605/2020 (Faculty of Health Sciences Human Research Ethics Committee, University of Cape Town), following informed consent/assent.

Consent for publication
The informed consent/assent accounts for the publication of our research data are available upon request.

Competing interests
The authors declare that they have no competing interests.

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B.2 SUPPLEMENTARY MATERIAL 1: ADDITIONAL CLINICAL INFORMATION

Table B.1: MADD-DS3 domains

Group	Rb-unresponsive (severe phenotype; n = 3)			Variably Rb-responsive (moderate phenotype; n = 8)								Rb-responsive (mild phenotype; n = 3)		
Patient	**P12**	**P8**	**P6**	**P13**	**P2**	**P3**	**P9**	**P10**	**P11**	**P1**	**P7**	**P4**	**P14**	**P5**
MADD-DS3 domains														
Age at onset	3	3	3	0	0	0	0	0	0	3	0	0	0	0
Congenital anomalies	0	0	6	0	0	0	0	0	0	0	0	0	0	0
Cardiac	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CNS	0	0	0	0	0	0	2.25	0	0	2.25	4.5	0	0	0
PNS	0	0	0	0	0	0	0	0	0	0	0	1.5	0	0
Liver	6	6	6	0	0	3	1.5	6	0	4.5	6	0	4.5	6
Muscle	6	3	6	3	3	3	3	0	3	1.2	3	4	3	2.4
Patient-reported outcome	9	9	9	0	0	0	0	3	6	3	9	0	0	3
Total MADD-DS3 score	**24**	**21**	**30**	**3**	**3**	**6**	**6.75**	**9**	**9**	**13.95**	**22.5**	**5.5**	**7.5**	**11.4**

Abbreviations: CNS: central nervous system; MADD-DS3: multiple acyl-CoA dehydrogenase deficiency-disease severity 3 (van Rijt *et al.,* 2019); n: number of patients; PNS: peripheral nervous system; Rb: riboflavin.

Table B.2: Clinical symptoms applicable to MADD-DS3 domains

Group	Rb-unresponsive (severe phenotype; n = 3)			Variably Rb-responsive (moderate phenotype; n = 8)								Rb-responsive (mild phenotype; n = 3)		
Patient	**P12**	**P8**	**P6**	**P13**	**P2**	**P3**	**P9**	**P10**	**P11**	**P1**	**P7**	**P4**	**P14**	**P5**
MADD-DS3 domains														
Congenital anomalies														
Hydronephrosis	U	U	+	–	–	–	–	–	–	–	–	–	–	–
Cardiac														
Patent foramen ovale	U	U	+	–	–	–	–	–	–	–	–	–	–	–
Pulmonary valve stenosis	U	U	+	–	–	–	–	–	–	–	–	–	–	–
CNS														
Epilepsia partialis continua (resolved)	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Delayed gross motor development	–	–	–	–	+	–	–	–	–	–	+	–	–	–
Cerebral white matter abnormality	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Migraine/paroxysmal headache	–	–	–	–	+	–	–	–	–	+	+	–	–	–
Learning disabilities	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Intellectual disabilities	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Encephalopathy	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Seizures	–	–	+	–	–	+	–	–	–	–	+	–	–	–
Drooling	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Stroke	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Coma	–	–	–	–	–	–	–	+	+	–	+	–	–	–
Lethargy	–	–	–	–	+	+	–	–	–	–	–	+	–	–
Liver														
Increased liver enzymes	–	–	+	–	–	–	–	+	–	–	+	–	–	–
Acute hepatic failure	–	–	–	–	–	–	–	–	–	–	+	–	–	+
Hepatic steatosis	–	–	–	–	–	–	–	–	–	+	+	–	–	+
Hepatomegaly	–	–	–	–	+	–	–	+	–	–	+	–	–	–
Hepatosplenomegaly	–	–	–	–	–	–	–	–	–	–	–	–	–	+

Table B.2: *Continued*

Group	Rb-unresponsive (severe phenotype; n = 3)			Variably Rb-responsive (moderate phenotype; n = 8)								Rb-responsive (mild phenotype; n = 3)		
Patient	**P12**	**P8**	**P6**	**P13**	**P2**	**P3**	**P9**	**P10**	**P11**	**P1**	**P7**	**P4**	**P14**	**P5**
MADD-DS3 domains														
Neuromuscular														
Beevor's sign	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Elevated CK	–	–	–	–	–	–	–	–	+	–	+	+	–	+
Facial weakness	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Neck muscle weakness	–	–	–	+	+	+	–	–	+	–	+	–	+	+
Susceptibility to fatigue	–	–	–	–	+	+	–	–	–	–	–	–	–	–
DM weakness upper limbs	–	–	–	–	+	–	–	–	–	–	–	+	–	–
DM weakness lower limbs	–	–	–	+	+	–	–	–	–	–	–	–	–	–
PM weakness upper limbs	–	–	–	+	+	–	–	–	–	–	–	+	+	–
PM weakness lower limbs	–	–	–	+	+	–	–	–	–	–	–	+	+	–
Weakness	–	–	–	–	+	–	–	–	–	–	+	+	–	+
Hypotonia	–	+	–	–	+	+	+	–	–	+	+	–	–	–
Myopathy	–	–	–	–	+	–	–	–	–	+	+	+	–	+
Pain/myalgia	–	–	–	–	–	+	–	+	–	+	–	–	–	–

Abbreviations: +: reported; CK: creatine kinase; CNS: central nervous system; DM: distal muscle; MADD-DS3: multiple acyl-CoA dehydrogenase deficiency-disease severity 3 (van Rijt *et al.,* 2019); n: number of patients; PM: proximal muscle; Rb: riboflavin; U: unknown.

Table B.3: Additional clinical symptoms

Group	Rb-unresponsive (severe phenotype; n = 3)			Variably Rb-responsive (moderate phenotype; n = 8)								Rb-responsive (mild phenotype; n = 3)		
Patient	**P12**	**P8**	**P6**	**P13**	**P2**	**P3**	**P9**	**P10**	**P11**	**P1**	**P7**	**P4**	**P14**	**P5**
Metabolic														
Hyperammonaemia	+	+	+	–	–	–	–	–	–	–	+	–	+	+
Metabolic acidosis	+	+	+	–	–	–	+	–	–	+	–	–	–	–
Aminoaciduria	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Hypoglycaemia	+	–	+	–	–	–	–	+	+	+	+	–	+	+
Lactic acidosis	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Ketosis	–	–	–	–	+	+	+	+	–	–	+	–	+	+
Gastrointestinal														
Chronic constipation	–	–	–	–	–	–	–	+	–	+	–	–	–	–
Abdominal pain	–	–	–	–	+	+	–	–	–	–	–	–	–	–
Diarrhoea	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Vomiting	–	+	–	–	+	+	–	–	+	–	–	–	+	–
Kidney														
Acute kidney injury	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Skeletal														
Lumbar hyperlordosis	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Scapular winging	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Osteopenia	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Other														
Intra-uterine growth restriction	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Restrictive ventilatory defect	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Obstructive sleep apnoea	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Recurrent infections	–	–	–	–	–	–	–	+	+	–	–	–	–	–
New-onset pancytopaenia	+	–	+	–	–	–	–	–	–	–	–	–	–	–
Astigmatism	–	–	–	–	–	–	–	+	–	–	–	–	–	–

Abbreviations: +: reported; n: number of patients; Rb: riboflavin.

B.3 SUPPLEMENTARY MATERIAL 2: ADDITIONAL METABOLIC INFORMATION

Table B.4: Urine organic acids

Status	Patient	Gender	Age	Upper reference value†	EMA	GA	2-MBGly	HGly	iVGly	iBGly	2-HGA	c4DC8	DC6	DC8	DC10
-	-	M + F	-	1: Birth-M12	10.97	4.80	2.00	1.20	10.00	3.50	-	15.80	56.23	38.40	119.31
-	-	M + F	-	2: <Y5	24.69	8.91	2.00	1.20	10.00	3.50	-	6.17	60.34	19.20	2.74
-	-	M + F	-	3: >Y5; <Y15	13.71	6.17	2.00	1.20	10.00	3.50	-	3.40	9.60	17.14	3.43
-	-	M + F	-	4: >Y15	6.86	5.49	2.00	2.00	10.00	3.80	-	0.00	61.71	5.49	-
-	-	M + F	-	5: Birth-M4	-	-	-	-	-	-	67.00	-	-	-	-
-	-	M + F	-	6: M4-Y2	-	-	-	-	-	-	49.00	-	-	-	-
-	-	M + F	-	7: Y2-Y10	-	-	-	-	-	-	35.00	-	-	-	-
-	-	M + F	-	8: >Y10	-	-	-	-	-	-	16.00	-	-	-	-
Untr	P1	M	M1 D14	1 + 5	36.61	36.93	1.24	29.92	0.96	-	117.53	8.32	93.16	63.19	38.71
Untr	P2	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Untr	P3	M	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Untr	P4	F	Y41 M9	4 + 8	26.62	2.37	0.47	1.18	1.34	-	50.80	1.35	21.61	1.29	-
Untr	P5	F	Y36 M6	4 + 8	154.35	675.58	30.02	43.05	151.77	30.52	312.03	47.77	400.34	-	3.03
Untr	P6	M	D3	1 + 5	200.27	1370.1	7.05	40.40	444.85	27.19	1611.9	51.59	677.00	21.06	7.69
Untr	P7	F	Y23 M5	4 + 8	0.16	86.49	0.36	10.63	4.76	16.93	54.31	21.29	126.97	1.58	1.42
Untr	P8	F	D6	1 + 5	-	2412.2	4.33	2.25	157.98	5.52	342.16	9.82	232.80	27.53	1.09
Untr	P9	M	M4	1 + 6	129.77	29.39	-	22.98	-	2.98	75.26	-	189.01	56.18	-
Untr	P10	F	Y7 M10	3 + 7	25.72	215.96	1.13	44.83	8.61	10.37	95.29	79.77	393.00	77.39	42.13
Untr	P11	M	Y2 M6	2 + 7	51.55	21.57	-	-	4.84	3.95	31.65	1.28	55.89	11.82	1.09
Untr	P12	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Untr	P13	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Untr	P14	F	Y26 M4	4 + 8	5.10	23.08	-	6.41	0.41	1.13	102.89	111.87	483.98	59.28	23.35

Table B.4: *Continued*

Status	Patient	Gender	Age	Upper reference value†	EMA	GA	2-MBGly	HGly	iVGly	iBGly	2-HGA	c4DC8	DC6	DC8	DC10
-	-	M + F	-	1: Birth-M12	10.97	4.80	2.00	1.20	10.00	3.50	-	15.80	56.23	38.40	119.31
-	-	M + F	-	2: <Y5	24.69	8.91	2.00	1.20	10.00	3.50	-	6.17	60.34	19.20	2.74
-	-	M + F	-	3: >Y5; <Y15	13.71	6.17	2.00	1.20	10.00	3.50	-	3.40	9.60	17.14	3.43
-	-	M + F	-	4: >Y15	6.86	5.49	2.00	2.00	10.00	3.80	-	0.00	61.71	5.49	-
-	-	M + F	-	5: Birth-M4	-	-	-	-	-	-	67.00	-	-	-	-
-	-	M + F	-	6: M4-Y2	-	-	-	-	-	-	49.00	-	-	-	-
-	-	M + F	-	7: Y2-Y10	-	-	-	-	-	-	35.00	-	-	-	-
-	-	M + F	-	8: >Y10	-	-	-	-	-	-	16.00	-	-	-	-
Tr	P1	M	Y11 M5	3 + 8	13.16	1.40	-	-	0.64	0.67	28.46	0.57	1.38	5.18	-
Tr	P2	F	Y29 M9	4 + 8	14.80	1.56	-	7.36	0.81	2.90	26.06	0.38	7.07	3.02	-
Tr	P3	M	Y25 M8	4 + 8	21.28	1.49	-	-	0.52	0.64	20.96	-	2.93	1.06	-
Tr	P4	F	Y45 M1	4 + 8	5.69	1.80	-	-	0.15	0.08	63.07	-	4.01	1.36	-
Tr	P5	F	Y40 M2	4 + 8	19.59	1.29	-	-	0.03	-	13.26	-	2.53	1.33	0.95
Tr	P6	M	D11	1 + 5	32.69	2915.5	-	-	62.67	2.10	1165.9	-	-	6.59	-
Tr	P7	F	Y22 M1	4 + 8	-	93.25	0.36	11.62	1.92	13.95	118.33	159.17	614.30	86.04	17.10
Tr	P8	F	D22	1 + 5	1053.4	2739.6	15.03	185.63	904.67	105.58	1441.2	21.48	615.20	128.52	342.49
Tr	P9	M	Y18 M9	4 + 8	40.01	8.91	0.25	33.57	2.60	7.10	58.26	17.10	87.39	24.09	7.35
Tr	P10	F	Y9	3 + 7	13.07	9.99	0.20	-	-	0.14	14.27	-	3.97	3.65	-
Tr	P11	M	Y8 M7	3 + 7	13.15	4.52	0.37	3.40	0.74	1.81	67.05	11.02	15.94	10.01	16.939
Tr	P12	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Tr	P13	F	Y41 M2	4 + 8	11.27	0.94	-	4.63	0.32	1.29	5.81	0.89	3.28	2.64	-
Tr	P14	F	Y26 M8	4 + 8	7.13	0.87	-	0.85	0.21	0.37	13.39	3.28	3.30	3.64	2.21

† Applied reference range is dependent on age. In-house reference ranges were used.

Values are given in mmol/mol creatinine. Abbreviations: 2-HGA: 2-hydroxyglutaric acid; 2-MBGly: 2-methylbutyrylglycine; c4DC8: *cis*-4-decenedioic acid; D and number: age in days; DC6: adipic acid; DC8: suberic acid; DC10: sebacic acid; EMA: ethylmalonic acid; F: female; GA: glutaric acid; HGly: *N*-hexanoylglycine; iBGly: *N*-isobutyrylglycine; iVGly: *N*-isovalerylglycine; M: male; M and number: age in months; n.d: not determined; Tr; treated; Untr: untreated; Y and number: age in years.

Table B.5: Urine acylcarnitines

Status	Patient	Gender	Age	Upper reference value†	C0	C4	C5	C5DC	C6	C8	C10
-	-	M + F	-	1: Newborn	4.89	0.84	0.38	0.63	0.23	0.2	0.1
-	-	M + F	-	2: Y1-Y5	13.93	0.19	0.25	0.04	0.13	0.15	0.08
-	-	M + F	-	3: Y6-Y17	4.74	0.77	0.29	0.46	0.11	0.15	0.09
-	-	M + F	-	4: ≥Y18	3.77	0.62	0.24	0.51	0.09	0.14	0.07
Untr	P1	M	Y1 M9	2	456.78	14.15	4.12	2.85	0.22	0.60	0.22
Untr	P2	F	Y1 M5	2	7.71	0.44	0.43	1.95	0.18	0.27	0.34
Untr	P3	M	D42	1	100.68	8.19	3.78	8.47	0.47	1.30	0.64
Untr	P4	F	Y41 M9	4	24.94	5.75	1.57	3.06	0.06	0.21	0.09
Untr	P5	F	Y36 M7	4	5.73	0.73	0.54	5.61	0.19	0.18	0.13
Untr	P6	M	D3	1	26.80	38.67	61.07	17.32	1.24	1.32	0.98
Untr	P7	F	Y3 M2	2	8.54	1.69	0.89	6.72	0.35	0.71	0.67
Untr	P8	F	9D	1	5.71	52.03	29.15	7.89	0.23	0.39	0.26
Untr	P9	M	Y1 M9	2	6.85	5.82	2.58	2.79	2.28	0.00	0.37
Untr	P10	F	Y7 M11	3	31.79	1.44	0.26	1.95	0.16	0.57	0.21
Untr	P11	M	Y2 M8	2	6.61	7.20	0.66	1.93	0.16	0.24	0.18
Untr	P12	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Untr	P13	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Untr	P14	F	Y26 M4	4	0.94	0.65	0.09	2.02	0.04	0.11	0.08
Tr	P1	M	Y11 M5	3	1.11	2.44	0.23	1.49	0.07	0.07	0.04
Tr	P2	F	Y29 M9	4	21.01	5.01	0.50	2.74	0.10	0.59	0.13
Tr	P3	M	Y25 M8	4	46.34	6.30	2.14	2.49	0.14	0.55	0.08
Tr	P4	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Tr	P5	F	Y40 M2	4	1.06	0.22	0.05	2.33	0.04	0.09	0.05
Tr	P6	M	D11	1	1202.20	623.42	2193.77	15.02	13.48	18.63	12.68
Tr	P7	F	Y13 M4	3	46.21	4.69	0.85	4.60	0.11	0.37	0.14

Table B.5: *Continued*

Status	Patient	Gender	Age	Upper reference value†	C0	C4	C5	C5DC	C6	C8	C10
-	-	M + F	-	1: Newborn	4.89	0.84	0.38	0.63	0.23	0.2	0.1
-	-	M + F	-	2: Y1-Y5	13.93	0.19	0.25	0.04	0.13	0.15	0.08
-	-	M + F	-	3: Y6-Y17	4.74	0.77	0.29	0.46	0.11	0.15	0.09
-	-	M + F	-	4: ≥Y18	3.77	0.62	0.24	0.51	0.09	0.14	0.07
Tr	P8	F	D22	1	8.91	24.15	15.22	4.24	1.79	1.63	0.27
Tr	P9	M	Y18 M9	4	8.09	9.34	0.51	2.10	0.06	0.25	0.05
Tr	P10	F	Y9	3	21.42	4.29	0.70	1.12	0.06	0.23	0.08
Tr	P11	M	Y8 M7	3	436.34	37.05	10.89	7.04	0.25	3.86	0.24
Tr	P12	F	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Tr	P13	F	Y41 M2	4	6.66	2.75	0.18	1.75	0.05	0.10	0.06
Tr	P14	F	Y26 M8	4	147.42	7.64	2.56	3.74	0.13	0.69	0.08

† Applied reference range is dependent on age (Mueller *et al.,* 2003).

Values are given in mmol/mol creatinine. Abbreviations: C0: free carnitine; C4: butyryl-/isobutyrylcarnitine; C5: isovalerylcarnitine; C5DC: glutarylcarnitine; C6: hexanoylcarnitine; C8: octanoylcarnitine; C10: decanoylcarnitine; D and number: age in days; F: female; M: male; M and number: age in months; n.d: not determined; Tr: treated; Untr: untreated; Y and number: age in years.

Table B.6: Urine amino acids

Status	Patient	Gender	Age	Reference range†	Sarcosine	4-OH-Pro	Pro
-	-	M	-	1	0-8.5	20-320	21-213
-	-	F	-	2	0-8.5	20-320	21-213
-	-	M	-	3	0-8.5	0-143	0-130
-	-	F	-	4	0-1.4	0-13	0-13
-	-	M	-	5	0-1.4	0-13	0-13
-	-	M	-	6	0-1.4	0-13	0-9
-	-	F	-	7	0-1	0-13	0-9
-	-	M	-	8	-	0-6	64-236
-	-	M	-	9	-	0-5	43-173
Untr	P1	M	Y1 M9	5	7.94	3.83	12.43
Untr	P2	F	Y1 M5	4	25.86	7.06	32.48
Untr	P3	M	D42	3	92.71	240.39	94.27
Untr	P4	F	Y41 M9	7	9.84	2.31	4.29
Untr	P5	F	Y36 M7	7	60.09	106.42	41.70
Untr	P6	M	D3	1	67.25	1004.76	1149.35
Untr	P7	F	Y3 M2	7	10.98	7.86	14.64
Untr	P8	F	D9	2	106.99	1407.46	1674.14
Untr	P9	M	Y1 M9	5	5.14	34.65	132.79
Untr	P10	F	Y7 M11	7	2.63	1.68	4.00
Untr	P11	M	Y2 M8	6	6.83	2.03	6.89
Untr	P12	F	n.d	n.d	n.d	n.d	n.d
Untr	P13	F	n.d	n.d	n.d	n.d	n.d
Untr	P14	F	Y26 M4	7	2.90	0.27	1.32
Tr	P1	M	Y11 M5	8	0.70	0.75	24.64
Tr	P2	F	Y29 M9	7	1.16	0.29	5.75
Tr	P3	M	Y25 M8	9	2.58	0.87	32.60
Tr	P4	F	n.d	n.d	n.d	n.d	n.d
Tr	P5	F	Y40 M2	7	0.35	0.23	9.20
Tr	P6	M	D11	1	93.18	510.92	4118.11
Tr	P7	F	Y13 M4	7	0.57	0.67	1.53
Tr	P8	F	D22	2	37.5	873.2	1505.58
Tr	P9	M	Y18 M9	9	3.15	0.31	10.89
Tr	P10	F	Y9	7	0.16	0.54	11.19
Tr	P11	M	Y8 M7	8	1.26	2.17	14.24
Tr	P12	F	n.d	n.d	n.d	n.d	n.d
Tr	P13	F	Y41 M2	7	0.14	0.21	4.03
Tr	P14	F	Y26 M8	7	0.12	0.13	9.30

† Applied reference range is dependent on age and gender. In-house reference ranges were used.

Values are given in mmol/mol creatinine. Abbreviations: 4-OH-Pro: 4-hydroxyproline; D and number: age in days; F: female; M: male: M and number: age in months; n.d: not determined; Pro: proline; Tr: treated; Untr: untreated; Y and number: age in years.

B.4 SUPPLEMENTARY MATERIAL 3: ADDITIONAL STRUCTURAL INFORMATION

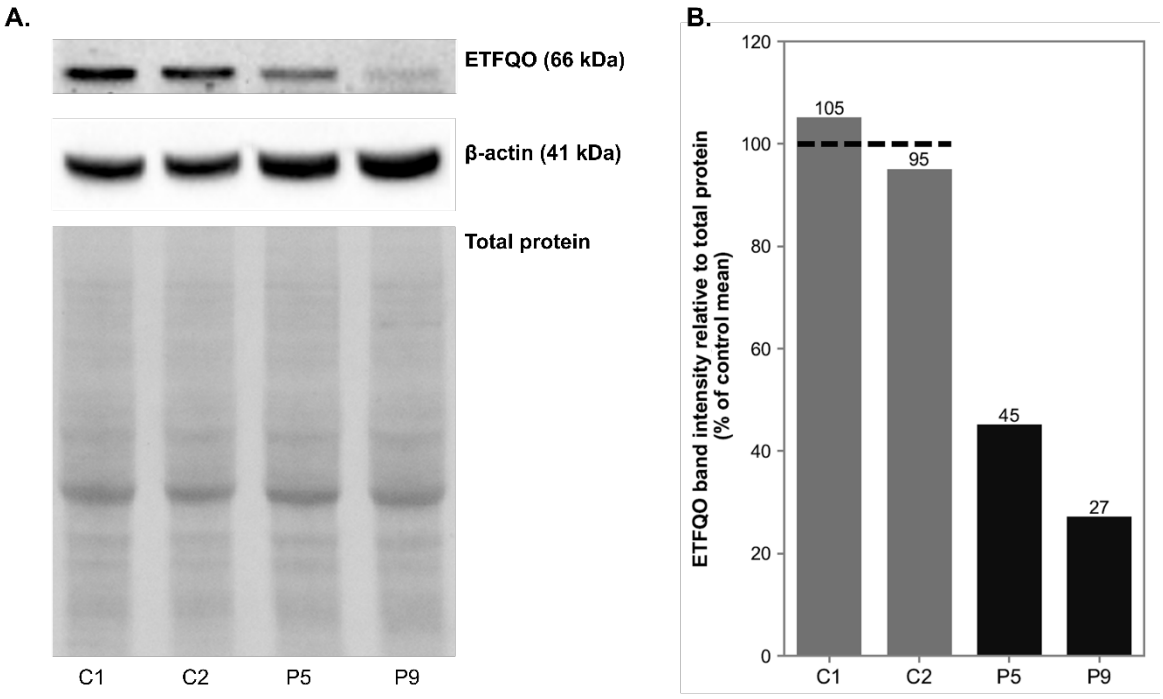

Figure B.1: Structural instability of ETFQO in skin fibroblasts of P5 and P9

(a) Cell lysate, prepared from primary skin fibroblasts of P5 (c.[1448C>T];c.[1448C>T]) and P9 (c.[1067G>A];c.[1448C>T]) as well as two healthy controls, C1 and C2 (matched in age, gender, and ethnicity to P9 and P5, respectively), were separated on a 10% SDS-PAGE gel and immunoblotted with primary antibodies against ETFQO (reported weight of the monomeric protein: 64 kDa) and β-actin (42 kDa). (b) Band volume intensities were normalised to the total protein content of each corresponding lane and quantified as a percentage, relative to the mean (100%) of both controls (n = 1). Abbreviations: ETFQO: electron transfer flavoprotein-ubiquinone oxidoreductase.

