

**Molecular Characterisation of
Erythropoietic Protoporphyrin
in South Africa**

**Michelle Parker B.Sc (Hons)
PRKMIC010**

**A DISSERTATION, SUBMITTED
TO THE UNIVERSITY OF CAPE TOWN
in fulfillment of the requirements for the degree
M.Sc (Med)**

**Lennox Eales Porphyria Laboratories of the MRC-UCT Liver Centre
Department of Medicine
Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN**

August 2006

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

Dedication

**To mom and dad,
your love carries me**

and

**The Lord Jesus Christ,
my all in all**

University of Cape Town

Psalm 121

I lift up my eyes to the hills -
where does my help come from?
My help comes from the LORD,
the Maker of heaven and earth.
He will not let your foot slip -
He who watches over you will not slumber;
indeed, He who watches over Israel
will neither slumber nor sleep.
The LORD watches over you -
the LORD is your shade at your right hand;
the sun will not harm you by day,
nor the moon by night.
The LORD will keep you from all harm -
He will watch over your life;
the LORD will watch over your coming and going
both now and forevermore.

Declaration

I, Michelle Parker, hereby declare that the work on which this dissertation is based is my original work (*except where acknowledgements indicate otherwise*) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purposes of research either the whole or any portion of the contents in any manner whatsoever.

Signature:..

Signed by candidate

Date:.....20/11/2006.....

Table of Contents

Table of Contents	i
List of Tables	vi
List of Figures	vii
Symbols and Abbreviations	viii
List of Suppliers	xi
Acknowledgements	xii
Abstract	xiv

CHAPTER 1: Introduction

1.1. Porphyrins	2
1.1.1. Structure	2
1.1.2. Properties	2
1.1.3. Porphyrin and haem biosynthetic pathway	3
1.2. The Porphyrrias	6
1.2.1 Diagnosis and treatment of the porphyrias	7

CHAPTER 2: FECH and EPP

2.1 Ferrochelatase	8
2.1.1. Catalytic mechanism	8
2.1.2. Protein characterisation	11
2.1.3. Structure	13
2.1.4. FECH gene	15
2.1.5. <i>FECH</i> pseudogene	16
2.2. EPP – Clinical aspects	17
2.2.1. History of EPP	17
2.2.2. Clinical presentation of EPP	18
2.2.3. Biochemical diagnosis of EPP	19
2.2.4. Pathogenesis of photocutaneous sensitivity in EPP	22
2.2.5. Pathogenesis of liver disease in EPP	23
2.3. EPP – Molecular aspects	24
2.3.1. Inheritance of EPP	24
2.3.2. EPP in the absence of the IVS3-48C polymorphism	28
2.3.3. Genetic heterogeneity of EPP	29
2.3.4. Genetic basis of EPP-related liver disease	30

2.4. EPP – Management	31
2.4.1. Photocutaneous sensitivity	31
2.4.2. Liver disease	33
2.4.3. General considerations	34
2.5. Animal studies and potential therapies	35
2.5.1. Animal models	35
2.5.2. Correction of the FECH deficiency	36

CHAPTER 3: Aims and Objectives

CHAPTER 4: Materials and Methods

4.1. Subject recruitment and control selection	42
4.2. Subject and family interview and blood sample collection	43
4.3. Biochemical diagnosis of EPP	44
4.3.1. Preliminary red blood cell porphyrin screening	44
4.3.2. Plasma fluorescence scanning	44
4.3.3. Porphyrin quantitative analysis	44
4.3.4. Determination of free and zinc-chelated protoporphyrin IX concentrations	45
4.4. DNA isolation	45
4.5. Analysis of the FECH gene	46
4.5.1. Fragment amplification by polymerase chain reaction	46
4.5.2. Polyacrylamide gel electrophoresis (PAGE)	48
4.6. Investigation of the IVS3-48T/C polymorphism	49
4.6.1. <i>Ita</i> I Restriction endonuclease analysis	49
4.7. Mutation detection and screening	50
4.7.1. Single-stranded conformational polymorphism analysis	50
4.7.2. Direct sequencing	51
4.7.3. Isolation of the mutant allele for sequencing	52
4.7.4. Restriction endonuclease analysis for identified sequence variations	52
4.8. Haplotype analysis	54
4.8.1. Microsatellite selection, design and amplification	54
4.8.2. Marker genotyping (allele identification and assignment)	56
4.8.3. Pedigree construction	56

CHAPTER 5: Results

5.1 Subject recruitment and control selection	58
5.2. DNA Isolation	58
5.3. Analysis of the FECH gene	59
5.3.1. <i>FECH</i> exon amplification	59
5.3.2. Mutation detection	61
5.3.3. Direct sequencing	61
5.3.4. Isolation of mutant alleles for sequencing	64
5.4 Investigation of the IVS3-48T/C polymorphism	66
5.5. Restriction endonuclease analysis for identified variations	68
5.5.1. Determination of the frequencies of the common polymorphisms identified	68
5.5.2. Determination of the frequencies of the primary mutations identified	69
5.6. Haplotype analysis	72
5.6.1. Microsatellite selection, design and amplification	72
5.6.2. Marker genotyping and pedigree construction	74
5.7. Clinical features	81
5.7.1. Patient presentation and perception of their disease	81
5.7.1.1. Age	81
5.7.1.2. Precipitants	81
5.7.1.3. Signs and symptoms	82
5.7.1.4. Precautionary measures and symptom alleviation	82
5.7.1.5. Liver disease	82
5.7.1.6. Family history	82
5.7.2. Biochemical diagnosis of EPP	86
5.7.2.1. Preliminary red blood cell porphyrin screening	86
5.7.2.2. Plasma fluorescence scanning	86
5.7.2.3. Porphyrin quantitative analysis	86
5.7.2.4. Determination of free and zinc-chelated protoporphyrin IX	92

CHAPTER 6: Discussion

6.1 Analysis of the FECH gene	95
6.1.1. Methodologies	95
6.1.2. Characterisation of the EPP cohort	96
6.1.2.1. <i>FECH</i> primary mutations and polymorphisms identified	97
6.1.2.2. Inheritance of disease-associated alleles	98
6.1.3. Screening for identified sequence variations	100
6.2. Haplotype analysis	101
6.3. Unresolved cases (families EPP11 and EPP8)	103
6.4. Patient presentation and perception of their disease	104
6.5. Biochemical diagnosis of EPP	106
6.5.1. Plasma fluorescence scanning	106
6.5.2. Porphyrin quantitative analysis	107

CHAPTER 7: Conclusions and Future Studies

APPENDICES

APPENDIX A	113
A.1. Patient correspondence and EPP information sheet	113
A.2. Consent form	121
A.3. Subject information sheet	124
APPENDIX B	125
APPENDIX C	127
C.1. Red blood cell porphyrin screening	127
C.2. Plasma fluorescence scanning	128
C.3. Porphyrin quantitative analysis	129
C.4. Determination of free and zinc-chelated protoporphyrin IX concentrations	132
APPENDIX D	133
D.1. Genomic DNA isolation	133
D.2. DNA purification	135
APPENDIX E	136
E.1. Primer selection for fragment amplification	136
E.2. Polymerase chain reaction (PCR)	137
APPENDIX F	138
F.1. Non-denaturing polyacrylamide gel electrophoresis	138

APPENDIX G	142
G.1. Restriction endonuclease analysis	142
G.2. Silver staining and visualisation	143
G.3. IVS3-48C polymorphism screening	144
APPENDIX H	145
H.1. MDE-PAGE	145
APPENDIX I	148
I.1. DNA purification from PCR product	148
I.2. Agarose gel electrophoresis and DNA purification from agarose	149
I.3. DNA purification from acrylamide	152
APPENDIX J	154
J.1. Marker genotyping	154
J.2. ROX-500 size standard	155
APPENDIX K	156

REFERENCES

List of Tables

Table 1.1. Summary of the clinical and molecular characteristics of the porphyrias	6
Table 4.1. Summary of primer sequences and melting temperatures for amplification of the exons of <i>FECH</i>	47
Table 4.2. SSCP running conditions	50
Table 4.3. Summary of restriction endonucleases used for mutation screening	53
Table 4.4. Summary of primer sequences, melting temperatures and minimum fragment sizes for amplification of microsatellite markers	55
Table 5.0. Genotype frequencies for the <i>FECH</i> IVS3-48T/C polymorphism	67
Table 5.1. <i>FECH</i> common polymorphisms identified in the South African EPP cohort	70
Table 5.2. <i>FECH</i> primary mutations identified in the South African EPP cohort	71
Table 5.3. Informativity of microsatellite markers	75
Table 5.4. Haplotype analysis of the <i>FECH</i> locus for families in which the 757_761delAGAAG deletion segregates as a familial trait	76
Table 5.5. Haplotype analysis of the <i>FECH</i> locus for families in which the 757_761delAGAAG deletion is absent	77
Table 5.6. Allele frequencies for the most common marker alleles associated with the 757_761delAGAAG deletion	78
Table 5.7. Subject perspective of the disease EPP	83
Table 5.8. Summary of clinical results for the study cohort	87
Table 5.9. Summary of zinc-chelated and free protoporphyrin IX concentrations	92

List of Figures

Figure 1.1. General porphyrin structure	2
Figure 1.2. Haem biosynthetic pathway in mammals	4
Figure 2.1. Schematic representation of the reaction mechanism employed by FECH	9
Figure 2.2. Space-filling and ribbon diagrams of FECH	10
Figure 2.3. Ribbon drawings of human FECH	14
Figure 2.4. Proposed mechanism of the pathogenesis of photocutaneous sensitivity in EPP	22
Figure 2.5. Schematic representation of the use of an alternative splice-site in the presence of the IVS3-48C polymorphism	26
Figure 5.1. PAGE (6%) of PCR amplification fragments for <i>FECH</i> exon 4	60
Figure 5.2. PAGE (6%) of PCR amplification fragments for <i>FECH</i> exon 7	60
Figure 5.3. Mutation detection and identification: <i>FECH</i> exon 3	62
Figure 5.4. Mutation detection and identification: <i>FECH</i> exon 4	62
Figure 5.5. Mutation detection and identification: <i>FECH</i> exon 7	63
Figure 5.6. Characterisation of 356_362delTTCAAGA deletion	65
Figure 5.7. PAGE (6%) of <i>FECH</i> exon 4A amplification products after <i>Ita</i> I restriction analysis	67
Figure 5.8. Frequencies of SNPs in the EPP and control cohorts	68
Figure 5.9. Schematic representation of the <i>FECH</i> gene on chromosome 18	73
Figure 5.10. Visualisation and optimisation of microsatellite marker <i>FECH</i> -MM1ac amplification products	74
Figure 5.11. Pedigree of Family EPP2	79
Figure 5.12. Pedigree of Family EPP8	80

Symbols and Abbreviations

A	Adenine
AIP	Acute intermittent porphyria
ALA	δ -aminolevulinic acid
APS	Ammonium persulphate
BCRP	Breast cancer resistance protein
BLAST	Basic local alignment search tool
Bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
cM	CentiMorgan
Co	Company
ddNTP	Dideoxynucleoside triphosphate
dH ₂ O	Distilled water
DHA	dihydroxyacetone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
e.g.	For example
EDTA	Ethylenediaminetetra-acetic acid
EPP	Erythropoietic protoporphyria
EtBr	Ethidium bromide
F	Forward
FECH	Ferrochelatase
<i>FECH</i>	Ferrochelatase gene
G	Guanine
h	Hours
HPLC	High performance liquid chromatography
I	Proband (index case)
Kb	Kilobase
L	Litre
LD	Linkage disequilibrium

Ltd	Limited
M	Molar
MDE	Mutation Detection Enhanced
Mg	Milligram
MgCl ₂	Magnesium chloride
min	Minutes
ml	Millilitre
mM	Millimolar
mm	Millimeter
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
nmol	Nanomole
NCBI	National Centre for Biotechnology Information
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBG	Porphobilinogen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCT	Porphyria cutanea tarda
Pg	Page
pH	Log H ⁺ ions
pmol	Picomole
PPIX	Protoporphyrin IX
PPOX	Protoporphyrinogen IX oxidase
R	Reverse
RBC	Red blood cell
RE	Restriction endonuclease
RFU	Relative fluorescent units
sec	Seconds
SA	South Africa
SNP	Single nucleotide polymorphism
SSCP	Single stranded conformational polymorphism
T	Thymine

t	Time
T _x	Temperature
T _a	Annealing temperature
T _i	Incubation temperature
TBE	Tris borate EDTA
TLC	Thin-layer chromatography
U	Unit
UCT	University of Cape Town
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
v	Volume
VP	Variegate porphyria
vs	Version
w	Weight
www	World wide web
ZPP	Zinc-chelated protoporphyrin IX
3'-OH	Three prime hydroxyl
°C	Degrees Celsius
[E]	Enzyme concentration
β	Beta
λ	Wavelength
λ _{ex}	Excitation wavelength
λ _{em}	Emission wavelength
Fe ²⁺	Ferrous iron
μg	Microgram
μl	Microlitre
μM	Micromolar
%	Percentage
±	Approximately
+	Positive
-	Negative

List of Suppliers

Acme Electric Corp., New York, UK
Amersham Pharmacia Biotechnology Inc., California, USA
Associated Chemical Enterprises, Johannesburg, SA
B & M Scientific, Cape Town, SA
BDH Chemicals Ltd., Poole, UK
BDH Laboratory Supplies, Poole, UK
Boeringer Mannheim, Germany
Cambrex Bio Science Rockland Inc., Rockland ME, USA
FMC BioProducts, Maine, USA
Greiner Labortechnik, Cape Town, SA
Hitachi Koki Co. Ltd., Japan
IDT-Integrated DNA Technologies Inc., Coralville IA, USA
Kimix Chemical and Laboratory Supplies, Cape Town, SA
Lab-line Instruments Inc., Melrose Park, Illinois USA
Laboratory and Scientific Equipment Co., Cape Town, SA
Merck Chemicals, Darmstadt, Germany
PE Applied Biosystems, Foster City, CA, USA
Pharmacia Biotech, Cambridge, UK
Promega, Madison WI, USA
Qiagen, Hilden, Germany
Roche Diagnostics Corp., Mannheim, Germany
Roche Diagnostics Pty Ltd., Randburg, SA
SAARCHEM-holpro Analytic, Johannesburg, SA
Sabax, Cape Town, SA
Scientific Industries Inc., New York, USA
Sigma Chemical Co., St Louis MO, USA
Spectra-Physics, California, USA
Spectrum Medical Industries, Los Angeles, USA
Stratagene, California, USA
UltraLum, California, USA
UVItec Ltd., Cambridge, UK

Acknowledgements

I would like to acknowledge the support and dedication of my supervisor, Associate Professor Peter Meissner, whose wisdom, patience and direction have been of great encouragement during the course of this project.

I would also like to acknowledge my co-supervisor, Dr. Anne Corrigan, for technical assistance and hours of pouring over results. Thank you too for the attention you gave to the little details of my dissertation, particularly punctuation.

Then, I would like to acknowledge my second co-supervisor, Professor Richard Hift, for advice on the clinical aspects of my project and for accompanying me on visitations to the EPP families for collection of blood samples and patient information.

A special thank you to my friends and colleagues in the Liver/Porphyrin Laboratories for support and encouragement, and for helping to keep a smile on my face during my studies.

In particular, I'd like to thank:

- Sister Doreen Meissner, for initial identification and recruitment of patients to the UCT Porphyria Laboratories.
- Lavinia Petersen, for her role in contacting and informing the patients.
- Brandon Davidson, for biochemical analysis of samples, specifically; preliminary RBC porphyrin screening, plasma fluorescence scanning and porphyrin quantitative analysis.
- Roger Duffet and Brent Jennings, for IT assistance.
- Surita Marais, for "brain-storming sessions" on both the practical and written aspects of my project.
- Maggie and Amina for all their effort to ensure that the lab is clean and stocked with equipment and most importantly – dH₂O.

I would like to thank the staff of the Division of Human Genetics, particularly Dr. George Rebello, Jeanne Rousseau and Alvera Vorster for advice and technical assistance with genotyping for my family studies.

I acknowledge financial support received from various sources, without which it would not have been possible to undertake these studies;

- UCT Liver scholarship
- Guy Elliot Trust
- University of Cape Town

I would especially like to thank certain members of my family and close friends who have been an inspiration and have held up my arms in prayer.

- My mom and dad, for building all the necessary qualities into my life that have allowed me to achieve what I have thus far.
- My sister, for always encouraging me and listening when I needed to talk.
- My Grandmother, Joan Rousseau, for all the "Saturday calls".
- My Grandfather, Tom Parker, for giving me R200 for University when I was only 9 years old which sparked a dream in my heart to achieve what I have this year.
- My friends, Erika, Monique, Alvera and Janine, for patience and tolerance and for the many prayers that have carried me over the years.

And most importantly to my Lord and Saviour, Jesus Christ, my Source and my Life.

Abstract

Erythropoietic protoporphyria (EPP) is a genetic disorder with a 'pseudodominant' mode of inheritance. Typically, patients present in childhood with photocutaneous sensitivity. This is a result of a defect in ferrochelatase (FECH), the terminal enzyme in haem biosynthesis, causing accumulation of protoporphyrin IX. In most cases, a single, primary *FECH* mutation is identified in EPP families, but unlike classic dominant inheritance, many mutation carriers are clinically asymptomatic. These individuals have approximately 50% FECH activity, while symptomatic individuals have a FECH activity of <50%. This has recently been attributed to a common polymorphism (IVS3-48C) that, when inherited in *trans* to a deleterious allele, leads to reduced *FECH* expression below a critical threshold limit and symptomatic EPP. This study characterised a cohort of South African EPP subjects from 17 families by identification and assessment of *FECH* sequence variations, including the disease-associated IVS3-48C polymorphism. PCR amplification and subsequent SSCP analysis of the coding region and surrounding intronic sequences of *FECH* was performed. Fragments corresponding to gel mobility shifts were sequenced to identify the variation. Restriction endonuclease analysis was employed to determine the frequencies of these variations, including the IVS3-48C polymorphism, in a study cohort of symptomatic and asymptomatic family members, and a matched control cohort. Ten sequence variations were identified, including the IVS3-48T/C polymorphism and 4 previously described common polymorphisms. The molecular basis of EPP was established for 15 of the 17 families. Unexpectedly, a 5bp deletion in exon 7 (757_761delAGAAG) was present in 12 of these families. The other mutations were family-specific and included 2 previously described splice-site mutations (IVS3+2T>G and IVS7+1G>A) and a novel 7bp deletion in exon 4 (356_362delTTCAAGA). A second novel variation (IVS10-61G/A), was shown to be a common polymorphism. The hypomorphic (IVS3-48C) allele appears to modulate the phenotypic expression of EPP in the South African EPP cohort as previously observed in other populations. In light of the above-mentioned results, the study investigated whether individuals positive for the 757_761delAGAAG deletion share a common ancestral allele with respect to *FECH*. Genotyping of the study cohort and 50 control samples for microsatellite markers surrounding *FECH* was completed for manual haplotype construction. A common restricted haplotype was confirmed in all families in which the 757_761delAGAAG deletion segregates as a familial trait, and for which complete family triads were available. This suggests a single mutational event for the 757_761delAGAAG deletion.

CHAPTER 1

Introduction – Porphyrins and the Porphyrins

University of Cape Town

CHAPTER 1

Introduction

The porphyrias are a group of metabolic disorders that result from enzymatic defects in the haem biosynthetic pathway. Most are inherited. Generally, 7 different types of porphyria can be distinguished and are conveniently classified according to the specific haem biosynthetic enzyme deficiency observed. The most commonly encountered forms of porphyria in South Africa include variegate porphyria (VP), acute intermittent porphyria (AIP) and the acquired form, porphyria cutanea tarda (PCT) (Eales and Dowdle 1968; Meissner and Hift 2005). The porphyrias are normally considered rare. However, in certain areas, genetic founder effects have given rise to a higher incidence of a particular type of porphyria [e.g. AIP in Northern Sweden (Lee and Anvret 1991) and VP in South Africa (Meissner et al. 1996; Warnich et al. 1996; Groenewald et al. 1998)].

Another form of inherited porphyria, erythropoietic protoporphyria (EPP), occurs in South Africa, although in our experience, of lower prevalence than is seen in some other parts of the world. To date there have been no molecular studies of EPP in South African patients. A few case reports at a clinico-biochemical level have been documented (Sweeney et al. 1963; Findlay et al. 1966; Leeming and Kramer 1968; van der Walt and Heyl 1971; Eales et al. 1978a; Eales et al. 1978b), and EPP is considered in the differential diagnosis of the porphyrias in South African laboratories (Day 1978).

The focus of this dissertation is the molecular characterisation of a cohort of South African EPP patients; thus EPP will be reviewed in chapter 2. However, as the porphyrias result from enzyme deficiencies in the haem biosynthetic pathway, with the consequent accumulation of porphyrin intermediates, it is pertinent to give a brief introduction to porphyrins and the haem biosynthetic pathway.

1.1. Porphyrins

1.1.1. Structure

Porphyrins are rigid, planar structures consisting of a macrocycle of four pyrrole rings interconnected by four methine (-C=) bridges (Figure 1.1) (Meissner et al. 2001). The four pyrrole rings have been designated A, B, C and D while α , β , γ and δ denote the four methine bridges (Fischer and Orth 1934; Moore et al. 1987a). There are eight positions for the attachment of side chains when the tetrapyrrole macrocycle is in the oxidised state, and depending on their precise configuration these give rise to different isomeric forms. The specific side chains present determine the physico-chemical properties of the porphyrin.

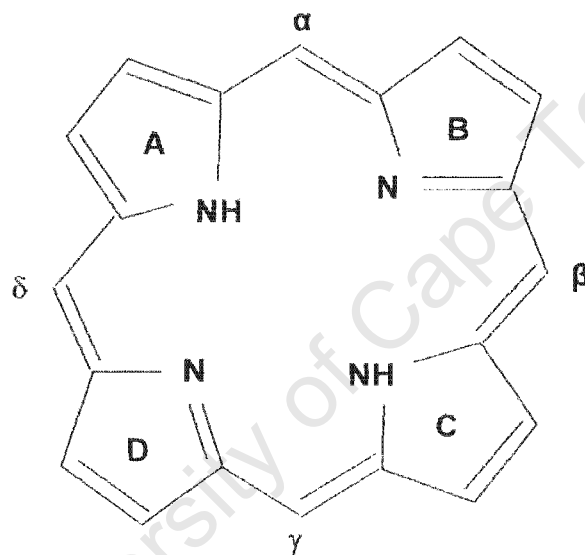


Figure 1.1. General porphyrin structure (from Moore et al. 1987a)

1.1.2. Properties

The complex ring structure of porphyrins has the capacity to bind metals, e.g. iron to form haem, magnesium to form chlorophylls and cobalt bound to corrins (modified porphyrin rings) used in vitamin B12 synthesis (Scott 1972; Battersby and McDonald 1975, Bissell and Schmid 1987).

These metalloporphyrins occur throughout the plant and animal kingdoms where they play essential roles in many processes, such as oxygen transport, electron transport, reduction of molecular oxygen and photosynthesis (Bissell 1985; Moore 1990).

Porphyrins, the "pigments of life" (Battersby et al. 1980), are highly coloured, conjugated compounds, which exhibit red fluorescence on absorption of near ultraviolet (UV) light (Battersby 1978; Meissner et al. 2001). The absorption spectra show absorption in the visible region of the electro-magnetic spectra with a major band at $\pm 400\text{nm}$ i.e. the Soret band (Soret 1883).

The property of fluorescence has been utilised to study porphyrins and the associated porphyrias (e.g. diagnosis through the measurement of excreted porphyrins) since the beginnings of porphyrin research. The origins of porphyrinology can be tracked to the acid treatment of haemoglobin to produce "iron-free haematin" which was purple-red in colour by Scherer, Berzelius and Mülner in the 1840s (Moore et al. 1987b). Porphyrinogens (hexahydroporphyrins) are unstable, colourless porphyrin intermediates in which the methine bridges are reduced to methylene bridges (Meissner et al. 2001). They do not fluoresce and are readily oxidised to their corresponding porphyrin on exposure to oxygen.

1.1.3. Porphyrin and haem biosynthetic pathway

Haem synthesis in mammals requires a well-defined, tightly controlled pathway and is accomplished by the sequential catalytic reactions of eight distinct enzymes (Figure 1.2). Synthesis is initiated in the mitochondrion, continues in the cytosol and is completed in the reducing environment of the mitochondrion once again.

The first step is the condensation of succinyl-CoA and glycine in the mitochondrion, catalysed by the pyridoxal phosphate-containing enzyme δ -aminolevulinic acid (ALA) synthase, to form ALA which is transported into the cytoplasm (Michal 1999). ALA dehydratase catalyses the condensation of two ALA moieties to produce porphobilinogen (PBG), the monopyrrole subunit of the porphyrin ring. Next, four molecules of PBG condense in a head-to-tail manner producing a linear tetrapyrrole intermediate, hydroxymethylbilane (Moore et al. 1987a). The enzyme responsible for this reaction is PBG deaminase.

Working concurrently with PBG deaminase, uroporphyrinogen III cosynthase performs the necessary intramolecular rearrangement and ring closure, to form uroporphyrinogen III via a spiro intermediate. Uroporphyrinogen decarboxylase yields coproporphyrinogen III by step-wise decarboxylation of the four acetate side chains of uroporphyrinogen III (Dailey 1997). Hydroxymethylbilane is converted non-enzymatically into uroporphyrinogen I if uroporphyrinogen III cosynthase is not present (Michal 1999). This isomer cannot be metabolised further and is lost to haem synthesis. The process returns to the mitochondrion where coproporphyrinogen III oxidase converts it to protoporphyrinogen IX by oxidative decarboxylation of the two propionate side chains to yield vinyl substituents on the two pyrrole rings. Protoporphyrin IX is formed by protoporphyrinogen IX oxidase (PPOX) via a 6-electron oxidation (Dailey 1997). Ferrochelatase (FECH), located in the inner mitochondrial membrane, catalyses the final step of haem biosynthesis by the insertion of iron into protoporphyrin IX to yield protoheme (Jones and Jones 1969; Meissner et al. 2001). FECH will be reviewed in more detail in the following section.