Appendix C

Additional methods and results from Chapter 3

C.1 ETHICS APPROVAL AND CONSENT/ASSENT TO PARTICIPATE

C.1.1 Ethics approval and documentation

The patients in this study were enrolled as part of the ICGNMD study, following informed consent/assent, with ethics approval numbers 19/LO/1796 [Health Research Authority (HRA) and Health and Care Research Wales (HCRW)], NWU-00966-19-A1 and NWU-00966-19-A1-01 [Health Research Ethics Committee (NWU-HREC) of the Faculty of Health Sciences, NWU], 296/2019 (Faculty of Health Sciences Research Ethics Committee, UP), B19/01/002 (Health Research Ethics Committee, SU), and 605/2020 and 196/2018 (Faculty of Health Sciences Human Research Ethics Committee, UCT), as relevant. The ethics approval documentation is shown below in Figures C.1–7.

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Professor Michael Hanna
Professor of Clinical Neurology, Consultant Neurologist
(Nat. Hosp. for Neurology & Neurosurgery), Director
UCL Queen Square Institute of Neurology, Director
MRC Centre for Neuromuscular Diseases
University College London NHS Hospital NHS Trust
MRC Centre for Neuromuscular Diseases
8-11 Queen Square
London
WC1N3BG

Email: hra.approval@nhs.net
HCRW.approvals@wales.nhs.uk

02 December 2019

Dear Professor Hanna

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**INTERNATIONAL CENTRE FOR GENOMIC MEDICINE IN NEUROMUSCULAR DISEASES (ICGNND)**
IRAS project ID:	**243406**
Protocol number:	**1.1**
REC reference:	**19/LO/1796**
Sponsor	**University College London**

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Figure C.1: Ethics approval: 19/LO/1796

Figure C.2: Ethics approval: NWU-00966-19-A1

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

27 November 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 27/11/2019, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Investigating neuromuscular diseases in South Africa: the ICGNMD study
Principal Investigator/Study Supervisor/Researcher: Prof FH van der Westhuizen
Student: -

Ethics number: N W U - 0 0 9 6 6 - 1 9 - A 1
(Institution – Study Number – Year – Status)

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Larger study
Commencement date: 27/00/2019
Expiry date: 30/00/2020

Risk: **Adult patients: Medium**
Adult control individuals: Minimal
Child patients: Category 2 – Greater than minimal risk with prospect of benefit
Child control individuals: Category 1 – No more than minimal risk

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of a six-monthly monitoring report and the concomitant issuing of a letter of continuation. Monitoring reports are due at the end of May and November annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:*
  - *Six-monthly on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*

  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the six-monthly monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Prof Petra Bester
Date: 2019.11.27 10:57:09 +02'00'

Chairperson NWU-HREC

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-HREC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2

Figure C.3: Ethics approval: NWU-00966-19-A1-01

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

04 March 2020

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 04/03/2020, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Biochemical and molecular genetic characterisation of MADD in a South African cohort
Principal Investigator/Study Supervisor/Researcher: Prof FH van der Westhuizen
Student: M Mereis - 23114126

Ethics number: N W U - 0 0 9 6 6 - 1 9 - A 1 - 0 1

Institution Study Number Year Status Sub-study

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Sub-study
Commencement date: 04/03/2020
Expiry date: 31/03/2021

Risk: **Adults – Medium**
Children: Category 2 – Greater than minimal risk with direct benefit

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of a six-monthly monitoring report and the concomitant issuing of a letter of continuation. Monitoring reports are due at the end of September and March annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:*
  - *Anually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*

- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Prof Petra Bester
Date: 2020.03.04 16:18:53 +02'00'

Chairperson NWU-HREC

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-HREC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2

Figure C.5: Ethics approval: B19/01/002

UNIVERSITEIT STELLENBOSCH UNIVERSITY

Approval Letter
Amendment

16/10/2019

Project ID: 8924

Ethics Reference No: B19/01/002

Project Title: Stellenbosch University Inherited Neuromuscular Disorders Registry and DNA Repository

Dear Dr. Francio Henning

We refer to your responses to modifications that were requested on your submission received 16/09/2019 11:53.

The Health Research Ethics Committee (HREC) reviewed and **approved** the amended documentation through an expedited review process.

The following amendment was reviewed and **approved**:

1. To make some or all of the study data available to the wider research community for appropriate research by submission of pseudonymised data to an access-controlled data archive and a clinical bioinformatics database.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your project ID 8924 and ethics reference number B19/01/002 on any documents or correspondence with the HREC concerning your research protocol.

Yours sincerely,

Mrs. Melody Shana

Coordinator

Health Research Ethics Committee 1

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the
World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects;the South African *Department of Health (2006). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition)*; as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.

Page 1 of 1

Figure C.4: Ethics approval: 296/2019

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Exoires 03/14/2020.

21 November 2019

Approval Certificate
New Application

Ethics Reference No.: 296/2019
Title: INTERNATIONAL CENTRE FOR GENOMIC MEDICINE IN NEUROMUSCULAR DISEASE:A SYSTEMS BIOLOGY APPROACH IN NEUROMUSCULAR OR NEUROGENETIC DISEASES

Dear Prof I Smuts

The **New Application** as supported by documents received between 2019-10-21 and 2019-11-20 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-11-20.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-11-21.
- Please remember to use your protocol number (296/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
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University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Figure C.6: Ethics approval: 605/2020

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room G50- Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: hrec-enquiries@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

12 October 2020

HREC REF: 605/2020

Prof J Wilmshurst
Paediatric Neurology
5th Floor, ICH Building
Red Cross War Memorial Children's Hospital
Email: Jo.wilmshurst@uct.ac.za
Student: mereismichelle@gmail.com

Dear Prof Wilmshurst

PROJECT TITLE: BIOCHEMICAL AND MOLECULAR GENETIC CHARACTERIZATION OF MULTIPLE ACYL-COA DEHYDROGENASE DEFICIENCY (MADD) IN A SOUTH AFRICAN COHORT-PHD CANDIDATE MICHELLE MEREIS-sub-study linked to R030/2018

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020 & 06 July 2020.

Approval is granted for one year until the 30 October 2021.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: - Michelle Mereis will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC/REF:605/2020sa

Figure C.7: Ethics approval: 196/2018

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338
Email: lamees.emiedi@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

08 October 2018

HREC REF: 196/2018

Mrs S Meldau
Chemical Pathology
Level 6
Falmouth building
Medical School

Dear Mrs Meldau

PROJECT TITLE: DETERMINATION OF THE POPULATION FREQUENCY OF THE GALNT3:c.484C> (p.Arg162Ter) PATHOGENIC VARIANT DETECTED IN TWO UNRELATED BLACK SOUTH AFRICAN PATIENTS WITH HYPERPHOSPHATEMIC FAMILIAL TUMORAL CALCINOSIS

Thank you for your response letter dated 08 October 2018 to the Faculty of Health Sciences Human Research Ethics Committee.

The HREC notes that all studies being utilised have been closed/updated.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 October 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Please quote the HREC reference number in all your correspondence.

HUMAN RESEARCH ETHICS COMMITTEE
04 FEB 2019
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

UNIVERSITY OF CAPE TOWN — FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

Form FHS006: Protocol Amendment

HREC office use only (FWA00001637; IRB00001938)		
☐ Approved	☐ Type of review: Expedited	☐ Full committee
This serves as notification that all changes and documentation described below are approved.		
Signature Chairperson of the HREC		Date 12/3/2019

Note: All major amendments must include a local **PI Synopsis** justifying the changes for the amendment. Please note that incomplete amendment submissions will not be reviewed.

Comments from the HREC to the Principal Investigator:
Thank you for confirming all aspects with satisfactory Ergebnisse open and closed studies
Note: The approval of this protocol amendment does not grant annual approval. Please complete the FHS016 / FHS017 form for annual approval at least one month before study expiration.

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)			
HREC REF Number	196/2018		
Protocol title	~~Determination of the population frequency of the GALNT3:c.484C>T(p.Arg162Ter) pathogenic variant detected in two unrelated Black South African patients with hyperphosphatemic familial tumoral calcinosis.~~ **New Title:** Determination of Cape Town based population allele frequencies of a number of inherited metabolic disease-causing pathogenic variants occuring on two or more alleles from non-consanguinous South African families.		
Protocol number (if applicable)	Version 2		
Principal Investigator	Surita Meldau		
Department / Office Internal Mail Address	Chemical Pathology, Level 6, Falmouth Building		
1.1 Is this a major or a minor amendment? (see FHS006hlp) Major (tick box) Minor (tick box)		✓ Major	☐ Minor
1.2 Does this protocol receive US Federal funding?		☐ Yes	✓ No

C.1.2 Consent/assent documentation

Patients were recruited via the four SA ICGNMD centres: Steve Biko Academic Hospital (UP), Tygerberg Hospital (SU), Red Cross War Memorial Children's Hospital, and Groote Schuur Hospital (UCT). For eligible patients located via these cites, as well as referrals from the UCT, informed consent/assent was obtained by the UP and SU, using the consent/assent documentation in Figures C.8–11.

Figure C.8: University of Pretoria's parental/legal guardian consent

Short title: ICGNMD_UP Protocol number 296/2019 ICDI.a Children May 2020 V2

PARENTAL OR LEGAL GUARDIAN INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD):

A Systems Biology Approach in Neuromuscular or Neurogenetic diseases in South Africa

Funded by: 1. UK Government's Medical Research Council (MRC)
2. Guarantors of Brain (Registered Charity 264139)

Chief Investigator: Professor Michael Hanna

Institution: University College London Queen Square Institute of Neurology

Principal Investigator: Professor Izelle Smuts

Institution: University of Pretoria

Contact details:

Daytime number/s: 012 354 5303 or 076 383 2286

After hours' number: 076 383 2286

Email: icgnmdup@gmail.com

Page 1 of 24

Short title: ICGNMD_UP Protocol number 296/2019 ICDI.a Children May 2020 V2

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

				:	
day	month	year		Time	

Dear Parent or Legal Guardian

Dear Mr. / Ms./Mrs._____

1. INTRODUCTION

We invite your child to participate in a research study. This information document will help you to decide if your child may participate. Before you agree that your child may take part, you should fully understand what is involved. If you have any questions that this document does not explain, please do not hesitate to ask your child's doctor.

It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your child's participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect your child negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially.

This project has been approved and monitored by Faculty of Health Sciences Research Ethics Committee and we collaborate with researchers from around the world.

Page 2 of 24

2. THE NATURE OF THE STUDY

Children and adults with conditions affecting their muscles, nerves, brain and often also many other organs have diseases called neuromuscular (NMD's) or neurogenetic diseases. It is sometimes very difficult to confirm a diagnosis and understand what causes it. In this study we want to try and find the different genetic mutations that may be linked to the disease and then we will do further research to understand how it causes the disease.

Genetic research is research that is based on studying the genetic material, also called DNA or RNA of individuals. The genetic material is obtained from a blood sample, saliva or biopsy specimen. A biopsy is a tiny piece of tissue that is cut out e.g. from a muscle to help doctors make diagnosis.

DNA has four different subparts (represented by the letters A, T, C and G). Combinations of these letters code for the instructions our bodies use. For example, some stretches of DNA code for genes. Genes are instructions to make particular products in our bodies. Other stretches of DNA control which genes are used and which are turned off, in a given time of life or part of the body. Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Every person's DNA code (or "genetic code") is unique.

3. WHAT IS A GENETIC CONDITION AND HOW WILL THIS STUDY LOOK AT THESE?

Sometimes, people can have variations in their DNA that cause medical conditions. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations. Finding which DNA variants cause a medical condition is potentially useful because it might contribute to finding genetic diagnoses and when new diagnoses are being made, this might allow clinicians to understand how a condition might be treated or if it might develop over time and also that may benefit people today, who suffer from particular diseases or conditions

This study is particularly interested in DNA changes which cause neurogenetic and neuromuscular conditions. Some changes are known, but there is still much to learn about how they actually cause problems in our bodies. Other changes have not yet been found, or there is not yet enough evidence that they cause a medical condition.

For genetic conditions, it is often important to compare the DNA code of someone with a medical condition with DNA from close relatives and unrelated, healthy people. This allows us to narrow down which DNA variants might be affecting a medical condition (e.g. by ruling out changes that people with a condition have in common with healthy people). Sometimes, researchers need to analyse thousands of different people's genetic and health data, from different population groups, to find which variants are responsible.

Finding which DNA variants cause a medical condition is potentially useful because:-

- It might contribute to finding genetic diagnoses for people with a neuromuscular/neurogenetic condition and their families;
- In helping to make new diagnoses, it might allow clinicians to understand how a condition might be treated or if it might develop over time.

4. WHAT IS THE PURPOSE OF THE STUDY?

Complex genetic NMDs often present with problems outside of the muscles and nerves, particularly when they occur in childhood. These can include developmental delay, behavioral issues, seizures, impaired coordination, movement problems, and memory disturbance.

The purpose of our research project is to identify genetic changes that cause neuromuscular and other neurogenetic diseases, and to improve our understanding of the clinical and genetic features of these conditions. We hope that this will allow us to work out better ways to diagnose and treat these disorders.

Up to 15,000 participants will be recruited globally at the participating centres, over a period of 5 years.

5. WHY HAS YOUR CHILD BEEN CHOSEN?

Your child has been chosen either because your doctor feels that his/her symptoms may be due to an underlying neuromuscular or neurogenetic disorder, or because he/she does not have symptoms suggestive of a neuromuscular or neurogenetic disorder and may or may not be related to an individual with symptoms suggestive of a neuromuscular or neurogenetic disorder. If your child is healthy and unrelated to someone with symptoms suggestive of a neuromuscular or neurogenetic disorder, your child results will act as a "control" data, providing a baseline against which we will compare the findings from people with a neuromuscular or neurogenetic disorder.

6. WHAT WILL HAPPEN TO YOUR CHILD IF HE/SHE TAKES PART?

If you decide that your child may to participate in the study, you will be asked to sign the attached consent form before any of the study assessments can take place or any of your data or samples can be used for the study.

6.1 Child with symptoms

If your child is taking part in the study because he/she has symptoms compatible with an underlying neuromuscular or neurogenetic condition you might be asked to attend a once-off study visit, in addition to your routine clinic appointment, lasting approximately 1-2 hours. At the visit we will ask for your consent to collect the following data and undertake the following assessments:

6.1.1 Demographic data

Age, gender and ethnicity will be documented.

6.1.2 Medical History

Extract the clinical data from the medical notes and clinical assessments from past and future routine outpatient visits, so they can be used in this study.

6.1.3 Physical examination, including neurological examination

This will include a thorough clinical examination including strength testing, balance etc.

6.1.4 Questionnaires

You might be asked to complete some standard questionnaires that we use as routine for specific conditions.

6.1.5 Videos and photographs

This may include videoing your child when walking, his/her eye movements, speech etc. The video or photographs may be used in SA or abroad for teaching, training, research, diagnostic/treatment purposes, and/or publication in medical journals, books, as lecture material. Images and videos will be securely stored. As this is optional, if you don't want to have a video or photos taken, your child can still take part in the study.

6.1.6 Blood tests

A blood sample (about 6 ml) to test your DNA and/or RNA for possible changes in the genetic code will be taken. We will look at the genes in which genetic mutations may cause symptoms of neuromuscular or neurogenetic diseases. This may also include sequencing your entire genome. The sample will be sent to North West University for initial sample preparation and then sent to the UK or Netherlands for analysis. You will be asked to donate your child's blood sample for future research. If you consent to it, the blood sample will be stored indefinitely at NWU and will be used for future research. As this is optional, you don't have to consent to it to be in the study. Results of genetic tests performed on a research basis are not routinely made available. Should a result of interest be found, we are now able to validate research genetic results and you would be invited back for testing on a clinical basis. If your child is not a patient at SBAH, we can arrange a follow up visit to discuss this,

otherwise your doctor can be informed. We keep details of any research results confidential.

We may also sent blood for special investigations routinely done in the diagnosis and follow up of the possible underlying condition suspected to assist us in making a diagnosis or assist in the decisions that need to be made in your treatment.

6.1.7 Urine sample

A urine sample may be taken to develop new indicator of disease severity (biomarkers) or new factor contributing to disease symptoms.

6.1.8 Skin biopsy

A skin biopsy may be taken if indicated. This may also be used to see how the skin cells work and give us more information about how the disease affects your child. The cells will be looked at under a microscope and grown as fibroblast cells to investigate how they work differently. Cells obtained from the skin may also be manipulated in the laboratory and transformed into other cell types (e.g. muscle or nerve cells) to investigate the mechanisms that cause the disease. These types of cells are called "induced pluripotent stem cells", or iPSCs.

The skin biopsy procedure involves taking a small sample of skin usually taken from just above the ankle but can also be taken from other sites that your doctor selects (like the top of the thigh or forearm). Local anaesthetic will be given, and when the area is numb a small (3mm diameter) piece of skin will be removed using a small punch. No stitches are needed, and the wound heals over 7-10 days, generally becoming nearly invisible over a 4-6 weeks. This is a safe and widely used procedure but rarely side effects such as allergy to local anaesthetic, difficulties with healing, bleeding or skin infections may occur. Alternatively, if your child previously had a skin biopsy performed as part of routine clinical care some of this tissue may remain stored, and we may be able to re-examine this tissue and perform additional tests on it to aid current and future research into mitochondrial disease without the need for a further skin biopsy.

6.1.9 Existing blood and tissue samples

Use any leftover diagnostic samples collected during past or future routine visits such as urine, saliva, blood, muscle and skin biopsies in this study.

In some instances we may use letters from clinical colleagues. If you attend clinic at SBAH this part of the visit may be done in the context of your routine appointment. If your child is not a patient at SBAH then we will arrange a research clinic visit at your convenience, or these details may be sent to us by your child's treating doctor.

6.2 CONTROL PARTICIPANT

If your child is taking part as a control participant (who may or may not be a relative of a participant with a neuromuscular or neurogenetic condition) you will be asked to attend a one-off study visit lasting approximately 1-2 hours. At the visit we will ask for your consent to collect the following data and undertake the following assessments:

6.2.1 Demographic data

Age, gender and ethnicity will; be documented.

6.2.2 Medical History

A medical history will be obtained.

6.2.3 Physical examination, including neurological examination

This will include a thorough clinical examination including strength testing, balance etc.

6.2.4 Questionnaires

You might be asked to complete some standard questionnaires that we use as routine for specific conditions.

6.2.5 Blood tests

A blood sample (about 6 ml) to test your child's DNA and/or RNA for possible changes in the genetic code will be taken. We will look at the genes in which genetic mutations may cause symptoms of neuromuscular or neurogenetic diseases. This may also include sequencing the entire genome. The sample will be sent to North West University for initial sample preparation and then sent to the UK for analysis. You will be asked to donate the blood sample for future research. If you consent to it, your child's blood sample will be stored indefinitely at NWU and will be used for future research. As this is optional, you don't have to consent to it to be in the study. Results of genetic tests performed on a research basis are not routinely made available. Should a result of interest be found, we are now able to validate research genetic results and you would be invited back for testing on a clinical basis. If you are not a patient at SBAH, we can arrange a follow up visit to discuss this, otherwise your doctor can be informedWe keep details of any research results confidential.

6.2.6 Urine sample

A urine sample may be taken to develop new indicator of disease severity (biomarkers) or new factor contributing to disease symptoms.

6.2.7 Skin biopsy

A skin biopsy may be taken if indicated. This may also be used to see how the skin cells work and give us more information about how the disease affects people. The cells will be looked at under a microscope and grown as fibroblast cells to investigate how they work differently. Cells obtained from the skin will also be manipulated in the laboratory and transformed into other cell types (e.g. muscle or nerve cells) to investigate the mechanisms that cause the disease.

The skin biopsy procedure involves taking a small sample of skin usually taken from just above the ankle but can also be taken from other sites that the doctor selects (like the top of the thigh or forearm). Local anaesthetic will be given, and when the area is numb a small (3mm diameter) piece of skin will be removed using a small punch. No stitches are needed, and the wound heals over 7-10 days, generally

becoming nearly invisible over a 4-6 weeks. This is a safe and widely used procedure but rarely side effects such as allergy to local anaesthetic, difficulties with healing, bleeding or skin infections may occur. Alternatively, if you have previously had a skin biopsy performed as part of routine clinical care some of this tissue may remain stored, and we may be able to re-examine this tissue and perform additional tests on it to aid current and future research into mitochondrial disease without the need for a further skin biopsy.

6.2.8 Existing blood and tissue samples

Use any leftover diagnostic samples collected during past or future routine visits such as urine, saliva, blood, muscle and skin biopsies in this study.

7. WHAT PROCEDURES WILL BE INVOLVED IN THIS RESEARCH?

7.1 Informed consent and/or assent (all participants)

7.2 Eligibility confirmation (all participants)

7.3 Demographic data collection (all participants)

7.4 Medical history (all participants)

7.5 Family history (all participants)

7.6 Blood test (all participants ≥ 16: optional for participants < 16)

Approximately 6 ml blood (1 teaspoons) for haematopoietic cells, plasma, serum and/or DNA isolation will be collected at a single visit. DNA isolation will be done using a standardised methodologies according to laboratory protocols. Blood may also be subjected to metabolic analyses at the NWU.

7.7 Saliva test

Approximately 2ml of saliva for DNA isolation will be collected, for example using an Oragene Saliva Kit.

7.8 Urine or other painless biological sample (if required)

Approximately 20-50 ml of urine will be collected in a pot by the patient during urination. Where necessary, urinary epithelial cells may be extracted and processed to generate DNA for mitochondrial genome sequencing. Urine may also be subjected to metabolic analyses at the NWU.

7.9 Lumbar puncture to obtain CSF (if indicated)

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

7.10 Skin biopsy (all participants: optional and only if required)

When appropriate, affected participants and control participants may have a skin biopsy collected to explore the cellular consequences of their gene mutations. Some unaffected subjects (estimated 0.1-1% of total, or ca 1-5 people) may be asked for a skin biopsy for comparison as a control. For this procedure a 2-4mm skin punch biopsy will then be taken under local anaesthetic from a limb site (either 5 cm above the medial malleolus, lateral thigh, upper arm or the forearm).

Skin biopsies will not be requested if the participant is planning to have surgery in the next 6 months. Patients older than 16 years and recruited into the study will have a skin biopsy at the study visit or during one of their outpatient appointments. The biopsy will leave a small scar approximately 4mm in diameter.

7.11 EMG and NCV (if indicated)

Nerve conduction studies and electromyography will only be done if it has not been done before and clinically may contribute to confirm the clinical diagnosis of affected nerves and/or muscle.

7.12 Muscle ultrasonography (if indicated)

It is a painless procedure where ultrasound machine is used to get an impression of the quality of the muscle.

7.13 Muscle biopsy (if indicated)

A muscle and/or relevant tissue biopsy will be done in some cases if the blood tests were inconclusive and if a relevant condition e.g. mitochondrial disease is suspected. The biopsies will be taken under general anaesthetics by a qualified paediatric surgeon or adult neurologist. For a muscle biopsy a 3-4 cm incision will be made on the thigh and a small piece of muscle about 1 cubic cm will be removed for possible histology, biochemical and genetic analyses. DNA extraction will be performed at the NWU, but the genetic analyses will be performed in the UK and Netherlands.

7.14 Recording photographs or videos of all or part of the body: optional and only if required

7.15 Clinical phenotyping using Human Phenotype Ontology (HPO) (affected participants only).

It is a standardised way to document symptoms and signs.

8. ARE THERE ANY RISKS INVOLVED IN GENETIC RESEARCH?

8.1 Blood tests

Blood tests do not pose a significant risk to your health. You may experience pain and/or bruising at the site on your arm where blood is taken. The use of a needle can carry some discomfort and a small risk of bruising, bleeding, fainting, and a very small risk of infection where the skin is broken. If you experience faintness you should lie down immediately to avoid possible injuries and then notify the study personnel.

8.2 Skin biopsy

The skin biopsy procedure involves taking a small sample of skin usually taken from just above the ankle but can also be taken from other sites that your doctor selects (like the top of the thigh or forearm). Local anaesthetic will be given, and when the area is numb a small (3mm diameter) piece of skin will be removed using a small punch. No stitches are needed, and the wound heals over 7-10 days, generally becoming nearly invisible over a 4-6 weeks. A biopsy care sheet will be given to you after the biopsy so you know how to care for the site. This is a safe and widely used procedure but rarely side effects such as allergy to local anaesthetic, difficulties with healing, bleeding or skin infections may occur.

Alternatively, if you have previously had a skin biopsy performed as part of routine clinical care some of this tissue may remain stored, and we may be able to re-examine this tissue and perform additional tests on it to aid current and future research into mitochondrial disease without the need for a further skin biopsy.

8.3 Muscle biopsy

There may be a risk of complications during anaesthetics, but the anaesthetist will be informed about precautions to take. Bleeding at the site and infection may occur. A scar may develop in the area where the biopsy is done.

8.4 Other risks

To the best of our ability, the data we collect about you in this will remain confidential. However, the risk of a breach of confidentiality is always possible. We will minimize any risk by anonymising the data we collect. All published reports or public presentations will also be also be anonymised. Additionally, any data which can identify you will be kept completely confidential and stored in password protected folders and files that will only be accessible to the research team at SBAH.

9. ARE THERE ANY BENEFITS TO YOUR TAKING PART IN THIS STUDY AND WILL YOU GET TOLD YOUR RESULTS?

If we are able to confirm a diagnosis, you will be informed and specific treatment, if available in SA, will be offered to your child.

If no specific diagnosis is made or if you are a control, there are no direct benefits to your child if you take part in this research. We hope that this study will generate a greater understanding of neuromuscular and neurogenetic diseases by possibly identifying underlying causes. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients with such conditions.

Your child's results will be made known to you **only if they indicate** that he/she have or may:

- Have a definite risk for developing a particular disorder.
- Have a condition or predisposition to developing a condition that is treatable or avoidable e.g. by a lifestyle modification.
- Need genetic counselling.

10. HOW LONG WILL YOUR CHILD'S BLOOD BE STORED AND WHERE WILL IT BE STORED?

The samples we collect from you during the study visits will initially be stored at SBAH. It will then be transported to the Mitochondria Research Laboratoryat NWU in Potchefstroom. The samples will be processed there, stored and some analyses will be done at the NWU. A part of the material will be sent to the UK and/or Netherlands for additional investigations that can only be performed overseas.

The cells in the blood may be grown to give us more information about how the disease affects your child.

You will be able to give your consent to donate your samples as a gift so they can be retained and used for future research. If you consent to it specifically, your samples will also be stored in a rare disease

biobank at Centre for Human Metabolomics, NWU, Potchefstroom and, in some cases, at the biobank of the Great Ormond Street Hospital, UK. Any new research will be reviewed by a research ethics committee but consent for future studies may only be required if the committee considers that the study is likely to substantially affect your interests. If your child already have had DNA, RNA or lymphoblasts stored then we may be able to use these without the need for a further blood test.

11. IF THE BLOOD IS TO BE STORED IS THERE A CHANCE THAT IT WILL BE USED FOR OTHER RESEARCH?

Your child's blood and other specimens will only be used for genetic research that is directly related to NMDs and neurogenetic disorders in as outlined to find new changes and to understand the mechanism of the disease.

Stored blood samples, RNA/DNA, skin fibroblasts, and myoblasts may possibly be used in future research to facilitate the development of treatments for neuromuscular and neurogenetic diseases, but then additional permission to do so must be obtained form from the Faculty of Health Sciences Research Ethics Committee at the University of Pretoria.

If you do not wish your child's blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

12. HOW WILL YOUR CONFIDENTIALITY BE PROTECTED?

All information regarding medical records will be treated as strictly confidential and will only be used for research purposes. Your child's medical records may be inspected by competent authorities and properly authorised persons. Your identity will remain confidential and only the local authorised personnel at SBAH will be able to identify your child if you need to be contacted.

The information and samples we have collected about your child may be shared with researchers at other academic organisations or private companies. If any information is released this will be done in a coded form so that confidentiality is strictly maintained.

If you agree to it, the researchers will inform your doctor that you are participating in the study.

13. WILL YOU OR THE RESEARCHERS BENEFIT FINANCIALLY FROM THIS RESEARCH?

Your child will not be paid to take part in this study.

Important information: In the unlikely event that this research leads to the development of a commercial application or patent in future studies, your child or your family will not receive any profits or royalties.

14. YOUR CHILD"S RIGHTS AS A RESEARCH PARTICIPANT

Your child's participation in this trial is entirely voluntary. Your child can refuse to participate or stop at any time without stating any reason. His/her withdrawal will not affect his/her access to other medical care.

15. WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

Results may be published in scientific journals and may be used as educational materials. Any of your child's data used in this manner would be anonymous. No identifying information would be released.

16. WHAT IF SOMETHING GOES WRONG?

If you have any concerns about any aspects of this study you may discuss these with Prof Smuts. If you remain unhappy and wish to go further and complain formally about any aspect of the way you or your

child have been approached or treated during the course of the study, you can do this inform the HREC. Please quote the project number at the top this consent form.

If your child is harmed by taking part in this research project, there are no special compensation arrangements. If your child is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns of this study, the normal complaints mechanisms should be available to you.

17. ETHICS APPROVAL

This research study was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it. It will also be conducted according to international and locally accepted ethical guidelines for research, namely the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes.*

18. INFORMATION

If I have any questions concerning this study, I should contact Prof Smuts or Dr Rammego.

Daytime number/s: 012 354 5303 or 076 383 2286

After hours' number: 076 383 2286

Email: icgnmdup@gmail.com

19. CONFIDENTIALITY

All information obtained during the course of this study will be regarded as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001. This will ensure confidentiality of information so collected. Only the researcher will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of all your records will be kept in a locked facility at Department of Paediatrics, SBAH, and University of Pretoria.

20. CONSENT TO PARTICIPATE IN THIS STUDY

I have read the information sheet dated _____ for this study. I have considered the information and have had adequate time to ask questions and my questions have been answered. I have no objections for my child to participate in this study.

I confirm that:

- The person requesting my consent for my child to take part in this study has told me about the nature and process, any risks or discomforts, and the benefits of the study.
- I have received a signed copy of this informed consent agreement.

I understand that:

- My child's participation is entirely voluntary and that I or my child are free to withdraw at any time without giving a reason, and without my child's medical care or legal rights being affected.
- I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results and that my child will not be identified in scientific publications or at conferences.
- I will not receive any profits if commercially valuable product(s) result from research using my child's data or samples.

I agree that:

- The data collected during the study and relevant sections of my child's medical notes (if applicable) may be looked at by his/her clinician and authorised members of their team, individuals from University of Pretoria and University College London, from regulatory authorities and from NHS Trusts, where it is relevant to my child taking part in this research. I give permission for these individuals to have access to my child's records.
- My child's data from his/her past or future clinical or diagnostic investigations can be used in this study (if applicable) and can be shared with approved international researchers working at other academic and government research institutes and also with approved commercial

companies (excluding my child's name, contact details, date of birth, and NHS and other hospital numbers).

(Approved commercial companies are companies approved by the research leader who are developing novel therapeutics for neuromuscular diseases to receive as much information as possible so that therapies have international benefit.)

- My child's tissue samples can be used to obtain his/her DNA and RNA, which will be stored for the duration of the study and will be used to generate study data.
- My child's personal, medical and genetic data can be deposited in online, controlled-access scientific databases, and my child's data can be studied by authorised researchers from around the world.
- If I or my child decide to withdraw from the study, the data already collected from me and my child will continue to be used in the study. The Study Team will destroy any DNA and biological samples that they hold under the Study ethics. However, I understand that any cell lines (including iPS cells) that have been created from my child's tissue will not be destroyed and information about them will be retained.

	YES	NO	INITIAL ↓
I agree for my child to take part in this study			
I agree for my child to have a skin biopsy			
I agree for my child to have a muscle biopsy			
I agree for my child to have another biopsy. Specify tissue:			
I agree that my child's tissue, in addition to his/her DNA and RNA, can be stored and used for research by the UP/NWU and UK study team, including for the generation of cell lines (which may include induced pluripotent cell lines, or iPSCs).			
I agree that my child's DNA, RNA and other tissue (including cell lines, which may include iPSCs) can be shared with approved international researchers working at other academic and government research institutes and also with approved commercial companies.			
I agree that photographs and/or videos taken during clinic visits can be used in this study. I understand that their use might lead to my child being personally identified by researchers who have access to his/her data.			
I agree that DNA and all tissue samples provided in this study (e.g. blood, urine, saliva samples and biopsies) and cell lines derived from these and may be stored indefinitely and used to support other research.			
	YES	NO	INITIAL ↓

I agree to be contacted personally by the research team in the future. I can be contacted by **(please circle)** e-mail phone			
Affected participants only I agree that genetic research findings which might be relevant to my child's symptoms can be sent to his/her clinician for further, formal checks, where appropriate.			
Related controls only I agree that genetic research findings which might be relevant to my child's "carrier status" can be sent to his/her clinician for further, formal checks, where appropriate or discussed with me.			
Affected participants and Related Controls only I understand that tests carried out on my child's samples may identify abnormalities which do not relate specifically to his/her (or his/her close relative's) condition ("additional findings"). I understand that any results of this kind will not actively be looked for, and will not be passed back to his/her clinician or myself if found.			
Unrelated controls only I understand that my child's clinician and myself will receive no personal results back from the Study.			
	YES	NO	INITIAL ↓

Affected participants** and **Related Controls only If I am unable to receive the results for findings about my child's condition that may be relevant to other members of my family you can contact: Name: Address/email/phone: Relationship to me:			

Parent/Legal Guardian's name (Print)	
Signature:	
Date	

Researcher's name (Print)	
Signature:	
Date	

When completed: original for research site file, 1 copy for the participant; 1 copy in medical notes (patients only)

AFFIRMATION OF INFORMED CONSENT BY AN ILLITERATE PARENT OR LEGAL GUARDIAN OF THE PARTICIPANT (If suitable)

I, the undersigned, _____, have read and have explained fully to the parent or legal guardian of_____, named _____ the parental or

legal guardian information and informed consent document, which describes the nature and purpose of the study in which I have asked the child's parent/legal guardian to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The child's parent or legal guardian indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and that the withdrawal will not affect the child's current and future medical care.

I hereby certify that the parent or legal guardian has agreed for his/her child to participate in this study.

Parent/Legal Guardian's name (Print)	
Signature	
Date	

Investigator's name (Print)	
Signature	
Date	

Name witnessed (Print)	
Signature:	
Date	

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD):

A Systems Biology Approach in Neuromuscular or Neurogenetic diseases in South Africa

Funded by: 1. UK Government's Medical Research Council (MRC)
2. Guarantors of Brain (Registered Charity 264139)

Chief Investigator: Professor Michael Hanna
Institution: University College London Queen Square Institute of Neurology

Principal Investigator: Professor Izelle Smuts
Institution: University of Pretoria

Contact details:
Daytime number/s: 012 354 5303 or 076 383 2286
After hours' number: 076 383 2286
Email: icgnmduo@gmail.com

Short title: ICGNMD_UP Protocol number 296/2019 ICD8 Adults
May 2020_V2

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

day	**month**	**year**	**Time**

Dear Prospective Participant

Dear Mr. / Mrs. _____

1. INTRODUCTION

You are invited to volunteer for a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially.

2. WHAT IS GENETIC RESEARCH?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva, urine or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis). Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously

Figure C.9: University of Pretoria's adult consent

discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

3. WHAT IS THE PURPOSE OF THE STUDY?

- Neuromuscular Diseases (NMDs) affect at least 17 million children and adults globally.
- They include many different disorders affecting muscle and nerve function, and account for approximately 25% of all non-infectious neurological diseases.
- Complex genetic NMDs often present with problems outside of the muscles and nerves, particularly when they occur in childhood. These can include developmental delay, behavioral issues, seizures, impaired coordination, movement problems, and memory disturbance.

The aim of our research project is to identify genetic changes that cause neuromuscular and other neurogenetic diseases, and to improve our understanding of the clinical and genetic features of these conditions. We hope that this will allow us to work out better ways to diagnose and treat these disorders.

Up to 15,000 participants will be recruited globally at the participating centres, over a period of 5 years.

4. WHY HAVE I BEEN CHOSEN?

You have been chosen either because your doctor feels that your symptoms may be due to an underlying neuromuscular or neurogenetic disorder, or because you do not have symptoms suggestive of a neuromuscular or neurogenetic disorder and may or may not be related to an individual with symptoms suggestive of a neuromuscular or neurogenetic disorder. If you are a healthy person unrelated to someone with symptoms suggestive of a neuromuscular or neurogenetic disorder, your results will act as a "control" data, providing a baseline against which we will compare the findings from people with a neuromuscular or neurogenetic disorder.

Short title: ICGNMD_UP Protocol number 296/2019 ICD8 Adults
May 2020_V2

5. WHAT WILL HAPPEN TO ME IF I TAKE PART?

If you decide to participate in the study, you will be asked to sign the attached consent form before any of the study assessments can take place or any of your data or samples can be used for the study.

5.1. Participant with symptoms

If you are taking part in the study because you have symptoms compatible with an underlying neuromuscular or neurogenetic condition you might be asked to attend a one-off study visit, in addition to your routine clinic appointment, lasting approximately 1-2 hours. At the visit we will ask for your consent to collect the following data and undertake the following assessments:

5.1.1. Demographic data

We will collect your age, gender and ethnicity.

5.1.2. Medical History

Extract the clinical data from your medical notes and clinical assessments from past and future routine outpatient visits, so they can be used in this study.

5.1.3. Physical examination, including neurological examination

This will include a thorough clinical examination including strength testing, balance etc.

5.1.4. Questionnaires

You might be asked to complete some standard questionnaires that we use as routine for specific conditions.

5.1.5. Videos and photographs

This may include videoing you walk, your eye movements, speech etc. The video or photographs may be used in SA or abroad for teaching, training, research, diagnostic/treatment purposes, and/or publication in medical journals, books, as

lecture material. Images and videos will be securely stored. As this is optional, if you don't want to have a video or photos taken, you can still take part in the study.

5.1.6. Blood tests

Approximately 20 ml blood (3.5 teaspoons) for haematopoietic cells, plasma, serum and/or DNA isolation will be collected at a single visit. DNA isolation will be done using a standardised methodologies according to laboratory protocols. Blood may also be subjected to metabolic analyses at the NWU. A blood sample to test your DNA and/or RNA for possible changes in the genetic code will be taken. We will look at the genes in which genetic mutations may cause symptoms of neuromuscular or neurogenetic diseases. This may also include sequencing your entire genome. Your sample will be sent to North-West University (NWU) for initial sample preparation and then sent to the UK for analysis. You will be asked to donate your blood sample for future research as part of a rare disease biobank. If you consent to it, your blood sample will be stored indefinitely at NWU and will be used for future research. As this is optional, you don't have to consent to it to be in the study. Results of genetic tests performed on a research basis are not routinely made available. Should a result of interest be found, we are now able to validate research genetic results and you would be invited back for testing on a clinical basis. If you are not a patient at SBAH, we can arrange a follow up visit to discuss this, otherwise your doctor can be informed. Some insurance companies ask whether you have had gene studies to see if you suffer from hereditary disease or if you have undergone genetic testing. We keep details of any research results confidential.

We may also sent blood for special investigations routinely done in the diagnosis and follow up of the possible underlying condition suspected to assist us in making a diagnosis or assist in the decisions that need to be made in your treatment.

5.1.7. Saliva test

Approximately 2ml of saliva for DNA isolation will be collected, for example using an Oragene Saliva Kit.

5.1.8. Urine sample

A urine sample may be taken to develop new indicator of disease severity (biomarkers) or new factor contributing to disease symptoms.

5.1.9. Lumbar puncture to obtain CSF (if indicated)

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

5.1.10. Skin biopsy

You may have a skin biopsy taken if indicated. This may also be used to see how your skin cells work and give us more information about how your disease affects you. The cells will be looked at under a microscope and grown as fibroblast cells to investigate how they work differently. Cells obtained from the skin may also be manipulated in the laboratory and transformed into other cell types (e.g. muscle or nerve cells) to investigate the mechanisms that cause the disease. These types of cells are called "induced pluripotent stem cells", or iPSCs.

The skin biopsy procedure involves taking a small sample of skin usually taken from just above the ankle but can also be taken from other sites that your doctor selects (like the top of the thigh or forearm). Local anaesthetic will be given, and when the area is numb a small (3mm diameter) piece of skin will be removed using a small punch. No stitches are needed, and the wound heals over 7-10 days, generally becoming nearly invisible over a 4-6 weeks. This is a safe and widely used procedure but rarely side effects such as allergy to local anaesthetic, difficulties with healing, bleeding or skin infections may occur. Alternatively, if you have previously had a skin biopsy performed as part of routine clinical care some of this tissue may remain stored, and we may be able to re-examine this tissue and perform additional tests

on it to aid current and future research into mitochondrial disease without the need for a further skin biopsy.

5.1.11. EMG and NCV (if indicated)

Nerve conduction studies and electromyography will only be done if it has not been done before and clinically may contribute to confirm the clinical diagnosis of affected nerves and/or muscle.

5.1.12. Muscle ultrasonography (if indicated)

It is a painless procedure where ultrasound machine is used to get an impression of the quality of the muscle.

5.1.13. Muscle biopsy

A muscle and/or relevant tissue biopsy will be done in some cases if the blood tests were inconclusive and if a relevant condition e.g. mitochondrial disease is suspected. The biopsies will be taken under general anaesthetics by a qualified paediatric surgeon or adult neurologist. For a muscle biopsy a 3-4 cm incision will be made on the thigh and a small piece of muscle about 1 cubic cm will be removed for possible histology, biochemical and genetic analyses. DNA extraction will be performed at the NWU, but the genetic analyses will be performed in the UK and Netherlands

5.1.14. Existing blood and tissue samples

Use any leftover diagnostic samples collected during past or future routine visits such as urine, saliva, blood, muscle and skin biopsies in this study.

In some instances you may be examined at home or we may use letters from clinical colleagues. If you attend clinic at SBAH this part of the visit may be done in the context of your routine appointment. If you are not a patient at SBAH then we will arrange a research clinic visit at your convenience, or these details may be sent to us by your treating doctor

5.2. CONTROL PARTICIPANT

If you are taking part as a control participant (who may or may not be a relative of a participant with a neuromuscular or neurogenetic condition) you will be asked to attend a one-off study visit lasting approximately 1-2 hours. At the visit we will ask for your consent to collect the following data and undertake the following assessments:

5.2.1. Demographic data

We will collect your age, gender and ethnicity.

5.2.2. Medical History

Extract the clinical data from your medical notes and clinical assessments from past and future routine outpatient visits, so they can be used in this study.

5.2.3. Physical examination, including neurological examination

This will include a thorough clinical examination including strength testing, balance etc.

5.2.4. Questionnaires

You might be asked to complete some standard questionnaires that we use as routine for specific conditions.

5.2.5. Saliva test

Approximately 2ml of saliva for DNA isolation will be collected, for example using an Oragene Saliva Kit.

5.2.6. Blood tests

Approximately 20 ml blood (3.5 teaspoons) for haematopoietic cells, plasma, serum and/or DNA isolation will be collected at a single visit. DNA isolation will be done using a standardised methodologies according to laboratory protocols. Blood may also be subjected to metabolic analyses at the NWU.

A blood sample to test your DNA and/or RNA for possible changes in the genetic code will be taken. We will look at the genes in which genetic mutations may cause symptoms of neuromuscular or neurogenetic diseases. This may also include sequencing your entire genome. Your sample will be sent to North-West University (NWU) for initial sample preparation and then sent to the UK for analysis. You will be asked to donate your blood sample for future research. If you consent to it, your blood sample will be stored indefinitely at NWU as part of a rare disease biobank and will be used for future research. As this is optional, you don't have to consent to it to be in the study. Results of genetic tests performed on a research basis are not routinely made available. Should a result of interest be found, we are now able to validate research genetic results and you would be invited back for testing on a clinical basis. If you are not a patient at SBAH, we can arrange a follow up visit to discuss this, otherwise your doctor can be informed. Some insurance companies ask whether you have had gene studies to see if you suffer from hereditary disease or if you have undergone genetic testing. We keep details of any research results confidential.

5.2.7. Urine sample

A urine sample may be taken to develop new indicator of disease severity (biomarkers) or new factor contributing to disease symptoms.

5.2.8. Skin biopsy

You may have a skin biopsy taken if indicated. This may also be used to see how your skin cells work and give us more information about how your disease affects you. The cells will be looked at under a microscope and grown as fibroblast cells to investigate how they work differently. Cells obtained from the skin will also be manipulated in the laboratory and transformed into other cell types (e.g. muscle or nerve cells) to investigate the mechanisms that cause the disease. These types of cells are called "induced pluripotent stem cells", or iPSCs.

The skin biopsy procedure involves taking a small sample of skin usually taken from just above the ankle but can also be taken from other sites that your doctor selects (like the top of the thigh or forearm). Local anaesthetic will be given, and when the area is numb a small (3mm diameter) piece of skin will be removed using a small punch. No stitches are needed, and the wound heals over 7-10 days, generally becoming nearly invisible over a 4-6 weeks. This is a safe and widely used procedure but rarely side effects such as allergy to local anaesthetic, difficulties with healing, bleeding or skin infections may occur. Alternatively, if you have previously had a skin biopsy performed as part of routine clinical care some of this tissue may remain stored, and we may be able to re-examine this tissue and perform additional tests on it to aid current and future research into mitochondrial disease without the need for a further skin biopsy.

5.2.9. Existing blood and tissue samples

Use any leftover diagnostic samples collected during past or future routine visits such as urine, saliva, blood, muscle and skin biopsies in this study.

6. WHAT PROCEDURES WILL BE INVOLVED IN THIS RESEARCH?

The following procedures will be followed and the details are discussed in Section5:

- Informed consent and/or assent (all participants)
- Eligibility confirmation (all participants)
- Demographic data collection (all participants)
- Medical history (all participants)
- Family history (all participants)
- Blood test (all participants ≥ 16: optional for participants < 16)
- Urine or other painless biological sample (if required)
- Lumbar puncture to obtain CSF (if indicated)
- Skin biopsy (all participants: optional and only if required)

- EMG and NCV (if indicated)
- Muscle ultrasonography (if indicated)
- Muscle biopsy (if indicated)
- Recording photographs or videos of all or part of the body: optional and only if required
- Clinical phenotyping using Human Phenotype Ontology (HPO) (affected participants only). It is a standardised way to document symptoms and signs.

7. ARE THERE ANY RISKS INVOLVED IN GENETIC RESEARCH?

7.1 Blood tests

Blood tests do not pose a significant risk to your health. You may experience pain and/or bruising at the site on your arm where blood is taken. The use of a needle can carry some discomfort and a small risk of bruising, bleeding, fainting, and a very small risk of infection where the skin is broken. If you experience faintness you should lie down immediately to avoid possible injuries and then notify the study personnel.

7.2 Skin biopsy

The skin biopsy procedure involves taking a small sample of skin usually taken from just above the ankle but can also be taken from other sites that your doctor selects (like the top of the thigh or forearm). Local anaesthetic will be given, and when the area is numb a small (3mm diameter) piece of skin will be removed using a small punch. No stitches are needed, and the wound heals over 7-10 days, generally becoming nearly invisible over a 4-6 weeks. A biopsy care sheet will be given to you after the biopsy so you know how to care for the site. This is a safe and widely used procedure but rarely side effects such as allergy to local anaesthetic, difficulties with healing, bleeding or skin infections may occur.

Alternatively, if you have previously had a skin biopsy performed as part of routine clinical care some of this tissue may remain stored, and we may be able to re-examine this tissue and perform additional tests on it to aid current and future research into mitochondrial disease without the need for a further skin biopsy.

7.3 Muscle biopsy

Pain, bleeding or infection may occur, but it is very rare. I scar may also develop.

7.4 Other risks

To the best of our ability, the data we collect about you in this will remain confidential. However, the risk of a breach of confidentiality is always possible. We will minimize any risk by anonymising the data we collect. All published reports or public presentations will also be also be anonymised. Additionally, any data which can identify you will be kept completely confidential and stored in password protected folders and files that will only be accessible to the research team at SBAH.

8. ARE THERE ANY BENEFITS TO YOUR TAKING PART IN THIS STUDY AND WILL YOU GET TOLD YOUR RESULTS?

If we are able to confirm a diagnosis, you will be informed and specific treatment, if available in SA, will be offered to you.

If no specific diagnosis is made or if you are a control, there are no direct benefits to you if you take part in this research. We hope that this study will generate a greater understanding of neuromuscular and neurogenetic diseases by possibly identifying underlying causes. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients with such conditions.