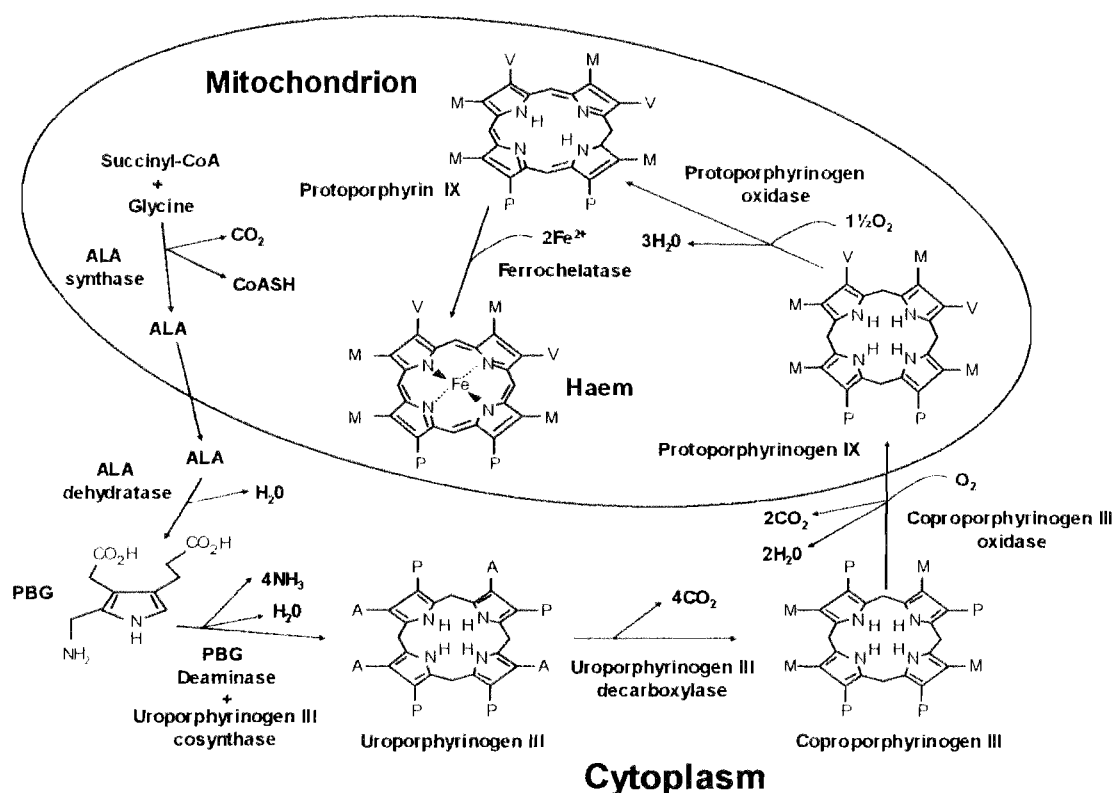


Figure 1.2. Haem biosynthetic pathway in mammals showing compartmentalisation reactions in the mitochondria and cytosol (from A. E. Medlock, PhD dissertation, University of Georgia, 2002)
Key: M: CH₃; V: CH-CH₂; A: CH₂-COOH; P: CH₂-CH₂-COOH

In mammals, haem synthesis is an efficient, tightly-regulated process that ensures homeostatic concentrations of haem in the body. Erythropoietic and hepatic cells are the primary sites of haem synthesis. It appears that synthesis in these two tissues is regulated differently, although in both cases primary regulation is at the level of ALA synthase (Moore et al. 1987c). Regulation and differential expression of other enzymes are likely to occur. The two isoforms of the ALA synthase enzyme used for non-erythroid haem synthesis (ALAS1) and erythroid haem synthesis (ALAS2) (Watanabe et al. 1983, 1984; Bishop et al. 1990) are encoded by two distinct mRNA molecules with different regulatory and untranslated regions (Cox et al. 1991). Haem itself activates regulatory mechanisms or inhibits ALA synthase directly, as is characteristic of feedback regulation (Andrew and Riley 1990). These mechanisms include repressing its synthesis and blocking the transport of synthesised ALAS1 into the mitochondria (Hamilton et al. 1991; Lathrop and Timko 1993), specifically in the liver and non-erythroid tissues of higher animals (Meissner et al. 2001). Earlier reports proposed that ALAS1 regulates haem at a translational level (Scholnick 1972) and at a transcriptional level (Srivastava 1988). However, studies by Roberts and Elder (2001) on the ALAS1 promoter, and by Guberman et al (2003) on transcription factor binding sites support transcriptional regulation of ALAS1.

ALAS2 regulation is different. Expression and regulation of erythroid haem synthesis are linked to differentiation events initiated by erythropoietic iron availability and globin chain production (May et al. 1995; Weiss et al. 1997). Regulation of both hepatic and erythroid haem synthesis is a complex issue and there is much additional information in the literature. However, as it is not directly relevant to this dissertation, this regulation is not discussed further.

1.2. The porphyrias

A deficiency of any enzyme in the haem biosynthetic pathway described above (section 1.1.3, pg. 3), may lead to a specific porphyria due to abnormal accumulation of the porphyrin intermediates and/or their precursors ALA and PBG (Nordmann and Puy 2002). Clinically, the porphyrias may present with acute neurological attacks and/or photocutaneous sensitivity (Elder et al. 1990). Hence the two most useful ways of classifying the porphyrias are by the site of their primary enzyme deficiency and the presence/absence of acute attacks (see Table 1.1).

Table 1.1. Summary of the clinical and molecular characteristics of the porphyrias

Disorder	Affected Enzyme	Clinical Effects	Inheritance
δ -Aminolaevulinate dehydratase deficiency porphyria	δ -Aminolaevulinate dehydratase	Acute attack	Autosomal recessive
Acute intermittent porphyria	Porphobilinogen deaminase	Acute attack	Autosomal dominant
Congenital erythropoietic porphyria	Uroporphyrinogen cosynthase	Photosensitivity	Autosomal recessive
Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Photosensitivity	Acquired ($\pm 75\%$) or autosomal dominant ($\pm 25\%$)
Hereditary coproporphyria	Coproporphyrinogen oxidase	Acute attack and/or Photosensitivity	Autosomal dominant
Variegate porphyria	Protoporphyrinogen oxidase	Acute attack and/or Photosensitivity	Autosomal dominant
Erythropoietic protoporphyria	Ferrochelatase	Photosensitivity	Autosomal recessive or complex (see section 2.3.1)

The most common inheritance pattern observed amongst the porphyrias is autosomal dominant inheritance. Recessive and complex forms of inheritance are encountered. Porphyria cutanea tarda is frequently acquired, though autosomal dominant inheritance is also possible (Badminton and Elder 2005; Meissner and Hift 2005). Rare cases of other forms of porphyria, acquired as a result of somatic mutations, have been described (Aplin et al. 2001; Goodwin et al. 2006).

It is important to note, however, that incomplete penetrance is a feature of the dominant forms of porphyria, and not all individuals express a clinical porphyric phenotype. Of these asymptomatic individuals, a proportion will exhibit biochemical porphyrin abnormalities, while others show no disease phenotype, despite carrying a deleterious gene for the specific haem enzyme (Elder et al. 1990; Kauppinen 2005). The porphyrias can therefore be considered “low-penetrance genetic conditions” (Badminton and Elder 2005). Both genetic and environmental factors underlying susceptibility and expression of the disease have been, and continue to be, active areas of research.

1.2.1 Diagnosis and treatment of the porphyrias

Accurate, timeous diagnosis of porphyria is important. Rational diagnostic approaches involve both clinical and laboratory investigations and are aimed at: a) establishing whether a patient has porphyria; b) assessing whether the presenting symptoms are due to the porphyria and c) correctly typing the porphyria in order to provide appropriate treatment and advice for the patient and family (Meissner and Hift 2005; Anderson et al. 2005). The most common laboratory investigations centre around the use of porphyrin and porphyrin precursor measurements, and the demonstration of a porphyria-related mutation in the relevant gene. Metabolite measurements include urinary ALA/PBG, faecal, urinary and plasma porphyrin concentrations and plasma fluorescence emission spectroscopy (“plasma fluoroscan”) (Meissner and Hift 2005). Management of the acute attack involves confirming the diagnosis, removal of any known precipitating factors (e.g. porphyrinogenic drugs), analgesia, fluid therapy and the only uniformly effective specific therapy; administration of haem arginate or haemin (Hift and Meissner 2005).

Management of the skin disease in porphyria attempts to minimise the accumulation of porphyrins, reduce exposure to UV light and avoid minor trauma. The skin disease of PCT is particularly rewarding to treat in that most cases can be considered “curable”, since removal of specific trigger factors, such as alcohol exposure and hepatic iron overload, is commonly followed by remission (Kirsch et al. 1998).

CHAPTER 2

Ferrochelatase and Erythropoietic Protoporphyrin

University of Cambridge

CHAPTER 2

FECH and EPP

Erythropoietic protoporphyria (EPP) is an inherited disorder resulting from a deficiency of ferrochelatase (FECH), the final enzyme of the haem biosynthetic pathway (Bonkowsky et al. 1975; de Goeij et al. 1975).

2.1 Ferrochelatase

Ferrochelatase (E.C. 4.99.1.1, protoheme ferro-lyase) is constitutively expressed in all cell types because of the demand for haem by haemoproteins including those involved in reduction-oxidation reactions such as the respiratory cytochrome complexes. FECH activity, however, is highest in the liver for cytochrome P450 synthesis, and in erythroblasts for haemoglobin synthesis during the final stages of erythropoiesis (Brenner and Fraser 1991; Tugores et al. 1994; Fujita 1997).

2.1.1. Catalytic mechanism

The FECH enzyme probably employs an ordered, sequential bi-bi reaction mechanism in which Fe^{2+} binds to the enzyme before the porphyrin and the two protons are abstracted from the porphyrin following release of haem (Figure 2.1) (Dailey and Fleming 1983; Burden et al. 1999).

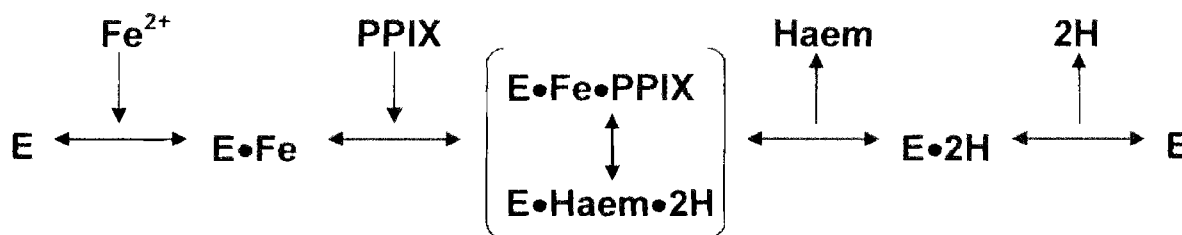


Figure 2.1. Schematic representation of the reaction mechanism employed by FECH (from Dailey and Fleming 1983)
Key: E: enzyme; PPIX; protoporphyrin IX

In the catalytic process, metal binding to FECH is followed by distortion of the porphyrin macrocycle into a non-planar structure (Franco et al. 2000) which facilitates porphyrin-metal chelation and the release of haem (Lavallee 1988; Blackwood et al. 1997; Ferreira 1999; Burden et al. 1999). Simultaneous tilting or doming of the four pyrrole rings of the porphyrin, resulting in the distortion of yeast FECH, was demonstrated by resonance Raman spectroscopy (Blackwood et al. 1998; Sellers et al. 2001). Crystal structures of FECH of both *Bacillus subtilis* (Al-Karadaghi et al. 1997; Lecerof et al. 2000) and human (Wu et al. 2001) support this proposed mechanism (Figure 2.1) and iron-binding and metallation amino acid residues have been identified (Figure 2.2).

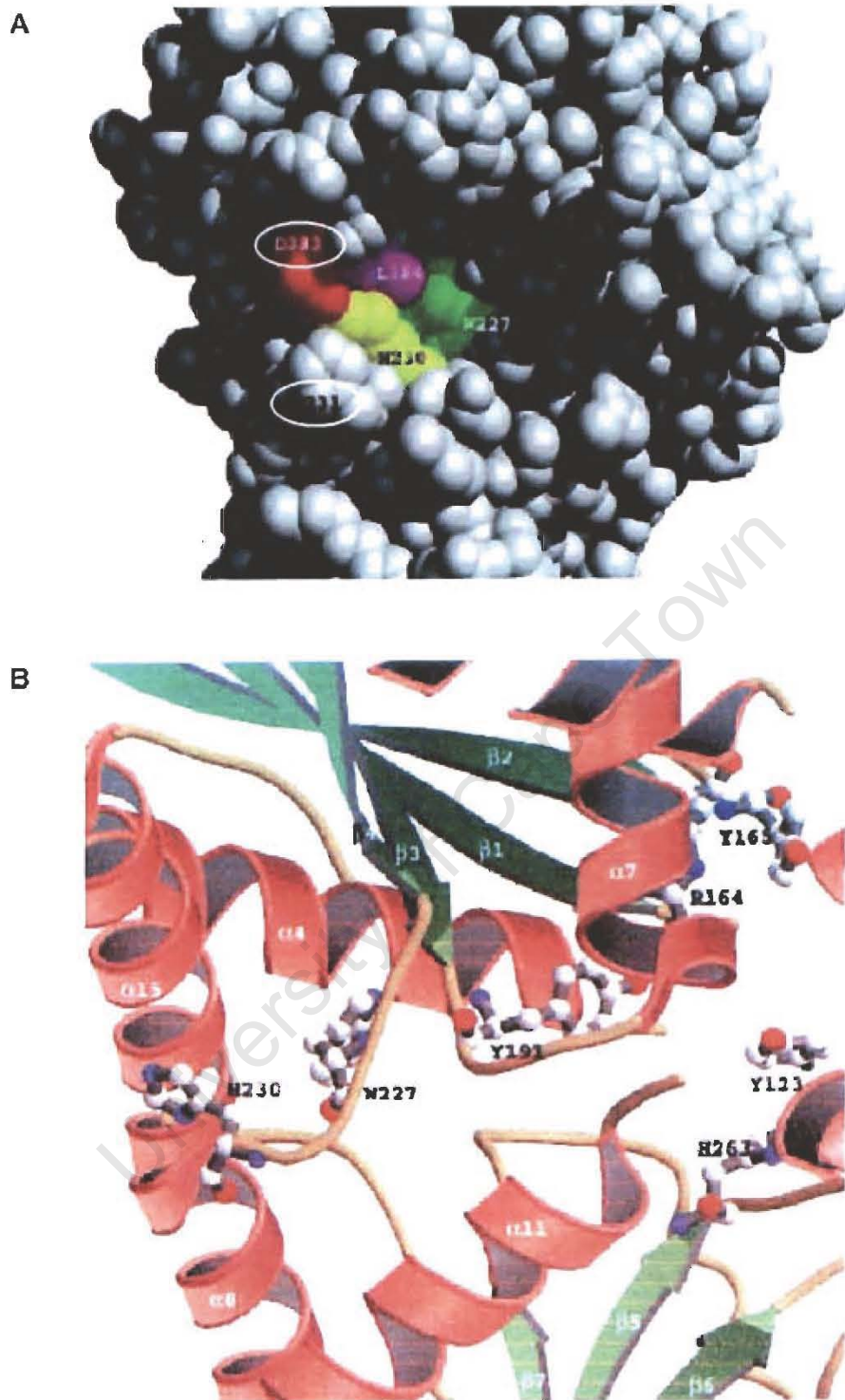


Figure 2.2. A) Space-filling diagram and B) ribbon diagram of FECH showing putative iron-binding and metallation residues; D383 and H231 (A) H263, R164 and Y165 (B) (from Sellers et al. 2001)

There is general consensus that macrocycle distortion is thermodynamically stable and will allow release of the product following catalysis (Dailey and Dailey 2003). Although this FECH mechanism is likely to be relatively conserved amongst species, the model for iron entry into the active site pocket may only apply to mitochondrial-associated eukaryotic FECHs and may differ amongst other species, particularly amongst soluble FECHs of some bacterial species (Sellers et al. 2001).

2.1.2. Protein characterisation

Ferrochelatase has been purified from various sources, including the yeast *Saccharomyces cerevisiae* (Camadro and Labbe 1988), rat (Taketani and Tokunaga 1981), mouse (Karr and Dailey 1988) and human liver (Mathews-Roth et al. 1987). Human FECH, a 423 amino acid protein, has a molecular mass of approximately 42kDa (Mathews-Roth et al. 1987). Radiation inactivation showed that the functional size of FECH in bovine liver is approximately 80kDa suggesting two subunits of approximately 40kDa (Straka et al. 1991), in contrast to *Bacillus subtilis* FECH which is a monomeric protein (Al-Karadaghi et al. 1997). Crystallisation of human FECH confirmed that the human enzyme is a homodimer (Burden et al. 1999; Wu et al. 2001).

Nuclear-encoded eukaryotic FECH is synthesised in the cytoplasm and then translocated to the matrix side of the mitochondrial membrane (Camadro and Labbe 1988). Translocation of FECH across the mitochondrial membrane requires a transmembrane potential (Karr and Dailey 1988). It is converted to the active form on proteolytic cleavage of a leader peptide of 30-80 amino acids (62 in human FECH) which is essential for intracellular targeting and localisation of FECH and, in turn, for optimal functioning of the enzyme (Nakahashi et al. 1990; Prasad and Dailey 1995; Cobbold et al. 2006).

Mammalian FECHs are distinct from yeast, plant and prokaryotic FECHs in that they contain a “uniquely coordinated”, novel prosthetic group, a [2Fe-2S] cluster (Cys-X₂₀₈-Cys-X₂-Cys-X₄-Cys^{*}) (Dailey et al. 1994; Ferreira et al. 1994; Wu et al. 2001). This cluster, although necessary for enzyme function, does not appear to be involved in catalysis and is distinct from the Fe²⁺ binding site (Dailey et al. 1994). It is however labile and degrades rapidly in the presence of nitric oxide, causing inhibition of FECH activity. A possible role for this regulation may be a defence mechanism against bacterial infection to decrease iron availability for the pathogen (Sellers et al. 1996). More recently, structural studies have suggested that the cluster may anchor the C-terminal domain of the monomer at the dimer interface thereby indirectly facilitating dimerisation (Wu et al. 2001).

Three highly conserved cysteine residues in human FECH are ligands for the [2Fe-2S] cluster (Crouse et al. 1996). These occur in a 30 to 50-residue C-terminal extension that is unique to eukaryotic FECH (Dailey et al. 1994), while the fourth cysteine lies approximately 200 residues towards the amino terminus (Sellers et al. 1998). Crystallisation and characterisation of human FECH and the [2Fe-2S] cluster revealed that the iron positions for the cluster are consistent with those observed for [2Fe-2S] clusters in other proteins (Burden et al. 1999).

* X represents any amino acid

The subscripted number represents the number of residues between coordinating Cys residues

2.1.3. Structure

The first 3-dimensional structure for FECH was determined for the *Bacillus subtilis* form of the protein. Two similar domains (consisting of four-stranded β -sheet flanked by α -helices) forming a cleft was suggested to be a potential binding site for protoporphyrin IX (Al-Karadaghi et al. 1997).

The first crystallisation of a recombinant human FECH was obtained by Burden et al in 1999 and was followed by the resolution of the structure to 2.0 Å (Wu et al. 2001). Each mature FECH monomer consists of amino acids 65-423 and a [2Fe-2S] cluster. The FECH dimer (Figure 2.3A) has a mass of approximately 86kDa and has 580 associated water molecules (Wu et al. 2001). Approximately 300 amino acids form a catalytic core and are conserved in all FECHs (Ferreira 1999). The entrance to the active site of FECH is lined with hydrophobic residues arranged linearly as hydrophobic ridges. A 12-residue hydrophobic lip in this region is thought to mediate membrane association. This positioning allows the poorly soluble porphyrin substrate and haem molecule to be transported via the mitochondrial membrane. A possible metal-binding site, facing the mitochondrial matrix, is suggested to be the initial binding site for ferrous iron at conserved residues His 231 and Asp 383, before being shuttled along a path of conserved residues (Trp 227 and Tyr 191) to the active site (Wu et al. 2001; Sellers et al. 2001).

The active site pocket (Figure 2.3B) is highly conserved and is hydrophilic. The most conserved residue is His 263 which is located in the active site of all known FECH proteins. Other conserved residues (Asp 340, Glu 343, Glu 347, His 341) line one side of the active site, forming an acidic path from the protein surface to the catalytic core. In addition, Arg 164 and Tyr 165 are conserved and probably lie on the opposite side of the protoporphyrin IX macrocycle to these residues (Wu et al. 2001). Site-directed mutagenesis experiments confirmed the essential role of these residues for FECH function since mutation of residues His 263, Asp 340 and Glu 343 resulted in no measurable FECH activity (Kohno et al. 1994; Sellers et al. 2001).

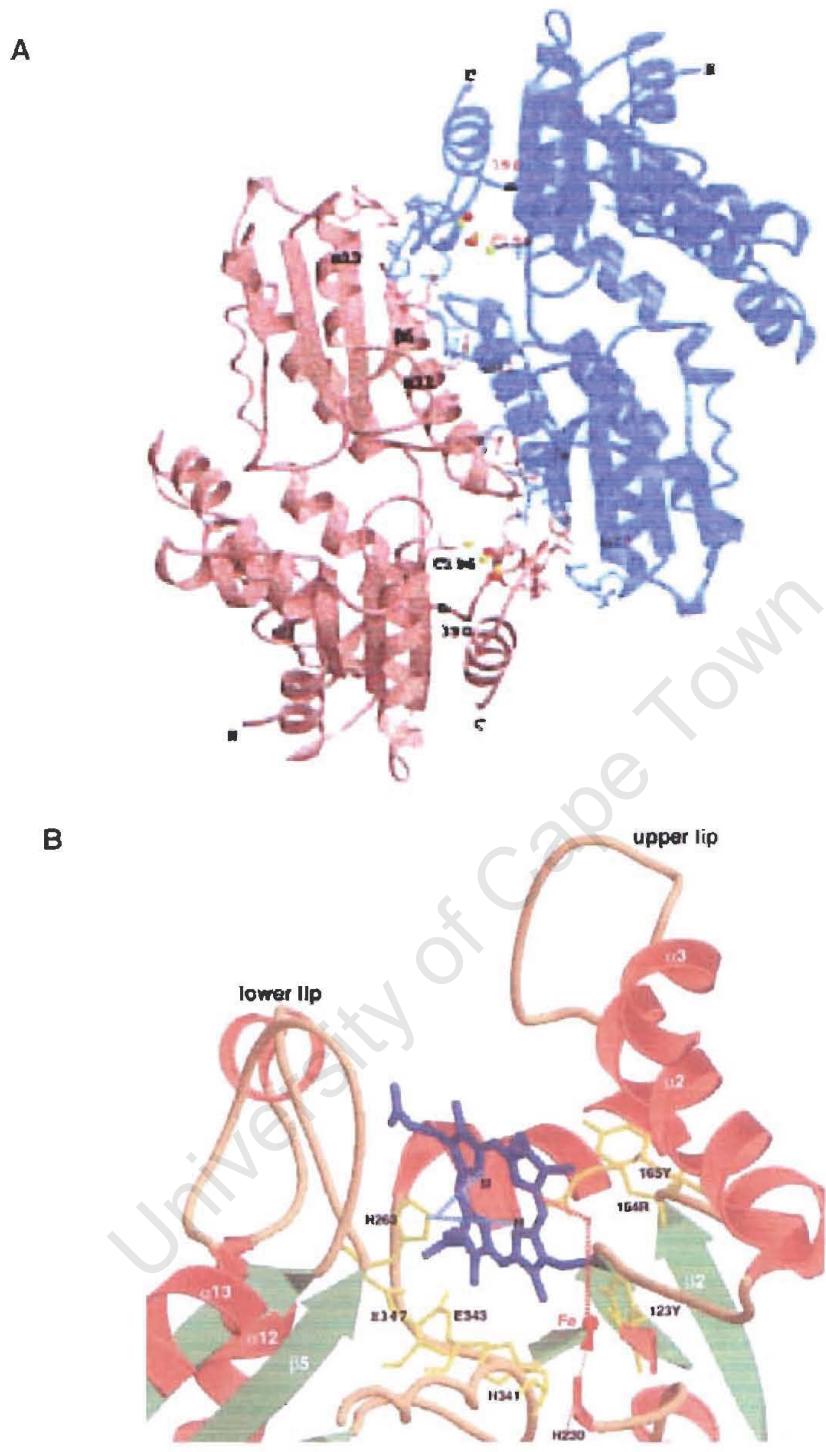


Figure 2.3. Ribbon drawings of human FECH showing: A) the structure of the dimer, including the positions of residue 390 of the C-terminal extension and C196 and; B) the active site pocket with key residues H263, E343 and H341 (from Wu et al. 2001; Sellers et al. 2001)

2.1.4. FECH gene

The gene for FECH was first cloned from *Saccharomyces cerevisiae* in 1990 (Labbe-Bois 1990) and in the mouse in 1991 (Brenner and Frasier 1991). Sequence homology among FECH genes is <10% (Wu et al. 2001). The human cDNA transcript was initially cloned and sequenced by Nakahashi et al in 1990. The transcript is approximately 2.5kb in length of which 1.3kb is coding (Taketani et al. 1992). Two mRNAs, transcribed from a single gene, with differences in their 3'-polyadenylation signals have been reported. It was suggested that the transcripts encode a housekeeping form expressed in all cells, and an erythroid-specific form (Nakahashi et al. 1990; Tugores et al. 1994; Taketani and Fujita 1995). Human FECH is the product of a single gene that maps to chromosome 18q21.3, spans approximately 45kb and consists of 11 exons (Brenner et al. 1992; Taketani et al. 1992). Unlike other enzymes of the haem biosynthetic pathway, *FECH* is transcribed from a single promoter in erythrocytes and other tissues (Taketani et al. 1992). This promoter lies in an area rich in CpG islands (>80% C+G nucleotides) and does not contain the usual TATA and CAAT boxes. Consensus *cis*-elements that are recognised by erythroid-specific factors including NF-E2 and GATA-1 and the ubiquitous Sp1 transcription factor are present instead, and may regulate differential expression of *FECH*. The activity of the promoter is 3-4 fold higher in erythroid cells, and is likely to be repressed by negative regulatory elements in the region 1kb upstream from the transcription start site (Tugores et al. 1994). The high C+G content of the proximal promoter region, extending into exon 1 and intron 1, is characteristic of many housekeeping genes. This region is highly accessible to regulatory proteins as it cannot be tightly packed into chromatin and can therefore be constitutively expressed (Tugores et al. 1994; Lewin 2000).

When conducting a molecular study of *FECH*, it is important to be aware of the existence of a *FECH* pseudogene to ensure that similar sequences are not erroneously amplified and mistaken for the expressed gene. The characteristics of this pseudogene will be described in detail in the following section.

2.1.5. *FECH* pseudogene

A pseudogene of approximately 30kb with overall nucleotide sequence identity of >80% to the functional *FECH* gene was identified by Whitcombe et al in 1994. No corresponding intronic sequences in the open reading frame were identified. The sequence does not encode a functional protein, as numerous insertions and deletions disrupt the reading frame of the transcript. Multiple termination codons are dispersed throughout the transcript and can be identified in all three reading frames. Fluorescence *in situ* hybridisation of metaphase chromosomes localised the pseudogene to 3p22-p23 (Whitcombe et al. 1994). Alignment of the *FECH* cDNA with the genomic sequence for this region of chromosome 3 revealed that exons 2-11 are closely homologous to the pseudogene with 82-93% nucleotide sequence identity but only 63% homology in the 5'-region, including exon 1. The pseudogene is thought to be the product of reverse transcription of human *FECH* mRNA which was subsequently transposed to chromosome 3. Accumulation of mutations in this transcript finally resulted in a non-functional, processed-type human *FECH* pseudogene on chromosome 3. No pseudogene transcripts were detected in human liver tissue or reticulocytes although it remains a possibility that low-level transcription may occur in other tissues.

2.2. EPP – Clinical aspects

Erythropoietic protoporphyria, unlike the other commonly encountered porphyrias, is characterised by the onset, in early infancy, of life-long photocutaneous sensitivity (DeLeo et al. 1996). The partial deficiency of FECH leads to accumulation of protoporphyrin IX, primarily in erythrocytes, plasma, skin and liver and is responsible for the clinical and pathological effects of the disease (de Goeij et al. 1975; Bloomer et al. 1991). Although previously thought to be more common in males, both sexes appear to be equally affected (Cripps and MacEachern 1971; Rufener 1987; Todd 1994). In South Africa, the prevalence of EPP has not previously been determined. However, 17 affected families have been documented by the Lennox Eales UCT Porphyria Laboratories, which is the major referral centre for porphyric patients in South Africa.

The world-wide incidence of EPP is considered low. There is an estimated incidence of 1:75000 (Went and Klasen 1984) in the Netherlands 1:130000 (Murphy et al. 1985) and 1:200000 (Elder et al. 1990), in the United Kingdom (UK), 1:79000 in Northern Ireland (Todd 1993, 1994) and at least 1:200000 in Sweden (Wiman et al. 2003). The largest cohorts of patients reported to date appear in the more recent literature, viz. in Finland (Kauppinen and Timonen 2005: 36 patients), the UK (Whatley et al. 2004: 105 patients; Holmes et al. 2006: 223 patients), France (Gouya et al. 2006: 241 patients), and the USA (Chen et al. 2002: 34 patients and Risheg et al. 2003: 52 patients).