Your personal results will be made known to you **only if they indicate** that you have or may:

- Have a definite risk for developing a particular disorder.
- Have a condition or predisposition to developing a condition that is treatable or avoidable e.g. by a lifestyle modification.
- Need genetic counselling.

9. HOW LONG WILL YOUR BLOOD BE STORED AND WHERE WILL IT BE STORED?

The samples we collect from you during the study visits will initially be stored at SBAH. It will then be transported to the Mitochondria Research Laboratory at NWU in Potchefstroom. The samples will be processed there, stored and some analyses will be done at the NWU. A part of the material will be sent to the UK and/or Netherlands for additional investigations that can only be performed overseas.

The cells in the blood may be grown to give us more information about how your disease affects you. The cells will be looked at under a microscope and grown as lymphoblast cells (a type of blood cell) to investigate how they work differently.

You will be able to give your consent to donate your samples as a gift so they can be retained and used for future research. If you consent to it specifically, your samples will also be stored in a rare disease biobank at Centre for Human Metabolomics, NWU, Potchefstroom and, in some cases, at the biobank of the Great Ormond Street Hospital, UK. Any new research will be reviewed by a research ethics committee, but consent for future studies may only be required if the committee considers that the study is likely to substantially affect your interests. If you already have DNA, RNA or lymphoblasts stored then we may be able to use these without the need for a further blood test.

10. IF YOUR BLOOD IS TO BE STORED IS THERE A CHANCE THAT IT WILL BE USED FOR OTHER RESEARCH?

Your blood and other specimens will only be used for genetic research that is directly related to NMDs and neurogenetic disorders in as outlined to find new changes and to understand the mechanism of the disease.

Stored blood samples, RNA/DNA, skin fibroblasts, and myoblasts may possibly be used in future research to facilitate the development of treatments for neuromuscular and neurogenetic diseases, but then additional permission to do so must be obtained form from the Faculty of Health Sciences Research Ethics Committee at the University of Pretoria.

If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

11. WILL YOU OR THE RESEARCHERS BENEFIT FINANCIALLY FROM THIS RESEARCH?

You will not be paid to take part in this study.

Important information: In the unlikely event that this research leads to the development of a commercial application or patent in future studies, you or your family will not receive any profits or royalties.

12. YOUR RIGHTS AS A RESEARCH PARTICIPANT

Your participation in this trial is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care.

13. WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

Results may be published in scientific journals and may be used as educational materials. Any of your data used in this manner would be anonymous. No identifying information would be released

14. WHAT IF SOMETHING GOES WRONG?

If you have any concerns about any aspects of this study you may discuss these with Prof Smuts. If you remain unhappy and wish to go further and complain formally about any aspect of the way you have been approached or treated during the course of the study, you can do this inform the HREC. Please quote the project number at the top this consent form.

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns of this study, the normal complaints mechanisms should be available to you.

15. ETHICS APPROVAL

This research study was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it. It will also be conducted according to international and locally accepted ethical guidelines for research, namely the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes.*

16. INFORMATION

If I have any questions concerning this study, I should contact Prof Smuts or Dr Rammego.

Daytime number/s: 012 354 5303 or 076 383 2286

After hours' number: 076 383 2286

Email: icgnmdup@gmail.com

17. CONFIDENTIALITY

All information obtained during the course of this study will be regarded as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001. This will ensure confidentiality of information so collected. Only the researcher will be able to identify you as participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of all your records will be kept in a locked facility at Department of Paediatrics, SBAH, and University of Pretoria.

CONSENT TO PARTICIPATE IN THIS STUDY

Name of Researcher: Prof I Smuts

Participant Identification Number for this study: _____

1: TAKING PART IN THE STUDY
This section is about the aspects of the study which are **not** optional. If you do not feel able to agree to them then you will not be asked to participate in the study.

I have read the information sheet dated _____ for this study. I have considered the information and my questions have been answered.

I understand that:

- My participation is entirely voluntary and that I am free to withdraw at any time without giving a reason, and without my medical care or legal rights being affected.
- I will not be identified in scientific publications or at conferences.
- I will not receive any profits if commercially valuable product(s) result from research using my data or samples.

I agree that:

- The data collected during the study and relevant sections of my medical notes (if applicable) may be looked at by my clinician and authorised members of their team, individuals from SBAH/UP, UCL from MRC(UK) from regulatory authorities and from NHS Trusts, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
- My data from my past or future clinical or diagnostic investigations can be used in this study (if applicable) and can be shared with approved international researchers working at other academic and government research institutes and also with approved commercial companies (excluding my name, contact details, date of birth, and other hospital numbers).

(Approved commercial companies are companies approved by the research leader who are developing novel therapeutics for neuromuscular diseases to receive as much information as possible so that therapies have international benefit.)

- My tissue samples can be used to obtain my DNA and RNA, which will be stored for the duration of the Study and will be used to generate Study data.
- My personal, medical and genetic data can be deposited in online, controlled-access scientific databases, and my data can be studied by authorised researchers from around the world.
- If I decide to withdraw from the study, the data already collected from me will continue to be used in the study. The Study Team will destroy any DNA and biological samples that they hold under the Study ethics. However, I understand that any cell lines (including iPS cells) that have been created from my tissue will not be destroyed and information about them will be retained.

Please initial here if you agree to take part in this study as control:	initials
Please initial here if you agree to take part in this study as affected individual:	initials

2: OPTIONAL PARTS OF THE STUDY	
This section is optional parts of the study. You can be in the study even if don't agree to them. Some of these aspects may not be relevant to you. You can ask your doctor or nurse if you are not sure which parts are relevant to you. **Please only initial the boxes that are relevant to you and if you agree.**	
I agree to my doctor being informed about my participation in this study (if applicable).	initials

I agree to have a skin biopsy (only applicable as discussed with the study doctor).	initials
I agree to have a muscle biopsy (only applicable as discussed with the study doctor).	initials
I agree that my tissue, in addition to my DNA and RNA, can be stored and used for research by the UK study team, including for the generation of cell lines (which may include induced pluripotent cell lines, or iPSCs).	initials
I agree that my DNA, RNA and other tissue (including cell lines, which may include iPSCs) can be shared with approved international researchers working at other academic and government research institutes and also with approved commercial companies.	initials
I agree that photographs and/or videos taken during clinic visits can be used in this study. I understand that their use might lead to me being personally identified by researchers who have access to my data.	initials
I agree that DNA and all tissue samples provided in this study (e.g. blood, urine, saliva samples and biopsies) and cell lines derived from these and may be stored indefinitely and used to support other research.	initials
I agree to be contacted personally by the research team in the future. I can be contacted by (please circle) e-mail phone	initials
3: RESULTS AND ADDITIONAL FINDINGS	
Affected participants only	YES NO

I agree that genetic research findings which might be relevant to my symptoms can be sent to my clinician for further, formal checks, where appropriate.	initials
Related controls only I agree that genetic research findings which might be relevant to my "carrier status" can be sent to clinician for further, formal checks, where appropriate.	YES NO initials
Affected participants *and* ***Related Controls only*** I understand that tests carried out on my samples may identify abnormalities which do not relate specifically to my (or my close relative's) condition ("additional findings"). I understand that any results of this kind will not actively be looked for, and will not be passed back to my clinician or myself if found.	initials
Affected participants *and* ***Related Controls only*** If I am unable to receive the results for findings about my condition that may be relevant to other members of my family you can contact: Name: Address/email/phone: Relationship to me:	YES NO initials
Unrelated controls only I understand that my GP and/or clinician and myself will receive no personal results back from the Study.	initials

PARTICIPANT INFORMATION AND ASSENT DOCUMENT FOR 7-18 YEARS FOR RESEARCH INVOLVING GENETIC STUDIES

International Centre for Genomic Medicine in Neuromuscular Diseases (ICGNMD):

A Systems Biology Approach in Neuromuscular or Neurogenetic diseases in South Africa

Funded by: 1. UK Government's Medical Research Council (MRC)

2. Guarantors of Brain (Registered Charity 264139)

Chief Investigator: Professor Michael Hanna

Institution: University College London Queen Square Institute of Neurology

Principal Investigator: Professor Izelle Smuts

Institution: University of Pretoria

Contact details:

Daytime number/s: 012 354 5303 or 076 383 2286

After hours' number: 076 383 2286

Email: icgnmdup@gmail.com

Figure C.10: University of Pretoria's child assent

Name of participant:	BLOCK CAPITALS
Signature:	
Date:	dd/mmm/yyyy

Received by:	BLOCK CAPITALS
Signature:	
Date:	dd/mmm/yyyy

Name witnessed (Print)	BLOCK CAPITALS	
Signature:		
Date	dd/mmm/yyyy	

When completed: original for research site file, 1 copy for the participant; 1 copy in medical notes (patients only)

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

day	**month**	**year**

:
Time

1. INTRODUCTION

My name Prof Smuts and my job is to do research on children with muscle and brain diseases. We want to know how children with these disease is the same as healthy children. I am going to explain this research to you and invite you to be part of this research study. You can choose whether or not you want to participate in this study. We have discussed this research study with your mom /dad/legal guardian and they know that we are also asking for your permission. If you are going to be part in this research, your mom/dad/legal guardian also has to agree. But if you do not wish to participate, you do not have to.

You may discuss anything on this form with your mom/dad/legal guardian or friends. You can decide whether to participate or not after you have talked it over. You do not have to decide immediately. There may be some words you don't understand or things that you want me to explain to you. Please ask me to stop at any time and I will explain.

2. WHAT IS RESEACRH?

Research is what we do to find new knowledge about subjects (and people). We use research studies to help us find more information about disease or illness. Research also helps us to find better ways of treating children who are sick.

3. WHAT IS THIS RESEARCH PROJECT ABOUT AND WHAT IS EXPECTED OF ME?

Children conditions affecting their muscles, nerves, brain and often also many other organs have diseases called neuromuscular (NMD's) or neurogenetic diseases. It is sometimes very difficult to confirm a diagnosis and understand what causes it. In this study we want to try and find the reason for the disease

in your cell where all the recipes for the processes in your body are. If we can find the mistake in the recipe that cause the disease, we will do further research to understand exactly why it happens. We call in genetic research and we get the cells with the recipes (DNA) in blood, saliva, urine, muscle or skin. We will ask you if we can examine you and ask you to allow is to take some in blood, saliva, urine, muscle or skin. Sometimes we may have to ask you for another test to find out what is the problem.

4. WHY HAVE YOU BEEN CHOSEN?

You are diagnosed with a muscle and or brain problem and we want to find out more about the reason why you got it and if we need to give you specific treatment. If you are healthy and not family to somebody with the disease your results will act as a "control" data, providing a baseline against which we will compare the findings from people with such a disease. If you have a brother or sister with the disease it can help us to compare your results with his/her results and try to understand more about the disease.

5. WHO IS DOING THE RESEARCH?

The doctors here at the Paediatric Neurology Clinic in SBAH, work together with other specialists in the country and overseas to try and find out how these diseases work. If we can find an answer it may help us to know how to treat them. It may also help to discover new medicine to help people with these diseases

6. WHAT WILL HAPPEN TO ME IN THIS STUDY?

We are going to ask your mom/dad/legal guardian questions about your symptoms, history and how you are. We will either see you in the clinic, but if you are very ill and we need to be admitted to the ward we will arrange it. You are going to be weighed on a scale and your height will be measured. You are going to be examined for clinical signs of muscle and brain problems, but it will not hurt. Your blood will be drawn by one of the doctor from a blood vein in your arm and it may hurt, but only for a short time. We will give your medication as usual or start medication if you need some to help you to become better.

7. CAN ANYTHING BAD HAPPEN TO ME?

It might be painful when we draw your blood or take a piece of skin or muscle, but the doctors need to get the samples so that it can be tested. Nothing bad can happen to you because of this research study. Three is a very small chance that other people may get to know who you are, but we try our best that it does not happen.

8. CAN ANYTHIN GOOD HAPPEN TO ME?

If we are able to find an answer, you will be informed and if there is other medicine that will help you, we will give it to you. If you are healthy nothing specific will happen to you, but your results may help other people.

9. ETHICS APPROVAL

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, Medical Campus, Tswelopele Building, Level 4-59, Telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee.

10. WILL ANYONE KNOW I AM IN THE STUDY?

Only the study personnel for the study will know that you are participating in the study.

11. WHO CAN I TALK TO ABOUT THE STUDY?

Prof I Smuts

Daytime number/s: 012 354 5303 or 076 383 2286

12. WHAT IF I DO NOT WANT TO DO THIS?

You do not have to participate in the study, even if your mom/ dad/ legal guardians have signed consent that you can participate.

You can also withdraw from the study at any time without getting in trouble.

You must just remember that you'll still be weighed and we need to draw blood, because it's part of the treatment at the ward or clinic.

13. CONSENT TO PARTICIPATE IN THIS STUDY

Do you understand this research study and are you willing to participate in it?	**YES**	**NO**
Do you understand that we will examine you, take blood and maybe other samples?	**YES**	**NO**
Has the researcher answered all your questions?	**YES**	**NO**
Do you understand that you can pull out of the study at any time without any one consequences?	**YES**	**NO**

You don't have to give us your answer now, take your time and read the rest of this form before you decide.

If you sign at the bottom it will mean that you have read this paper, and that you would like to be in this study.

	Your Name	**Person Obtaining Consent**	**Parent / Guardian / Nurse As Witness**
Name Please Print			
Signature			
Date			

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

Stellenbosch University Inherited Neuromuscular Disorders Registry and DNA Repository

REFERENCE NUMBER: B19/01/002

PRINCIPAL INVESTIGATOR: Dr Franclo Henning

ADDRESS:
Division of Neurology, Tygerberg Academic Hospital / Stellenbosch University

CONTACT NUMBER:
Work: 021 938 5500
Cell: 079 5240851

You are being invited to take part in an inherited neuromuscular disorders (NMDs) registry and DNA repository. Please take some time to read the information presented here, which will explain the details of this project. Please ask the registry staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the registry and/or DNA repository at any point, even if you initially do agree to take part.

This registry and DNA repository has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

Why have you been invited to take part?

You have been invited to take part in this registry and DNA repository because you (possibly) have an inherited neuromuscular disorder. These disorders affect muscles, the nerves in your arms and legs, or the junction between these nerves and your muscles, and are inherited. It is important to note that the fact that it is in an inherited disorder does not mean that one of your parents should be affected, as many disorders are inherited in a way where you need both genes for the specific protein to be affected in order to have the disorder. Each of the parents could therefore carry one abnormal gene without actually being affected, and only if their child inherits both abnormal genes (one from each parent) will he/she be affected. This is called an autosomal recessive pattern of inheritance.

What is the registry about?

A registry is a collection of clinical information from patients such as yourself, which we collect over time and store together in one place. This is not a trial of any new treatment or therapy, it is merely collecting the normal clinical information that we gather as part of your normal (routine) care. Types of information that we collect would be: details about your symptoms, our findings on clinical examination, and the results of any tests we would normally do, such as blood tests, genetic tests, muscle biopsy or nerve conduction studies.

We have a lot of reasons to keep a registry. Firstly, by keeping this information together we are able to monitor how our patients with inherited neuromuscular conditions are doing, and how well we are treating them. Secondly, we will be able to see if our patients with inherited neuromuscular conditions are different from patients in other parts of the country / world. Lastly, there is still a lot we don't know about inherited neuromuscular conditions such as yours. By keeping a registry we will be able to design and implement research projects that may help to answer some of these questions.

Health professionals will be able to access the information recorded in four ways – please tick the boxes if you agree to one, two, or all four of these options:

- ☐ The main team (led by Dr Henning) will be allowed to look at the information in order to make sure that we are treating patients appropriately at Tygerberg Hospital and to see how well our patients are doing (compared to national and international standards). This in essence is a form of quality control.
- ☐ Secondly, researchers may ask us to provide them with some of your information as part of a research project on a condition like yours. Before your information is released, the University's Human Research Ethics Committee and the doctors managing the registry must first approve the researcher's request. Please note that the researchers will not have access to any of your personal information, and will only be supplied with information relevant to their research project.
- ☐ Thirdly, information may be shared with local and international researchers as part of multicentre research collaborations. This is a "give and take" scenario where researchers from different universities and countries pool their research in order to get a better overall idea of the characteristics of conditions. An example of this would be if we decide to pool our findings with colleagues from a university in the UK, and analyse our combined findings. This is done because, in research, there is strength in numbers. In other words, the more participants we use to make conclusions, the more reliable the conclusions will be. Again, please note that these researchers will not have access to any of your personal information.
- ☐ Lastly, in line with our aims to follow international best practice for open data sharing between researchers, we would like to eventually make the information available to the wider research community. This will include information about the clinical findings (what we found on clinical examination, for example the degree of weakness, which muscle groups are affected etc.), as well as results of genetic analysis. This information will be uploaded into data archives that researchers can access to gain information about the clinical and genetic aspects of inherited disorders. Such databases are invaluable to help researchers decide whether newly discovered genetic variations may be responsible for a specific disorder. Because inherited disorders are not common, such data sharing greatly enhances the scientific community's knowledge about these disorders. Again,

Figure C.11: Stellenbosch University's consent

please note that this will not contain ANY information that would make it possible for you to be identified.

Currently, only patients seen at Tygerberg Hospital and our referring hospitals will be included in the registry.

It is important to note that your information will be de-identified before being stored in our registry. This means that all personal and identifying information, e.g. your name, date of birth, residential address, email address and details of family members will be removed). All your information will be stored under a registry number, and only the investigators at Tygerberg Hospital will have access to your personal identifying information, which will be stored separately and securely.

Once we have collected information from a number of patients, we may decide to publish some of this information as a way of sharing our findings with doctors or researchers in other centres. If we do so, the publication will not include any of your personalised information (i.e. it will be de-identified). By agreeing to be part of this registry, you are also agreeing to allow us to publish this information, provided there is none of your personal identifying information in it.

Additionally, we will occasionally contact you telephonically to assess how you are doing. By agreeing to be part of this registry, you are consenting to allow us to call you from time to time to see how you are doing.

What is the DNA repository about?

Genetic material, also called DNA, is found in every cell in the human body. Our genes determine what we look like, but abnormalities (also known as mutations) in our DNA may also lead to certain disorders, referred to as inherited disorders. Some disorders are fully explained by genetic abnormalities, while others are associated with the effects of multiple genes in combination with lifestyle and/or environmental factors (i.e. the genetic abnormality on its own does not cause the disorder). These disorders are called complex disorders.

The purpose of the DNA repository is for us to collect and store genetic DNA from people with inherited or complex disorders, in order to test the DNA for abnormalities that may cause, or predispose to, these disorders. This testing will most likely be done at a later stage, once DNA has been obtained from a sufficiently large group of people. Because of the cost of genetic analysis, it may be significantly cheaper to have at least some of the DNA testing done in an overseas laboratory as part of a large group of samples. If this is the case, you will <u>not</u> be responsible for the export and transport costs, and the costs will be covered by research funding.

It is important to take note of the following:

- If you agree to donate a DNA sample, approximately 5 ml (1 teaspoon) of blood will be taken from a vein in your arm in the standard manner. The blood will then be processed and the DNA will be extracted for storage.
- **The DNA will be stored in the Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University under the care of Dr Craig Kinnear.**
- **All DNA samples will** be de-identified and will be marked only with your registry number.
- Your DNA will be stored indefinitely.

- Your DNA will only be used for genetic research in the field of inherited neuromuscular disorders and no other conditions, e.g. cardiovascular disorders.
- You are under no obligation to disclose your participation in this genetic research to any individual or company, e.g. insurance company.
- Neither you nor the researchers will benefit financially from the genetic research.

Will I receive the results of my DNA analysis?

If you so desire, we can give you the results of the DNA analysis if and when they become available. However, before you would be able to receive the results, you would need to undergo proper explanation and counselling. There are a number of implications to be taken into account when making this decision. Firstly, on a personal level, receiving a genetic diagnosis has potential implications for employment, insurance, family planning and the possibility of stigmatization. Secondly, a genetic diagnosis has important implications for family members. For example, it is important to realise that a genetic diagnosis for yourself practically implies the same genetic diagnosis for children, siblings or parents who have the same symptoms. It is therefore important to realise that, if you decide to receive the results, you will also need to decide whether to disclose this to your family members.

Please indicate whether you would like to receive the results of genetic analysis, with appropriate genetic counselling, if and when they become available:

☐ YES
☐ NO

What will your responsibilities be?

Whenever you attend our clinic, we ask that you make it known to the doctors seeing you that you are a registry patient. You will not be required to come for extra visits or have extra tests performed, we will only be doing for you what we would normally do for patients with your condition.

Please also note, if you agree to join our registry and DNA repository, it does not mean that you are agreeing to join any future research project. If we do start a new research project for people with your condition, you may be invited to participate, and you will need to provide special consent before we include you in a project. This means that your participation in future research would be entirely voluntary, and separate from this registry. Any further studies will be explained to you in great detail, and the decision to participate will be entirely yours.

Will you benefit from taking part in this registry and DNA repository?

There will be no direct benefits to you should you agree to participate. We will, however, appreciate it if you decide to do so, as it will allow us to assess and improve our clinical service as a whole, as well as help us learn more about your condition.

Are there in risks involved in your taking part in this registry and DNA repository?

Drawing blood is a somewhat painful procedure, and there is a slight risk of prolonged bleeding from the puncture wound. This is, however, usually only a real risk in patients with bleeding abnormalities. We are committed to keep all names and personal

information a secret, but there is a very small possibility that someone may illegally gain access to this information.

If you do not agree to take part, what alternatives do you have?

If you do not agree, you will not be biased against in any way. We will still provide you with same care we would normally provide.

Who will have access to your medical records?

All information collected will be treated as confidential and protected. If it is used for research, a publication or a thesis, your identity will remain unknown (de-identified). Only investigators will be allowed access to your information.

As stated above, only the main team of doctors and researchers (who have fulfilled all the requirements we stated in this document - see the tick boxes on page 2) will be able to look at the information. Additionally, members of the Stellenbosch University HREC (Health Research Ethics Committee) may need to inspect research records to monitor compliance.

How long will my records and/or DNA be kept, and can they be deleted or destroyed?

Your records will kept indefinitely by our division, however if you would like your information removed from the registry or your DNA sample withdrawn, you may contact the investigators at any time and request the removal. You may do so verbally or in writing. Please keep in mind that although your information will be removed from the registry, information that has already been used for research purposes (e.g. information published in a medical journal as part of a study) will remain accessible to people who read these medical journals. However, as stated above your identity will not be known as this information will be de-identified.

Will you be paid to take part in this registry and DNA repository and are there any costs involved?

No, you will not be paid to take part in this registry, and there will be no additional costs involved for you, if you do take part. As per normal, you will still be required to settle any hospital bills (if applicable) for the care you receive. If, for any reason, you are requested to attend hospital for a visit for research purposes and not as part of your standard care, you will be compensated for your time, inconvenience and expenses.

Is there any thing else that you should know or do?

- You can contact **Dr Franclo Henning** at telephone **021 938 5500** if you have any further queries or encounter any problems.
- You can contact the **Health Research Ethics Committee at 021-938 9207** if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …… agree to take part in the **Stellenbosch University Inherited Neuromuscular Disorders Registry and DNA repository** and I give consent:

- to allow my clinical information to be collected as part of the registry
  YES☐ NO☐
- that my records may be stored indefinitely
  YES☐ NO☐
- to donate a DNA sample for storage and future research.
  YES☐ NO☐
- that my DNA sample may be stored indefinitely.
  YES☐ NO☐
- that some of my DNA may be sent overseas for analysis. This sample will be destroyed when analysis is complete.
  YES☐ NO☐
- to be contacted telephonically from time to time by the registry team, if necessary
  YES☐ NO☐
- to be contacted if a new research project becomes available for which I may be eligible
  YES☐ NO☐

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this registry and DNA repository is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the registry or withdraw my DNA sample at any time and will not be penalised or prejudiced in any way.

Signed at (*place*) ……. on (*date*) ……. 2005.

……
Signature of participant

Declaration by investigator

I *(name)* …… declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the registry and DNA repository, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ..... on (*date*) ..... 2005.

.....
Signature of investigator

Declaration by interpreter

I (*name*) ..... declare that:

- I assisted the investigator (*name*) ..... to explain the information in this document to (*name of participant*) ..... using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... on (*date*) .....

.....
Signature of interpreter

Inherited NMD ICF ENG V4, 12 Sep 2019

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C.2 PARTICIPANT RECRUITMENT AND SAMPLING

C.2.1 MADD study cohort

This study was designed with the purpose of characterising the clinical, metabolic, genetic (*in vivo*), and functional (*in vitro*) aspects of MADD in a Southern African cohort. Patients were recruited retrospectively or prospectively based on their medical records, as described in Section 3.4.1. In addition, a project awareness campaign was launched to promote the study to a larger and more diverse audience. This included a public interest article and advertisement in the electronic magazine of Rare Disease South Africa (RDSA)—a non-profit organisation advocating for and providing support to individuals affected by IEM in SA (Bisschoff, 2022; RDSA, 2023). The study was also promoted on the website of PLIEM and the NWU, the social platforms of PLIEM and RDSA, as well as healthcare providers who are registered or in contact with RDSA, PLIEM, and the Southern African Society for Human Genetics (SASHG) (NMP, 2023; RDSA, 2023; SASHG, 2022). During the recruitment process and prior to informed consent/assent, potential participants were informed about the study, its aims and objectives, as well as participant expectations. Individuals of all ages, genders, and self-reported ethnicities were allowed to consent/assent, and eligibility for inclusion included a Southern African nationality and clinical and metabolic diagnosis of

MADD. By contrast, individuals with a known HIV positive status were excluded from the study, as the human immunodeficiency virus (HIV) infection and antiretroviral treatments are known to mimic a variety of neurological symptoms (McArthur *et al.*, 2005)[37].

Informed consent (adults and parents/guardians) or verbal assent (children) was obtained either electronically or in person for each proband and their affected or unaffected first-degree relatives (the latter were included for genetic confirmation purposes). Hereafter, deidentified personal and demographic details, family history, and medical history were collected and entered on the secure ICGNMD REDCap platform for each proband and their affected first-degree relatives, by the paediatric/adult neurologist(s) from the relevant centre (Harris *et al.*, 2019). This was followed by a comprehensive physical assessment and the collection of new samples. It should be noted that consent/assent also included permission for the use of pre-existing samples, where available. Sample types and their use in this study are shown in Table C.1.

Table C.1: Sample types used in this study and their purpose

Sample type	Pre-existing/new	Participant status	Analysis	Objective
Whole blood (option 1)	New	Living	Genetic	1.2–1.5
Saliva (option 2) †	New	Living		
Urine	Pre-existing	Deceased		
Urine (untreated patients)	Pre-existing	Living/deceased	Metabolic	1.2
Urine (treated patients)	New/pre-existing	Living/deceased		
Skin fibroblasts	New	Living	Functional	2.1–2.3

† P10 was the only patient who opted to give a saliva sample instead of whole blood for genetic analyses.

C.2.2 Allele frequency estimation

To estimate the allele frequency of selected *ETFDH* variants in SA, (i) DBS samples and (ii) isolated DNA were used, respectively. The samples included an equal distribution of healthy male and female newborns and were selected by randomisation. DBS cards (stored in the CHM Biobank) were initially collected via heel prick for the purpose of NBS at the CHM, while the DNA samples were extracted from umbilical cord DBSs of newborns who had been included in the thyroid NBS program of the Red Cross War Memorial Children's Hospital. It should be noted that all samples were originally tested at request of the parents/guardians and not referred for NBS by a healthcare professional on the suspicion of an IEM.

[37] HIV status was confirmed verbally, and HIV testing was not carried out at the point of sample collection.

Accordingly, all included samples tested negative for IEMs. As per the stipulations of the specific ethics application of each facility, reconsent for the purpose of this study was not required. As discussed in Sections 3.4.4 and 3.7, samples were collected from the four largest population groups in SA, as shown in Table C.2.

Table C.2: Number of samples collected from each of the four largest population groups in SA.

Sample type	Represented geographical region	Centre	Population group	s (c.[1067G>A])	s (c.[1448C>T])
Heel prick DBS cards	Northern SA	NWU	• African	750	750
			• White SA	750	750
			• Mixed	594	594
			• Indian	750	750
Umbilical cord DBS DNA	Southern SA	UCT	• African	258	729
			• Mixed	481	735

Abbreviations: DBS: dried blood spot; DNA: deoxyribonucleic acid; NWU: North-West University; s: number of available samples; SA: South Africa; UCT: University of Cape Town.

C.3 DNA ISOLATION AND WHOLE EXOME/GENE PANEL SEQUENCING

C.3.1 DNA isolation

Depending on the type of sample collected/available, DNA was isolated using either the FlexiGene® DNA kit (for whole blood or urine; 51206, Qiagen Science, Hilden, DE) or Quick-DNA™ Miniprep Plus kit (for saliva; D4069, Zymo Research, Inqaba Biotec, Pretoria, SA) according to the manufacturer's instructions. For those participants from whom only urine was available, samples were prepared as follows: Urine pellets were collected from 4 mL of sample following centrifugation (4,000 ×*g*, 10 min). The pellets were resuspended in 5 mL of 5 mM EDTA disodium salt dihydrate (1644, Sigma Aldrich) dissolved in nuclease-free water, to aid the solubilisation of salts and contaminants (Sokup, 2011). Following steps 1–3 of the "Isolation of DNA from 1–2 × 10^6 Cultured Cells" protocol (FlexiGene® DNA Handbook, 1080350 01/2014), the residual particulates were pelleted by centrifugation (15,000 ×*g*, 1.5 min) and discarded. The remainder of the steps proceeded according to the manufacturer's instructions and DNA was eluted following overnight incubation at 22°C.

C.3.2 Sequencing

WES analysis (conducted at Macrogen Europe, Amsterdam, NL) and bioinformatics (performed at UCL) were conducted on the isolated DNA of probands P1, P2, P5, and P9–P13 as described by Wilson *et al.*

(2023) (WES analysis was not performed for P3 and P4, as these patients are relatives of P2). The mean coverage, suspected clinical phenotype, and PanelApp[38] applied upon consideration of the clinical symptoms are shown in Table C.3 (Martin *et al.*, 2019). Variants identified by WES analysis and relevant to the patient phenotype, are listed in Table C.4.

Table C.3: WES and Bioinformatics criteria

Patient	Gender	ICGNMD centre	Mean Coverage	Phenotype	PanelApp applied
P1	Male	SBAH	37,06	MM	• Rhabdomyolysis and metabolic muscle disorders_1.57
P2	Female	SBAH	52,30	MM	• Rhabdomyolysis and metabolic muscle disorders_1.57
P5	Female	SBAH	50,81	MM	• Rhabdomyolysis and metabolic muscle disorders_1.57
P9	Male	SBAH	47,93	MM	• Mitochondrial disorders_2.54
P10	Female	SBAH	41,99	MM	• Mitochondrial disorders_2.54
P11	Male	SBAH	49,97	MM	• Mitochondrial disorders_2.54
P12	Female	SBAH	51,78	MM	• Rhabdomyolysis and metabolic muscle disorders_1.57
P13	Female	TBH	58,27	MM	• Congenital muscular dystrophy_2.2 • Congenital myopathy_2.6 • Undiagnosed metabolic disorders_1.486

Abbreviations: ICGNMD: International Centre for Genomic Medicine in Neuromuscular Diseases; MM: metabolic myopathy; P: patient; SBAH: Steve Biko Academic Hospital; TBH: Tygerberg Hospital.

Table C.4: WES results of probands P1, P2, P5, and P9–P13

Patient	Gene	HGVSc	HGVSp	GT	DP	Max AF
P1	*ETFDH*	ENST00000511912.6: c.[1067G>A]	ENSP00000303552.5: p.[Gly356Glu]	0/1	37	n.a
	ETFDH	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	0/1	43	0,0001163
P2	*ETFDH*	ENST00000511912.6: c.[287dupA*]	ENSP00000303552.5: p.[Asp97Glyfs*24]	0/1	62	n.a
	ETFDH	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	0/1	75	0,0001163
P5	*ETFDH*	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	1/1	67	0,0001163
P9	*ETFDH*	ENST00000511912.6: c.[1067G>A]	ENSP00000303552.5: p.[Gly356Glu]	0/1	43	n.a
	ETFDH	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	0/1	66	0,0001163
P10	*ETFDH*	ENST00000511912.6: c.[1067G>A]	ENSP00000303552.5: p.[Gly356Glu]	0/1	44	n.a
	ETFDH	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	0/1	52	0,0001163
P11	*ETFDH*	ENST00000511912.6: c.[1067G>A]	ENSP00000303552.5: p.[Gly356Glu]	0/1	37	n.a
	ETFDH	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	0/1	69	0,0001163
P12	*ETFDH*	ENST00000511912.6: c.[976G>C]	ENSP00000303552.5: p.[Gly326Arg]	0/1	36	n.a
	ETFDH	ENST00000511912.6: c.[1067G>A]	ENSP00000303552.5: p.[Gly356Glu]	0/1	60	n.a
P13	*ETFDH*	ENST00000511912.6: c.[740G>T]	ENSP00000303552.5: p.[Gly247Val]	0/1	40	0,0000088
	ETFDH	ENST00000511912.6: c.[1448C>T]	ENSP00000303552.5: p.[Pro483Leu]	0/1	83	0,0001163

Symbols and abbreviations: /: unphased genotype; AF: allele frequency; DP: depth; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; GT: genotype; HGVS: human genome variation society nomenclature;

[38] PanelApp is a curated and crowdsourced resource which was used by the ICGNMD to develop standardised virtual gene panels to identify those genes in which variants would be relevant considering the observed phenotype (Martin *et al.*, 2019).

HGVSc: nucleotide change (according to HGVS) aligned to the coding deoxyribonucleic acid reference sequence; HGVSp: amino acid change (according to HGVS) aligned to the protein reference sequence; Max AF: maximum allele frequency (gnomAD v2.1.1); n.a: not applicable; P: patient.

Following a clinical and metabolic diagnosis of MADD, genetic analysis for proband P14 was performed via Invitae (California, USA) by NGS using a gene panel to evaluate 27 genes associated with fatty acid oxidation disorders. Figure C.12 depicts the results obtained from the gene panel. The sequencing data displays all variants encountered in the 27 genes. Once compared to the patient's phenotype and metabolic profile, the only variants of relevance were those detected in *ETFDH*. Since MADD is an autosomal recessive disorder, variants occurring in *ETFDH* can only be considered potentially causative if encountered as a homozygous or compound heterozygous genotype. This was the case for all the probands sequenced, thereby confirming the clinical and metabolic diagnoses of MADD.

GENE	VARIANT	ZYGOSITY	VARIANT CLASSIFICATION
ETFDH	c.1448C>T (p.Pro483Leu)	homozygous	PATHOGENIC
CPT2	c.1838G>C (p.Gly613Ala)	heterozygous	Uncertain Significance
HADHA	c.785A>C (p.Lys262Thr)	heterozygous	Uncertain Significance

Figure C.12: Gene panel sequencing results of proband P14

The homozygous *ETFDH* c.[1448C>T] variant corresponds to the phenotype and metabolic profile of the patient. Abbreviations: *CPT2*: carnitine palmitoyltransferase 2 gene; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; *HADHA*: hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha gene.

C.4 SEQUENCE VALIDATION AND SEGREGATION ANALYSES

To confirm the presence of the *ETFDH* variant(s) detected by WES or gene panel sequencing of each proband, Sanger sequencing of the forward (5'–3') and reverse (3'–5') DNA strands of PCR-amplified regions was conducted at CAF (Stellenbosch, SA). The process entailed the following: First, the region of DNA spanning each variant was amplified, as described in Section 4.2.3 and successful amplification was confirmed by agarose gel electrophoresis. Next, the PCR amplicons were cleaned up using the AMPure XP bead-based reagent (A63882, Beckman Coulter, Centurion, SA) as per the manufacturer's instructions. Cycle sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing kit (4337458, Applied Biosystems, Massachusetts, USA) along with the applicable primer pairs listed in Table 4.2,

according to the manufacturer's instructions. Hereafter, samples were cleaned up via SDS-treatment and application to a Sephadex® G-50 fine column (17004203, Cytiva), after which the purified DNA was sequenced on a 3730xl DNA Analyzer (A41046, Applied Biosystems).

Data processing entailed Sanger trace alignment of the .abi files to the *ETFDH* gene of the human reference genome (Build GRCh38.p13) via the SAGE alignment tool (Tracy v0.7.5; GEAR-Genomics, 2023; Rausch *et al.,* 2020). To determine the segregation of each variant, Sanger sequencing of the specific variant(s) was also performed on the DNA of each parent (and siblings), where available, and the sequencing data, listed in Table C.5, was compared to that of the proband.

Table C.5: Sanger sequencing results of probands and their first-degree relatives

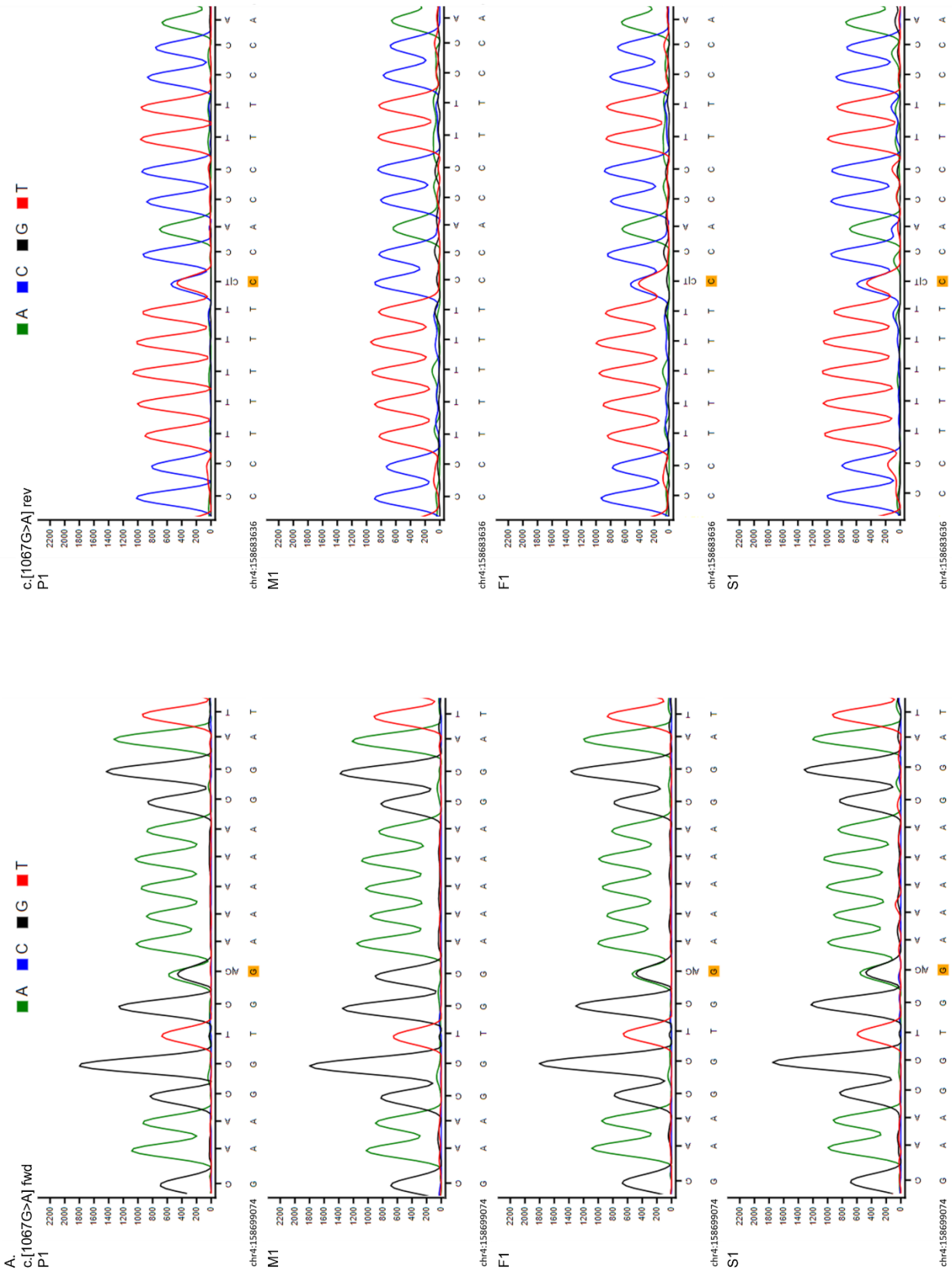
Proband	Relative	Figure	*ETFDH* variant 1	*ETFDH* variant 2
P1		C.13.a–b	**c.[1067G>A];**	**c.[1448C>T]**
	M1		-	c.[1448C>T]
	F1		c.[1067G>A]	-
	S1		c.[1067G>A]	-
P2		C.13.c–d	**c.[287dupA*];**	**c.[1448C>T]**
	P4 (mother)		c.[1448C>T];	c.[1448C>T]
	F2		c.[287dupA*]	-
	P3 (sibling)		c.[287dupA*];	c.[1448C>T]
P5		C.13.e	**c.[1448C>T];**	**c.[1448C>T]**
P6		C.13.i	**c.[1067G>A];**	**c.[1067G>A]**
	M6		c.[1067G>A]	-
	F6		c.[1067G>A]	-
P7		C.13.j–k	**c.[1067G>A];**	**c.[1448C>T]**
	M7		c.[1067G>A]	-
	F7		-	c.[1448C>T]
	S7		c.[1067G>A]	-
P8		C.13.l	**c.[1067G>A];**	**c.[1067G>A]**
	M8		c.[1067G>A]	-
	F8		c.[1067G>A]	-
	S8		c.[1067G>A]	-
P9		C.13.m–n	**c.[1067G>A];**	**c.[1448C>T]**
	M9		-	c.[1448C>T]
	F9		c.[1067G>A]	-
	S9		-	-
P10		C.13.o–p	**c.[1067G>A];**	**c.[1448C>T]**
	M10		c.[1067G>A]	-
	F10		-	c.[1448C>T]
P11		C.13.q–r	**c.[1067G>A];**	**c.[1448C>T]**
	M11		-	c.[1448C>T]
	F11		c.[1067G>A]	-
P12		C.13.s–t	**c.[976G>C];**	**c.[1067G>A]**
	M12		-	c.[1067G>A]
	F12		c.[976G>C]	-
P13		C.13.f–g	**c.[740G>T];**	**c.[1448C>T]**
P14		C.13.h	**c.[1448C>T];**	**c.[1448C>T]**

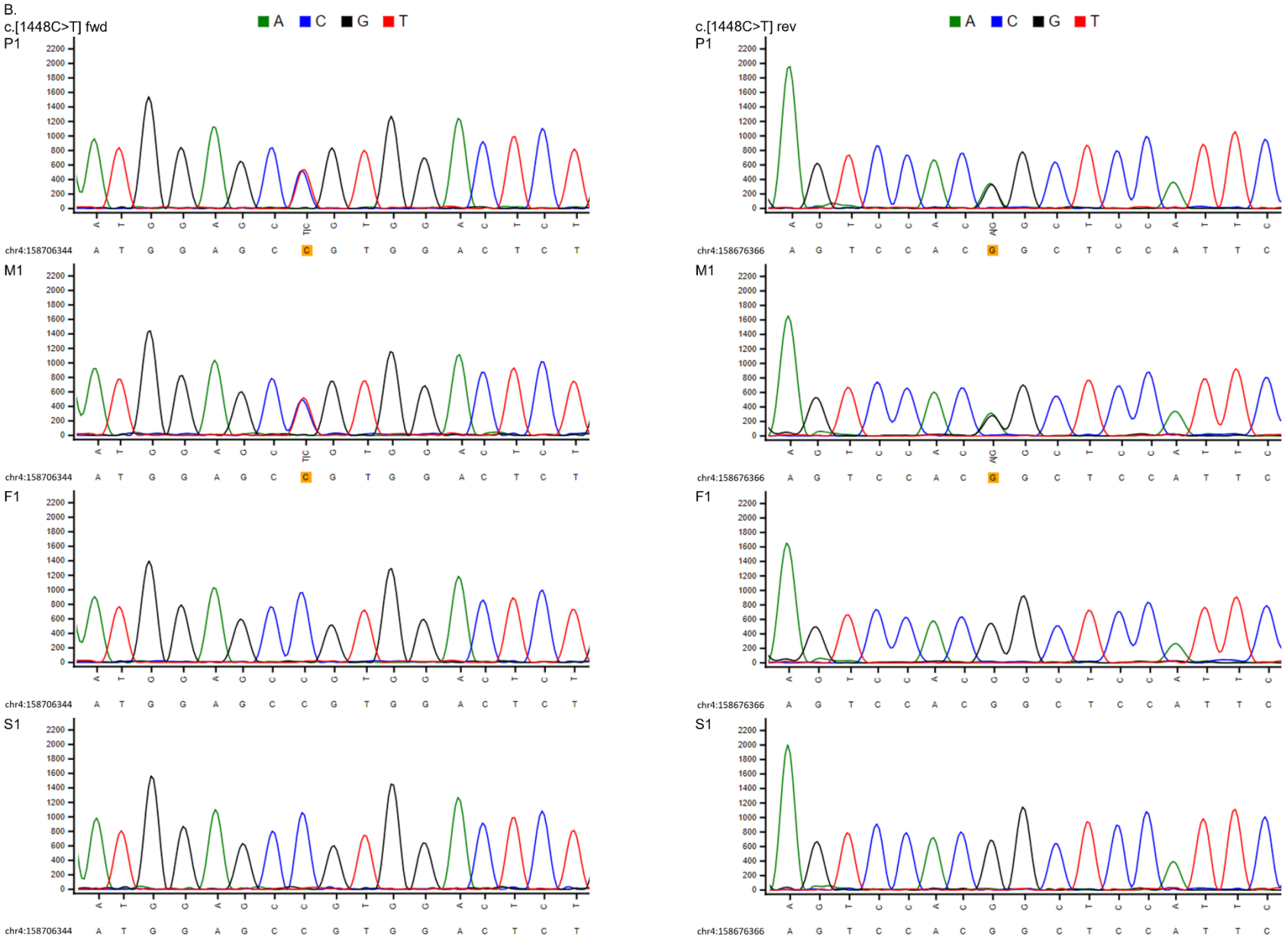
Symbols and abbreviations: *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; F: father; M: mother; P: patient; S: sibling.

The electropherograms of each family are depicted in Figures C.13.a–t and confirm the presence of the *ETFDH* variants identified by WES and gene panel sequencing (Appendix C.3). In cases where the proband is homozygous, the variant is present on one allele of each parent, whereas the parents of compound heterozygous probands each carry one of the two variants on a single allele. While no DNA was available for P7, it was possible to provide the most likely genetic diagnosis (homozygous *ETFDH* c.[1067G>A];c.[1067G>A]) via the Sanger sequence data of her parents (both heterozygous for *ETFDH* c.[1067G>A]). Likewise, Sanger sequencing enabled the retrospective diagnosis of P4 (homozygous *ETFDH* c.[1448C>T];c.[1448C>T]), who had not previously been diagnosed clinically with MADD.