2.2.1. History of EPP

EPP was first reported in 1953 by Kosenow and Treibs and named and defined by Magnus et al in 1961. The patient observed by Magnus et al (1961) presented initially with solar urticaria, and had experienced symptoms since the age of eight. Skin photosensitivity was greatest in the Soret band 400-410nm with erythema, itching and development of weals. Urinary porphyrins were normal. Both the red cells and faeces contained increased amounts of protoporphyrin IX and coproporphyrin.

The first South African case of EPP, an 11 year old male of European ancestry, was observed in Cape Town in 1957, and was reported by Sweeney et al in 1963.

2.2.2. Clinical presentation of EPP

The clinical symptoms observed in EPP include photosensitivity to visible light and, in rare cases ($\pm 1-7\%$), fatal liver disease with cholelithiasis (Frank and Doss 1989; Baart de la Faille et al. 1991; Nordmann 1992; Cox 1997; Meerman 2000; A. Anstey, Cardiff – personal communication 2005). Patients describe erythema, oedema, pruritus and a burning sensation following short exposure to visible light (DeLeo et al. 1976). A common observation among EPP patients is a decreasing tolerance to sunlight on subsequent exposures, called the “priming phenomenon” (Poh-Fitzpatrick 1989). In contrast to other photosensitive porphyrias, cumulative damage is minor and is restricted to elliptical scarring on the hands and face or thickening of the skin on the knuckles and bridge of the nose. The lack of obvious permanent features may lead to diagnosis being delayed for several years (Todd 1994; Varma 2000).

Less common symptoms associated with the disease include painful changes in the fingernails and toenails, extreme temperature sensitivity, chills, and petechiae (Rufener 1987). Several case reports attest to the association of anaemias with EPP (Leone 2000; Davenport 2004), and larger studies support this. Went and Klasen found that, in a cohort of 200 EPP subjects, the mean haemoglobin concentration was 1.5g/dL lower than the mean concentration in their unaffected relatives. In a recent study in the UK, one third of a cohort of 223 EPP individuals were found to be anaemic (A. Anstey and M. Badminton, Cardiff - personal communication 2006). Mild hypochromic microcytic anaemia is reported to occur in approximately 25% of EPP patients (Mathews-Roth 1974; Elder 1990; Cox et al. 1998; Lecha 2003). Romeo (1977), in a review of the porphyrias, refers to a frequent, mild hemolytic anaemia in EPP but this is unsupported by data. Baart de la Faille et al (1991) reported that 50% of a personal cohort of Dutch patients had microcytic anaemia with iron depletion. He reported evidence that the circulating erythrocytes appeared to represent a young population, possibly the result of mild photohaemolysis.

Key et al (1992) reported two cases in whom severe haemolytic anaemia appeared to complicate advanced liver disease, and which was further exacerbated by the operative procedure associated with liver transplantation. Indeed, in the Cardiff study of 223 EPP individuals referred to above, anaemia was independently associated with liver disease in EPP. Both poor absorption of iron from the gut and inadequate haemoglobin synthesis have been suggested as possible mechanisms.

The anaemia observed in EPP appears to be due to deficient iron utilisation rather than a deficiency of iron itself (Cox et al. 1998) and Rademakers et al (1993) observed mitochondrial iron deposition in patient erythroblasts to support this. Brun et al (1988) showed that protoporphyrin IX concentrations are lower in mature erythrocytes than in reticulocytes. He suggests that protoporphyrin IX may dissociate from haem binding sites on the globin molecule in erythrocytes as they mature resulting in lower haemoglobin concentrations and therefore anaemia.

An assessment of the psychosocial aspects of EPP in 12 patients revealed that the patients had gradually learned how much sun they could tolerate (Rufener 1987). Typically, they would move indoors or into the shade whenever they sensed they would burn. However, school pupils found this more difficult as they felt they could not excuse themselves repeatedly from certain activities. Detachment from their surroundings was reported, as well as changes in personality, including nervousness, tension, aggressiveness, hypersensitivity and suicidal ideation.

2.2.3. Biochemical diagnosis of EPP

Biochemical diagnosis of EPP is based primarily on the presence of elevated levels of protoporphyrin IX in erythrocytes, blood plasma and faeces (Elder et al. 1990; Bonkovsky and Barnard 1998; Thunell et al. 2000; Murphy 2003). An explanation for this accumulation of protoporphyrin IX is detailed further in sections 2.2.4 and 2.2.5.

A rapid-screening test for raised erythrocyte protoporphyrin IX concentrations, which included ether/acetic acid separation followed by hydrochloric acid extraction of porphyrins from red blood cells (RBC), with subsequent visualisation under UV light, was described in 1965 by Rimington and Cripps. Since then there have been many variations on this, some qualitative and some quantitative, but all have utilised protoporphyrin IX spectral properties. Today high performance liquid chromatography (HPLC) methods with fluorescence detection (Gray et al. 1977; Guo et al. 1991) and fluorescence methods based on Piomelli (1977) are routinely employed for assay of RBC protoporphyrin IX and other blood or excreted porphyrins, as appropriate.

The plasma "fluoroscan", first described by Poh-Fitzpatrick (1980), is an efficient front-line test to determine increased porphyrin concentrations in blood plasma by fluorescence spectroscopy and is important for the differential diagnosis of porphyrias (VP, EPP and PCT/AIP) (Pathak and West 1982; Meissner and Hift 2005). Plasma is diluted in phosphate-buffered saline and then scanned at an emission wavelength of 570-650nm, using a fluorescence spectrophotometer set at a fixed excitation wavelength of 405nm. An emission maximum peak at 632nm is indicative of increased protoporphyrin IX and therefore EPP (Elder et al. 1990). In VP, which is also associated with the retention of protoporphyrin IX, the emission peak occurs at 625nm, apparently because in this condition the porphyrin is bound to a peptide (Lamola et al. 1981). A tightly bound protein porphyrin complex has been isolated from the plasma of a patient with VP and fluorescence studies by Lamola (1982) show that when free protoporphyrin IX is bound to haemoglobin (probably not at the active site), fluorescence is emitted at 626nm (Longas and Poh-Fitzpatrick 1982). However, membrane-bound protoporphyrin IX has an emission maximum at 634nm (Lamola 1982).

In South Africa it has been shown that plasma "fluoroscanning" is more sensitive and specific than faecal porphyrin determination for the diagnosis of VP (Meissner and Hift 2005). However, not all symptomatic individuals with EPP display a plasma peak at 632nm and in some samples the peak is shifted to ± 625 nm which is more typical of VP. Quantitative analysis of erythrocyte porphyrins, and/or DNA analysis, will however make the distinction between VP and EPP (Atkins and Nedorost 2003; Meissner and Hift 2005).

It is important to note that erythrocyte protoporphyrin IX may be raised in other conditions, including lead poisoning and iron deficiency anaemia (Bonkovsky and Barnard 1998). However, in these cases the predominant porphyrin species observed is zinc-chelated protoporphyrin IX (ZPP) rather than the free protoporphyrin IX characteristic of EPP (Lamola and Yamane 1974). In cases of lead intoxication ZPP accumulates in erythrocytes because lead inhibits FECH by interfering with the binding of iron to protoporphyrin IX (Bailey and Needham 1986; Dailey 1990). Zinc is a good substrate for FECH and in iron-deficiency anaemia it can be used as a substitute for Fe^{2+} when this is in short supply (Dailey et al. 2000). Zn^{2+} competes with Fe^{2+} for the chelatase at low iron levels, resulting in the incorporation of Zn^{2+} into protoporphyrin IX (Camadro and Labbe 1982; Camadro et al. 1984).

Accumulation of ZPP in the above conditions is not associated with photosensitivity (Rimington and Cripps 1965; Piomelli et al. 1975; Haeger-Aronson 1982). ZPP does not diffuse out of the erythrocytes into the plasma (Lamola et al. 1975) and thus no photosensitivity occurs as there is no porphyrin accumulation in the skin; in contrast to EPP where free protoporphyrin IX readily diffuses into the plasma and the skin. The absence of raised faecal levels of protoporphyrin IX in individuals with lead toxicity and iron-deficiency anaemia supports this explanation (Piomelli et al. 1975).

2.2.4. Pathogenesis of photocutaneous sensitivity in EPP

Photosensitivity associated with EPP occurs as a direct consequence of the accumulation of porphyrin intermediates, specifically protoporphyrin IX, in RBCs and the skin (Harber et al. 1982). During erythropoiesis a high rate of haem synthesis occurs. The partial block at the level of FECH results in an accumulation of protoporphyrin IX in maturing RBCs in the bone marrow. Protoporphyrin IX may diffuse from these cells as they circulate in the blood stream, and become bound to, amongst other proteins, haemopexin and albumin in the plasma (Schothorst et al. 1970; Lamola 1982). In addition, light-induced transfer of protoporphyrin IX from RBCs to endothelial cells may occur because of their close proximity to dermal capillaries (Brun 1990; Brun and Sandberg 1991). Violet light can penetrate the skin and reach the upper dermal layers. When excess porphyrins in the endothelial cells are exposed to this light, particularly in the Soret band (400-410nm), they absorb radiant energy; raising the ground state orbital electrons of protoporphyrin IX to an "excited", unstable singlet state and subsequently to their triplet state (Todd 1994) (see Figure 2.4). The stable triplet state of the "excited" protoporphyrin IX allows it to react with ground-state molecular oxygen, and reactive oxygen species are produced, which may induce cell damage and/or lysis primarily via membrane lipid peroxidation (Goldstein and Harber 1972; Girotti 1990; Williams et al. 1994; Takeshita et al. 2004). Photodamage may not be confined to loss of cellular membrane integrity. Protoporphyrin IX was observed to accumulate specifically in lysosomes of fibroblasts, and lysosomal membranes are also targets for photo-oxidation. Further damage includes cross-linking of proteins and oxidative damage of nucleic acids (Slater and Riley 1966; Schothorst et al. 1977).

In addition to the direct consequences, “excited” protoporphyrin IX has also been found to activate the complement system leading to mast cell activation, generation of inflammatory mediators and chemotaxis of polymorphonuclear leukocytes (Lim et al. 1984; Poh-Fitzpatrick 1985; Meerman 2000). Continuous endothelial damage and subsequent repair accounts for the thickened and waxy appearance of the skin, due to the generation of new basal lamina (Hönigsmann et al. 1976; Lim 2005). Thus these mechanisms are likely to explain the typical burning sensation, swelling, itchiness and redness described by EPP patients on exposure to sunlight (Todd 1994).

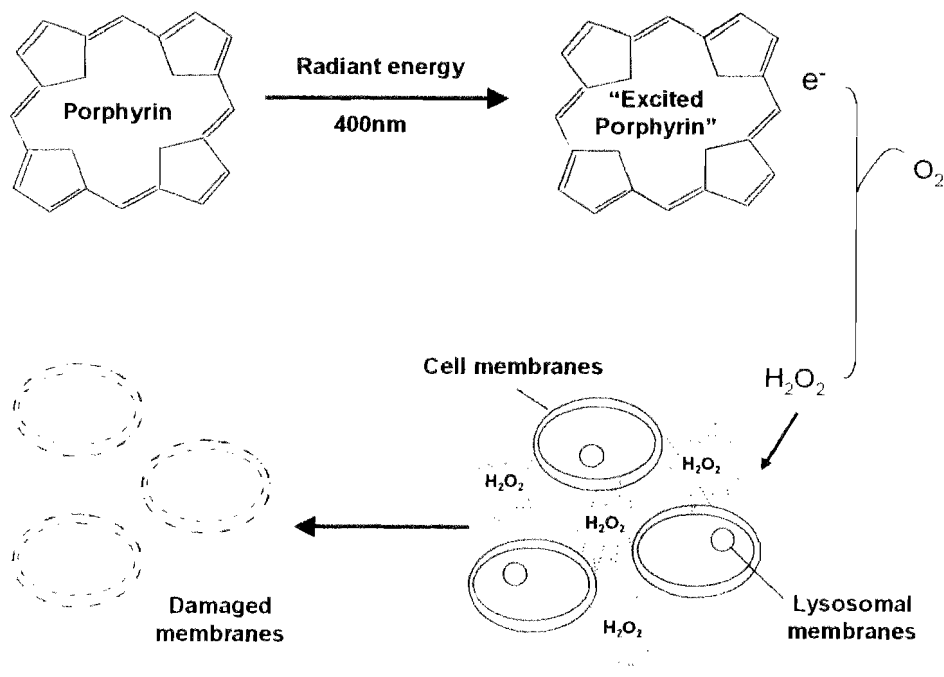


Figure 2.4. Proposed mechanism of the pathogenesis of photocutaneous sensitivity in EPP (from DeLeo et al. 1976)

2.2.5. Pathogenesis of liver disease in EPP

As mentioned earlier, a proportion of individuals with EPP, approximately 1-7%, develop liver disease (Doss and Frank 1989; Baart de la Faille et al. 1991; Nordmann 1992; Cox 1997; Meerman 2000; A. Anstey, Cardiff – personal communication 2005). This appears to result from the accumulation of protoporphyrin IX in the liver (Bloomer and Enriquez 1982). Protoporphyrin IX is not water-soluble and therefore cannot be excreted by the kidneys. The liver excretes the majority of the protoporphyrin IX via the bile and therefore high levels of protoporphyrin IX can be detected in the faeces. However, due to the high biliary concentration of protoporphyrin IX, crystallisation may occur, resulting in cholelithiasis with resultant biliary obstruction (Meerman 2000). In severe cases, intracellular protoporphyrin IX accumulation of the bile ducts with resulting clinical sequelae has been observed in Kupffer cells, hepatocytes and biliary canaliculi (Komatsu 2000a).

The porphyrin excretory spectrum appears to change in EPP individuals with end-stage liver disease. Normally, the porphyrin profile in urine is normal and high levels of protoporphyrin IX are recorded only in the faeces. In patients with advanced liver disease, urinary uroporphyrin and coproporphyrin concentrations are also significantly elevated while faecal uroporphyrin, coproporphyrin and mesoporphyrin concentrations are raised, in addition to the increased protoporphyrin IX (Gray et al. 1977; Frank and Doss 1991).

2.3. EPP – Molecular aspects

Incomplete penetrance is a feature of many of the porphyrias, including AIP and VP (Wang et al. 1995). There has, however, been long-standing debate over the mode of inheritance of EPP. In 1984 Went and Klasen proposed a three-allele system to explain the inconsistent inheritance of EPP. In their study of 200 patients, representing 91 Dutch families, they concluded that the genetic model of disease pointed to a recessive mode of inheritance, yet the pattern and presence of an occasional fluorescent red cell within family members was autosomal dominant in nature. Thus the mode of inheritance of EPP has been described as autosomal dominant with incomplete clinical penetrance (10-20%) (OMIM +17700). Indeed, the apparent discordance in disease manifestation between the patient and carrier parent has been particularly puzzling in many EPP studies, at least until recently.

2.3.1. Inheritance of EPP

An autosomal dominant disease, by definition, requires a single deleterious allele for disease expression. Where an enzyme is defective as a result of a dominant disorder, one would expect to measure an approximate 50% reduction in catalytic activity, as the wild type allele would continue to produce normal protein. However, individuals who are heterozygous for *FECH* mutations exhibit $\pm 50\%$ normal activity and are typically asymptomatic (Gouya et al. 1996; Cox et al. 1998), while symptomatic EPP patients consistently show 15–25% *FECH* activity even in the absence of clear evidence of compound heterozygosity (Brenner et al. 1992; Schneider-Yin 2000).

That said, a few cases have been reported in which EPP is inherited in a true autosomal recessive manner, as a result of compound heterozygosity (Lamoril et al. 1991; Sarkany et al. 1994a; Poh-Fitzpatrick et al. 2002; Whatley et al. 2004). The prevalence of true recessive inheritance of EPP in the UK and France has been reported as 3% and 4%, respectively (Whatley et al. 2004; Gouya et al. 2006).

An early mechanism proposed for the difference in *FECH* activity between symptomatic and asymptomatic individuals with, apparently, a single mutated allele is a dominant-negative effect from the deleterious *FECH* allele. As *FECH* is a homodimer, it is possible that the holoenzyme may only be active if it contains two wild type subunits. The presence of a mutant subunit in the dimer would inactivate the wild type subunit

resulting in total loss of activity (Brenner et al. 1992; Wu et al. 2001). This has been debated (Sarkany et al. 1994b; Wang et al. 1994; Gouya et al. 1996). Indeed, Ohgari et al (2005) showed that a dimer consisting of one mutant and one wild type FECH molecule is active, the degree of activity depending on the mutation. However, enzyme activity was less than 50% of a wild type homodimer, suggesting that only the wild type subunit functioned in catalysis. Najahi-Missaoui and Dailey (2005) confirmed that wild type/mutant type heterodimers have between 0 and 50% enzyme activity. They proposed that a subset of *FECH* mutations, particularly those that disrupt the [2Fe-2S] cluster, or are present at the dimer interface, may give the EPP clinical phenotype via this dominant-negative mechanism, even in the absence of the disease-associated IVS3-48C polymorphism (the role of this polymorphism in EPP will be described below). Whether this mechanism operates in EPP families has yet to be established.

More recently, the >50% reduction in FECH activity in symptomatic patients with EPP has been ascribed, in the majority of families, to the co-inheritance of a 'low-expression allele' in *trans* to a deleterious allele (Gouya et al. 1996). These authors confirmed that symptomatic disease was associated with the inheritance of a specific allele from the non-carrier parent in *trans* to the allele associated with the deleterious mutation. They confirmed that this allele resulted in reduced expression of *FECH* giving <25% normal FECH activity in the symptomatic individual with a *FECH* mutation, in contrast to 50% activity in asymptomatic siblings carrying the same mutation.

Later, they were able to find an association between this 'low-expression' allele and a specific haplotype [-251G; IVS1-23T; IVS2 μ satA9] which, in turn, led to more accurate risk calculation for genetic counseling (Gouya et al. 1999).

Interestingly, an individual who inherits two 'low-expression' alleles does not show any clinical or biochemical evidence of EPP (Gouya et al. 1999; Schneider-Yin et al. 2000). They concluded that the inheritance of this 'low-expression' allele is a requirement for symptomatic EPP, suggesting that when FECH activity falls below a critical threshold excess protoporphyrin IX cannot be cleared by the liver and photosensitivity results (Gouya et al. 1999). This mechanism of disease has been observed for hereditary elliptocytosis, where the degree of clinical manifestation is increased when the α -spectrin gene is expressed at a low level in *trans* to a mutated allele (Alloisio et al. 1991; Gouya 1999).

Currently, the modulation of the penetrance of EPP by this wild type 'low-expression' *FECH* allele has been found to be directly associated with a single intragenic polymorphism (IVS3-48C) that was present in 38/40 French EPP patients investigated by Gouya et al in 2002 and confirmed in larger cohorts in 2004 and 2006.

This hypomorphic allele, which is estimated to be the result of a single mutational event that occurred 60 000 years ago (Gouya et al. 2006), was found to have a frequency of 11% in the French population. The presence of the IVS3-48C polymorphism in intron 3 prompts the use of a cryptic acceptor splice site 63 bases upstream from the normal splice site (Figure 2.5.). This cryptic splice site is used constitutively whether the allele carries a "T" or a "C" at this position. However, the ratio between correctly spliced mRNA molecules and incorrectly spliced mRNA molecules changes from 80:20 to 60:40 when the "C" is present. Aberrantly spliced mRNA molecules are degraded by the nonsense-mediated decay mechanism because a premature stop codon is created. Thus, wild type ferrochelatase concentrations are reduced to below a critical threshold of 35% as a result (Gouya et al. 2002, 2006).

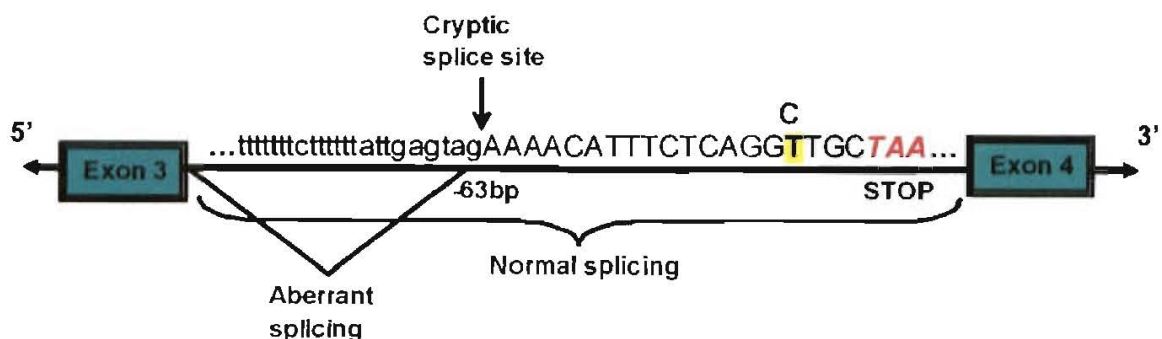


Figure 2.5. Schematic representation of the use of an alternative splice-site in the presence of the IVS3-48C polymorphism (Gouya et al. 2002)

It is interesting that in 1994, Wang et al had suggested an association of a polymorphism with the pathogenesis of EPP, the IVS1-23T polymorphism. Henriksson et al (1996) confirmed that this IVS1-23T allele occurred more frequently among EPP patients than among the normal Finnish population. This polymorphism has subsequently been found to be in linkage disequilibrium with the IVS3-48C polymorphism (Gouya 1999), which accounts for the association.

The presence of a hypomorphic *FECH* allele containing this IVS3-48C polymorphism in individuals presenting with EPP, and its role in clinical expression of the disease, has been confirmed in a few populations, including people of French (Gouya et al. 1999, 2002), Swiss (Schneider-Yin et al. 2001), North American (Wang et al. 1999; Chen et al. 2002; Risheg et al. 2003), Swedish (Wiman et al. 2003), British (Whatley et al. 2004) and Japanese origin (Yasui et al. 2002; Onaga et al. 2004) as well as one Israeli patient (Schoenfeld et al. 2003).

Recent studies by Gouya et al (2006) have shown that the frequency of this IVS3-48C polymorphism differs amongst populations. The highest frequency was observed in Japan (43%) and the lowest in West Africa (<1%). These authors propose that the prevalence of symptomatic EPP in a population would be determined by the frequency of the IVS3-48C polymorphism in the respective population (Gouya et al. 2006; Nakano et al. 2006).

Interestingly, only a few black (African-American) EPP cases have been reported to date (Todd 1994; Baran and Juhlin 2002; Elliot et al. 2004) and no case of EPP in a Black African subject has been reported (Gouya et al. 2006). The prevalence of EPP in Japan has not been precisely determined; however, a study of this nature has been proposed in the light of the reported high prevalence of the IVS3-48C polymorphism in Japan (Nakano et al. 2006).

The terminology used to describe the inheritance of EPP has been confusing. Thus terms such as 'complex mode of inheritance' and 'compound heterozygous disorder' (Risheg et al. 2003), 'pseudodominant' (Gouya et al. 2006), 'autosomal dominant' and 'autosomal recessive' have been used (Cox et al. 1998). However, as mentioned earlier, in contrast to a classical autosomal recessive disorder, homozygosity for the IVS3-48C polymorphism does not cause disease (Badminton and Elder 2005). Thus it is probably best to regard EPP as a 'pseudodominant' condition in which penetrance is modulated by expression of the *FECH* allele inherited in *trans* to a deleterious allele.

2.3.2. EPP in the absence of the IVS3-48C polymorphism

Though co-inheritance of the IVS3-48C allele appears to accompany most cases of EPP, cases have been reported where the IVS3-48C allele is absent (Risheg et al. 2003; Wiman et al 2003), and other genetic, epigenetic and/or acquired factors are predicted to play a role in modulating the phenotype. Recessive inheritance is the most common explanation for disease expression in the absence of the IVS3-48C polymorphism as, even in cases where a second mutation is not identified, it is possible that more stringent screening would reveal other *FECH* variations (Whatley et al. 2004). The dominant-negative mechanism described above is a further possibility. Hypermethylation or point mutations affecting the SP1 binding site of the *FECH* promoter have also been reported to modulate disease phenotype (Onaga et al. 2004; Di Pierro et al. 2005). Lastly, acquired, late-onset EPP has been described in an individual with myelodysplastic syndrome in whom there was deletion of the long arm of chromosome 18, which included the *FECH* gene, in haematopoietic cells with subsequent clonal expansion (Aplin et al. 2001). Late-onset EPP has also been associated with myelogenous leukemia and myeloproliferative disorder and is likely to be the result of acquired somatic *FECH* mutations in bone marrow (Goodwin et al. 2006).

2.3.3. Genetic heterogeneity of EPP

EPP is characterised by allelic heterogeneity of the *FECH* gene (Taketani and Fujita 1995). The Human Gene Mutation database (<http://www.hgmd.org>) records 88 mutations identified in the *FECH* gene to date (15/06/06). Analysis of these mutations reveals three main categories: 1) Nucleotide substitutions (38%), which include both missense and nonsense mutations caused by single base changes in the coding region, 2) Frameshift mutations (32%), from insertions or deletions, that may result in premature stop codons and 3) Splice-site mutations (27%), which cause exon-skipping. The remaining mutations (3%) include one gross deletion, one complex rearrangement and a single regulatory mutation.

Early reports suggested exon skipping to be a common genetic defect in EPP (Wang et al. 1993). In particular, the loss of exons 2, 4 or 6 is predicted to cause frameshifts resulting in premature stop codons, whereas skipping of exons 3, 5, 7, 8, 9 or 10 would not disrupt the reading frame. In reality, the product is likely to be non-functional regardless of which exon is skipped, as loss of any of exons 3-11 of *FECH* resulted in an inactive enzyme that lacks a [2Fe-2S] cluster. This is most likely a result of significant altering of the structure of the protein so that the cluster cannot be coordinated (Sellers et al. 1998).

As of 2005 \pm 80% of all identified mutations were 'null-allele' mutations in which either no enzyme is produced or the resulting protein is non-functional (Ohgari et al. 2005). Mutations are scattered throughout the gene and no confirmed mutation 'hot-spots' have been identified for *FECH* (Imoto et al. 1996; Schneider-Yin et al. 2000). However, haplotype analysis of a 'g(+5)-a' transition in exon 1 in 4 American families of European ancestry revealed three distinct haplotypes associated with this mutation suggesting a 'hot-spot' in this region (Wang 1999).

The disease-causing mutations that have been identified, referred to as primary mutations in this dissertation, are largely family-specific. The first report of a *FECH* mutation occurring in more than one family was in Northern Ireland. Todd et al (1993) reported a deletion of a single base (40delG) found in 3 out of 6 families studied, and suggested that this was the most common mutation in the UK. A Swiss study reported that the mutations identified in their population were mostly shared by more than one family (Rüfenacht et al. 1998). Recently, specific mutations have been described in a number of different countries around the world e.g. IVS3+2T/G in the UK, USA and France (Sarkany et al. 1994; Bloomer et al. 1998; Chen et al. 2002; Gouya et al. 2006). Two *de novo* mutations for EPP, 'Ala155-Pro' and '1122delT', were reported by Henriksson et al (1996) and Wang et al (1996).