Figure C.13.a–t: Electropherograms of probands and their first-degree relatives

See caption after Figure C.13.t

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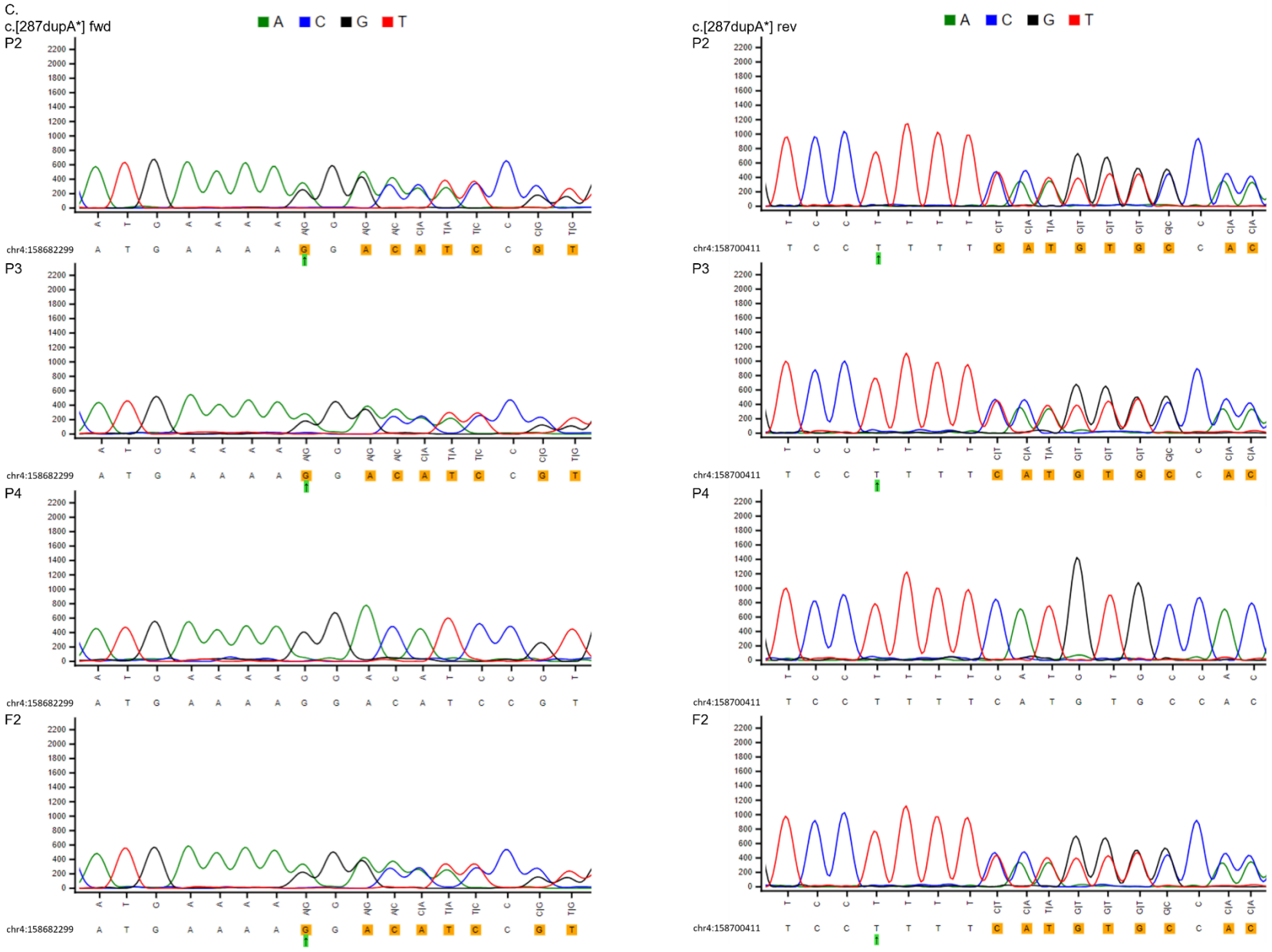
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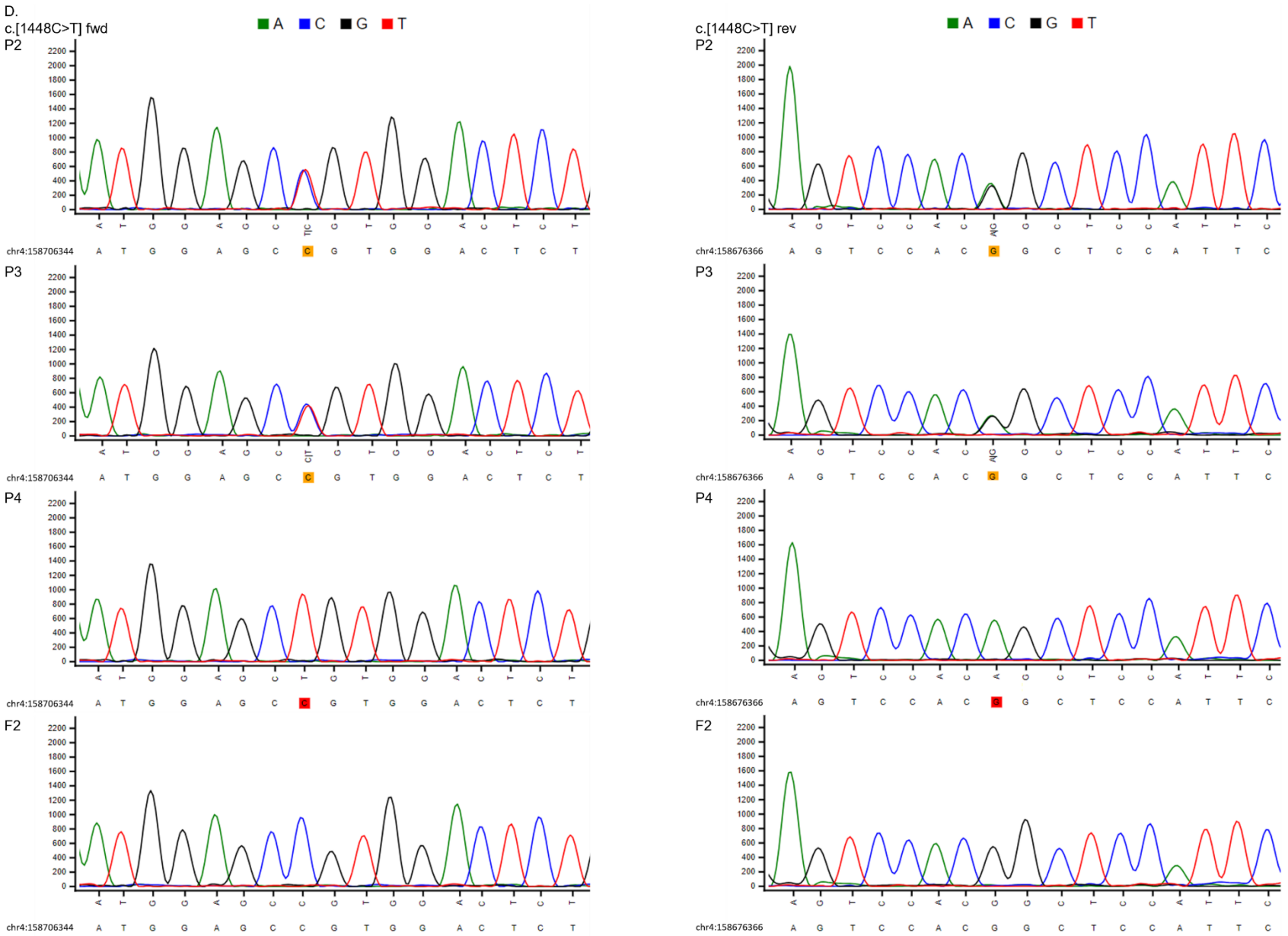
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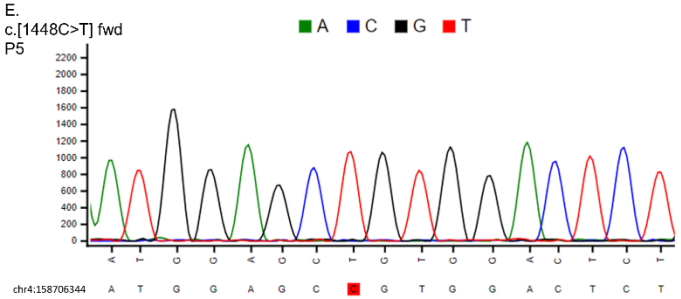

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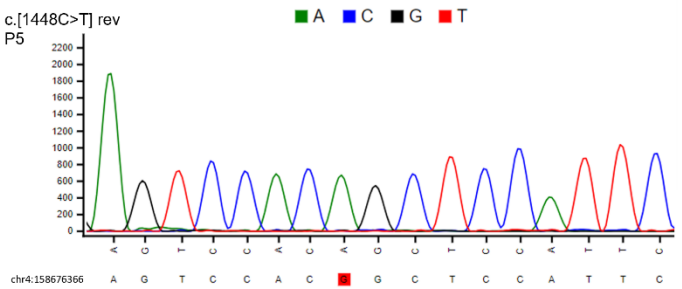

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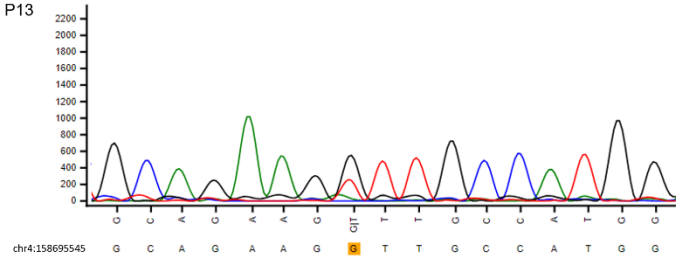

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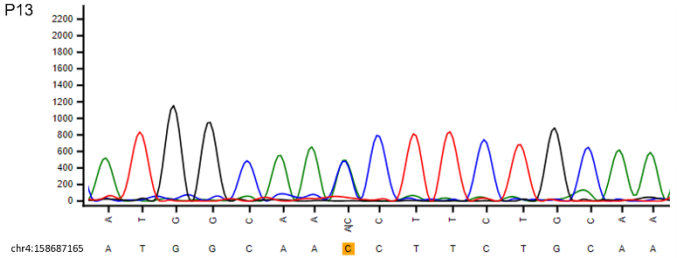

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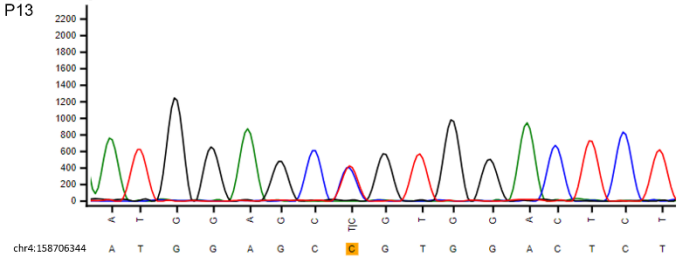

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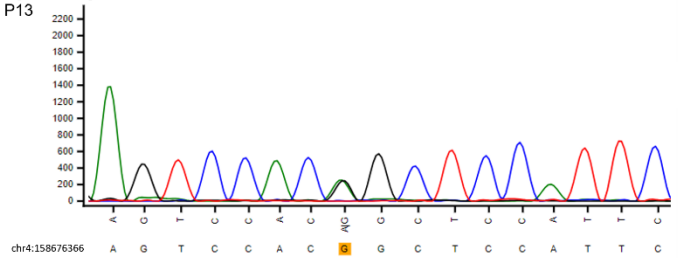

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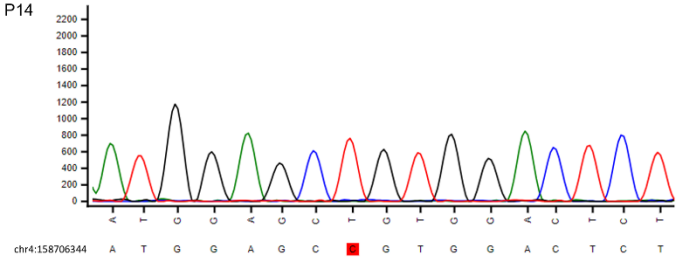

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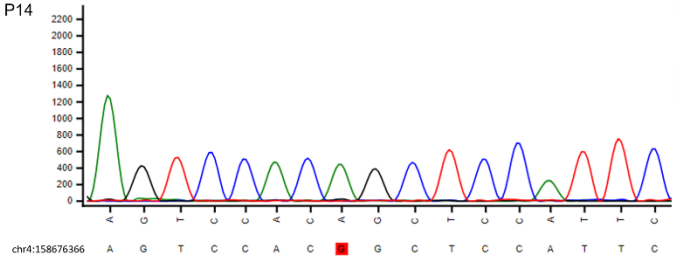