2.3.4. Genetic basis of EPP-related liver disease

A contentious question is whether liver disease, which is far less common than photosensitivity in patients with EPP, is associated with a particular genetic predisposition. The onset in adolescence of fulminant hepatic failure in two affected siblings with recessive EPP led Sarkany et al (1994) to suggest that liver disease is associated with compound heterozygosity. However, this does not seem to be a prerequisite, nor a common association for the development of liver disease in EPP (Bloomer et al. 1998). Further support for a higher risk of liver disease in recessive inheritance of EPP has been provided by Whatley et al (2004) in a study conducted on 105 randomly selected EPP cases. Other studies have proposed that a 'null-allele' mutation in the deleterious *FECH* allele may also predict hepatic involvement (Chen et al. 2002; Schoenfeld et al. 2003). It is also possible that acquired factors may contribute to reduced *FECH* activity during progression of liver disease (Bloomer et al. 1998, 2006) and researchers may need to investigate loci apart from *FECH* to explain susceptibility to EPP liver disease. Recent investigations include DNA microarray analysis of liver tissue from individuals who underwent a liver transplant in order to investigate hepatic gene expression (Bloomer et al. 2005). Similarly, attempts to link the 'severity' of a mutation to *FECH* activity and to liver disease have not yielded conclusive results.

A single case in a Japanese patient with EPP and fatal liver failure has been described in which it is possible that altered methylation patterns in the *FECH* promoter region, in *cis* to the IVS3-48C polymorphism, led to further reduced expression of the wild type allele (Onaga et al. 2004). Hypermethylation near a transcription initiation site is proposed to inhibit gene transcription by inhibiting basal transcription machinery (de Smet et al. 1999). Thus, epigenetic inheritance of the hypermethylated promoter, in addition to a primary mutation and the IVS3-48C polymorphism, may have been an additional factor in the expression of this patient's disease. Indeed, very low levels of normal *FECH* transcript were observed in this patient (Onaga et al. 2004).

In general, although attempts have been made to correlate a specific *FECH* genotype with the liver-disease phenotype, with a view to predicting the risk of serious liver damage in EPP individuals, the link remains elusive. Thus it appears that detailed characterisation of the genetic defect responsible for EPP in an individual cannot directly assist in allowing prevention or early detection of liver disease. However, such studies are essential for collecting data other information necessary to work towards a better understanding of the genotype–phenotype relationship in EPP.

2.4. EPP – Management

2.4.1. Photocutaneous sensitivity

Treatment of EPP skin disease primarily involves measures to limit sun exposure, such as topical barrier sunscreens, protective clothing and window filters (Meerman 2000; Kauppinen 2005). Narrow band UV B therapy may improve tolerance to sunlight (Bart de la Faille et al. 1991; Roelandts 1995). The only systemic medication that is effective in EPP for sun protection is β -carotene (Mathews-Roth 1970; Alemzadeh and Feehan 2004). The beneficial effects of β -carotene occur through two probable mechanisms. Firstly, it acts as a free radical scavenger, decreasing oxidative damage, and secondly, it causes colouration of the skin helping to create a 'light barrier' (Krook and Haeger-Aronsen 1982). However, the efficacy of this treatment is variable amongst patients (Schneider-Yin et al. 2000).

Anstey (2002) showed that β -carotene absorbs only weakly at 410nm and can therefore not be considered an 'optical filter'. Many conventional topical sun creams and lotions offer very little, if any, protection as they do not block visible light of the relevant wavelength (DeLeo et al. 1976; Mathews-Roth 1977). Tolerance to light depends largely on the individual patients, who quickly learn the amount of exposure they can withstand (Rufener 1987). They are encouraged to maintain short regular exposures to sunlight, as complete avoidance reduces their tolerance (Bart de la Faille et al. 1991).

Alternative agents for treatment have been tried. Antihistamines, antimalarials and both topical and systemic corticosteroids have been tested in the treatment of EPP, but with little success (DeLeo et al. 1976). Other systemic treatments include adenosine monophosphate, inosine, indomethacin, vitamins C and E, pyridoxine and oral zinc (DeLeo et al. 1976; Todd 1994). However, none of these were evaluated by controlled studies and conflicting results were obtained. A review of both vitamin E (α -tocopherol) and β -carotene treatment concluded that more experimental work needed to be conducted to determine the efficacy of α -tocopherol, but that β -carotene might be useful in EPP (Anstey 2002). The potential efficacy of dihydroxyacetone (DHA) that results in brown colouring of the skin was discussed by Todd in 1994. Excellent results were achieved by Fusaro and Runge (1970) with this treatment in 7 patients and it was recommended that DHA be combined with the application of topical sunscreens. Terfenadine, an H1 receptor antagonist, inhibited the immediate flare response elicited in response to exposure to blue light in seven patients. Although the localised 'erythematous response' was not altered, they suggested that this might be used in the treatment of EPP as it functions via a different mechanism to β -carotene (Farr et al. 1990). After the initial report of the use of cysteine to ameliorate photosensitivity in EPP patients (Roberts and Mathews-Roth 1993) a 3-year placebo-controlled phase III trial provided some evidence for the efficacy of cysteine in increasing the tolerable exposure-time of patients to light. It was offered as an alternative to β -carotene for patients who either do not benefit from this treatment, or who are unwilling to develop carotenoderma (Mathews-Roth and Rosner 2002). There has been a single report on an EPP patient where dietary fish oils (omega-3 marine triglycerides) were beneficial in reducing the length of time that the symptoms persisted after exposure (Chakrabarti and Tan 2002).

A second carotenoid, canthaxanthine, has been used to ameliorate EPP, often in combination with β -carotene. Phenoro[®], which contains canthaxanthine and β -carotene, was used with some success for approximately 10 years. However, patients were found to develop crystalline deposits in the retina after using Phenoro[®] for a number of years, and this treatment was suspended.

It is unclear whether iron therapy helps or hinders EPP patients. Milligan et al (1988) reported four cases of patients treated with oral iron who showed striking deterioration in the symptoms and biochemistry of their EPP; withdrawal of therapy was associated with improvement. Holmes et al (2006) discuss the place of iron supplementation in the management of anaemic patients with EPP (A. Anstey and M. Badminton, Cardiff – personal communication 2006). The literature is contradictory, with reports both supporting (Gordeuk et al. 1986; Mathews-Roth 1974) and refuting (Milligan et al. 1988; McClements et al. 1990) treatment. In their own patients (Holmes et al. 2006), 6 of 18 patients receiving iron supplementation reported an increase in symptoms, 2 an improvement and 10 were unchanged.

2.4.2. Liver disease

EPP patients are encouraged to visit their physician regularly for liver function tests. Protoporphyrin IX concentrations should be checked every 6-12 months (Lecha 2003), which is essential for the early detection of liver disease, although hepatobiliary complications may still go unnoticed until the late stages of disease as patients with liver damage may present with normal liver enzymes (Meerman 2000; Kauppinen 2005). In patients with protoporphyrin-induced liver dysfunction, measures to reduce production and enhance excretion of protoporphyrin IX may slow or halt the progress of liver disease. These include reducing the blood/plasma protoporphyrin IX concentration by plasmapheresis (Do et al. 2002), increasing the haem concentration by iron loading or haem arginate infusion or by increasing alimentary excretion of protoporphyrin IX with oral sorbents such as activated charcoal cholestyramine (Cox et al. 1998; Frank and Doss 1991). Treatment with α -tocopherol in a case of liver cirrhosis appeared to be beneficial in treating an EPP patient described by Komatsu et al (2000b). Protoporphyrin IX concentrations decreased, suggesting that α -tocopherol may be involved in porphyrin metabolism.

Red blood cell transfusion, to suppress haem synthesis, has not generally been accepted as a treatment for EPP (Rufener 1987; Todd 1994). In a study of two patients with liver disease, Eichbaum et al (2005) reported that this treatment was more effective than plasmapheresis at reducing circulating RBC protoporphyrin IX concentrations, although neither prevented progression to liver failure.

Administration of chenodeoxycholic acid was observed to result in reduced concentrations of faecal and biliary protoporphyrin IX, suggesting that this treatment may decrease the production of protoporphyrin IX, specifically in the liver (van Hattum et al. 1986). Even though EPP patients do not suffer from acute attacks, it is particularly important for them to avoid alcohol and cholestatic or hepatotoxic medication, to minimise liver damage (Rufener 1987 and Kauppinen 2005). Alcohol was found to reduce FECH activity, potentially resulting in greater accumulation of protoporphyrin IX and an increase in the risk of liver disease (Doss et al. 2000). When these measures fail, liver transplantation becomes the only option (Nordmann 1992; Cox et al. 1998).

If surgery is required, preparatory steps are necessary to reduce porphyrin concentrations and consequent photosensitivity. Plasmapheresis and haem therapy (to filter out porphyrins and reduce haem synthesis, respectively) were used to stabilise a patient requiring a liver transplant both before and after the transplant (Do et al. 2002). Special precautions must be taken to protect individuals from harsh theatre lights. These include the use of special filters to block harmful wavelengths and minimise exposure of internal organs to the lights (Shehade et al. 1991; Todd and Burrows 1992; Yotsumoto et al. 2003).

2.4.3. General considerations

In all cases correct and early diagnosis of EPP is essential for effective management. All clinical or suspected diagnoses should be confirmed in the laboratory. The mainstay of EPP laboratory diagnosis is RBC protoporphyrin IX concentration. This has been discussed previously (section 1.2.1, pg. 7). Mutation detection may confirm a diagnosis of EPP, and is the only reliable way to identify carriers of a deleterious gene in families where the mutation is known (Henriksson et al. 1996). It is now clear that the IVS3-48C polymorphism is highly significant in determining clinical expression of EPP. Therefore the determination of the IVS3-48C polymorphism status of individuals from EPP families (i.e. families with a primary *FECH* mutation) is important.

For example, the identification of a non-carrier parent who is homozygous for the IVS3-48C polymorphism simplifies genetic counselling and the determination of risk as, in this case, there is a 50% risk of having an affected child. This is important in cases where parents would choose not to have children rather than take the risk of having a child affected with EPP. Prenatal diagnosis by determination of the primary *FECH* mutation and IVS3-48C polymorphism status is not considered appropriate, as termination of pregnancy is not regarded as justified in a disease where a normal life-span can be expected (Morris et al. 2002).

2.5. Animal studies and potential therapies

2.5.1. Animal models

In early models, protoporphyria was induced in albino mice by the administration of griseofulvin (a *FECH* inhibitor) followed by repeated exposure to long-wave UV light in the 410nm range. Mice presented with thick, waxy lesions that were clinically, histologically and microscopically identical to the skin lesions observed in EPP (De Matteis and Gibbs 1975; Hönigsmann et al. 1976). This model has subsequently been used to investigate gene expression profiles (mRNA levels) of *FECH*, *ALAS* and haem oxygenase-1 and is reported as appropriate for studying the pathogenesis of EPP-associated skin and liver disease (Inafuku et al. 1999; Plösch et al. 2002; Gant et al. 2003; Takeshita et al. 2004).

The first genetically determined mouse model for human EPP was obtained from a chemical mutagenesis experiment using ethylnitrosourea (Tutois et al. 1991). The mutation was later characterised as a methionine to lysine substitution at amino acid position 98 in *FECH* (Boulechfar et al. 1993). Transmission of the disease was recessive, with homozygotes displaying photosensitivity and liver dysfunction with jaundice from an early age. *FECH* activities in the homozygotes were <10% of the normal control (Tutois et al. 1991). The severity of the phenotype obtained in this model was of limited relevance to the more commonly encountered and less severe presentation of human EPP, but it did provide the first mouse model for the study of phenotypes associated with liver disease (Bloks et al. 2001; Abitol et al. 2005; Davies et al. 2005). Indeed, these mice have been further investigated (see section 2.5.2, pg. 36) with a view to better understanding the pathophysiology of EPP by performing bone marrow transplant experiments between EPP and normal mice (Pawliuk et al. 2005).

Further transgenic mouse models have been generated to analyse the promoter region of *FECH* in order to investigate the regulation of *FECH* concentrations in erythroid and non-erythroid tissues. An extended region 4kb 5'-upstream of the *FECH* gene was found to correspond to a DNase 1 hypersensitive site and is necessary for increased expression levels in erythroid tissue (Magness et al. 1998). A second EPP mouse model has been established, in which a *FECH* exon 10 deletion, a common mutation in human EPP, was introduced into the mouse by gene targeting (Magness et al. 2002). These mice displayed *FECH* activity near the threshold for phenotypic expression and could be of relevance to human EPP studies by providing insight into the contribution of environmental and genetic factors to the expression of the disease.

A knock-out mouse model for the breast cancer resistance protein (BCRP/ABCG2) mouse homologue (*Bcrp1*) has shown that this protein may protect against dietary-induced protoporphyria. Mice presented with increased concentrations of protoporphyrin IX and displayed photosensitivity when fed with pheophorbide a (a chlorophyll break-down product). The authors suggest that BCRP may be involved in handling of endogenous porphyrins and, specifically, the clearance of excess protoporphyrin IX as this molecule is structurally similar to pheophorbide a (Jonker et al. 2002). A genetic defect in the BCRP gene may represent another mechanism whereby the phenotypic expression of EPP may be modulated in the absence of the IVS3-48C polymorphism (Zhou et al. 2005).

2.5.2. Correction of the *FECH* deficiency

Gene therapy, the transfer of wild type *FECH* genes or cDNA to target cells to correct the genetic defect associated with EPP, may be possible in the future. Long-term cure of photosensitivity in EPP mice was obtained using pre-selective gene therapy, though with no improvement in liver damage (Pawliuk et al. 1999). This method was later successfully refined to enable the use of fluorescence-based selection of genetically corrected cells, eliminating the need for potentially harmful gene markers (Fontenellas et al. 2001). More recently, Richard et al (2001) reported correction of skin photosensitivity in the above EPP mouse model using an erythroid-specific lentiviral vector, excluding the need for pre-selective gene therapy.

In 2004, Richard et al described a dual gene therapy in which the correction and in vivo expansion of deficient transduced hemopoietic stem cells was obtained, resulting in an increase in normal RBCs and complete correction of skin photosensitivity, thereby improving the efficacy of this procedure still further.

There is some evidence that bone marrow transplantation may be beneficial in the treatment of EPP (Poh-Fitzpatrick et al. 2002). An individual with recessive EPP and acute myelogenous leukemia showed marked reduction in circulating protoporphyrin IX concentrations, and a concomitant reduction in photosensitivity after receiving bone marrow from her brother who was a carrier of only one *FECH* mutation. In light of this, a recent study showed that bone marrow transplantation from wild type mice resulted in minimal skin photosensitivity and no liver disease in EPP mice 16-months post-transplantation (Pawliuk et al. 2005). They showed that normal *FECH* activity in hepatic and dermal cells is sufficient for amelioration of these symptoms and suggest that more localised treatment of exposed areas may allow patients to engage in outdoor activities. However, this remains controversial (Elder 2005; LeBoulche and Mathews-Roth 2005), and further investigation will be necessary to ascertain whether symptomatic relief from localised increase in *FECH* concentration is possible in humans.

LeBoulche and Mathews-Roth (2005) conclude that "the ideal way to treat EPP is to replace or correct the deleterious ferrochelatase gene in haematopoietic stem cells", using the methods described by Pawliuk et al (1999) and Poh-Fitzpatrick et al (2002) mentioned above. Presently the risks involved in these procedures, however, do not warrant their use in diseases such as EPP that are rarely life-threatening. In future we anticipate that techniques to reduce these risks will be identified and that gene or bone marrow transplant therapies will be made available for individuals suffering from EPP.

CHAPTER 3

Aims and Objectives

University of Cape Town

CHAPTER 3

Aims and Objectives

The molecular basis of EPP in South African families has not been established to date. This is probably because symptomatic EPP is relatively rare, and research has been directed towards VP, which is uniquely common in South Africa. Although rare, the diagnosis and management of this condition, within the South African context, would be better facilitated by a systematic characterisation of EPP families.

3.1. Initial aim:

The overall aim was to evaluate EPP in a cohort of South African families at a molecular (genotypic) level. This involved:

- a) the identification of sequence variations in the *FECH* gene in EPP subjects, to determine which are likely to be responsible for EPP in each family.
- b) the investigation of the IVS3-48C polymorphism, reported to result in reduced expression of the *FECH* allele, and its association with EPP in the South African cohort of symptomatic individuals.

Thus, the initial objectives were:

- i. to assess previously and newly diagnosed symptomatic EPP subjects associated with our Centre in Cape Town, and to obtain relevant information and biochemical data, where appropriate;
- ii. to identify *FECH* sequence variations in EPP subjects and establish family-specificity, and the likelihood that the variation(s) is/are disease-associated;
- iii. to employ restriction endonuclease analysis in order to assess the frequencies of identified variations, including the IVS3-48T/C polymorphism, in a cohort of symptomatic and asymptomatic family members and a matched control cohort.

In the light of results obtained in pursuing the above objectives, we investigated the possibility that the 757_761delAGAAG *FECH* mutation identified was the result of a single ancestral event.

3.2. The aim of this additional study was therefore:

To establish whether individuals heterozygous for the 757_761delAGAAG deletion shared a common haplotype across *FECH* and, if so, to determine the prevalence of the alleles associated with the haplotype in a control population.

Thus the objectives were:

- i. to identify microsatellite markers across *FECH* and complete genotyping of a cohort of symptomatic and asymptomatic family members and a matched control cohort for these markers;
- ii. to construct pedigrees for manual allele segregation determination and haplotype analysis for identification of the haplotype associated with the 757_761delAGAAG deletion in each family;
- iii. to compare these haplotypes with those associated with the other, distinct disease-causing mutations that were identified;
- iv. to assess the frequencies of the alleles associated with the most common haplotype in a control cohort.

CHAPTER 4

Materials and Methods

University of Cape Town

CHAPTER 4

Materials and Methods

4.1. Subject recruitment and control selection

The Lennox Eales Porphyria Laboratories of the University of Cape Town is the primary referral centre for the diagnosis and management of all forms of porphyria in South Africa. The index subjects of the EPP families in this study were originally referred to this Centre for assessment by Prof Richard Hift (Clinical Director). They were, for the purposes of this study, identified from the UCT Porphyria Laboratories database, hospital records and correspondence received by the Centre. Where family members had not already been screened for EPP, blood samples were obtained and tested biochemically. Subsequent to our initial selection, further EPP subjects referred to the Centre during the course of the study were included in the EPP cohort on positive screening.

The study received Ethics approval in November 2004 from the UCT Research Ethics Committee (408/2004). Inclusion of subjects in the EPP cohort was based on clinical and biochemical criteria. The clinical criteria included: a) a history of photocutaneous sensitivity beginning in childhood and b) burning, swelling, itching and/or redness of the skin after exposure to visible light, often continuing for a number of days. The presence of elliptical scarring and thickening of the skin, particularly of exposed areas, was an additional indicator for diagnosis of EPP. The primary biochemical criterion was a raised erythrocyte protoporphyrin IX concentration exceeding 1000nmol/L.

The EPP cohort, consisting of all symptomatic individuals evaluated, was later extended to include available asymptomatic family members and designated the "study cohort".

DNA samples of control subjects were available in the Porphyria Laboratories as they had been collected previously for use in an unrelated study and anonymised. Thus, a control cohort of 100 samples was randomly selected out of an ethnically matched group (white South Africans of European descent), for inclusion in this study.

4.2. Subject and family interview and blood sample collection

EPP subjects and their families living in the Cape Town region were visited in their homes in order to collect samples and subject information. Informed consent (Appendix A) from each individual contributing blood samples was obtained in accordance with the UCT Ethics Committee which adheres to the Declaration of Helsinki, October 2000. Strict regulations for patient confidentiality were adhered to. Subject information (Appendix A) was recorded in a password-protected data sheet for sole use by individuals directly involved in the project.

In addition to completing the consent form and subject information sheet, symptomatic individuals were subjected to a structured interview using a questionnaire (Appendix B).

Individuals residing outside the Cape Town region were asked to visit a pathology laboratory where samples were drawn and forwarded to the Porphyria Laboratories. The consent form and information sheets were mailed to these subjects for completion and return to the Porphyria Laboratories.

University of Cape Town

4.3. Biochemical diagnosis of EPP

4.3.1. Preliminary red blood cell porphyrin screening

The fluorescent property of porphyrins provides a rapid screening method for their identification in various tissues, including blood. Generally, the higher the concentration of porphyrin in the sample, the greater the fluorescence observed. Whole blood collected in 5ml EDTA tubes was screened for increased red blood cell porphyrin concentration in the presence of UV light (Appendix C).

4.3.2. Plasma fluorescence scanning

Plasma fluoroscanning is a sensitive, efficient screening method for detecting increased porphyrin concentrations in the plasma and differentiating between the porphyrias (Poh-Fitzpatrick 1980). The basis of this method is, as above, that of porphyrin fluorescence and relies on the property of different porphyrins to fluoresce at different wavelengths. Diluted plasma was scanned using a fluorescence spectrophotometer from 580nm to 700nm and a peak at 630-635nm was indicative of increased protoporphyrin IX (Appendix C). The area under the peak represented the protoporphyrin IX concentration of the sample.

4.3.3. Porphyrin quantitative analysis

Extracted, esterified porphyrins were separated by thin-layer chromatography using standard methodology developed and used routinely in the Porphyria Laboratories (Day 1978; Hift et al. 2004). The method is based on the fluorescence of porphyrins when exposed to light with a wavelength of ~400nm (Moore et al. 1987). Packed red blood cells were esterified by incubation in 5% H₂SO₄ in methanol. Porphyrins were then extracted with chloroform as detailed in Appendix C. Porphyrin quantitation was performed by photodensitometric fluorescence scanning and compared with standard porphyrin mixtures of known concentration. Results were expressed in nmol/L.

4.3.4. Determination of free and zinc-chelated protoporphyrin IX concentrations in EPP blood samples

Samples with elevated red blood cell protoporphyrin IX concentrations may contain both free and zinc-chelated protoporphyrin IX. EPP is specifically associated with increased concentrations of free protoporphyrin IX. The proportion of free protoporphyrin IX to zinc-chelated protoporphyrin IX in a sample was determined by extraction with acetone, as described by Hart and Piomelli (1981). This method reliably distinguishes between free and zinc-chelated protoporphyrin IX based on their distinct emission wavelength (Appendix C).

4.4. DNA isolation

Molecular methods used to characterise genetic factors in disease generally depend on the extraction of high-quality DNA. Isolation of DNA is based on: 1) lysis of the cell membrane and the nuclear envelope; 2) removal of contaminating molecules e.g. proteins, and 3) precipitation of DNA using alcohol (Jones et al. 1994).

Blood samples collected in 5ml EDTA tubes were initially stored at 4°C and processed as soon as possible. High-purity genomic DNA isolations were performed using the Wizard Genomic DNA purification kit. The protocol, as supplied in the kit, was followed, with minor modifications as detailed in Appendix D. The isolated DNA was quantified, and the purity determined. Stock DNA solutions and remaining blood samples were stored at -70°C.

An isoamyl alcohol:chloroform "clean-up" of previously isolated and stored DNA samples was necessary in order to obtain high-quality DNA for this study (Appendix D).

4.5. Analysis of the FECH gene

4.5.1. Fragment amplification by polymerase chain reaction

The objective of PCR is to generate large quantities of a specific DNA fragment *in vitro* for further analysis. It is based on the complementarity of DNA that allows copies of a single-stranded DNA fragment to be made in the presence of a polymerase. Synthesised primers complementary to the 5'- and 3'-ends of the fragment provide the free 3'-OH groups for the addition of deoxynucleoside triphosphates (dNTPs) complementary to the template strand, and the start and end-points for amplification, respectively.

The chromosome 18 genomic DNA sequence (NT_025028) on which the FECH gene lies, as well as the protein (P22830) and mRNA (NM_000140) sequences, were obtained from the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>). Annotation of the gene was performed using the annotation program ANNOTV9 (designed by Dr George Rebello, Division of Human Genetics, UCT)

Primers for the amplification of each of the 11 exons of *FECH* were designed using the Primer3 software package (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All fragments were between 250-350bp in length, included the intron-exon boundaries and, where possible, were placed 60bp into the intron on either side of the exon. The criteria for primer selection are listed in Appendix E. The annealing specificity of newly designed primers was evaluated by searching for genome-wide sequence similarity using the standard nucleotide NCBI BLAST (Basic Local Alignment Search Tool) (<http://www.ncbi.nlm.nih.gov/blast>).

Optimum annealing temperatures (Table 4.1) were determined using a temperature gradient of 51-65°C on the Robocycler Thermal Cycler. Amplification of all 11 *FECH* exons was subsequently performed. PCR reactions were set up as described in Appendix E. PCR amplification for the majority of *FECH* exons consisted of an initial cycle of denaturation at 95°C for 1 min (stage 1), followed by 35 cycles of denaturation for 30 sec at 95°C, annealing at T_a (Table 4.1) for 30 sec and extension at 72°C for 30 sec (stage 2). A final extension at 72°C of 7 min for 1 cycle completed the reaction (stage 3).

Table 4.1. Summary of primer sequences and melting temperatures for amplification of the exons of *FECH*, and resultant fragment sizes

PCR fragment	Primer sequence (forward and reverse)	Primer length (bp)	Product size (bp)	*Ta (°C)
Exon 1	5'-gagcgggcttctagctca-3'	18		
	5'-cgcgacagaccacttac-3'	18	349	59
Exon 2	5'-atgctctgaggacctt-3'	18		
	5'-ctattggtgtctgcatcg-3'	18	260	53
Exon 3	5'-agagtccaccctggaaga-3'	18		
	5'-gctgctgttaaccattaccag-3'	22	306	55
Exon 3c	5'-CTCATGACACTTCCTATTCAGCAg-3'	24		
	5'-gctgctgttaaccattaccag-3'	22	145	53
Exon 4A	5'-caagagagctggctattgtc-3'	20		
	5'-TTCATCCAGCAGCTTCAC-3'	18	297	57
Exon 4B	5'-AGAGCAGTACCGCAGGAT-3'	18		
	5'-gataacgcctggagaccta-3'	19	267	55
Exon 5	5'-gttcttgttctggcattg-3'	20		
	5'-tccttcctcagtagac-3'	22	311	57
Exon 6	5'-gcagttacctgcttgaact-3'	20		
	5'-agaaggacatccacaaacc-3'	19	296	53
Exon 7	5'-ttgaggggtgctctgagat-3'	19		
	5'-aatggaagctggacccat-3'	18	300	53
Exon 7c	5'-ttgaggggtgctctgagat-3'	19		
	5'-tacgtaagaacatttctgggat-3'	23	235	53
Exon 8	5'-attggtgcaggagacaga-3'	18		
	5'-gttatggaagagcctgacc-3'	19	342	57
Exon 9	5'-aggggtactacagaccgtca-3'	20		
	5'-gacaccgtacatgcaaac-3'	18	348	57
Exon 10	5'-ccttttctcatttgattcag-3'	20		
	5'-ctgcaagagtttctcagagg-3'	20	148	55
Exon 11	5'-ggaaaggaagatgctgtaga-3'	20		
	5'-CGGAGGTATCTGGAGGTT-3'	18	350	55

Note: *Ta = Annealing temperature

Exonic sequences given in uppercase characters

Exon 3c and 7c denote primer sequences designed to create restriction sites for screening (see section 4.7.4)

Only 30 cycles were required for stage 2 of the PCR reaction for amplification of *FECH* exons 7 and 9. Amplification of *FECH* exon 1 required the addition of 2% dimethyl sulfoxide (DMSO) to the reaction mixture as the region is particularly GC-rich.

4.5.2. Polyacrylamide gel electrophoresis (PAGE)

Separation, identification and purification of DNA fragments may be achieved by agarose or polyacrylamide gel electrophoresis (PAGE). DNA is separated primarily on the basis of size; however conformational variations do affect the migration of DNA through polyacrylamide gels, and will be discussed later (section 6.1.1). PAGE is most effective for separating small fragments, as these gels have a higher resolving power than agarose gels. Polyacrylamide gels are formed by polymerisation of monomers of acrylamide to produce long chains in the presence of free radicals. These become cross-linked by N,N'-methylenebisacrylamide and the porosity of the gel is determined by the extent of cross-linking and the length of the chains (Sambrook et al. 1990). An electric field is set up across the gel and negatively charged DNA fragments move through the pores of the gel in the direction of the current. Larger fragments are retarded, while smaller fragments can move more quickly through the gel, allowing separation of these fragments. Visualisation of these fragments is achieved by staining the gel with ethidium bromide (EtBr). This dye becomes intercalated into the DNA helix and fluoresces when exposed to UV light (Sharp et al. 1973).