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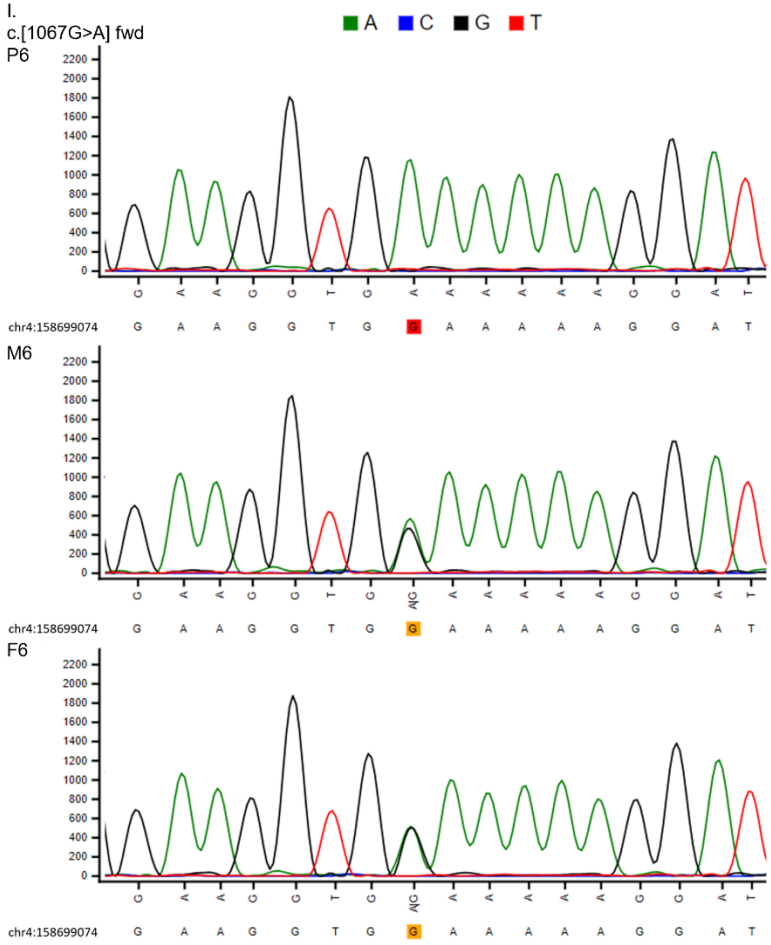
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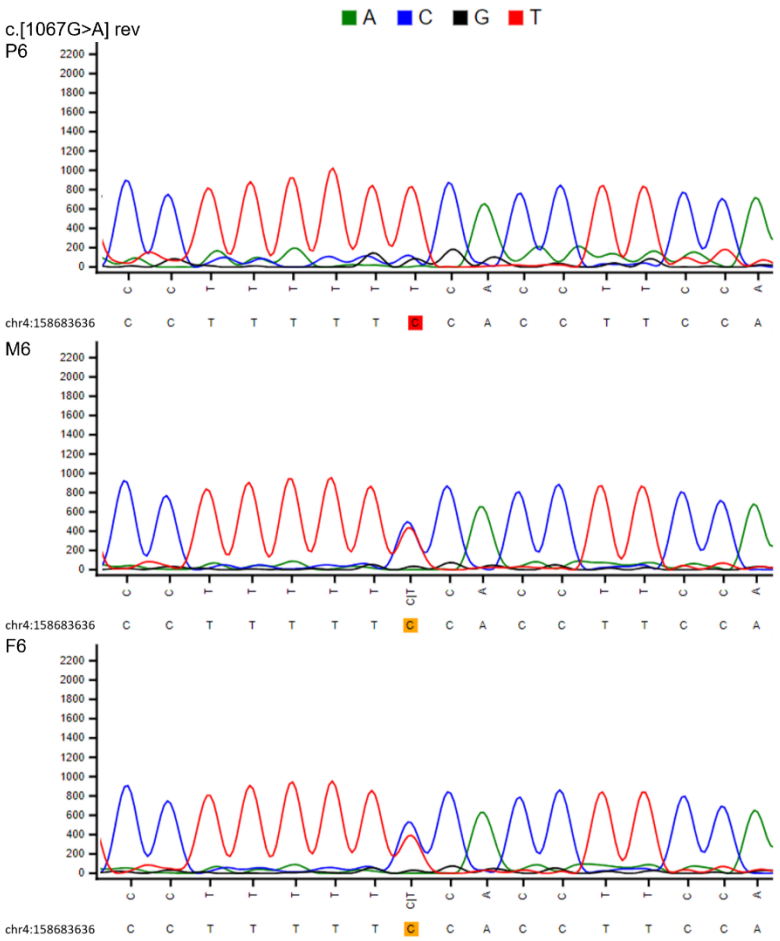
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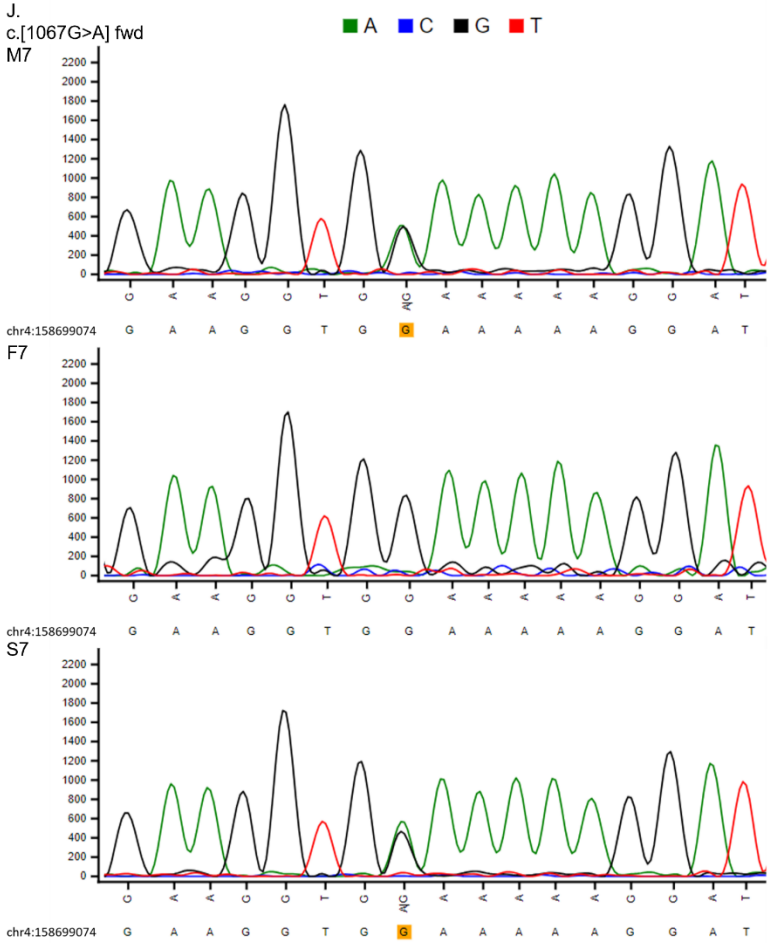
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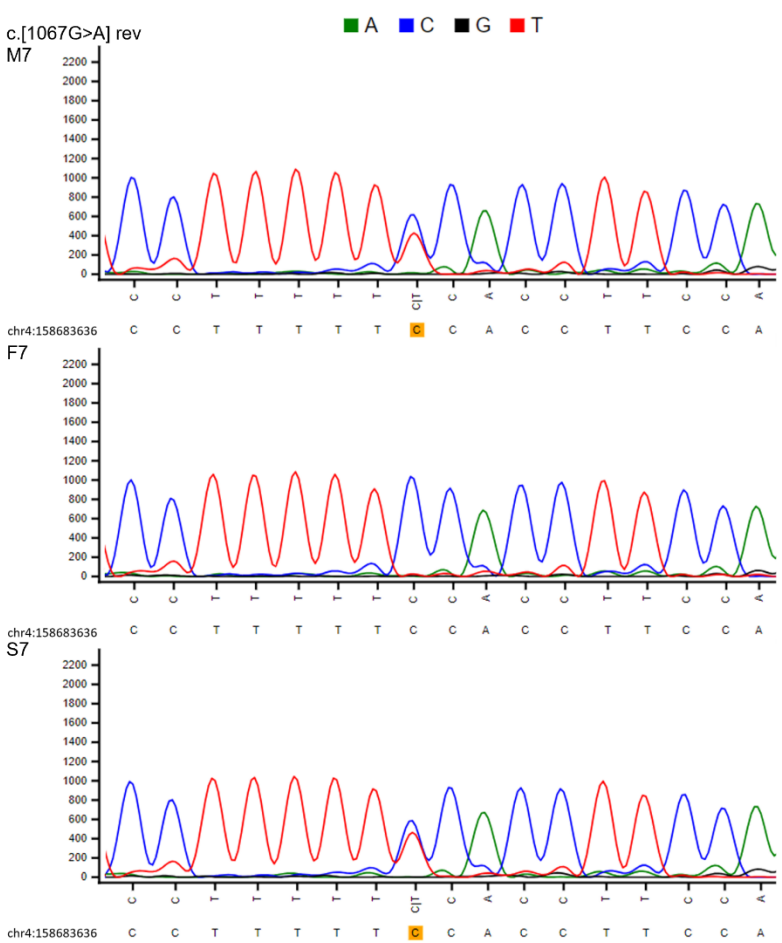
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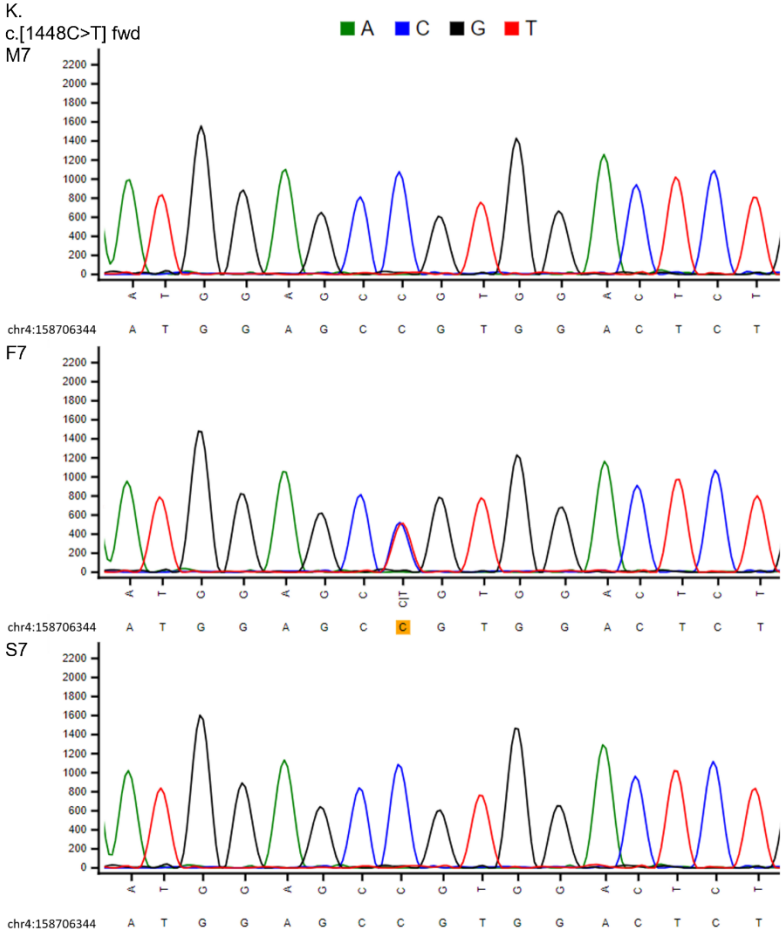
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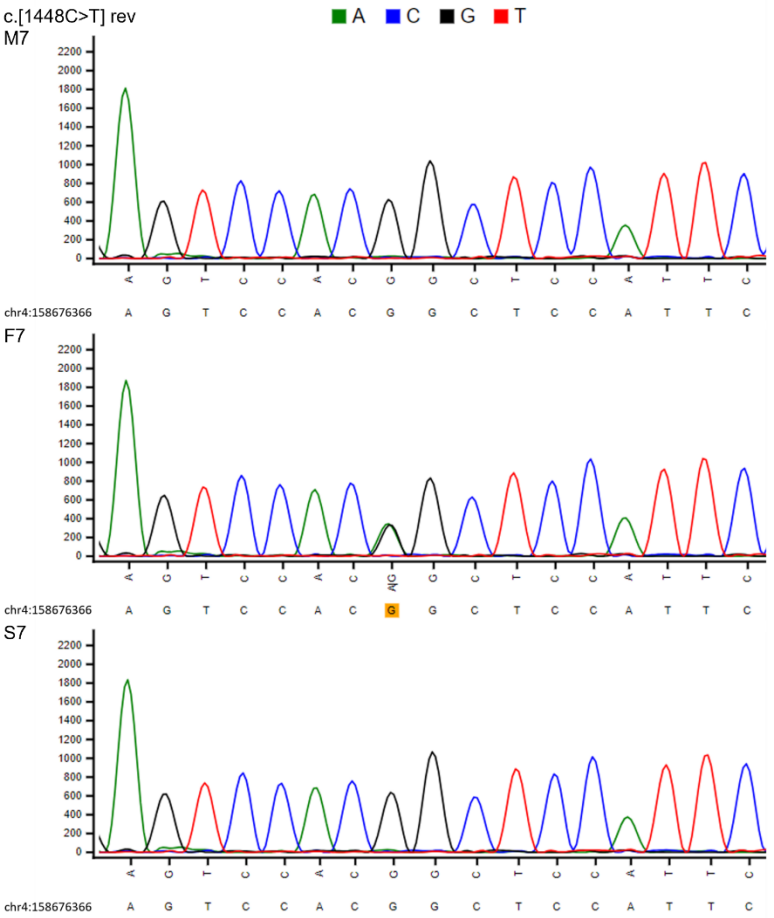
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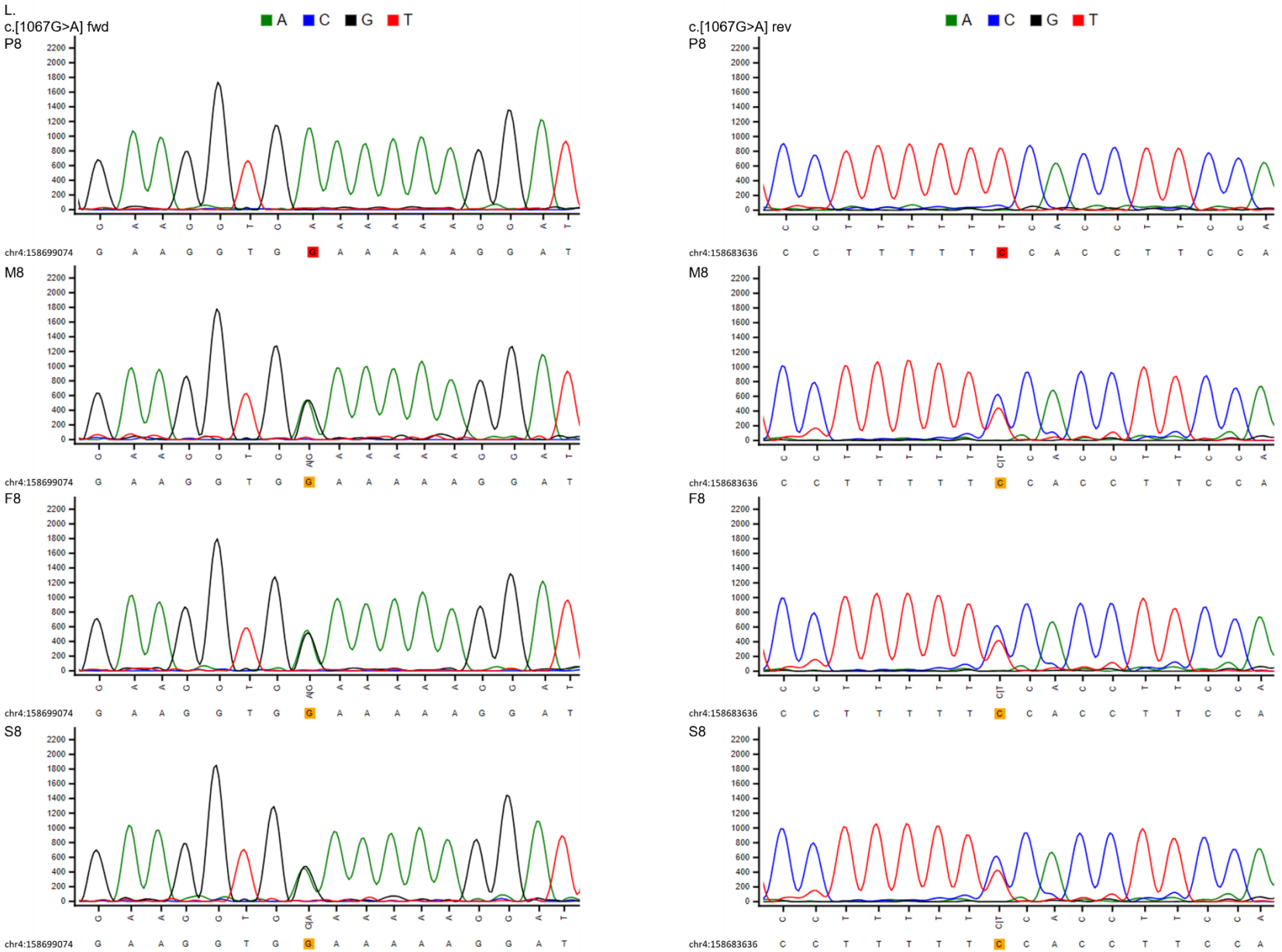
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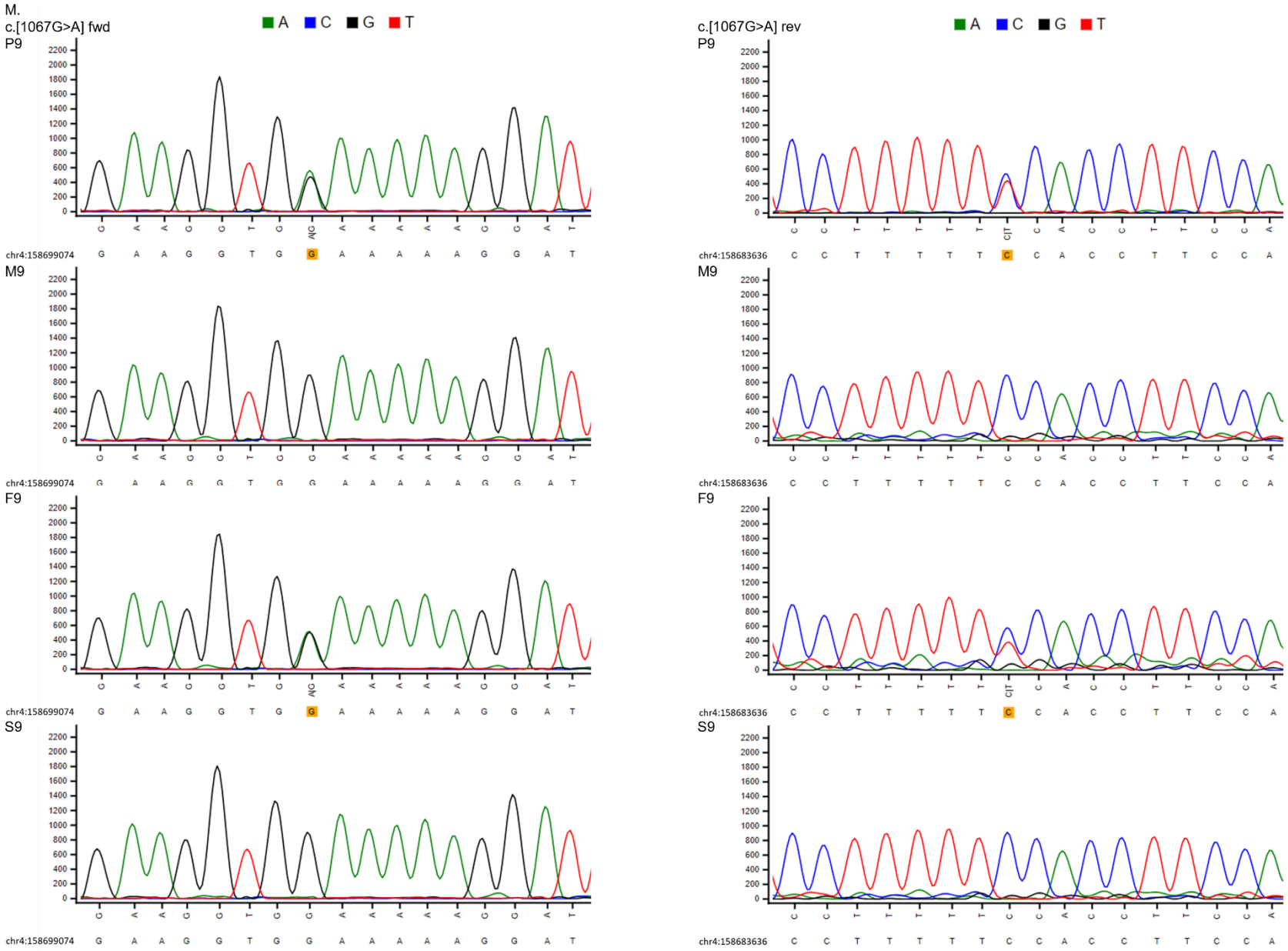
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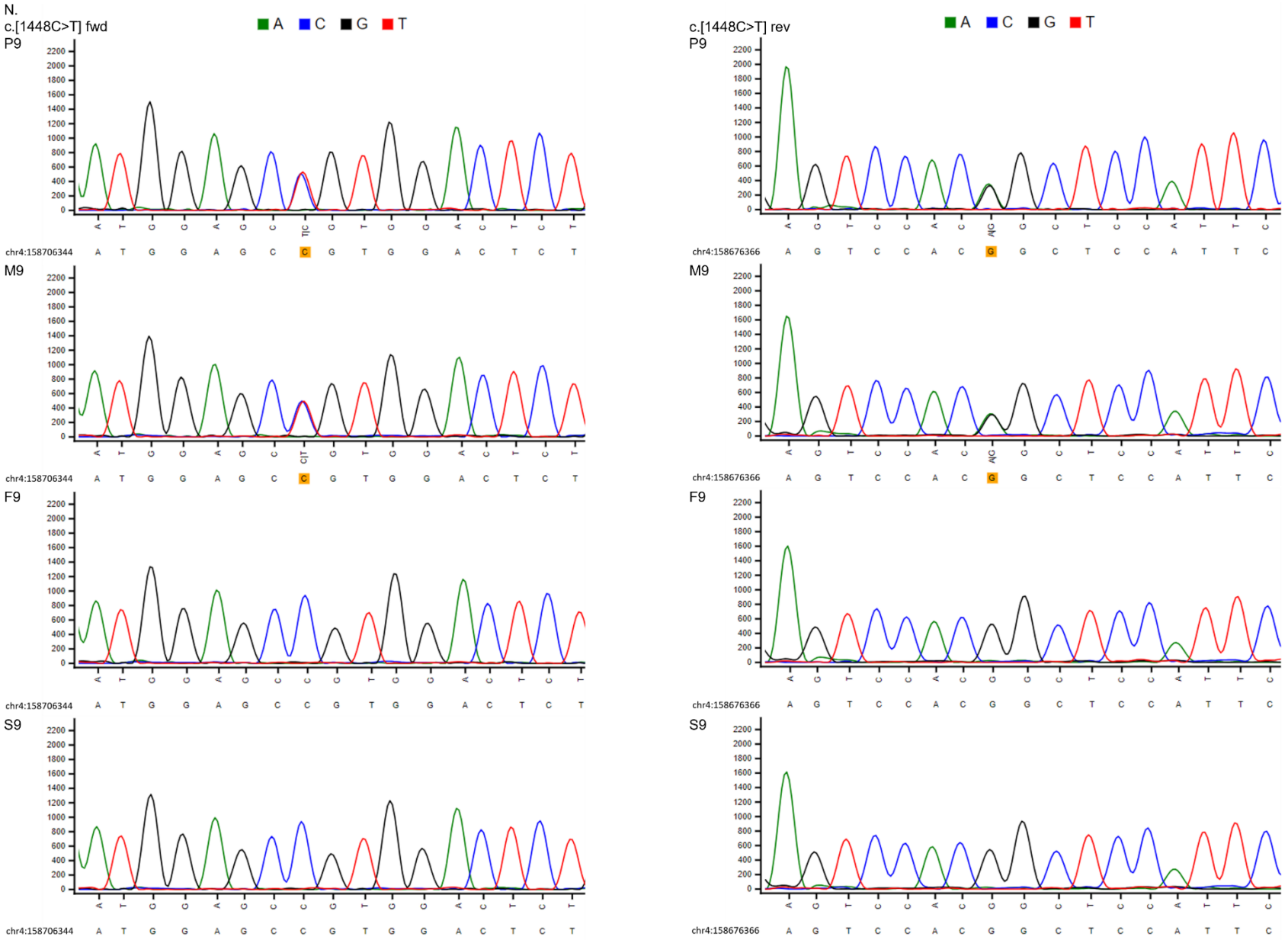
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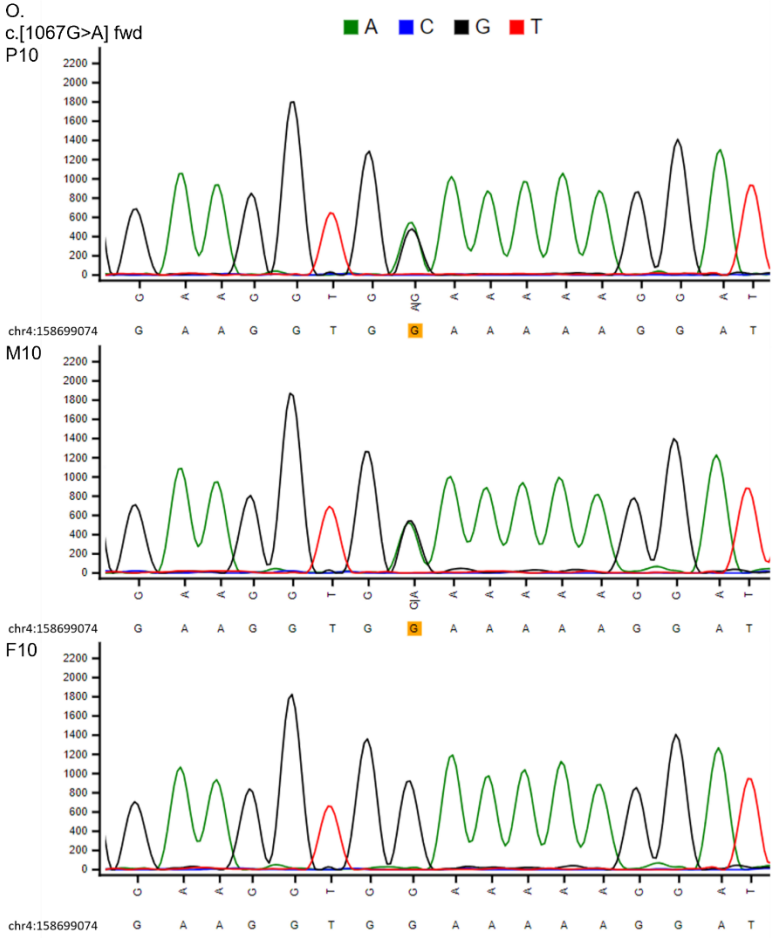
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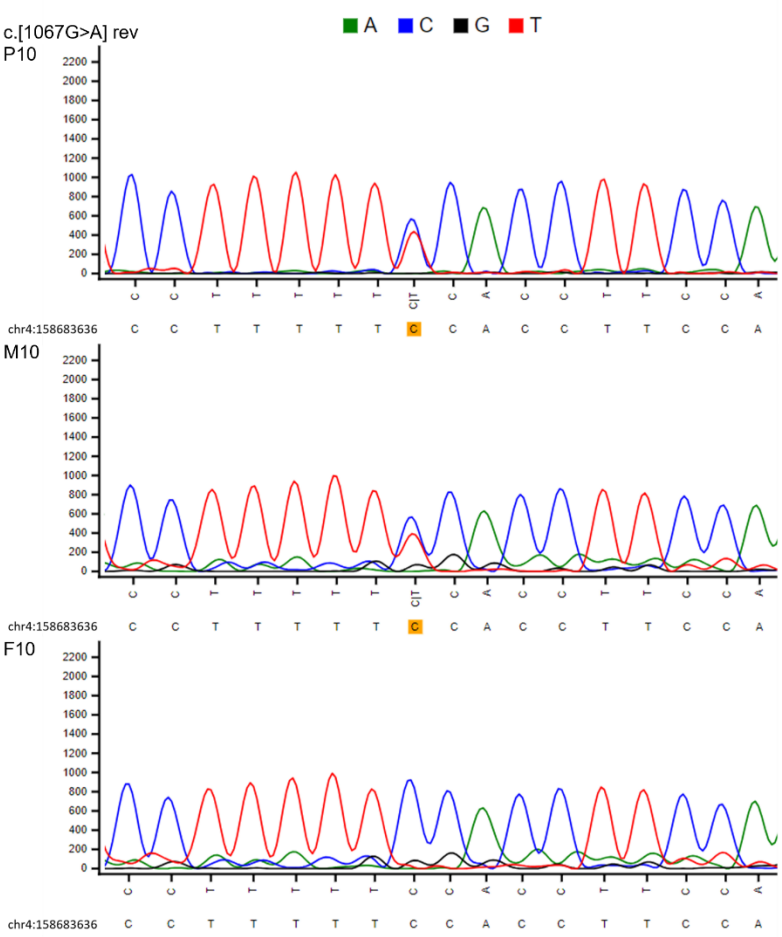
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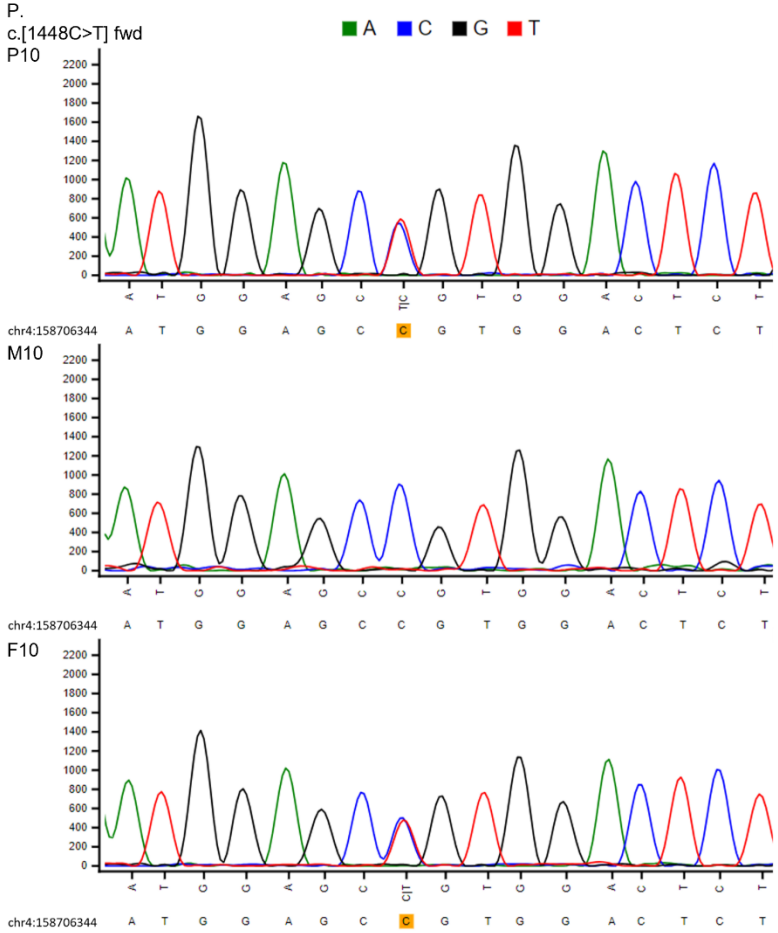
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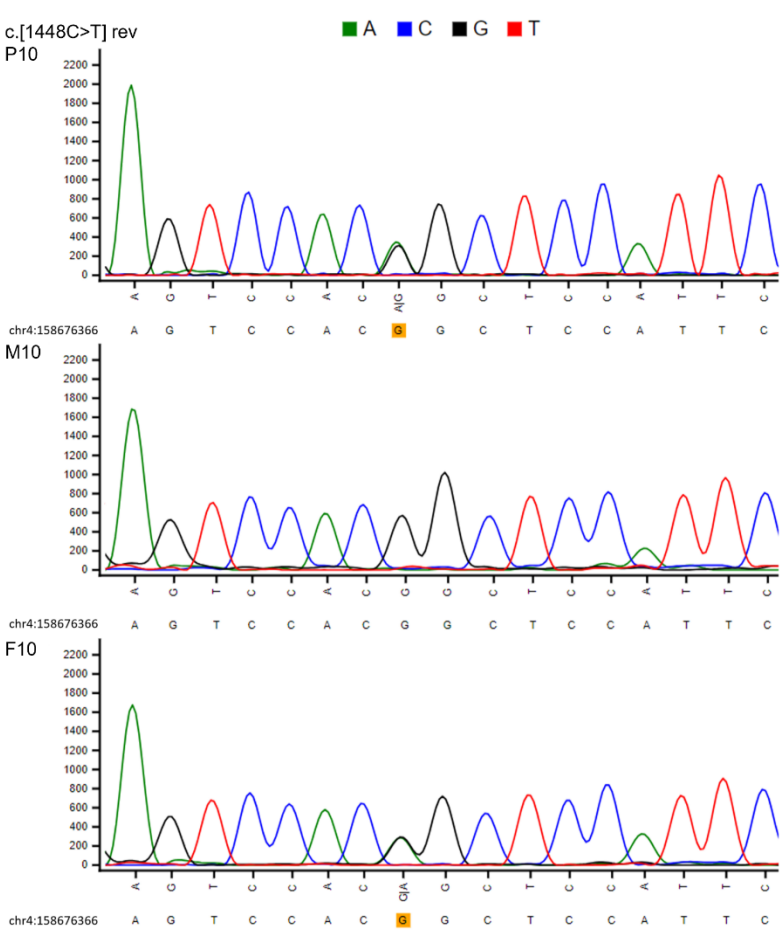
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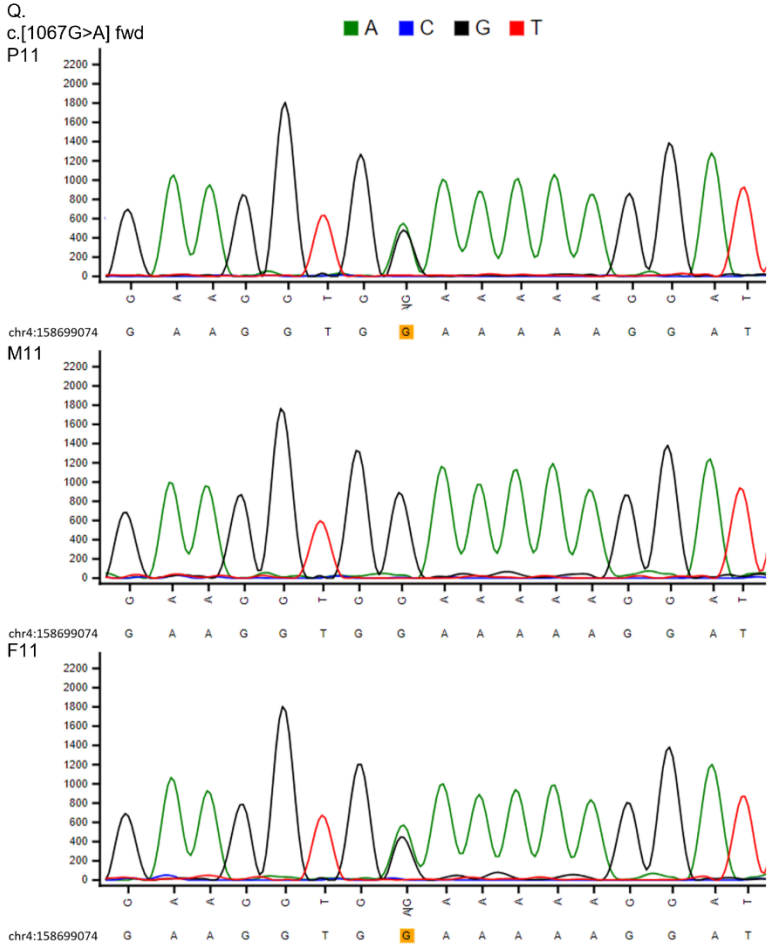
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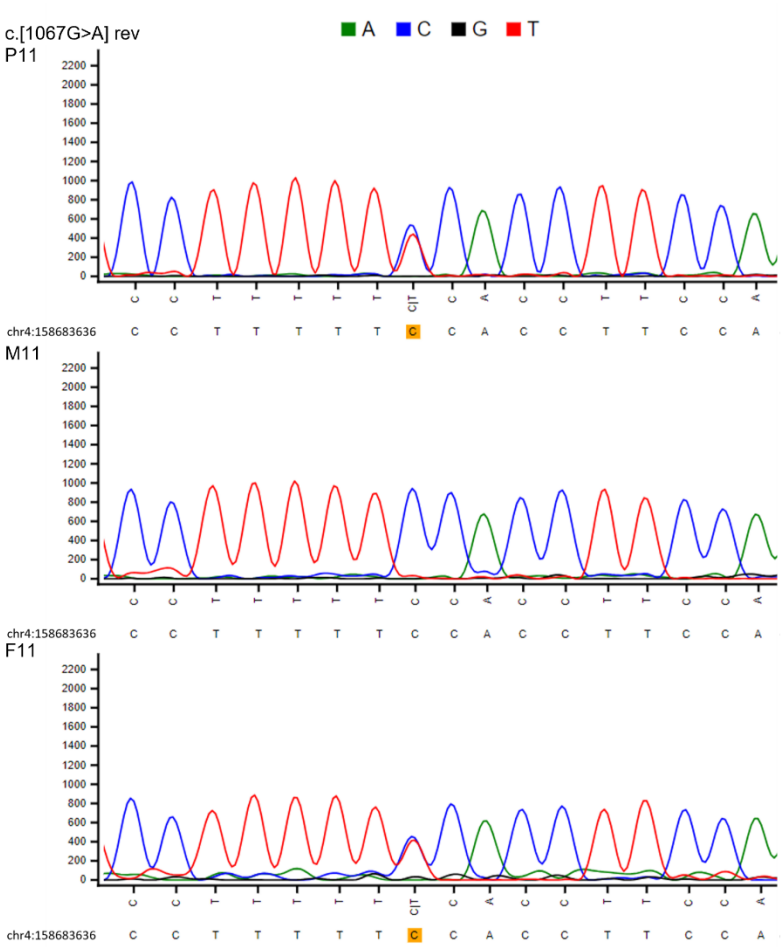
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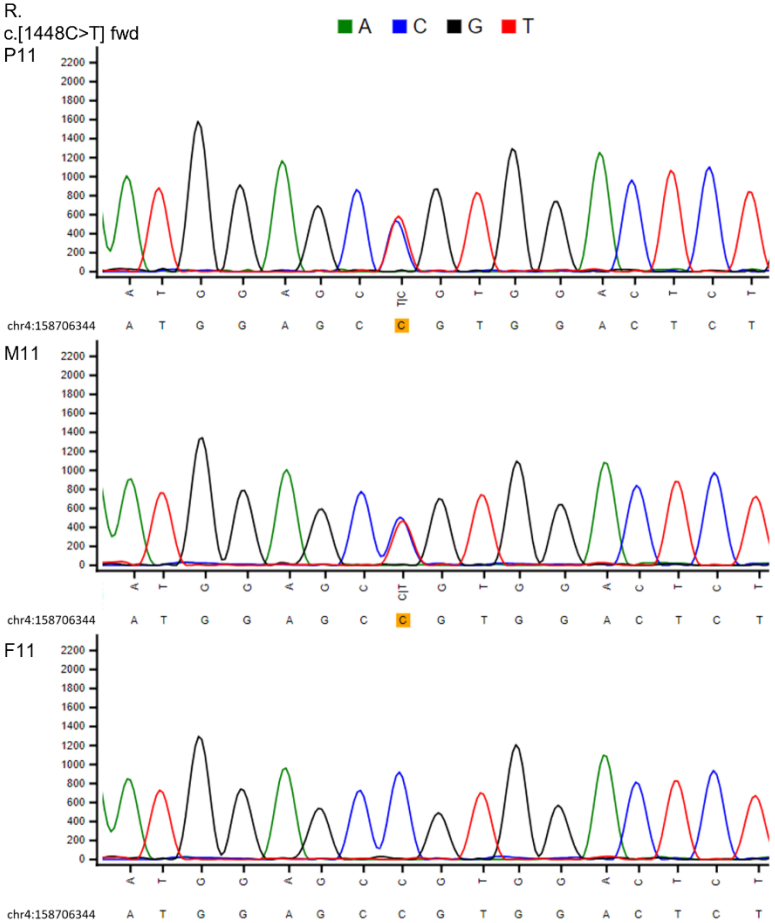
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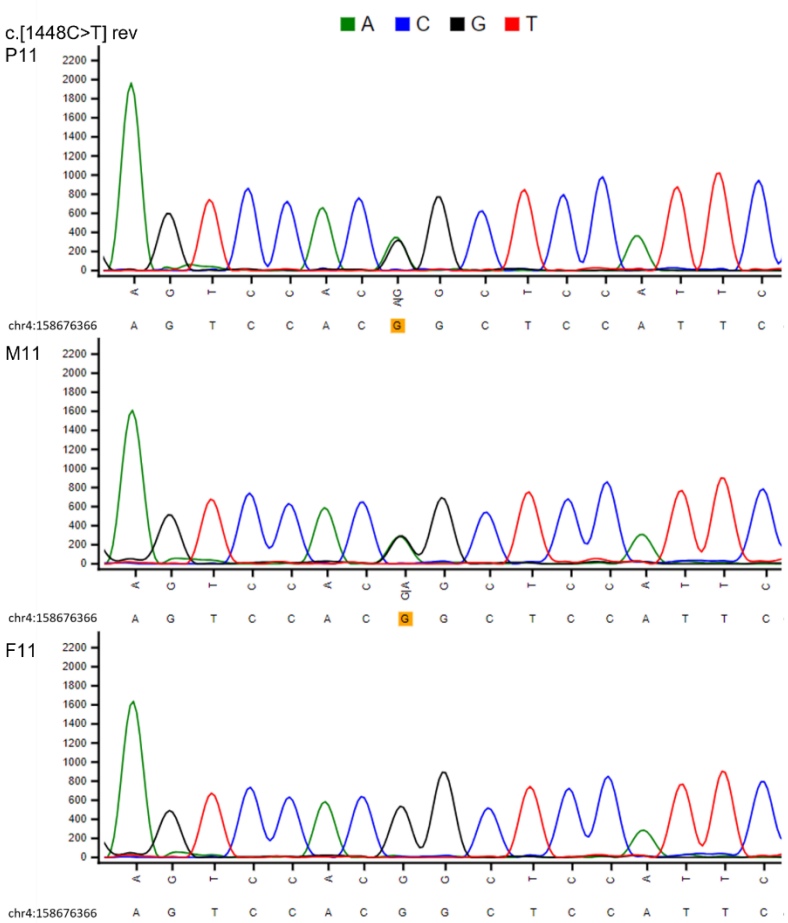
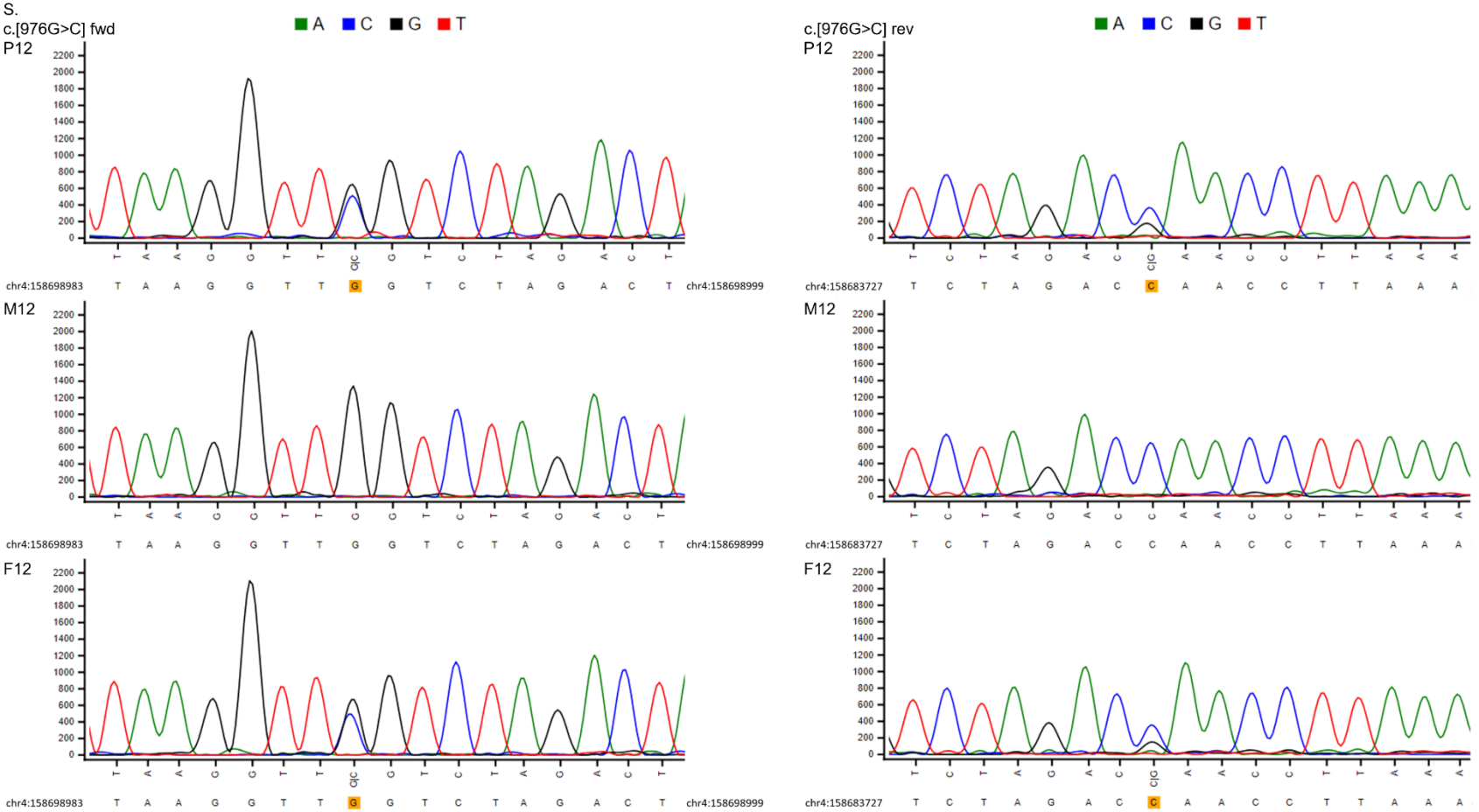
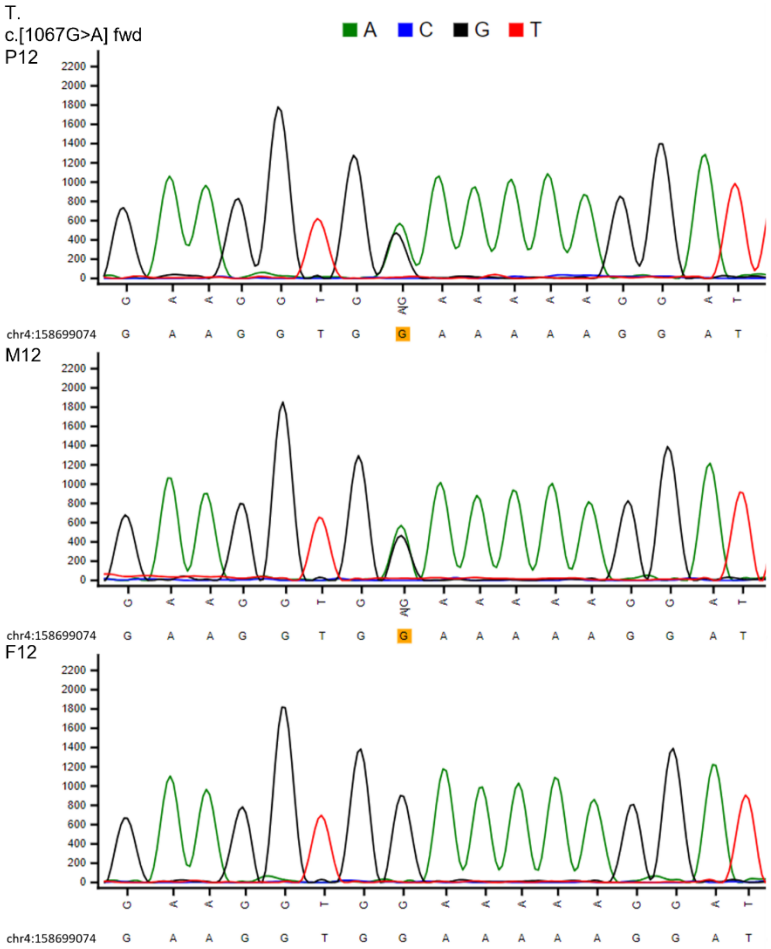
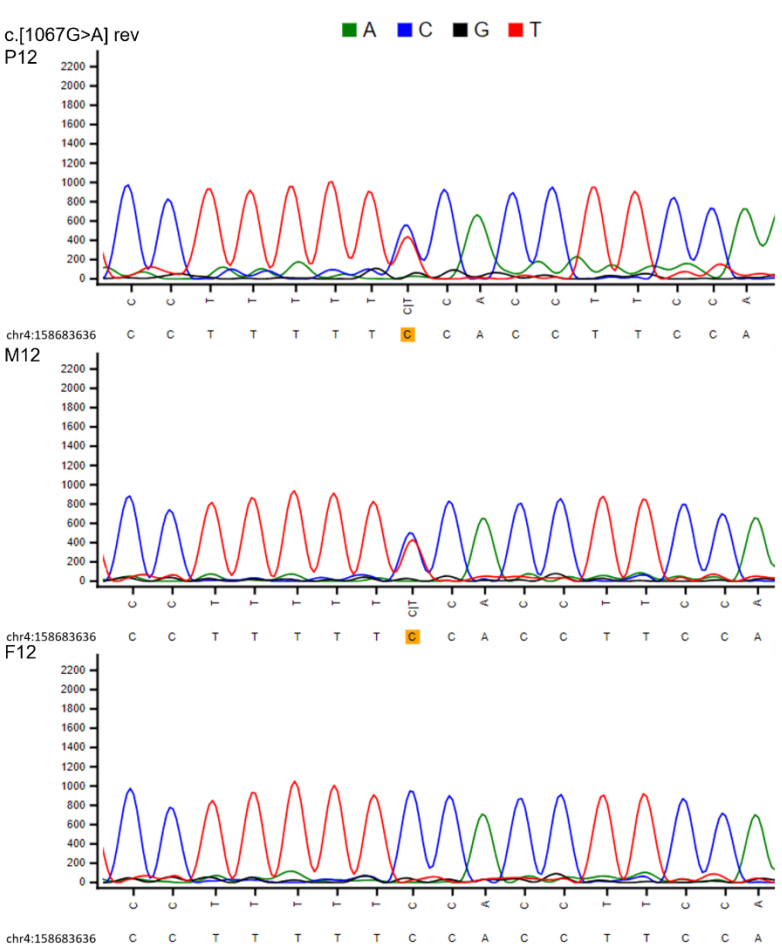