PCR products are routinely resolved by PAGE in the Porphyria Laboratories (Appendix F). A 100bp DNA ladder and a negative control (blank) for each amplified exon were included on each gel. Visualisation of PCR products was performed using UV illumination.

4.6. Investigation of the IVS3-48T/C polymorphism

4.6.1. *I*ta I Restriction endonuclease analysis

Restriction endonuclease analysis may be used for rapid screening of a cohort for previously described sequence variations. This method is based on the ability of certain enzymes to recognise and cleave specific palindromic sequences (restriction sites) in double-stranded DNA. Restriction endonuclease analysis is therefore applicable to variations that either create or abolish restriction sites by providing a base that either completes or interrupts the recognised sequence. Digestion of genomic DNA with an appropriate enzyme that cleaves the DNA at such a restriction site will provide information as to whether the variation is present at that site or not, by observing the number of restriction fragments.

PCR products amplified for exon 4 (amplification fragment 4A) were subjected to *I*ta I restriction endonuclease analysis (Appendix G) after confirming that this enzyme was appropriate (Webcutter3; www.carolina.com). The digestion products were separated using PAGE as described in Appendix F. A single undigested exon 4A PCR product, a positive control and two molecular weight markers, the 100bp DNA Ladder and 25bp DNA Step Ladder, were resolved on the same gel. Gels were visualised by silver staining (Appendix G). Samples in the matched control cohort were screened for this polymorphism using *I*ta I restriction endonuclease analysis. This allowed the determination of the frequency of the IVS3-48C polymorphism in the control population.

4.7. Mutation detection and screening

4.7.1. Single-stranded conformational polymorphism analysis

An effective method for mutation detection within specific exonic fragments is single-stranded conformational polymorphism (SSCP) analysis (Appendix H). Double-stranded DNA fragments are denatured and separated using a MDE (Mutation Detection Enhanced) PAGE system. Mobilities of distinct fragments differ because of the different conformations assumed by single-stranded fragments. Sequence variations therefore result in unique banding patterns on the gel that distinguish mutant from wild type fragments. As the sensitivity of SSCP decreases with increasing fragment length, it is important that amplification fragment length be taken into consideration (Orita et al. 1989; Sheffield et al. 1993). In our experience, variations have been detected in fragments ≤ 350 bp using this method.

Amplification fragments for exons 1-11 of *FECH* were screened by SSCP analysis for sequence variations. Three controls were included on each gel as well as a previously tested positive control where possible. The analysis was performed in both the absence and presence of 10% glycerol, and the running conditions are described in Table 4.2.

Table 4.2. SSCP running conditions

Fragment size (bp)	Voltage (V)	Running time (h)	
		+glycerol	-glycerol
260 – 290	270	17	15
290 – 320	300	19	15.5
320 – 360	350	23	17

Banding patterns were visualised by silver staining as described in Appendix G, but staining times were increased to 20 min (solution 1) and 10 min (solution 2) for the higher concentration acrylamide (MDE).

4.7.2. Direct sequencing

Direct sequencing of a DNA fragment allows the determination of the precise base composition of the fragment. It is based on PCR amplification, gel electrophoresis and fluorescent labeling of dideoxynucleoside triphosphates (ddNTPs) that lack a 3'-OH group. A PCR reaction is carried out using either the forward or reverse primer and includes ddNTPs producing amplification fragments of differing lengths, depending on where each specific ddNTP is incorporated. Electrophoresis of fragments through a polyacrylamide gel or capillary matrix separates the fragments according to size, and the termination base of each fragment is "read" by laser as it migrates, giving the exact sequence of the fragment.

Direct sequencing was carried out for all samples that exhibited a distinctive banding pattern on SSCP analysis. Prior to sequencing, a DNA "clean-up" of each sample was performed. For those amplification fragments not requiring further manipulation, the "clean-up" was performed directly using a GFX PCR DNA and gel band purification kit as described in Appendix I. Samples that could not be purified directly from the PCR product were subjected to agarose/polyacrylamide gel electrophoresis (Appendix I) and the relevant band excised and purified (Appendix I). Direct sequencing was carried out by the University of Stellenbosch Core DNA Sequencing Facility using the ABI 3130XL Genetic Analyser Big Dye Terminator vs 3.1 kit, on the ABI PRISM 3130XL automated sequencer (PE Applied Biosystems). Sequencing was performed in both the forward and reverse direction, and analysed using the program BioEdit vs 7.0.0 (Tom Hall, Isis Pharmaceuticals, Inc.).

4.7.3. Isolation of the mutant allele for sequencing

In order to further characterise the primary mutation (356_362delTTCAAGA) identified in exon 4 for sample EPP5.1, isolation of the mutant allele was required. This was achieved using the endonuclease *Hinf* I which digested the wild type allele but not the mutant allele for amplification fragment 4A as the deletion abolishes the restriction site.

The PCR reactions for the amplification of fragment 4A for sample EPP5.1 was as described previously (Appendix E). The template DNA was increased to 100ng and 35 cycles of amplification were employed. The restriction endonuclease analysis was performed as described for *Ita* I in Appendix G. The full PCR reaction (50µl) was digested with 8U of *Hinf* I. Digests were incubated at 37°C overnight. PAGE (Appendix F) at 80V for 4 h was performed to separate digestion products. DNA from gel bands that were likely to represent the mutant allele alone was isolated from polyacrylamide using the Qiaex II gel extraction kit as detailed in Appendix I. Recovered DNA for sample EPP5.1 was re-amplified by PCR (Appendix E). New amplification products for sample EPP5.1 were separated by PAGE (Appendix F) and samples that appeared to be free of heteroduplexes were purified for direct sequencing (Appendix I).

4.7.4. Restriction endonuclease analysis for identified sequence variations

The principle of restriction enzyme analysis has been outlined in section 4.6.1. PCR amplification fragments of *FECH* exons 2, 3, 4, 7, 9 and 11 from the study and control cohorts were subjected to appropriate restriction endonuclease analysis (Appendix G). This included screening for identified sequence variations (IVS1-23C/T, R96Q, IVS3+2T>G, 356_362delTTCAAGA, 757_761delAGAAG, IVS7+1G>A, P266P, P307P and IVS10-61G/A) and the initial screening for the known polymorphism (IVS3-48T/C) with *Ita* I as described above (section 4.6). Recommended conditions for each endonuclease were optimised (Table 4.3). When the sequence variation identified did not fall within a recognisable restriction site, primer sequences were selected to create one. The insizer on zeon program (<http://zeon.well.ox.ac.uk/git-bin/insizer>) was used to design these. Fragments amplified using these primers are denoted 3c and 7c in Table 4.1 and endonucleases used to screen for these changes are indicated by an asterisk in Table 4.3.

Alw26 I and *Bts* I digests required the addition of 1% BSA (Promega). Endonuclease-digested products were separated using PAGE as described in Appendix F and section 4.5.2. Electrophoresis was performed at 100V for 3-4 h. *Mbo* II, *Cac8* I and *Alw26* I digests were visualised by EtBr staining as described in Appendix F to reduce ambiguous results obtained from incomplete digestion. Staining time was extended to 20 min to accommodate lower DNA concentrations. The remainder of the gels were visualised by silver staining (Appendix G). The frequency of each of the variations identified in the study and control cohorts was determined. Probability (p) values comparing the EPP to the control cohorts were calculated using Yates chi-squared test on the Statistica-7 software package (University of Cape Town, ICTS).

Table 4.3. Summary of restriction endonucleases used for mutation screening

Sequence variation	Exon/ intron	Restriction enzyme	Restriction site	[E] (U)	Incubation Ti (°C) t (h)		Supplier
IVS1-23C/T	1	<i>Cac8</i> I	5'-GCN'NGC-3'	3	37	O/N	New England Biolabs
R96Q	3	<i>Alw26</i> I	5'-GTCTCN ₁ -3'	2	37	O/N	MBI Fermentas
IVS3+2T>G	3	* <i>Bts</i> I	5'-GCAGTGNN-3'	2	55	O/N	New England Biolabs
IVS3-48T/C	3	<i>Ita</i> I	5'-GCNGC-3'	1	37	2	Roche
	3	<i>Fnu4</i> H1	5'-GC'NGC-3'	1	37	2.5	New England Biolabs
356_362del TTCAAGA	4	<i>Hinf</i> I	5'-GANTC-3'	1.5	37	O/N	Promega
P266P	7	<i>Hsp92</i> II	5'-CATG-3'	1.5	37	O/N	Promega
IVS7+1G>A	7	* <i>Rsa</i> I	5'-GTAC-3'	3	37	O/N	Promega
757_761del AGAAG	7	<i>Mbo</i> II	5'-GAAGA(N) ₈ -3'	2	37	2	Promega
P307P	9	<i>Bst</i> XI	5'-CCANNNNN'NTGG-3'	2	55	O/N	Promega
IVS10-61G/A	10	<i>Bsm</i> FI	5'-GGGAC(N) ₁₀ -3'	1.5	65	O/N	New England Biolabs

*Modified restriction enzyme site

[E] = Endonuclease concentration

Ti = Incubation temperature

t = Incubation time

O/N = Overnight

h = Hours

4.8. Haplotype analysis

Microsatellite markers (short tandem repeat nucleotide units – see 6.2) in the immediate vicinity of a gene are likely to be inherited as a single unit. Genomic regions between these markers can therefore be investigated for association with disease (linkage analysis) or to investigate the presence of a founder mutation in a population. Affected individuals with a mutation at the same locus, or those carrying a mutation resulting from a single ancestral event, will have the surrounding sequence in common. The smaller the region between two microsatellite markers, the less the chance that recombination has occurred in the region and, in the case of a founder mutation, the more likely it is to be shared between mutation carriers. Microsatellite markers vary with respect to their repeat length and can therefore be distinguished by PCR amplification of the sequence because of the different fragment sizes obtained.

4.8.1. Microsatellite selection, design and amplification

Bioinformatics is a fundamental part of both the design and initial stages of a study such as this. Essential information regarding genes of interest and flanking regions may be obtained, allowing accurate design of primers, microsatellite markers and other components necessary for genetic analysis.

Published microsatellite markers D18S381 and D18S858 were identified from the NCBI database (<http://www.ncbi.nlm.nih.gov>). Interrogation of the Genome Database (<http://www.gdb.org>) revealed that these markers were unsuitable in repeat sequence (mixed, interrupted repeat) and repeat length, respectively. Another published marker (X69298) submitted by Whitcombe and Cox in 1992 lies within the gene, and has a maximum heterozygosity of 0.68. A Clustalw multiple sequence alignment of the given amplification fragment with the *FECH* pseudogene on chromosome 3 revealed high homology with a region of this gene. Primers were designed within regions of least similarity, but neither this, nor extensive PCR optimisation, could increase specificity for this marker. Thus, alternative microsatellite markers were chosen. A search was conducted for all (CA)_n dinucleotide repeats within 300kb either side of the *FECH* gene using the Tandem Repeats Finder program vs 2.02 (Gary Benson, Department of Biomathematical Sciences, Mount Sinai School of Medicine). Criteria for the selection of the (CA)_n dinucleotide repeats to be used as polymorphic markers were as follows: the number of CA repeats (minimum 15), the constitution of the surrounding sequence (e.g.

GC-content and complex repeats), and proximity to *FECH*. Primers resulting in a fragment smaller than 360bp, containing the repeat sequence, were designed for each marker using the software, Primer3 (see section 4.5.1). Primers utilised are shown in Table 4.4. The criteria for primer selection was as described in Appendix E. A map of the relative positions of the dinucleotide (CA)_n repeat microsatellite markers, as obtained from the Ensembl website (<http://www.ensembl.org>), is given in Figure 5.9 (section 5.6.1).

Table 4.4. Summary of primer sequences, melting temperatures and minimum fragment sizes for amplification of microsatellite markers surrounding *FECH*

Marker	Primer sequence	Primer length (bp)	Minimum product size (bp)	T _a (°C)
<i>FECH-MM1ac</i>	*F1: 5'-cagttcctccgtgcatgc-3'	18	276	65
	R1: 5'-cacaaatagctgggcctacag-3'	21		
<i>FECH-MM2gt</i>	*F3: 5'-cctctaacttcagatgacacc-3'	21	167	57
	R3: 5'-acagagtgagatgtgagatcc-3'	21		
<i>FECH-MM3gt</i>	*F2: 5'-gacttgtagacacatcactc-3'	20	262	59
	R2: 5'-ttcaccaggtgtagtggac-3'	19		

F denotes the forward primer, R denotes the reverse primer

***FAM labeled**

Forward primer sequences were labeled with a fluorescent tag (5' 6-FAM) while the reverse was unlabelled. The PCR reaction for amplification of these exons was carried out as described in Appendix E and section 4.5.1. Annealing temperatures (T_a) were as listed in Table 4.4 and stage 2 consisted of 30 cycles of amplification. PCR was performed using the hot-top facility on the thermal cycler in the absence of mineral oil, as this was a requirement for sample analysis using the ABI PRISM 3100 automated sequencer. PCR amplification products were subjected to PAGE (Appendix F) to visually determine the dilution factor to be used for each sample. Samples used for PCR optimisation were subjected to agarose gel electrophoresis to determine the specificity of the primers (see section 5.6.1).

4.8.2. Marker genotyping (allele identification and assignment)

A cohort of 83 individuals, consisting of EPP subjects and family members (the study cohort), and 50 controls were genotyped for the selected microsatellite markers. DNA was unavailable for individual EPP8.3 for haplotype analysis. Genotyping was performed on the ABI PRISM 3100 automated sequencer (Wenz et al. 1998). Results were analysed using the ABI PRISM GeneMapper software vs 3.0.

4.8.3. Pedigree construction

Pedigrees were constructed for each of the families using Cyrillic Pedigree Editor vs 2.0.2 (Chapman CJ 1993-1996, Cherwell Scientific Publishing Ltd Oxford, UK). Haplotypes were constructed manually for all pedigrees in which chromosome phase could be determined. Where parental genotypes were not known, the most probable haplotypes were inferred. Probability (p) values comparing haplotype allele frequencies in the EPP and control cohorts were calculated using Yates chi-squared test on the VassarStats: web site for statistical computation (<http://faculty.vassar.edu/lowry/VassarStats.html>).

CHAPTER 5

Results

University of Cape Town

CHAPTER 5

Results

5.1 Subject recruitment and control selection

We investigated 28 symptomatic EPP subjects, defined as subjects with positive biochemical evidence of EPP and a convincing history of photosensitivity, representing 17 apparently unrelated families. Of these families, 14 had previously been identified and were recorded in the UCT Porphyrria Database. A further 3 families were identified during the course of this study.

An additional 58 asymptomatic family members, mostly siblings or parents of the symptomatic individuals, were recruited for study. In 2 families, 3 generations were available for study. Our study cohort therefore had a total of 86 subjects, of whom 28 were symptomatic (EPP cohort), and for which 84 DNA samples were available.

As the primary aim of this investigation was to characterise the South African EPP cohort at a molecular level, the results of genetic analyses will be presented first, followed by the clinical information and biochemical data for the study cohort.

5.2. DNA Isolation

DNA (200 – 1000ng/μl) with a purity of 75-95% was obtained for the majority of samples; the minimum purity achieved being >65%. Prompt handling of blood samples gave the highest yields, and required fewer repetitions of critical steps in the protocol. In some cases, previously stored DNA required a “clean-up” purification, which resulted in lower yields (30-300ng/μl) but much-improved purity.

5.3. Analysis of the FECH gene

5.3.1. FECH exon amplification

Amplification of the coding region of *FECH* was achieved for all samples in the EPP cohort. For each exon, we encountered a few samples from the control and study cohorts which failed to amplify adequately, in most cases as a result of poor quality DNA. The number of samples suitable for analysis for each exon ranged from 77 to 96 for the control cohort and from 66 to 84 for the study cohort.

Highly specific PCR products of good intensity, as observed on PAGE, were obtained in all cases except for exon 1. The high GC-content of the sequence surrounding exon 1 made primer selection difficult, and weak amplification was only achieved with the addition of 2% DMSO. All further attempts to improve this amplification proved unsuccessful.

Heteroduplex formation was observed on amplification of a number of samples from the study cohort for exons 4 and 7 (Figures 5.1 and 5.2). This was useful as a first-line screening for heterozygous deletions in these exons. Lanes 2-6 (Figure 5.1) and lanes 2-5 (Figure 5.2) show samples that were later proven to be heterozygous for a minor deletion. Lane 7 (Figure 5.1) and lane 6 (Figure 5.2) contain homozygous wild type samples for the respective exons. Gel bands representing PCR amplification products did not always correspond to marker bands of the same size, as seen in Figures 5.1, 5.2 and subsequent gel figures. In each case the PCR fragment was confirmed to be correct by direct sequencing, and the anomaly, an artifact induced by the use of PAGE, is further explained in section 6.1.1.

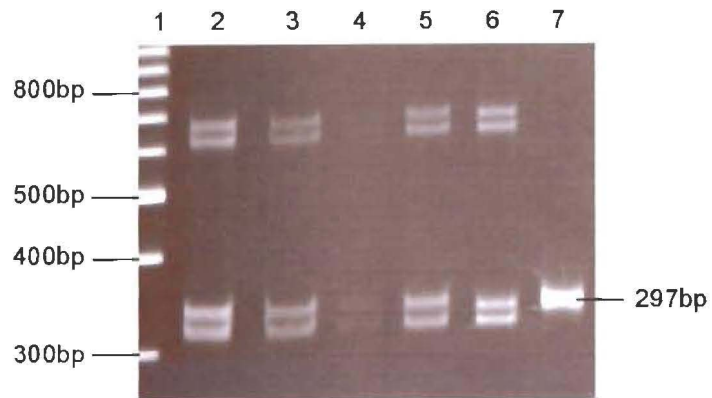


Figure 5.1. PAGE (6%) of PCR amplification fragments for *FECH* exon 4 visualised by EtBr staining. Heteroduplexes observed in lanes 2-6
 Lane 1 - 100bp DNA Ladder; Lane 2 - Subject EPP5.1; Lane 3 - EPP5.1;
 Lane 4 - EPP5.2; Lane 5 - EPP5.2; Lane 6 - EPP5.3; Lane 7 - EPP5.4

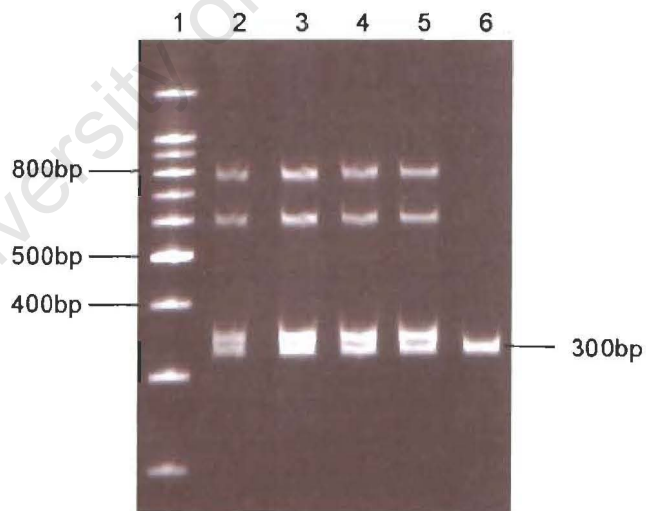


Figure 5.2. PAGE (6%) of PCR amplification fragments for *FECH* exon 7 visualised by EtBr staining. Heteroduplexes observed in lanes 2-5
 Lane 1 - 100bp DNA Ladder; Lane 2 - Subject EPP12.1; Lane 3 - EPP12.1;
 Lane 4 - EPP12.2; Lane 5 - EPP12.2; Lane 6 - control (C35)

5.3.2. Mutation detection

Previously amplified samples from EPP subjects for the 11 *FECH* exons were successfully subjected to SSCP analysis. The initial screen, which excluded glycerol in the MDE gel mixture, revealed conformational variations in exons 4A, 7, 9 and 11. The addition of 10% glycerol to the gel mixture on subsequent analyses allowed detection of these same variations, with slightly different banding patterns, and additional mobility shifts in exons 2, 3, 4B, 5, 7 and 9. For example, SSCP analysis of exon 7 with glycerol produced 6 distinct banding patterns, whereas only 3 distinct banding patterns were observed on analysis in the absence of glycerol.

Conformational variations were not observed in the other exons under these conditions. In the case of exon 4B and exon 5, direct sequencing (section 5.3.3 below) did not identify a sequence variation despite the conformational change.

SSCP analysis results for *FECH* exon 1 were inconclusive as a result of weak PCR amplification. Although a single band was observed for each sample on PAGE, a smear was repeatedly obtained on SSCP analysis and banding patterns could not be distinguished.

5.3.3. Direct sequencing

Representative samples for each amplification fragment that illustrated a mobility shift were sequenced. Ten variations were identified. These included the known IVS3-48T/C polymorphism, and four additional previously identified polymorphisms (R96Q, P266P, P307P and IVS1-23C/T), which are reported to have no effect on *FECH* expression or activity (rs1041951, rs536765, rs536560, rs7243988 and rs577152).

Sequence analysis corresponding to the remaining 5 mobility shifts, identified 4 primary (likely disease-causing) mutations in 15 of our 17 families, and one novel variation described below. Figures 5.3A, 5.4A and 5.5A show the various banding patterns observed on SSCP analysis of exons 3, 4 and 7, respectively. Sequencing electropherograms for the primary mutation/s observed in each exon that correspond to specific mobility shifts in each case are also shown (Figures 5.3B, 5.4B, 5.5B and C).

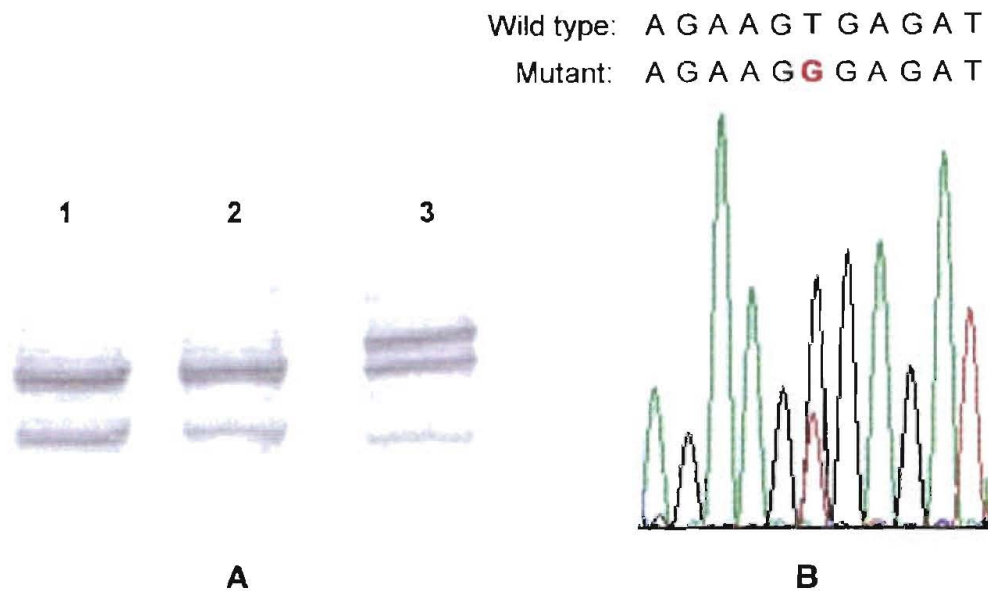


Figure 5.3. Mutation detection and identification: *FECH* exon 3

A) SSCP analysis indicating a mobility shift in lane 3

Lane 1 – EPP3.7 and Lane 2 – EPP9.4: Homozygous IVS3+2T;

Lane 3 – EPP13.1: Heterozygous IVS3+2T>G

B) Direct sequencing (forward primer) for EPP13.1: IVS3+2T>G mutation

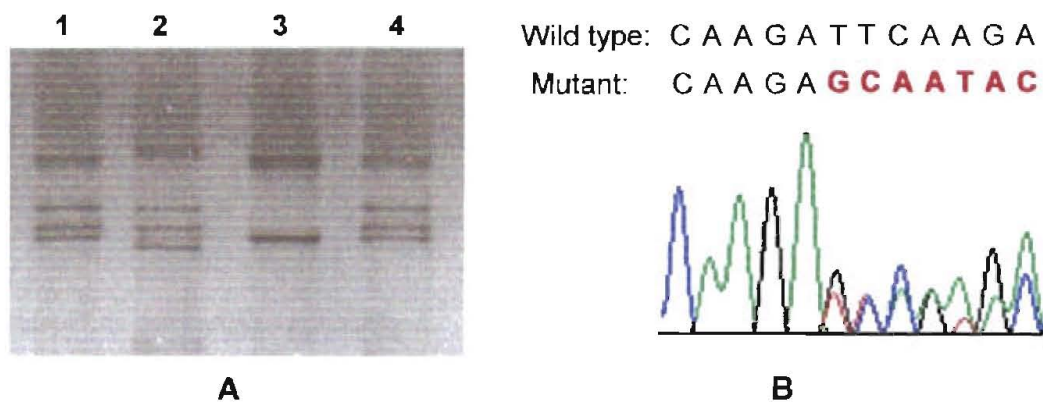


Figure 5.4: Mutation detection and identification: *FECH* exon 4

A) SSCP analysis indicating mobility shifts in lanes 1, 2 and 4

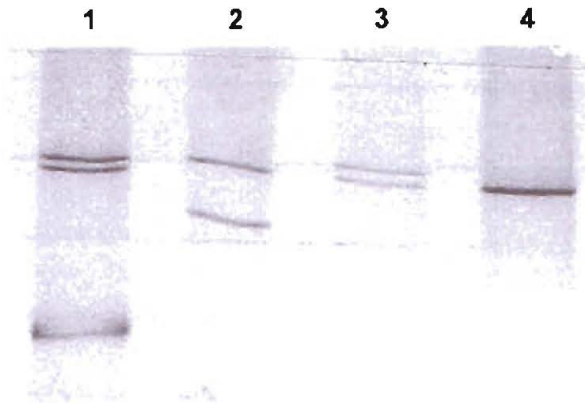
Lane 1 – EPP4.1: Heterozygous IVS3-48T/C;

Lane 2 – EPP5.1: Heterozygous 356_362delTTCAAGA deletion and IVS3-48T/C;

Lane 3 – EPP6.1: Homozygous IVS3-48T and does not carry the deletion;

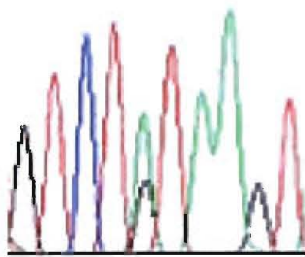
Lane 4 – EPP6.2: Genotype as for lane 1

B) Direct sequencing (forward primer) for EPP5.1: 356_362delTTCAAGA deletion

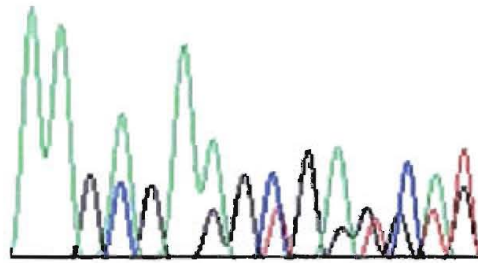


A

Wild type: GTCTGTAAGT AAGAGAAGCGAGGTG
 Mutant: GTCTATAAGT AAGCGAGGTGGTCAT



B



C

Figure 5.5. Mutation detection and identification: FECH exon 7

A) SSCP analysis showing mobility shifts in lanes1-3

Lane 1 – EPP6.1: Heterozygous 757_761delAGAAG, P266P;

Lane 2 – EPP6.2: Heterozygous P266P;

Lane 3 – EPP7.1: Heterozygous IVS7+1G>A;

Lane 4 – EPP7.2: Homozygous wild type

B) Direct sequencing (forward) for EPP7.1: IVS7+1G>A

C) Direct sequencing (forward) for EPP6.1: 757_761delAGAAG

Unexpectedly, the same 5bp deletion in exon 7 (Figure 5.5C) was identified in 12 of our families. We refer to this primary mutation as 757_761delAGAAG* which probably corresponds to a previously reported mutation (Henriksson et al. 1996). However, as it was not possible to determine the exact point of deletion of this mutation (section 5.3.4 below), we could not confirm that the position of these two deletions is identical, although it is likely.

Three family-specific primary mutations were identified:

- a novel 7bp deletion in exon 4 (356_362delTTCAAGA*) (Figure 5.4B)
- a previously identified splice-site mutation: IVS7+1G>A
(Nakahashi et al. 1993, Figure 5.5B)
- a previously identified splice-site mutation: IVS3+2T>G
(Sarkany et al. 1994, Figure 5.3B).

The remaining mobility shift observed corresponded to a second novel variation (IVS10-61G/A), identified in intron 10. We found this to be a common polymorphism with a frequency of 13% in our control cohort. Tables 5.1 and 5.2 summarise the variations identified in our South African EPP cohort.

5.3.4. Isolation of mutant alleles for sequencing

Further characterisation of the two deletions (356_362delTTCAAGA and 757_761delAGAAG) identified in this study was required to determine their exact positions. The most conclusive way to determine this would be to obtain the sequence of the mutant allele alone. In the case of exon 4A (EPP5.1), complete digestion of the normal allele was obtained by incubation of the sample with the restriction endonuclease *Hinf* I (section 4.7.4). Isolation and re-amplification of the mutant allele alone allowed sufficient product to be purified for sequencing (Figure 5.6A).

* The point of deletion cannot be determined because of the repetitive nature of the surrounding sequence. The name therefore corresponds to the most 3' set of possible deleted bases as per naming conventions suggested by den Dunnen and Antonarakis (2001).

Unfortunately, due to the nature of the sequence surrounding the deletion, the sequencing results (Figure 5.6B) were still ambiguous and the exact position of the deletion could not be determined. Hence the deletion was named as per den Dunnen and Antonarakis (2001). Isolation of the mutant allele for exon 7 (EPP2.1) was unsuccessful.

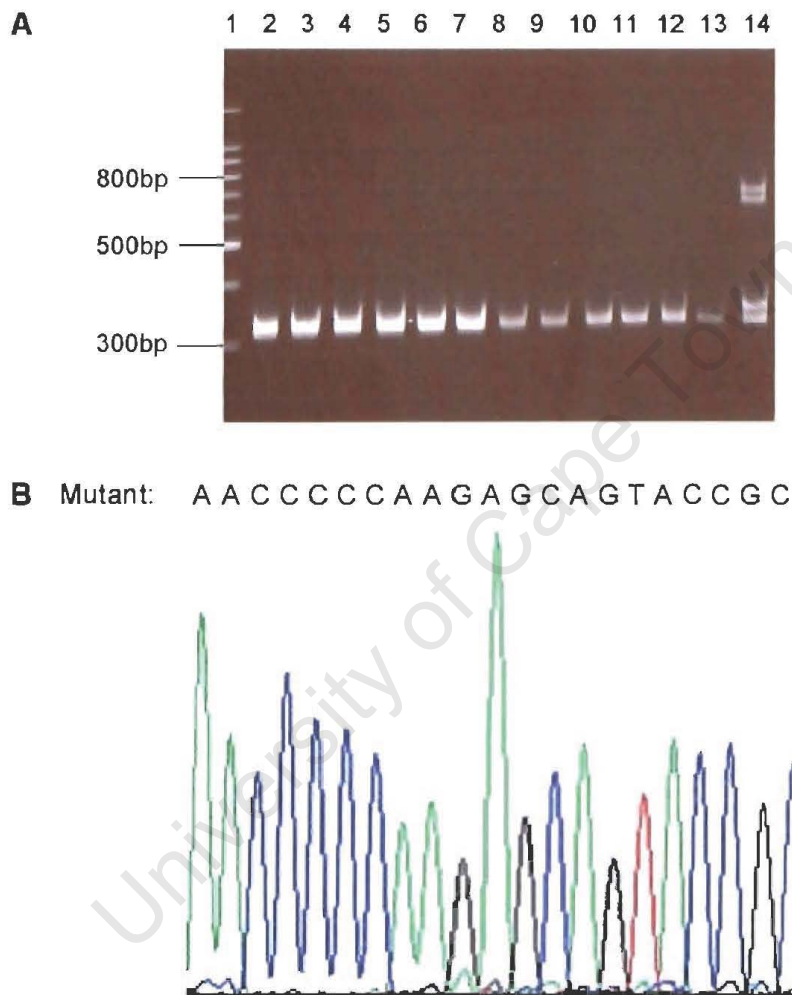


Figure 5.6. Characterisation of 356_362delTTCAAGA deletion identified for EPP5.1 (fragment 4A)

- A) PAGE of re-amplified PCR products after *Hinf*I digestion
 Lane 1 – 100bp DNA ladder; Lane 2 to 13 – amplification products of the mutant allele alone; Lane 14 – undigested sample
 B) Direct forward sequencing of the isolated mutant allele

5.4 Investigation of the IVS3-48T/C polymorphism

Exon 4A amplification products for the study cohort and a matched control cohort were screened using *I*ta I restriction endonuclease. We confirmed the suitability of *I*ta I with the Webcutter3 program (Appendix G). Direct sequencing confirmed the presence of the IVS3-48C polymorphism (NCBI database: rs2272783) in sample EPP2.1 (Appendix G), and we subsequently used this sample as a positive control for IVS3-48T/C polymorphism heterozygosity.

Figure 5.7 shows *I*ta I restriction analysis on a 6% polyacrylamide gel. The undigested exon 4A amplification fragment was 297bp in size. Samples homozygous for the IVS3-48T allele had a single *I*ta I cutting site on digestion (product size: 286 + 11bp). The presence of the IVS3-48C polymorphism, however, created an additional cutting site (product size: 169 + 117 + 11bp). We were able to distinguish heterozygous samples by the presence of three bands on the gel (286 + 169 + 117bp). The 11bp fragment was not visible as the band becomes too diffuse during electrophoresis due to its small size. The bands observed at approximately 600bp in lanes 12, 13, 14 and 17 are the result of heteroduplex formation as described for the PCR amplification of these samples (section 5.3.1). The digest was repeated for these samples, with the same result.

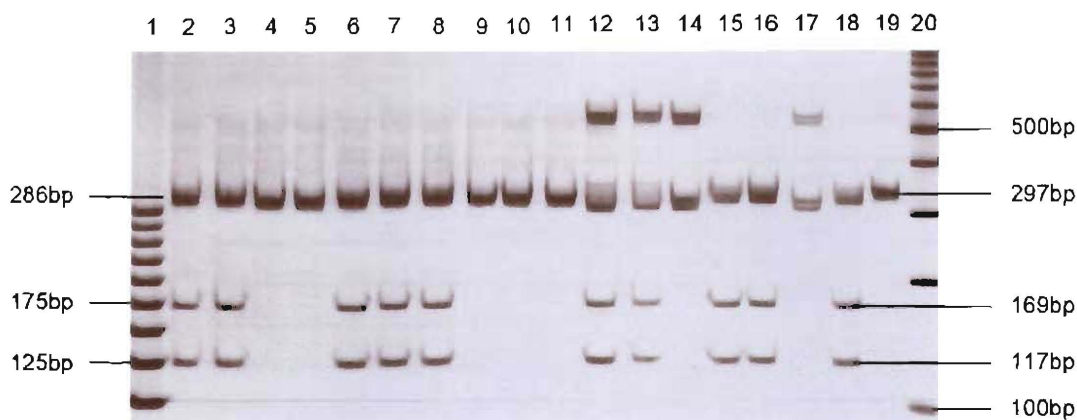


Figure 5.7. PAGE (6%) of *FECH* exon 4A amplification products after *Ita* I restriction analysis, visualised by silver staining

Lane 1 – 25bp Step DNA Ladder; Lane 2 – EPP14.1; Lane 3 – EPP14.2;
 Lane 4 – EPP14.3; Lane 5 – EPP14.4; Lane 6 – EPP14.5; Lane 7 – EPP15.1;
 Lane 8 – EPP15.2; Lane 9 – EPP15.3; Lane 10 – EPP15.4; Lane 11 – EPP15.5;
 Lane 12 – EPP5.1; Lane 13 – EPP5.2; Lane 14 – EPP5.3; Lane 15 – EPP5.4;
 Lane 16 – EPP5.5; Lane 17 – EPP5.6; Lane 18 – EPP2.1 (positive control);
 Lane 19 – EPP14.1 (undigested); Lane 20 – 100bp DNA Ladder
 Lanes 2, 3, 6, 7, 8, 12, 13, 15, 16 and 18: Heterozygous IVS3-48T/C polymorphism
 Lanes 4, 5, 9, 10, 11, 14 and 17: Homozygous IVS3-48T allele

The IVS3-48C polymorphism was present in 15/17 families (88%) and in 22/28 members of the EPP cohort (a frequency of 39%: 22/56 chromosomes). The frequency of the IVS3-48C polymorphism in the study cohort was 27% (45/168 chromosomes). In contrast, the IVS3-48C polymorphism was found to have a frequency of 9% (16/180 chromosomes) in the control cohort. Table 5.0 lists the respective frequencies of the IVS3-48T/C genotypes observed for each of the cohorts.

Table 5.0. Genotype frequencies for the *FECH* IVS3-48T/C polymorphism

IVS3-48T/C Genotypes	EPP cohort	Study cohort	Control cohort
TT	6/28 (21%)	40/84 (48%)	79/93 (85%)
TC	22/28 (79%)	43/84 (51%)	12/93 (13%)
CC	0/28 (0%)	1/84 (1%)	2/93 (2%)

5.5. Restriction endonuclease analysis for identified sequence variations

Screening for all sequence variations identified in the EPP cohort, including previously identified common polymorphisms, was performed using the appropriate restriction endonucleases to determine their frequencies in the EPP cohort, the study cohort and the control cohort, respectively.

5.5.1. Determination of the frequencies of the common polymorphisms identified

Figure 5.8 compares the single nucleotide polymorphism (SNP) frequencies in the EPP cohort with those in the control cohort. In the EPP cohort, the frequencies of the most commonly encountered SNPs (IVS3-48C and IVS1-23T) both equaled 39%. The IVS3-48C polymorphism had the lowest frequency in the control cohort (9%) as mentioned above, whereas the IVS1-23T polymorphism was more common in this cohort at 22%. The other SNPs identified had lower frequencies in the EPP cohort than in the control cohort.

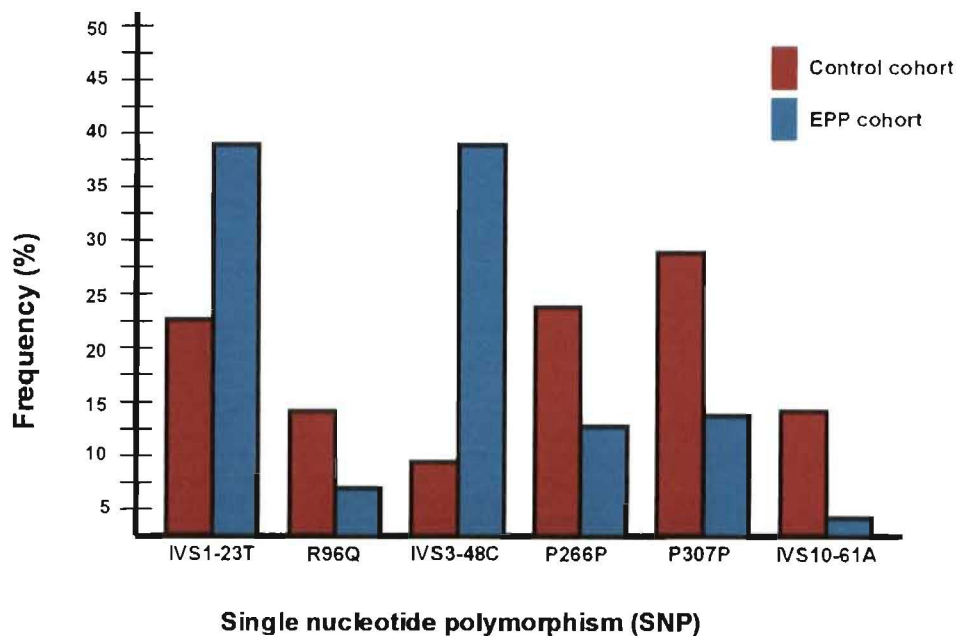


Figure 5.8. Frequencies of SNPs in the EPP and control cohorts

Table 5.1 provides a summary of the common polymorphisms identified in the South African EPP cohort and their respective frequencies.

5.5.2. Determination of the frequencies of the primary mutations identified

Three of the 4 primary mutations identified by SSCP analysis and direct sequencing, 356_362delTTCAAGA, 757_761delAGAAG and IVS7+1G>A were absent in our control cohort, confirming a frequency of <1% (150 chromosomes screened) characteristic of disease-associated mutations (Table 5.2). The frequencies of these mutations in the EPP cohort were 4% and 2% for the family-specific mutations (356_362delTTCAAGA and IVS7+1G>A) respectively, and 30% for the 757_761delAGAAG deletion. When calculated as a frequency within the study cohort the frequencies were 5% and 4% for the family-specific mutations (356_362delTTCAAGA and IVS7+1G>A) respectively, and 35% for the 757_761delAGAAG deletion. The fourth primary mutation, the IVS3+2T>G splice-site mutation, has not as yet been investigated.

University of Cape Town

Table 5.1. *FECH* common polymorphisms identified in the South African EPP cohort

SNP	Cohort frequencies (%)			*p	Effect	References
	EPP	Study	Control			
IVS1-23T	22/56 (39)	63/168 (38)	41/184 (22)	0.018	Exon 2 skipping or [None]	Nakahashi et al. 1992; [Wang et al. 1994]
R96Q	4/56 (7)	11/136 (8)	25/192 (13)	0.34	None	SNP (rs1041951)
IVS3-48C	22/56 (39)	45/168 (27)	16/180 (9)	<0.0001	↓ <i>FECH</i> expression	Gouya et al. 2002 SNP (rs2272783)
P266P	7/56 (13)	25/160 (16)	36/154 (23)	0.125	None	SNP (rs536765)
P307P	8/56 (14)	26/132 (20)	49/178 (28)	0.067	None	SNP (rs536560)
IVS10-61A	2/56 (4)	6/132 (5)	24/180 (13)	0.073	Unknown	Novel polymorphism (This study)

Key: *p values compare polymorphism frequency in the EPP and control cohorts and are calculated using Yate's χ^2 test.

Table 5.2. *FECH* primary mutations identified in the South African EPP cohort

Primary mutation	Cohort Frequencies (%)			Sequence alteration	Reported relationship to liver disease	References
	EPP	Study	Control			
IVS3+2T>G	ND	ND	ND	Exon 3 skipping	Present	Sarkany et al. 1994; Bloomer et al. 1998; Chen et al. 2002
356_362del TTCAAGA	2/59 (4)	4/168 (2)	0/176 (0)	Premature stop codon	ND	Novel
757_761del AGAAG	17/56 (30)	29/168 (17)	0/160 (0)	Premature stop codon	Present	Henriksson et al. 1996; Chen et al. 2002
IVS7+1G>A	1/56 (2)	3/156 (2)	0/163 (0)	Exon 7 skipping	None	Nakahashi et al. 1993

Key: ND = Not determine

5.6. Haplotype analysis

5.6.1. Microsatellite selection, design and amplification

The 757_761delAGAAG deletion was identified in 17 symptomatic individuals (deletion subgroup) from 12 families in our cohort. In order to investigate this further, we undertook haplotype studies to determine whether a common ancestral allele is present in these families. Online genetic databases were consulted in order to annotate chromosome 18 genomic sequence with regard to *FECH*. Three microsatellite markers (*FECH-MM1ac*, *FECH-MM2gt* and *FECH-MM3gt*) were selected on the basis of their repeat length, surrounding sequence and proximity to *FECH*, to follow allele segregation. A map of the relative positions of the markers to the 757_761delAGAAG deletion is shown in Figure 5.9.

University of Cape Town

Amplified samples used for PCR optimisation (T1) were subjected to agarose gel electrophoresis after PCR amplification to determine the specificity of the primers (electrophoresis of *FECH-MM1ac* is shown in Figure 5.10). A bright, single band was observed for each marker, indicating that primer specificity was optimal. Samples amplified under these conditions were used for genotyping.

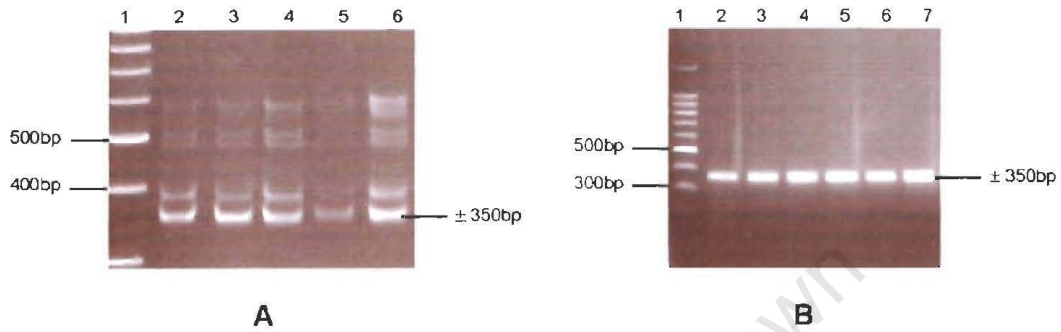


Figure 5.10. Visualisation and optimisation of microsatellite marker *FECH-MM1ac* amplification products by (A) polyacrylamide (6%) and (B) agarose (2%) gel electrophoresis, visualised with ethidium bromide staining.

A) Lane 1 - 100bp DNA Ladder; Lane 2 - EPP1.1; Lane 3 - EPP2.1; Lane 4 - EPP2.2; Lane 5 - EPP2.3; Lane 6 - EPP2.4

B) Lane 1 - 100bp DNA Ladder; Lanes 2 to 7 - Test sample (T1) amplified using a temperature gradient (51- 61°C)

5.6.2. Marker genotyping and pedigree construction

The study cohort and 50 randomly selected matched controls were genotyped for the three microsatellite markers (*FECH-MM1ac*, *FECH-MM2gt* and *FECH-MM3gt*). Each marker gave a distinct profile on the ABI PRISM 3100 automated sequencer that allowed assignment of alleles according to size in base pairs. As these marker profiles were representative of dinucleotide repeat sequences, a size change of two base pairs, or multiples thereof, indicated a new allele, and a new allele number was assigned accordingly. Marker *FECH-MM1ac* was more complex to analyse in that, in addition to the dinucleotide repeat sequence, it contained a short trinucleotide repeat region. Alleles of only one base pair difference were observed, indicating that this region was also unstable. Care was taken in assigning allele numbers for this marker as profiles were difficult to interpret and even a slight offset between runs caused profile peaks to fall within adjacent allele bins.

Fragments were allocated into "bins" by the genotyping analysis program according to their size – those that are close in size, e.g. those differing by only a single base, are sometimes difficult to assign correctly. Thus reference samples were used to delineate fragment sizes and allow the correct alleles to be determined.

Genotyping results confirmed that the three newly-designed microsatellite markers were highly informative (Table 5.3). Maximum heterozygosity was $\geq 90\%$ and marker *FECH-MM2gt* was the least heterozygous.

Table 5.3. Informativity of microsatellite markers

Microsatellite marker	Marker size range (bp)	*Number of alleles	Maximum heterozygosity
<i>FECH-MM3gt</i>	297-325	15	0.93
<i>FECH-MM1ac</i>	326-358	17	0.94
<i>FECH-MM2gt</i>	205-223	10	0.90

*268 chromosomes screened

Table 5.4 summarises the genotyping results for the probands of the 12 families in which the 757_761delAGAAG deletion segregates as a familial trait. In each case, only marker genotypes for the allele associated with the 757_761delAGAAG deletion are given except for EPP6.1 and EPP17.1, where allele phase could not be determined, and both sets of genotypes are included. The two symptomatic siblings from family EPP2 (EPP2.1 and EPP2.2) are both included in the table as they carry distinct haplotypes as a result of a recombination event in the current generation. In each of the other families, symptomatic siblings had the same set of genotypes for the allele associated with the 757_761delAGAAG deletion. The most common haplotype observed [4;C;8;G] was present in at least 15/17 (88%) of the deletion subgroup. A complete haplotype [4;C;8;G;4] was observed in at least 8/17 (47%) of this subgroup and other samples only differed in the *FECH-MM2gt* allele. Corresponding genotypes (highlighted in bold type) for samples EPP6.1 and EPP17.1, where phase is unknown, were observed and the common haplotype is therefore likely to be present.

Table 5.4. Haplotype analysis of the *FECH* locus for families in which the 757_761delAGAAG deletion segregates as a familial trait (+ = present)

Subject	<i>FECH</i> -		<i>FECH</i> -		<i>FECH</i> -	
	MM3gt	IVS1-23C/T	MM1ac	757_761delAGAAG	IVS10-61G/A	MM2gt
2.1	4	C	8	+	G	*7
2.2	4	C	8	+	G	4
4.1	4	C	8	+	G	4
9.1	4	C	8	+	G	4
10.1	4	C	8	+	G	4
12.1	4	C	8	+	G	4
15.1	4	C	8	+	G	4
16.1	4	C	8	+	G	4
1.1	4	C	8	+	G	7
3.1	4	C	8	+	G	2
14.1	4	C	8	+	G	2
6.1	4/4	C	8/16	+	G/A	4/7
17.1	4/3	C/T	8/1	+	G	4/3

Column 1 lists the individual subjects and the other columns record the genotype of each marker (microsatellites and polymorphisms) associated with the 757_761delAGAAG deletion.

Further support for the significance of the common haplotype observed for subjects carrying the 757_761delAGAAG deletion comes from the observation that this haplotype is not associated with any of the family-specific mutations identified in our EPP cohort (Table 5.5). Subject EPP16.1 contrasts the common haplotype with those associated with the 356_362delTTCAAGA, IVS7+1G>A and IVS3+2T>G mutations, respectively.

Although the SNPs correspond, the marker alleles are not consistent with those of the common haplotype. The range of marker *FECH*-MM2gt alleles is as observed in the above table.

Table 5.5. Haplotype analysis of the *FECH* locus for families in which the 757_761delAGAAG deletion is absent (+ = present; – = absent)

Subject	<i>FECH</i> -		<i>FECH</i> -		IVS10-61G/A	<i>FECH</i> -
	MM3gt	IVS1-23C/T	MM1ac	757_761delAGAAG		
16.1	4	C	8	+	G	4
5.1	6	C	1	–	G	2
7.1	6	C	11	–	G	4
13.1	1	C	12	–	G	7

Genotyping of a randomly-selected, matched control cohort was performed in order to determine the frequency of each of the alleles identified and particularly those alleles associated with the 757_761delAGAAG deletion, namely allele 4 for *FECH*-MM3gt, allele 8 for *FECH*-MM1ac and allele 4 for *FECH*-MM2gt (Table 5.5). All three alleles had low frequencies in the control cohort with allele 8 of *FECH*-MM1ac being the highest at 22%. Allele 4 of marker *FECH*-MM3gt and allele 4 of marker *FECH*-MM2gt had frequencies of only 11% and 10%, respectively in the control cohort.

A subset of 28 asymptomatic family members was used as a more closely matched control group (family control cohort) in order to assess the same allele frequencies. There is strikingly little difference between these frequencies and those obtained for the general control cohort. Allele 8 for *FECH*-MM1ac and allele 4 of marker *FECH*-MM3gt were present at 18% and allele 4 of marker *FECH*-MM2gt was at a frequency of 7% in this subset. The other *FECH*-MM2tg alleles associated with the 757_761delAGAAG deletion in the deletion subgroup, namely alleles 2 and 7, constitute 46% of the alleles observed in the random control cohort. The low frequencies of these alleles when compared to the EPP cohort suggest that the common haplotype is more prevalent than would be expected by chance (Table 5.6).

Table 5.6. Allele frequencies for the most common marker alleles associated with the 757_761delAGAAG deletion

Marker	Allele frequencies (%)			*p
	Deletion subgroup	Random control	Family control	
<i>FECH</i> -MM3gt – 4	20/34 (59)	11/100 (11)	10/56 (18)	<0.0001
<i>FECH</i> -MM1ac – 8	22/34 (65)	18/82 (22)	10/56 (18)	<0.0001
<i>FECH</i> -MM2gt – 4	11/34 (32)	10/100 (10)	4/56 (7)	0.0047

*p values compare allele frequency in the deletion subgroup and random control cohort and are calculated by χ^2 analysis (Yates)

Pedigrees for all 17 families were constructed, and genotyping results included on the pedigrees for haplotype analysis (Appendix K). At least two generations were present for the majority of families, allowing phase determination of alleles. Two examples of pedigrees (Figure 5.11 - EPP2 and Figure 5.12 - EPP8) are shown, demonstrating allele segregation in two generations. EPP2 illustrates the general mode of inheritance of alleles associated with the primary and secondary (IVS3-48C) mutations confirmed in 13 of the 17 EPP families. This pedigree also shows a single intra-familial recombination event (EPP2.1). The pedigree EPP8 shows the atypical presentation of EPP in this family as the disease is inherited in a highly penetrant fashion. The IVS3-48C polymorphism is absent and genotyping results did not reveal a single allele shared by all symptomatic individuals. A primary mutation was not identified in *FECH* for this family and haplotype analysis shows no linkage to *FECH* as the major EPP-associated locus in this family. The haplotype [4; T; 6; 3] was identified in three of the symptomatic individuals who also appear to have a more severe phenotype, and it is likely that the *FECH* locus may act as a modifier of EPP in this family.

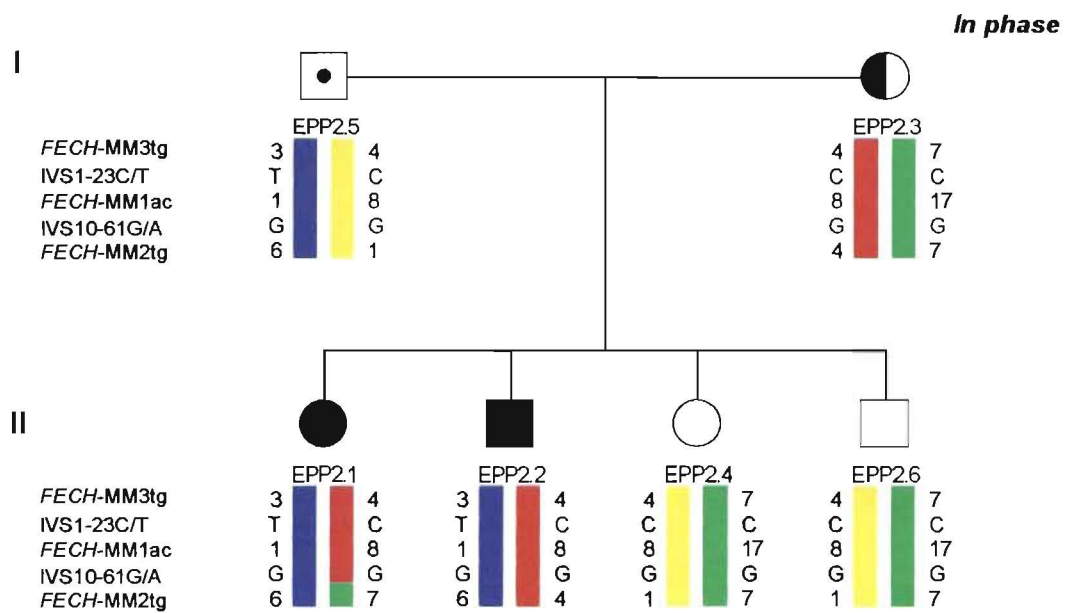


Figure 5.11. Pedigree of Family EPP2 illustrating the general mode of inheritance of alleles associated with the primary: 757_761delAGAAG (red bar) and secondary: IVS3-48C (blue bar) mutations. Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot respectively. Note the recombinant EPP2.1.