c.[1448C>T] rev
A C G T
P11
M11
F11
chr4:158676366
A G T C C A C G G C T C C A T T C

S.


c.[976G>C] fwd
P12
M12
F12
A C G T
chr4:158698983 T A A G G T T G G T C T A G A C T chr4:158698999
c.[976G>C] rev
P12
M12
F12
A C G T
chr4:158683727 T C T A G A C C A A C C T T A A A

T.


c.[1067G>A] fwd
P12
A C G T
chr4:158699074 G A A G G T G G A A A A A A G G A T
M12
chr4:158699074 G A A G G T G G A A A A A A G G A T
F12
chr4:158699074 G A A G G T G G A A A A A A G G A T


c.[1067G>A] rev
P12
A C G T
chr4:158683636 C C T T T T T C C A C C T T C C A
M12
chr4:158683636 C C T T T T T C C A C C T T C C A
F12
chr4:158683636 C C T T T T T C C A C C T T C C A

The Sanger data from the forward and reverse DNA strands are depicted. Altered nucleotides are highlighted in yellow (heterozygous variant), red (homozygous), or green (insertion). (a) *ETFDH* c.[1067G>A] and (b) c.[1448C>T] genotype of proband P1 and relatives, (c) *ETFDH* c.[287dupA*] and (d) c.[1448C>T] genotype of proband P2 and relatives (including P3 and P4), (e) *ETFDH* c.[1448C>T] genotype of proband P5, (f) *ETFDH* c.[740G>T] and (g) c.[1448C>T] genotype of proband P13, (h) *ETFDH* c.[1448C>T] genotype of proband P14, (i) *ETFDH* c.[1067G>A] genotype of proband P6 and relatives, (j) *ETFDH* c.[1067G>A] and (k) c.[1448C>T] genotype of proband P7's relatives, (l) *ETFDH* c.[1067G>A] genotype of proband P8 and relatives, (m) *ETFDH* c.[1067G>A] and (n) c.[1448C>T] genotype of proband P9 and relatives, (o) *ETFDH* c.[1067G>A] and (p) c.[1448C>T] genotype of proband P10 and relatives, (q) *ETFDH* c.[1067G>A] and (r) c.[1448C>T] genotype of proband P11 and relatives, and (s) *ETFDH* c.[976G>C] and (t) c.[1067G>A] genotype of proband P12 and relatives. Abbreviations: chr: chromosome; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; F: father; fwd: forward strand; M: mother; P: patient; rev: reverse strand; S: sibling.

C.5 ALLELE FREQUENCY ASSAYS

PCR–RFLP analysis was conducted at the NWU and UCT to estimate the allele frequency of *ETFDH* c.[1067G>A] and c.[1448C>T] (the two most frequently occurring variants in the Southern African MADD cohort) in the four largest population groups of SA. The type and number of samples amplified per population group, as well as the centre responsible are described in Table C.2.

C.5.1 Sample power calculation

To determine the allele frequency in the four largest population groups of SA with sufficient statistical power, it was determined that 750 samples should be analysed per population group. This sample size was calculated based on previous allele frequency estimations of IEMs in Southern Africa (Meldau *et al.,* 2018; van der Watt *et al.,* 2010), as well as the well-known formula provided in Daniel & Cross (2013). The accuracy of this calculation was confirmed using the Scalex SP (v1.0.1) calculator (Naing *et al.,* 2022). By following this approach, a frequency of 0.7% (± 0.5%) can be estimated with 90% confidence.

C.5.2 Samples and methodology

To screen for the (i) c.[1067G>A] and (ii) c.[1448C>T] variants, the following quantity of samples were used per reaction: an (i) eighth (corresponding to 0.2 ± 0.05 µL) and a (ii) quarter (corresponding to 0.4 ± 0.1 µL) of a saturated 3 mm heel prick DBS card and (i and ii) 10 ng umbilical cord DBS DNA. To interpret each batch of reactions, a homozygous (1 ng single-stranded DNA per reaction) and wildtype

(10 ng DNA per reaction) control were included. Depending on the centre of analysis [(a) NWU or (b) UCT], each PCR reaction consisted of either (a) 1× Phire Tissue Direct PCR Master Mix (F170L, Thermo Scientific™), or (b) 0.25 U GoTaq® G2 Flexi DNA polymerase (M780A, Promega, Wisconsin, USA), 1× Green GoTaq® Flexi Buffer (M891A, Promega), $MgCl_2$ (A351B, Promega) and 0.2 mM deoxynucleotide triphosphate (dNTP) mix (R0192, Thermo Scientific™), along with nuclease-free water and 0.2 µM of the corresponding forward and reverse primers (listed in Table 4.2). Thermal cycling was performed on (a) a T100™ thermal cycler (1861096, Bio-Rad) or (b) GeneAmp® PCR System 2700 (4322620, Applied Biosystems, Massachusetts, USA) and cycling conditions included initial denaturation at (a) 98°C for 5 min or (b) 98°C for 2 min, followed by (a) 34 or (b) 35 cycles of denaturation at (a) 98°C for 5 s or (b) 94°C for 30 s, annealing at the respective T_a for (a) 5 s or (b) 30 s and extension at 72°C for (a) 20 s or (b) 45 s, with a final extension step at 72°C for (a) 1 min or (b) 3 min.

The genotype of each reaction was determined by RFLP analysis using either 0.2 U HphI (ER1102, Thermo Scientific™) or 0.2 U AluI (FD0014, Thermo Scientific™), along with ~1 µg PCR product, nuclease water and 1× the respective reaction buffer, Buffer B (BB5, Thermo Scientific™) or FastDigest Buffer (B64, Thermo Scientific™). RFLP products were separated by electrophoresis at 4 V/cm on a 2% (w/v) agarose gel and visualised by ethidium bromide staining (5 µg/mL) and UV detection. As depicted in Figure C.14, the c.[1067G>A] variant creates an HphI restriction site, while the c.[1448C>T] variant creates a second AluI restriction site. The resulting fragments of each genotype are shown in Table C.6. The allele frequency was estimated as shown in Equation C.1:

$$p = f(AA) + 1/2\, f(AB) \tag{C.1}$$

Where, p represents the allele frequency of the variant, AA the ratio of homozygous affected samples per total number of samples per population group, and AB the ratio of heterozygous (carrier) samples per total number of samples per population group.

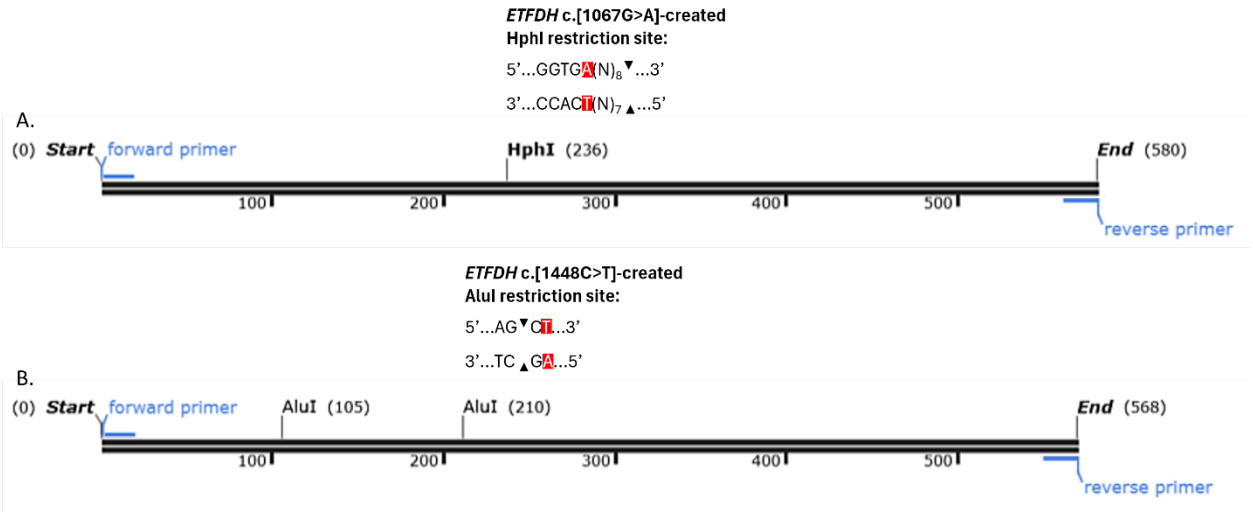


Figure C.14: Schematic representation of the RFLP reactions to screen for *ETFDH* c.[1067G>A] and c.[1448C>T]

The primer positions, length of the PCR-products and restriction sites are shown. (a) In the presence of *ETFDH* c.[1067G>A], an HphI restriction site is created at nucleotide 236, while (b) *ETFDH* c.[1448C>T] creates a second AluI restriction site at nucleotide 210. Abbreviations: *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; RFLP: restriction fragment length polymorphism.

Table C.6: Expected RFLP-product fragments for each genotype of *ETFDH* c.[1067G>A] and c.[1448C>T]

***ETFDH* variant**	**Restriction enzyme**	**Restricted band sizes (bp)**		
		Wildtype	**Heterozygous**	**Homozygous**
c.[1067G>A]	HphI	**580**	580	344
			344	**236**
			236	
c.[1448C>T]	AluI	**463**	568	**358**
		105	463	210
			358	105
			210	
			105	

Units and abbreviations: bp: base pairs; *ETFDH*: electron transfer flavoprotein-ubiquinone oxidoreductase gene; RFLP: restriction fragment length polymorphism.

Appendix D

Language certificates

EDITING EXCELLENCE

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editingexcellencepotch@gmail.com

This is to certify that the degree, Doctor of Philosophy in Science with Biochemistry,

titled,

Biochemical and molecular genetic characterisation of MADD in a Southern African cohort

of

Michelle Bisschoff

has been edited by

Valerie Viljoen,

Editing Excellence

Chapters 1, 2, 4 and 5; Appendix C and Abstract were edited.

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19 November 2023

To whom it may concern

Dear Sir or Madam,

Confimation of Language Edit completed on behalf of the PhD thesis of Michelle Bisschoff

This letter serves as formal confirmation that a language and grammar edit was completed by Dr Graham Baker of Kerlick Academic Services on Chapter 3 and Appendix B of the PhD thesis, submitted to North-West University, entitled "Biochemical and molecular genetic characterisation of MADD in a Southern African cohort" by Michelle Bisschoff.

However, the student is responsible for effecting the edits, and for the grammatical correctness of the documents before hand-in.

Yours sincerely

Graham Baker D.Phil. (Oxon.)
Language Specialist
Kerlick Academic Services (Pty) Ltd