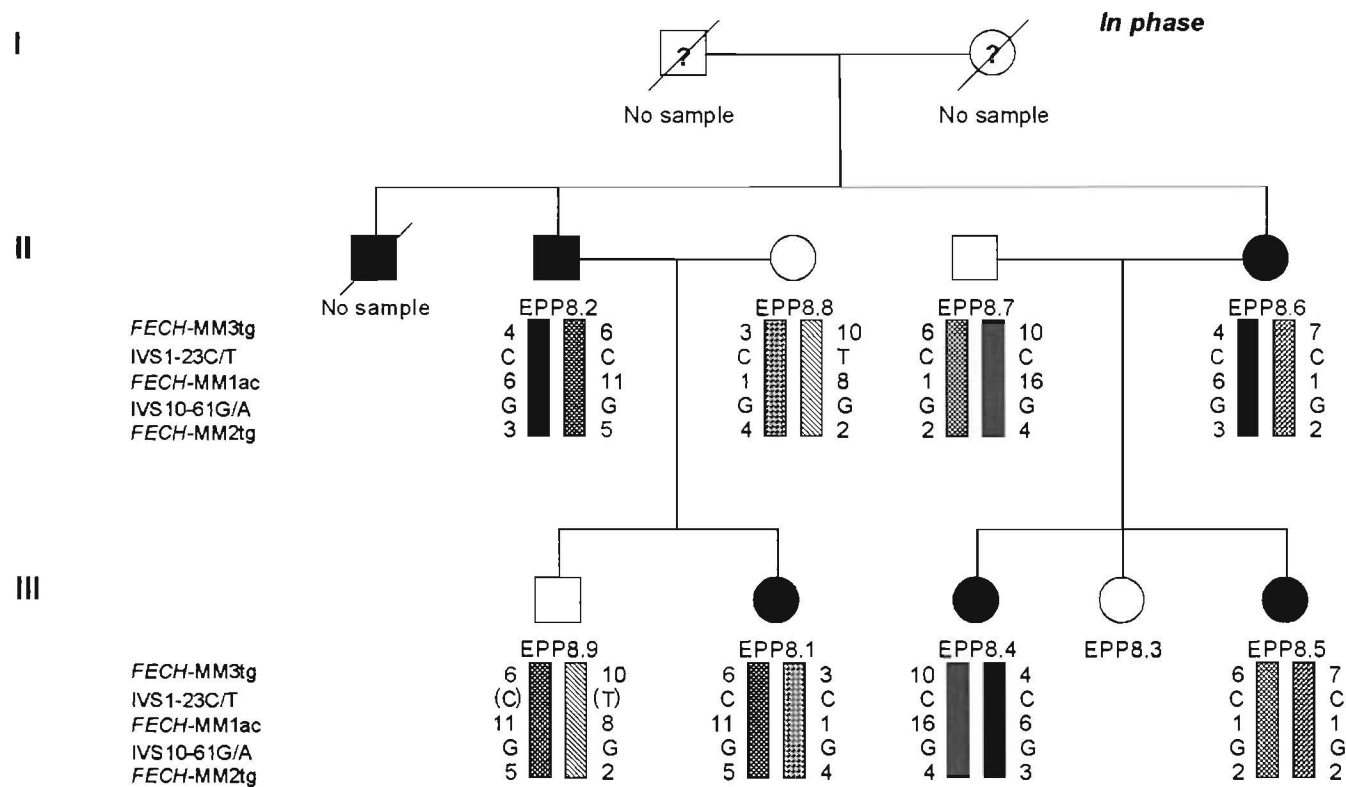


Figure 5.12. Pedigree of Family EPP8 showing atypical presentation of EPP in this family as the disease is inherited in a highly penetrant fashion (patterns differentiate haplotypes). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Inferred genotypes are given in brackets. A line through a symbol indicates a deceased individual. '?' = phenotype unknown.

5.7. Clinical features

5.7.1. Patient presentation and perception of their disease

Six families (40 subjects) were visited in their homes and blood samples collected. The questionnaire was completed by 16 of the 28 symptomatic individuals and the results are summarised in Table 5.7. We observed a slightly higher proportion of male symptomatic individuals compared to female [17:11 (60.7%)] (95% CI, 40.7 to 77.8%).

5.7.1.1. Age

The mean age of symptomatic individuals was 32.7 years (range: 13-79 years). The age of onset of EPP was predominantly between 9 months and 6 years, although symptoms were first observed in individuals up to the age of 10. The majority (56%) were diagnosed between 5 and 13 years. However, some children were younger, and two individuals were only diagnosed at 26 and 31 years, respectively. Two individuals were unsure of the age of onset of symptoms and diagnosis.

5.7.1.2. Precipitants

The majority of patients (75%) were affected only by direct sunlight and/or the reflection thereof. However, 19% said that fluorescent lights affected them after exposure to sunlight. In addition, one individual was sensitive to incandescent light after exposure to sunlight while another was sensitive to all types of light. The amount of exposure to light that could be tolerated varied and differed seasonally. Most individuals (94%) were more severely affected in summer, and 69% could tolerate at most 30 min of sunlight provided that no recent previous exposure to sunlight had occurred. Up to 4 h of sunlight could be tolerated by 38% of the individuals if they stayed in the shade, or if it was a cool day in early summer. In winter, the maximum exposure time ranged from 2 to 8 h. However, one individual claimed to be more severely affected in the winter months, and not more than 30 min of sunlight could be tolerated. The length of time symptoms persisted depended on the amount of sun exposure and could be up to a week, the most common being 12 h - 2 days.

5.7.1.3. Signs and symptoms

The general description of the sensation experienced after exposure to light included a burning, tingling pain often accompanied by heat. Swelling and redness of the exposed areas were described by 31% and 19%, respectively. Only one subject described the sensation as itchy, as opposed to a burning pain, and two individuals mentioned the appearance of a rash. Scarring on the hands and face, and thickening of the skin on the knuckles and bridge of the nose, was described by 69% and 56% of the patients, respectively.

5.7.1.4. Precautionary measures and symptom alleviation

The major precautionary measures taken by patients were avoiding sunlight (94%) and wearing protective clothing (81%). Ice, cold water or compresses, and cold, wet cloths were the most common means utilised to obtain symptomatic relief from the burning pain. Other methods include medication, staying in the shade or complete darkness, physically rubbing affected areas, e.g. the hands, to soothe the pain and applying body lotion.

5.7.1.5. Liver disease

With regard to liver disease, two individuals had gallstones and one had been investigated for a liver complaint. None had however been diagnosed as having EPP-associated liver disease, although patients were not intensively investigated in this study as this was not the purpose of the project. One deceased member of a single family (EPP8) had earlier been documented as having EPP-associated liver disease.

5.7.1.6. Family history

Of the 16 symptomatic subjects who completed the questionnaire, 6 subjects, representing 3 families, reported a previous family history of photosensitivity (38%).

Table 5.7. Subject perspective of the disease EPP

No.	Question	Most common response (%)	Other responses (%)
1.	Do you suffer from any undue photosensitivity?	Yes, very sensitive: 16/16 (100)	None: 0/16 (0)
Which of these (or other) types of light seem to have the most severe effect on your skin?			
2.	Sunlight?	Only sunlight: 12/16 (75)	Most severe: 4/16 (25) And reflection thereof: 2/16 (13)
3.	Fluorescent lights?	No: 13/16 (81)	Only after exposure to sunlight: 3/16 (19)
4.	Other types of light?	No: 14/16 (88)	All other types of light: 1/16 (6) Incandescent (after exposure): 1/16 (6)
5.	Can you describe the sensation you experience after exposure to light?	Burning: 13/16 (81) Pain: 7/16 (44) Heat: 6/16 (38) Tingling: 6/16 (38) Swelling: 5/16 (31)	Redness: 3/16 (19) Irritating: 3/16 (19) Rash: 2/16 (13) Itching: 1/16 (6) Blisters: 1/16 (6)
6.	Can you give an indication of the maximum tolerance you have to light exposure? i.e. how long can you spend in the sun without experiencing any symptoms?	Summer: Up to 30 min: 11/16 (69) Winter: More tolerance: 9/16 (56%)	Summer: 1-2 h: 2/16 (13) Less tolerance (not specified): 3/16 (19) Early summer/shade: 1-4 h: 4/16 (25) Winter: 2-8 h: 4/16 (25) Not affected: 1/16 (6)

No.	Question	Most common response (%)	Other responses (%)
7.	Does the length of exposure, type of light etc that you are sensitive to vary during the year or is the severity of symptoms constant throughout the year?	More severely affected in summer months: 15/16 (94)	One patient suffers more in the winter sun: 1/16 (6)
8.	For what length of time do the symptoms persist?	12 h-2 days: 10/16 (63)	3-5 days: 5/16 (31) More than a week: 1/16 (6)
9.	How do you attempt to relieve the symptoms described above?	Ice Cold water Cold, wet cloths Compresses etc: 14/16 (88)	Medication: 3/16 (19) Shade/darkness: 3/16 (19) Body lotion: 2/16 (13) Rubbing, sleep or a fan: 1/16 (6)
10.	Do you experience any other physical symptoms after light exposure or that you think may be attributed to porphyria?	Yes: 10/16 (63)	Bruising, brittle or fragile skin: 3/16 (19) Anaemia and low platelet count: 3/16 (19) Slow wound healing: 2/16 (13) Fatigue: 1/16 (6) Nausea, anxiety: 1/16 (6) Insomnia: 1/16 (6)
11.	What specific precautions do you take to limit exposure to sunlight or to protect yourself when you are exposed?	Avoid sunlight: 15/16 (94) Protective clothing: 13/16 (81)	Sun cream: 3/16 (19) Umbrella: 1/16 (6) Sensitivity to weather conditions: 1/16 (6)

No.	Question	Most common response (%)	Other responses (%)
12.	Have you noticed any scarring or thickening of your skin?	Scars: 11/16 (69) Thickening of the skin: 9/16 (56)	None: 3/16 (19)
13.	In which areas is this most apparent?	Hands and knuckles: 14/16 (88) Face (nose, lips, ears): 9/16 (56)	Feet: 4/16 (25) Neck, arms and elbows: 3/16 (19)
14.	At what age did you/a family member first become aware of the photosensitivity?	9 months-6 years: 9/16 (56)	7-10 years: 5/16 (31) 2 unsure
15.	At what age were you diagnosed?	5-13 years: 9/16 (56)	9 months-4 years: 3/16 (19) 26 and 31 years: 2/16 (13) 2 unsure
16.	Can you describe the level at which you find the disease debilitating? i.e. to what extent does it affect your lifestyle?	Moderately/severely: 12/16 (75) Socially: 8/16 (50) Lifestyle: 5/16 (31)	Minimal/mildly: 4/16 (25) Career: 4/16 (25) Worse when younger: 4/16 (25) Sport: 2/16 (13)
17.	Have you ever suffered from gall stones?	No: 14/16 (88)	Yes: 2/16 (13)
18.	Have you ever been investigated for a liver complaint?	No: 15/16 (94)	Yes: 1/16 (6)
19.	Is there a previous family history of photosensitivity?	No: 10/16 (63)	Parent: 4/16 (25) Cousin: 2/16 (13)

5.7.2. Biochemical diagnosis of EPP

5.7.2.1. Preliminary red blood cell porphyrin screening

All 28 symptomatic EPP subjects screened positive for increased red blood cell porphyrins (data not shown).

5.7.2.2. Plasma fluorescence scanning

Blood samples from 23 of the 28 symptomatic EPP subjects, were subjected to plasma fluorescence scanning. Of these, 19 had a plasma porphyrin peak ranging between 625nm and 632nm whereas the remaining 4 did not (Table 5.8). Depending on the amount of fluorescence observed, plasma protoporphyrin IX concentrations were noted as +/-, + or ++, indicative of low to high concentrations of plasma protoporphyrin IX. None of the 46 asymptomatic family members scanned exhibited a plasma porphyrin peak.

5.7.2.3. Porphyrin quantitative analysis

Table 5.8 shows the red blood cell protoporphyrin IX concentrations in the study cohort as determined by quantitative thin-layer chromatography. All 28 symptomatic EPP subjects had raised protoporphyrin IX concentrations with a mean of 15843nmol/L ranging from 2730nmol/L to 50000nmol/L. In all cases this was above the 1000nmol/L control reference range routinely used in our laboratory. Of these, 25 had concentrations above 4000nmol/L used by certain other international laboratories. In addition, 24 asymptomatic individuals in the study cohort had concentrations above 1000nmol/L. In addition, Table 5.8 records the disease status (symptomatic or asymptomatic) and carrier status (for the IVS3-48C polymorphism and the primary mutations) as determined from genetic analyses (sections 5.3-5.5) for subjects in the study cohort.

Table 5.8. Summary of clinical results for the study cohort, reflecting disease status, carrier status with respect to *FECH* disease-associated variations, plasma scanning results and RBC protoporphyrin IX concentrations

Subject (I = Proband)	Disease status	Carrier status		*Plasma porphyrin scan	RBC protoporphyrin IX (nmol/L) (Normal = <1000) ^
		IVS3-48C	Primary mutation		
EPP 1.1 (I)	Symptomatic	+ -	757_761delAGAAG	625nm +/-	23723
EPP 1.2	Symptomatic	+ -	757_761delAGAAG	630nm +	25252
EPP 1.3	Asymptomatic	+ -	-	Normal	1550
EPP 1.4	Asymptomatic	--	-	Normal	99
EPP 1.5	Asymptomatic	--	-	Normal	2170
EPP 1.6	Asymptomatic	--	-	Normal	453
EPP 1.7	Asymptomatic	--	-	Normal	104
<hr/>					
EPP 2.1 (I)	Symptomatic	+ -	757_761delAGAAG	630nm +/-	5160
EPP 2.2	Symptomatic	+ -	757_761delAGAAG	625nm +	21646
EPP 2.3	Asymptomatic	--	757_761delAGAAG	Normal	1271
EPP 2.4	Asymptomatic	--	-	Normal	264
EPP 2.5	Asymptomatic	+ -	-	Normal	1526
EPP 2.6	Asymptomatic	--	-	Normal	1470

Subject (I = Proband)	Disease status	Carrier status		*Plasma porphyrin scan	RBC protoporphyrin IX (nmol/L) (Normal = <1000) ^
		IVS3-48C	Primary mutation		
EPP 3.1 (I)	Symptomatic	+ -	757_761delAGAAG	Not done	23200
EPP 3.2	Symptomatic	+ -	757_761delAGAAG	630nm ++	50000
EPP 3.3	Asymptomatic	+ -	-	Normal	520
EPP 3.4	Asymptomatic	--	757_761delAGAAG	Normal	750
EPP 3.5	Asymptomatic	+ -	-	Not done	892
EPP 3.6	Asymptomatic	--	-	Normal	263
EPP 3.7	Asymptomatic	--	-	Normal	633
<hr/>					
EPP 4.1 (I)	Symptomatic	+ -	757_761delAGAAG	630nm +/-	23733
EPP 4.2	Symptomatic	+ -	757_761delAGAAG	Normal	23800
EPP 4.3	Asymptomatic	+ -	-	Normal	800
EPP 4.4	Asymptomatic	+ -	-	Normal	525
EPP 4.5	Asymptomatic	--	757_761delAGAAG	Normal	5612
EPP 4.6	Asymptomatic	+ -	-	Normal	10177
EPP 4.7	Asymptomatic	--	-	Normal	1600
<hr/>					
EPP 5.1 (I)	Symptomatic	+ -	356_362delTTCAAGA	625nm +	7200
EPP 5.2	Symptomatic	+ -	356_362delTTCAAGA	625nm +	3500
EPP 5.3	Asymptomatic	--	356_362delTTCAAGA	Normal	670
EPP 5.4	Asymptomatic	+ -	-	Normal	250
EPP 5.5	Asymptomatic	+ -	-	Normal	257
EPP 5.6	Asymptomatic	--	356_362delTTCAAGA	Normal	1750

Subject (I = Proband)	Disease status	Carrier status		*Plasma porphyrin scan	RBC protoporphyrin IX (nmol/L) (Normal = <1000)
		IVS3-48C	Primary mutation		
EPP 6.1 (I)	Symptomatic	--	757_761delAGAAG	No new sample	12027 (old sample)
EPP 6.2	Symptomatic	+ -	-	No new sample	3917 (old sample)
EPP 6.3	Asymptomatic	?	Unknown (no DNA)	No new sample	7876 (old sample)
<hr/>					
EPP 7.1 (I)	Symptomatic	+ -	IVS7+1G>A	630nm +/-	24640
EPP 7.2	Asymptomatic	+ -	-	Normal	518
EPP 7.3	Asymptomatic	--	IVS7+1G>A	Normal	3208
EPP 7.4	Asymptomatic	+ -	-	Normal	904
EPP 7.5	Asymptomatic	--	IVS7+1G>A	Normal	1596
<hr/>					
EPP 8.1 (I)	Symptomatic	--	None identified	Normal	5644
EPP 8.2	Symptomatic	--	None identified	630nm +	11083
EPP 8.3	Asymptomatic	--	None identified	No new sample	5912 (old sample)
EPP 8.4	Symptomatic	--	None identified	625nm +/-	9343
EPP 8.5	Symptomatic	--	None identified	Normal	8138
EPP 8.6	Symptomatic	--	None identified	625nm +/-	27285
EPP 8.7	Asymptomatic	--	None identified	Normal	195
EPP 8.8	Asymptomatic	--	None identified	Normal	97
EPP 8.9	Asymptomatic	--	None identified	Normal	95

Subject (I = Proband)	Disease status	Carrier status		*Plasma porphyrin scan	RBC protoporphyrin IX (nmol/L) (Normal = <1000) ^
		IVS3-48C	Primary mutation		
EPP 9.1 (I)	Symptomatic	+ -	757_761delAGAAG	Normal	2730
EPP 9.2	Symptomatic	+ -	757_761delAGAAG	630nm +/-	5500
EPP 9.3	Asymptomatic	+ -	-	Normal	158
EPP 9.4	Asymptomatic	--	-	Not done	212
EPP 9.5	Asymptomatic	+ -	-	Not done	93
EPP 9.6	Asymptomatic	--	757_761delAGAAG	Not done	95
EPP 9.7	Asymptomatic	++	-	Normal	1760
EPP 10.1 (I)	Symptomatic	+ -	757_761delAGAAG	625nm +/-	24383
EPP 10.2	Asymptomatic	+ -	-	Normal	4736
EPP 10.3	Asymptomatic	--	757_761delAGAAG	Normal	1677
EPP 10.4	Asymptomatic	+ -	-	Normal	1467
EPP 11.1 (I)	Symptomatic	+ -	None identified	No new sample	7553 (old sample)
EPP 11.2	Symptomatic	?	Unknown (no DNA)	No new sample	8250 (old sample)
EPP 12.1 (I)	Symptomatic	+ -	757_761delAGAAG	625nm +	14840
EPP 12.2	Asymptomatic	--	757_761delAGAAG	Normal	1908
EPP 12.3	Asymptomatic	--	757_761delAGAAG	Normal	2240
EPP 13.1 (I)	Symptomatic	+ -	IVS3+2T>G	630nm +/-	19283
EPP 13.2	Asymptomatic	+ -	-	Normal	385
EPP 13.3	Asymptomatic	--	IVS3+2T>G	Normal	870
EPP 13.4	Asymptomatic	+ -	-	Not done	179

Subject (I = Proband)	Disease status	Carrier status		*Plasma porphyrin scan	RBC [rotoporphyrin IX (nmol/L) (Normal = <1000) ^
		IVS3-48C	Primary mutation		
EPP 14.1 (I)	Symptomatic	+ -	757_761delAGAAG	Not done	19840
EPP 14.2	Asymptomatic	+ -	-	Not done	3333
EPP 14.3	Asymptomatic	--	757_761delAGAAG	Not done	10120
EPP 14.4	Asymptomatic	--	-	Not done	992
EPP 14.5	Asymptomatic	+ -	-	Not done	1400
EPP 15.1 (I)	Symptomatic	+ -	757_761delAGAAG	630nm +	13147
EPP 15.2	Asymptomatic	+ -	-	Normal	269
EPP 15.3	Asymptomatic	--	757_761delAGAAG	Normal	846
EPP 15.4	Asymptomatic	--	-	Normal	760
EPP 15.5	Asymptomatic	--	757_761delAGAAG	Normal	1275
EPP 16.1 (I)	Symptomatic	+ -	757_761delAGAAG	630nm +	22667
EPP 16.2	Asymptomatic	+ -	-	Normal	279
EPP 16.3	Asymptomatic	--	757_761delAGAAG	Normal	1755
EPP 16.4	Asymptomatic	--	757_761delAGAAG	Normal	264.
EPP 17.1 (I)	Symptomatic	+ -	757_761delAGAAG	630nm ++	4375
EPP 17.2	Asymptomatic	--	-	Normal	817

+ - = Heterozygous for the IVS3-48T/C polymorphism

EPP = Respective EPP families

-- = Homozygous for the IVS3-48T allele

? = Disease status unknown or uncertain

++ = Homozygous for the IVS3-48C allele

^ = See section 6.5.2

*+/- to ++ = a qualitative indicator of plasma porphyrin concentration

5.7.2.4. Determination of free and zinc-chelated protoporphyrin IX concentrations in blood samples from EPP subjects

Analysis of whole blood samples from EPP subjects to determine the free and zinc-chelated protoporphyrin IX concentrations was performed by fluorescence spectrometry (Table 5.9). Free protoporphyrin IX concentrations were higher than zinc-chelated protoporphyrin IX concentrations in 20 of the 23 EPP subjects tested, while 3 subjects in family EPP8 (EPP8.1, EPP8.4 and EPP8.5) had zinc-chelated protoporphyrin IX concentrations that were up to two-fold higher than the free form. Samples were unavailable for testing for 5 further subjects.

Table 5.9. Summary of zinc-chelated and free protoporphyrin IX concentrations in blood samples from EPP subjects. Results in relative fluorescent units (RFU)

Subject	Zinc-chelated protoporphyrin IX (RFU λ_{em} 588nm)	Free protoporphyrin IX (RFU λ_{em} 632nm)
Control (n = 5)	Range: 14-54	Range: 1-32
EPP 1.1	50	479
EPP 1.2	71	201
EPP 2.1	No sample	No sample
EPP 2.2	No sample	No sample
EPP 3.1	53	230
EPP 3.2	64	480
EPP 4.1	37	118
EPP 4.2	24	43
EPP 5.1	66	407
EPP 5.2	58	85
EPP 6.1	No sample	No sample
EPP 6.2	No sample	No sample
EPP 7.1	102	523
EPP 8.1	195	103
EPP 8.2	336	412
EPP 8.4	305	192
EPP 8.5	126	58
EPP 8.6	476	538

Subject	Zinc-chelated Protoporphyrin IX (RFU λ_{em} 588nm)	Free Protoporphyrin IX (RFU λ_{em} 632nm)
EPP 9.1	46	247
EPP 9.2	60	155
EPP 10.1	43	396
EPP 11.1	No sample	No sample
EPP 12.1	64	155
EPP 13.1	52	220
EPP 14.1	61	189
EPP 15.1	65	461
EPP 16.1	44	221
EPP 17.1	73	317

CHAPTER 6

Discussion

University of Cape Town

CHAPTER 6

Discussion

The significant advances that have been made in the past five years to explain the inheritance pattern observed in EPP have led to a new interest in determining the genetic status of EPP subjects in various population groups. No other studies of this nature in EPP subjects in South Africa have been reported to date. This discussion will be presented in keeping with the results section. Genetic analyses will be discussed first, followed by the clinical information and biochemical data.

6.1 Analysis of the FECH gene

6.1.1. Methodologies

In this study, the use of PAGE to visualise amplified PCR products was useful as a first-line screening method for the two heterozygous deletions identified. PAGE exploits the potential of acrylamide to distinguish between hetero- and homoduplexes. Heteroduplexes may arise during the final stages of PCR amplification of genomic DNA where two different alleles for the fragment of interest are present (Upchurch et al. 2000; Thompson et al. 2002). Heteroduplexes migrate more slowly than their corresponding homoduplexes as a result of a more open double-stranded configuration surrounding the mismatched bases. Heteroduplex formation was seen on analysis of the amplification product of exon 7 for subjects carrying the 757_761delAGAAG deletion and of exon 4A for subjects carrying the 356_362delTTCAAGA deletion (Figures 5.1 and 5.2). Any possible confusion of heteroduplex bands with non-specific priming was resolved by including wild type control DNA samples for PCR optimisation. The presence of multiple amplification bands such as these is only an indicator of a possible sequence variation and products were still subjected to SSCP analysis and sequencing for verification and identification. Heteroduplex formation has been observed previously on analysis of the FECH gene when a heterozygous deletion of exon 9 was identified in a cDNA sample (Wang et al. 1995).

One difficulty associated with the separation of nucleic acids by PAGE is the incorrect sizing of fragments as they are not only separated according to size but also conformation. Some amplification fragments separated on acrylamide do not migrate with the expected molecular weight marker fragment because of "conformational bending of the helix duplex" (Marini et al. 1982; Lareu et al. 1998). Sequencing is then necessary to determine whether the correct fragment has been amplified or not.

Single stranded conformational polymorphism analysis is a relatively efficient, cost effective method for mutation detection. An 80% mutation detection rate for SSCP has been reported for fragments ± 200 bp (Hayashi 1992; Sheffield et al. 1993) and mutations that are not revealed under specific running conditions may be detected by altering such conditions. In our study, the addition of 10% glycerol to the SSCP gel allowed detection of variations that were otherwise not observed. The sensitivity of this method for mutation detection was demonstrated by the analysis of *FECH* exon 7. Six distinct banding patterns were observed on the gel (4 of these are shown in Figure 5.5A) representing combinations of 3 variations, namely 757_761delAGAAG, IVS7+1G>A and P266P. The latter is a polymorphism and both the homozygous and the heterozygous states were observed in this set of samples.

6.1.2. Characterisation of the EPP cohort

One of the objectives of this study was to ascertain the prevalence of the *FECH* IVS3-48C polymorphism in the South African EPP cohort. This polymorphism was present in 15 of the 17 EPP families in this study and in 9% of the matched control cohort. This high prevalence in the EPP cohort strongly supports a role for this polymorphism in modulation of the penetrance of EPP, as proposed by Gouya et al (2002), in population groups exhibiting this polymorphism. This result was not entirely unexpected in that all these families are of European ancestry. The IVS3-48C polymorphism will be further discussed where relevant. The absence of the IVS3-48C polymorphism in one family (EPP8) demonstrates that other mechanisms must exist for the clinical expression of EPP as has been reported on a few previous occasions (Aplin et al. 2001; Onaga et al. 2004; Di Pierro et al. 2005; Goodwin et al. 2006). We were unable to detect any disease-associated mutations in the *FECH* gene for this family. Compound heterozygosity is therefore not a likely mechanism of disease in this case. In addition, heterozygosity for certain polymorphisms across *FECH* was demonstrated, excluding the possibility of a whole gene deletion.

6.1.2.1. *FECH* primary mutations and polymorphisms identified

The South African EPP cohort is characterised by some allelic heterogeneity. Of the 10 variations observed in the cohort, only 4 are likely to be disease-causing, the rest being common *FECH* polymorphisms. The low allelic heterogeneity can be explained by the observation that 71% (12/17) of the families in our cohort carry the same *FECH* mutation namely, 757_761delAGAAG. This probably corresponds to a previously reported 5bp deletion in exon 7 (751delGAGAA) described in Finland by Henriksson et al (1996), and subsequently observed by Chen et al (2002) and Gouya et al (2006) (c751delGAGAA and c757delAGAAG) in the USA and France, respectively. The difficulties encountered when a single mutation is assigned multiple names, highlights the importance of the adoption of a common nomenclature for sequence variations so as to facilitate easier mutation tracking around the world. The 757_761delAGAAG deletion results in a frameshift, producing a truncated protein of 321 amino acids.

The other three disease-associated sequence variants identified were family-specific, and included one novel deletion (356_362delTTCAAGA), and 2 donor splice-site mutations, IVS3+2T>G, previously identified in the UK, USA and France (Sarkany et al. 1994; Chen et al. 2002 and Gouya et al. 2006) and IVS7+1G>A, previously identified in Japan and France (Nakahashi et al. 1993 and Gouya et al. 2006).

We predict that the 356_362delTTCAAGA deletion results in a frameshift and a truncated protein of 141 amino acids. Although characterisation of this mutation at mRNA or protein level was not performed as part of this study, it is likely to be the disease-causing mutation in this family as the resulting transcript is not expected to produce a functional *FECH* protein. Furthermore, the deletion was identified in both the mother and grandmother of the proband, who were asymptomatic as they were negative for the IVS3-48C polymorphism.

The IVS3+2T>G splice-site mutation is reported to result in exon 3 skipping (deletion of amino acids 66-105) and an R65S amino acid change (Sarkany et al. 1994; Chen et al. 2002).

The IVS7+1G>A mutation results in skipping of the 99bp of exon 7 with the loss of amino acids 236-268 in the final protein product (Nakahashi et al. 1993). Although the skipping of this exon does not result in a frameshift, the transcript is likely to produce an inactive enzyme presumably as a result of significant structural variations (Sellers et al. 1998).

A single novel polymorphism was identified in our South African EPP cohort, namely IVS10-61G/A (frequency: 4%). It was found to have a prevalence of 13% in the matched control cohort and does not appear to be disease-associated ($p = 0.073$). The prevalence of the other previously identified polymorphisms (Table 5.1) ranges from 9-28% in the control cohort. The IVS3-48C polymorphism was significantly associated with EPP in our cohort ($p < 0.0001$) as observed in other populations.

6.1.2.2. Inheritance of disease-associated alleles

Seventeen symptomatic subjects from 12 families in our South African EPP cohort carry the 757_761delAGAAG deletion. Of the 17, 12 subjects from 8 of the 12 families were confirmed to have inherited the IVS3-48C polymorphism in *trans* to the primary mutation. Asymptomatic carrier parents and siblings of symptomatic individuals from these 8 families, in contrast, had an IVS3-48T allele in *trans* to the mutant allele. It is probable that the polymorphism is similarly inherited in three other families and haplotype analysis (section 5.6) supports this for two of these (EPP1 and EPP10). However, as both parental DNA samples were unavailable for phase determination of chromosomes in the third family (EPP17), one cannot definitively conclude that the polymorphism is inherited in *trans* to the primary mutation.

One seemingly symptomatic individual in family EPP6 carries the 757_761delAGAAG deletion but lacks the IVS3-48C polymorphism. Surprisingly, his apparently symptomatic brother is positive for the IVS3-48C polymorphism but negative for the primary mutation. Unfortunately we were unable to obtain repeat blood samples from these two brothers for confirmatory testing and there was a paucity of clinical/family data accompanying the original blood samples on which our diagnoses were made. Based on the genotyping results, neither subject would be predicted to exhibit symptomatic EPP; but clearly repeat testing is appropriate before any conclusions are drawn.

The IVS1-23T polymorphism has previously been reported as potentially disease-associated (Nakahashi et al. 1992) despite the acknowledgement that it is a common polymorphism (Wang et al. 1994; de Rooij et al. 1996). This was found to be true indirectly, in that the functional disease-associated IVS3-48C polymorphism, which was subsequently identified, is in linkage disequilibrium with the IVS1-23T polymorphism (Gouya et al. 1999). Our results confirm that these polymorphisms are in linkage disequilibrium (Lewontin (1964) $D' = 1$; [IVS1-23C; IVS3-48C] allele absent in the cohort). A single asymptomatic individual carries an [IVS1-23T; IVS3-48T] allele in *trans* to the 757_761delAGAAG deletion, excluding a direct association of the IVS1-23T polymorphism with disease expression, and providing additional support for the role of the IVS3-48C polymorphism in disease expression.

The above inheritance pattern, i.e. coinheritance of the IVS3-48C polymorphism in *trans* to the primary mutation, was also observed in the 3 other families in which we identified distinct primary mutations (EPP5, EPP7, EPP13). Thus, in total, 11 of 17 families followed the expected mechanism for disease expression as described by Gouya et al (2002). In 3 further families the same mechanism is likely to be operational, although not confirmed, as both the IVS3-48C polymorphism and a specific primary mutation are present. Disease expression in 1 family (EPP6) cannot be explained with the current results, despite the presence of a primary mutation. Furthermore, there were 2 families in which we were unable to detect the primary mutation responsible for EPP in the symptomatic individual. The IVS3-48C polymorphism is present in one of these families (EPP11) but is absent in all 5 symptomatic individuals in the other family (EPP8). Family EPP8 is therefore potentially of great interest, and warrants further study.

6.1.3. Screening for identified sequence variations

Restriction endonuclease analysis provided a fast, reliable method of screening for previously identified sequence variations, including those identified in this study, that are common in the population. For example, the prevalence of the 757_761delAGAAG deletion in our cohort suggests that it is likely to be present in other South African EPP families. Thus, the most valuable application of *Mbo* II restriction analysis was to initially screen newly identified families for this mutation. Those found to be positive were excluded from further unnecessary and time-consuming mutation detection.

In addition, restriction endonuclease analysis was effectively used to determine the prevalence of observed sequence variations in the study and control cohorts (Tables 5.1 and 5.2) and to confirm that the 4 suspected primary mutations identified were absent in more than 150 normal control chromosomes.

An important application of restriction endonuclease analysis is to identify asymptomatic carriers. This is necessary for genetic counseling of family members where the family-specific mutation has been identified. In the context of EPP, accurate risk assessment involves screening for both the primary mutation and the IVS3-48C polymorphism. The risk of having a child affected with EPP, when one parent is a mutation carrier and the other has either the IVS3-48C polymorphism or a second *FECH* mutation, is 25% with a 50% chance of being a carrier of one of the disease-associated alleles. The risk decreases significantly when the spouse of a primary mutation carrier does not carry the IVS3-48C polymorphism. Gouya et al (2006) have suggested that the prevalence of EPP in a population is related to the prevalence of the polymorphism in that population. For example the prevalence of the IVS3-48C polymorphism in West Africa is <1% and few cases of symptomatic EPP have been reported in Black Africans. It is therefore probable that, although EPP is rare in South Africa, the number of 757_761delAGAAG mutation carriers is higher than documented in this study because symptomatic disease is dependent on the prevalence of the IVS3-48C polymorphism (9%).

6.2. Haplotype analysis

The prevalence of the 757_761delAGAAG deletion in a subset of the South Africa EPP cohort, and the fact that they are all Afrikaans-speaking individuals, is suggestive of a common ancestor. In the light of previous reports of this mutation in this population it is most likely that the founder was an immigrant from Europe (Henriksson et al. 1996; Gouya et al. 2006). It was not possible to establish a common genealogy for these families but it was considered worth further exploration.

Thus, in order to investigate the possibility of a single ancestral event for the 757_761delAGAAG deletion, we assessed the genomic sequence to identify microsatellite markers for haplotype analysis. Microsatellite markers account for $\pm 3\%$ of the human genome and have an average density of ± 1 in 2kb (Lander et al. 2001; Chang et al. 2005). They are generally unstable although they remain stable in pedigrees because of a relatively low germline mutation rate (1×10^{-4} mutations per locus per gamete) (Banchs et al. 1994). Depending on their sequence variation and length they can be highly polymorphic. A microsatellite is considered polymorphic if it has a heterozygosity (the probability that an individual will be heterozygous at a given locus) of $>10\%$. SNP genotyping is becoming increasingly popular for haplotype analysis (Daly et al. 2001; Lin et al. 2005). Our decision to use microsatellites and not SNPs was based on the greater informativity (higher heterozygosity) of microsatellite markers. In contrast to microsatellites, each SNP only has two possible alleles and a higher number of SNPs are therefore needed to form a haplotype block that can distinguish one *FECH* allele from another.

Published microsatellite markers (X69298, D18S381 and D18S858) were found to be unsuitable because of interrupted repeat sequence and low heterozygosity. Therefore, three new microsatellite markers spanning a region of ± 700 kb across *FECH* were designed. Symptomatic individuals and family members were genotyped for these three markers, to determine whether a common haplotype was shared among individuals carrying the 757_761delAGAAG deletion. The maximum heterozygosity of the selected markers (where frequencies of all alleles are considered equal) ranged from 90-94%, indicating that they are highly informative.

A restricted haplotype [4; C; 8; G] that excludes marker *FECH*-MM2tg was observed in 88% of symptomatic individuals carrying the 757_761delAGAAG deletion (Table 5.4) and a complete haplotype [4; C; 8; G; 4] was present in 47%. These haplotypes were shown to be identical by descent as they were present in asymptomatic mutation carriers. Haplotype phase for 2 individuals was uncertain because of a lack of parental DNA samples. However, alleles corresponding to the complete haplotype were present in each of these samples, and it is likely that they do indeed share this haplotype.

It is important to note that the variability in the haplotypes of this cohort only involves the most 3'-marker *FECH*-MM2tg. This marker lies within 300kb downstream of the *FECH* gene and numerous recombination events are not expected within such a short physical distance. Indeed, the recombination rate across this region is reported as approximately 1% for published markers (http://www2.marshfieldclinic.org/RESEARCH/GENETICS/Map_Markers/maps). A single recombination event was observed in this cohort for individual EPP2.1, suggesting that recombination may have been a mechanism involved in the variability of this marker. This would suggest that, provided the deletion was produced by a single ancestral event, this mutation is likely to be very old as the haplotype block is small. Many years would be needed to accumulate sufficient recombination events that could lead to the observed variability in marker *FECH*-MM2tg. Alternatively, this single intra-familial recombination may suggest a recombination hotspot in this region which would account for the marker variability but would decrease the time span necessary for such mutations to accumulate.

As mentioned previously, microsatellite markers are unstable and the variability of sequence length is more likely to be the result of insertion-deletion loops that arise from 'slippage' of the polymerase during replication (Ellegren 2004). Polymerase 'slippage' is the result of dissociation of the template DNA strand and newly synthesised strand during replication. Misalignment of strands on re-association results in unpaired bases forming a loop that, if not repaired, causes reduction or expansion of the repeat sequence (Prolla 1998).

A comparison of the haplotype associated with the 757_761delAGAAG deletion and those associated with the 3 family-specific primary mutations supports the presence of a distinct common haplotype. In the case of the "non-757_761delAGAAG deletion carriers", the alleles at each locus, except for *FECH*-MM2tg, were not consistent with the [4; C; 8; G; 4] haplotype.

To investigate the prevalence of each allele of the common haplotype, 50 randomly selected, matched control individuals were genotyped for these markers. Respective allele frequencies observed were; 11% for allele 4 (*FECH*-MM3gt), 22% for allele 8 (*FECH*-MM1ac) and 10% for allele 4 (*FECH*-MM2gt) in this cohort. A subset of 28 family members provided a more closely, ethnically-matched control group. The frequencies of the above alleles were found to be similar to that of the control cohort. The low frequencies of these alleles when compared to the EPP cohort suggest that the [4; C; 8; G; 4] haplotype is more prevalent than would be expected by chance ($p < 0.005$). These results suggest the presence of a common ancestral allele for the 757_761delAGAAG deletion.

6.3. Unresolved cases (families EPP11 and EPP8)

The primary (disease-causing) mutation was not established for two symptomatic individuals in our cohort. The first is family EPP11 where the IVS3-48C polymorphism was found to be present on a single allele. This family, originally from Canada, is unavailable for further investigation and the proband is since deceased.

It is possible that not all sequence variations may have been detected by the SSCP method, particularly in the case of exon 1, where the amplification fragment was relatively long, and the high GC-content resulted in smearing of the band(s) on PAGE. Where necessary, exon 1 amplification fragments were subjected to direct sequencing to overcome such a problem but no sequence variations were identified.

In the second family (EPP8), who are of Jewish ancestry, the IVS3-48C polymorphism was not observed. Although this family falls outside the scope of the present study, it is of great interest, as a symptomatic individual died of liver disease, most likely associated with EPP (Eales et al. 1978a, b). Family EPP8 is distinct from the other EPP families in that the disorder appears to act in a highly penetrant fashion, and there are more symptomatic individuals than is usually observed.

Haplotype analysis of the symptomatic individuals in family EPP8 suggested that *FECH* could be excluded as the gene responsible for EPP in this family, as symptomatic individuals did not share a single haplotype associated with *FECH*. Thus it appears that although a *FECH* defect may have a role in modifying the phenotype, the primary mutation does not lie at this locus.

6.4. Patient presentation and perception of their disease

A total of 28 symptomatic EPP individuals are currently documented in the UCT Porphyria Laboratories database. Based on this information and the mid-year (2006) population estimates for South Africa (<http://www.statssa.gov.za/PublicationsHTML/P03022006/html/P03022006.html>; accessed 06/08/06) we estimate the prevalence of EPP in South Africa to be 0.6 per million and 6.4 per million in the white South African population, specifically.

Our questionnaire revealed that the majority of our EPP subjects experienced their disease similarly to those reported in other studies. As with the subjects reported by Rufener (1987) our subjects were grateful to be able to discuss their disease with health professionals with specialist porphyria knowledge. In many cases this was their first opportunity to do so. Subjects readily discussed their disease and were eager to find out more about the pathogenesis of EPP, as most had received little information on diagnosis. Most subjects were diagnosed before their teens, which is also the experience of other populations as reported in the literature (Todd 1998; Schneider-Yin 2000; Kauppinen 2005).

Our observation that slightly more males were affected than females is in keeping with other reports (Schmidt et al. 1974; Cox et al. 1998), although this appears not to be considered significant and, in recent years, has not drawn much comment. Interestingly, in our patient interviews most men appeared to be more severely affected and perceived their symptoms to be worsening with age, whereas women thought that their disease activity had improved with advancing age. However, there could be many reasons for this, and the aims and nature of our study do not allow us to speculate or reach a definitive conclusion.

Sensitivity to sunlight was the most frequent and dominant symptom that the subjects attributed to the porphyria. Other symptoms, not specified on the questionnaire but which were reported by the subjects, included slow wound-healing and bruising, fragile skin, fatigue, insomnia and anaemia. All these have previously been reported on occasion (Sweeney et al. 1963; Baart de la Faille et al. 1991; Todd 1994; Murphy 2003).

The photocutaneous sensitivity observed in our EPP cohort appeared similar to that described in the literature (Baart de la Faille et al. 1991; Lecha 2003). In general, few physical signs were observed except for the characteristic elliptical scarring on the hands, feet and arms of subjects and thickening of the skin on the knuckles. Less affected areas were the face, particularly the nose, lips and ears, the neck and elbows.

Subjects reported taking precautions to minimise exposure to light by avoiding sunlight and wearing protective clothing, such as hats. One family found applying sunscreen helpful, but most individuals did not report any benefit. The methods used by subjects to obtain symptomatic relief (mainly ice and cold water) were similar to those reported elsewhere (Todd 1994; Schneider-Yin et al. 2000).

Subjects perceived their disease as moderately to severely limiting, as their lifestyle had to be adapted to avoid activities that required them to be in the sun for any length of time. Those who reported their disease to be only minimally or mildly limiting felt that their lives had been more severely affected when they were younger, and their disease had influenced their choice of career and recreational and sporting activities. However, all felt they had been able to adapt to their disease.

6.5. Biochemical diagnosis of EPP

6.5.1. Plasma fluorescence scanning

Initial screening for EPP using plasma fluorescence scanning has proven to be an effective method for identifying individuals with increased plasma porphyrin concentrations (Poh-Fitzpatrick 1980; Hift et al. 2004). Fluoroscanning for 23 of the symptomatic individuals in our cohort identified 83% by the presence of a plasma porphyrin peak in the 625-632nm range. A peak at ± 630 nm was observed for 49% of these individuals, as expected, and is indicative of increased protoporphyrin IX concentrations and therefore EPP (Elder et al. 1990). However, a peak at ± 625 nm is usually associated with VP and 35% of these individuals presented with a peak in this range. "Normal" plasma scan profiles were observed for 17% of these symptomatic individuals and were generally associated with lower protoporphyrin IX concentrations (2700-8200nmol/L).

There have been various explanations given for both the lack of a plasma porphyrin peak and the presence of a peak at 625nm in EPP samples. It is presumed that a high concentration of protoporphyrin IX in the RBCs results in diffusion of this molecule into the surrounding plasma down the concentration gradient (Schothorst et al. 1970). On this basis, relatively low protoporphyrin IX concentrations in the red blood cells may not produce a strong-enough concentration gradient, resulting in a "normal" plasma porphyrin fluorescence scan. In 3 of our subjects, a normal scan was observed for samples that had high red blood cell protoporphyrin IX concentrations (EPP4.2: 23800nmol/L; EPP4.6: 10177nmol/L and EPP14.3: 10120nmol/L). There is no obvious explanation for this and we were unable to obtain repeat blood samples. Interestingly, only EPP4.2 was symptomatic and EPP4.6 was found to be heterozygous for the IVS3-48T/C polymorphism but lacked her family-specific *FECH* mutation. EPP14.3 was a primary mutation carrier.

One suggested reason for the maximum plasma emission peak at 625nm rather than the more common 632nm makes reference to bound protoporphyrin IX (e.g. to albumin) observed in VP (Lamola 1982; Elder et al. 1990). It has been speculated that this may occur as a consequence of the time elapsed prior to sample analysis or the handling of samples during transportation (Elder, Porphyrin conference 2005). This underlines the importance of fresh samples and rapid analysis in the laboratory. This was not always achievable in our study as a proportion of the samples were collected from areas geographically distant from our laboratory in Cape Town.

6.5.2. Porphyrin quantitative analysis

Quantitative analysis confirmed that all symptomatic EPP subjects had raised protoporphyrin IX concentrations (range: 2730nmol/L–5000nmol/L, mean: 15843nmol/L). In our laboratory we generally consider 1000nmol/L to be the upper limit of normal (Day 1978). In the literature the upper limit of normal is variously reported as 1900nmol/L (Gouya et al. 2006), 2000nmol/L (Sil et al. 2004), 4000nmol/L (www.porphyrin-europe.com) or a total erythrocyte porphyrin of 0.4-1.7 μ mol/L with >90% protoporphyrin IX (Goodwin et al. 2006; Wood et al. 2006). It should be noted however, that protoporphyrin IX concentration determination varies between laboratories, and these reference ranges correspond to different methods. It may therefore not be appropriate to equate these directly.

Symptomatic primary *FECH* mutation carriers consistently showed increased protoporphyrin IX concentrations, as expected. In addition, 65% (11/17) of asymptomatic carriers had concentrations above 1000nmol/L (range: 1271–10120nmol/L, mean: 2947nmol/L) or, using an upper limit of normal as 4000nmol/L, 1/17 (6%). This has also been observed previously and highlights the fact that raised RBC protoporphyrin IX concentrations alone do not correlate with presence/absence of clinical disease. The majority of family studies reveal normal to mildly raised protoporphyrin IX concentrations for asymptomatic *FECH* mutation carriers (Sarkany et al. 1994; Imoto et al. 1996; Henriksson et al. 1996; Wang et al. 1997; Frank et al. 1999; Chen et al. 2002; Risheg et al. 2003; Bloomer et al. 2005) making molecular techniques necessary for unequivocal detection of carriers (Rufenacht et al. 1996). Interestingly, a RBC protoporphyrin IX concentration above the normal limit (1760nmol/L) was observed for a single, asymptomatic individual who was homozygous for the IVS3-48C polymorphism (EPP9.7).

In our families, with one exception, the elevated protoporphyrin IX concentrations were shown to be predominantly in the free form. This correlates with the accepted biochemical presentation of EPP (Elder 1990). The exception was family EPP8, who showed a higher proportion of zinc-chelated to free protoporphyrin IX in 3 subjects, yet EPP photodermatitis sensitivity is present. In other conditions where zinc-protoporphyrin IX accumulation is a feature, such as iron-deficiency anaemia, photosensitivity is absent. This is the only family in which liver disease was recorded and, interestingly, this family has been shown previously to carry a glucose-6-phosphatase deficiency (Eales et al. 1978a, b). Symptomatic individuals in this family report anaemia and a positive response to iron therapy however this does not explain the elevated free protoporphyrin IX concentrations. This is one of two families in which we were unable to identify a *FECH* mutation and represents an atypical form of EPP possibly similar to that observed in a family by Wilson and De Rooij (personal communication 2005). Clearly, further investigation of this family is indicated but, as mentioned previously, this was outside the scope of the present study.

CHAPTER 7

Conclusions and Future Studies

University of Cape Town

CHAPTER 7

Conclusions and Future Studies

7.1. In conclusion:

1. Ten *FECH* sequence variations were identified in the South African EPP cohort
 - a) Of these, four were regarded as primary mutations as they were absent in a matched control cohort, and three (IVS3+2T>G; 757_761delAGAAG; IVS7+1G>A) had been previously reported as disease-causing. The fourth (356_362delTTCAAGA) was novel and requires further investigation. However the nature of the sequence variation (a 7bp deletion resulting in a frameshift) supports a disease-causing status for this mutation.
 - b) Of the remaining 6 sequence variations, 5 were previously identified single nucleotide polymorphisms, including the IVS3-48C polymorphism, and were confirmed to be common in the matched control cohort. We also identified one novel common polymorphism (IVS10-61G/A) which was present at a frequency of 13% in the matched control cohort.
2. The IVS3-48C polymorphism was observed in 15 of the 17 South African EPP families. Coinheritance of this polymorphism in *trans* to a defective *FECH* allele was confirmed in 11 of these families. The mechanism of modulating the phenotypic expression of EPP, as proposed by Gouya et al (2002), therefore appears to be operational in the South African cohort for the majority of families. This polymorphism had a frequency of 9% in the matched control cohort.

3. The 757_761delAGAAG deletion appeared significant in a local context as it was present in 12 of the 17 families in the EPP cohort. Haplotype analysis across the FECH gene revealed a common haplotype associated with the 757_761delAGAAG deletion in which the specific alleles are present at a higher frequency than in the matched control cohort. We speculate that the 757_761delAGAAG deletion is the product of a single mutational event and suggest that these EPP subjects are likely to be descended from a common ancestor.

7.2. Future studies

1. For a truly complete genetic characterisation of our cohort, it may be deemed necessary to investigate the regulatory (i.e. promoter) and untranslated regions of *FECH*.
2. Characterisation of the novel 356_362delTTCAAGA deletion (family EPP5) should be undertaken at an mRNA or protein level to confirm its likely pathogenicity. For example, quantitation of the mRNA by reverse transcription or protein expression studies may show whether or not FECH concentrations are below a critical threshold limit as routinely observed in symptomatic EPP.
3. Parental DNA samples necessary to complete 3 of the families could, where possible, be obtained for complete family haplotype analysis and to determine chromosomal phase, in order to confirm that the IVS3-48C polymorphism is inherited in *trans* to the defective allele in these families.
4. A further microsatellite marker could be designed between *FECH*-MM1ac and *FECH*-MM2gt and genotyping completed to attempt to obtain a smaller haplotype block that is shared by all 757_761delAGAAG deletion carriers.

5. Further investigation of family EPP8 is essential. Although the *FECH* locus does not appear to be the primary disease-associated site in this family, a role for *FECH* in modifying disease expression is indicated. The biochemistry of EPP in this family should be more thoroughly investigated by assaying the *FECH* functional activity in affected individuals, to determine whether or not a reduced *FECH* activity is present.

If a *FECH* deficiency is confirmed to play a role in disease manifestation, then mutation detection should be completed for the uninvestigated regions of the gene, including screening for major deletions (e.g. an entire exon which will not be identified using PCR-based mutation detection methods). A second method, namely restriction fragment length polymorphism analysis by southern blotting, may also be employed. Hypermethylation of the *FECH* promoter has been described (Onaga et al 2004) and has been shown to reduce *FECH* expression, apparently increasing the severity of EPP and is possibly associated with liver disease in a single individual. This could be investigated as a possibility in family EPP8. Failing any clear *FECH* anomaly it would be prudent to consider a second disease-associated locus that, when mutated, would result in the EPP phenotype, i.e. a phenocopy. As EPP has generally not been described as heterogeneous with respect to the disease-associated locus such a finding would have a significant impact on the molecular understanding of EPP, particularly as 3 of the 5 symptomatic individuals in this family reportedly had some degree of liver disease.

Appendices

University of Cape Town

APPENDIX A
Patient Information and Consent

A.1. Patient correspondence and EPP information sheet



LIVER RESEARCH CENTRE

OF THE MEDICAL RESEARCH COUNCIL (MRC)
AND THE UNIVERSITY OF CAPE TOWN



Medical School, University of Cape Town, Observatory, 7925

Tel. +27-21-4066332, Fax: +27-21-4479670 or 4486815; E mail: RJH@liver.uct.ac.za

Date:

Address:

.....

Name:

Re: Genetic Studies in Erythropoietic Protoporphyrin

As you are no doubt aware, erythropoietic protoporphyria (EPP) is a rare form of porphyria associated with abnormal sensitivity to sunlight. Attached you will find an information sheet about the condition. Currently we are aware of 15 families with EPP in South Africa. We have carried out diagnostic and genetic investigations in 14 patients representing 12 of these families, including your own. (You may recall having provided us with blood samples for these genetic investigations a number of years ago, or we may have approached you more recently for samples.)

When we first approached our families with a view to carrying out this investigation, we were not in a position to perform all the necessary research ourselves. Recently however, we have formed a team to carry out the appropriate tests. The project is being carried out by Miss Michelle Parker, our postgraduate student, with technical supervision from Dr Anne Corrigall, acting under the direction of Professors Meissner and Hift.

EPP is a genetic illness. You may have read that it is an autosomal dominant condition, which implies that only one parent need carry a faulty gene for the disease to manifest in their children. Over recent years it has become apparent that this is not entirely true. Research performed in Europe has suggested that the disease only becomes clinically apparent in people who inherit a primary mutation from one parent, but who simultaneously inherit a so-called "low expression allele" from the other parent. In essence, this means that the second gene cannot fully compensate for the gene carrying the primary mutation.

Michelle's study is designed to see whether this applies to our South African families as well. Furthermore, she is searching for the primary mutations responsible for EPP in our South African families.

To this point we have turned up some interesting findings. Of the 12 families, we have successfully identified a primary mutation in 9. The remaining 3 have proved more difficult, and this work will continue. Seven of the 9 families carry the same mutation. One of our plans is to test the hypothesis that these families may all share a common ancestor.

Ten of the 12 families carry the same "low expression allele" described from Europe, suggesting that the mechanism reported in those European patients operates in most of our South African families as well. Two families appear atypical; in one we can find neither a primary mutation nor the low expression allele, whereas the second requires further investigation.

We hope that you will find this explanation interesting and potentially relevant to your family. We intend contacting you in the next few weeks, either by telephone or in person. It will be our pleasure to explain in more detail the relevance of these findings to you and your family and to answer any questions you may have. We may ask for your permission and cooperation in providing us with the information and samples we need to understand your condition further.

This would be a matter of:

- Answering a few questions about any symptoms experienced by members of your family
- Providing us with the information we need to put together a limited family tree
- Allowing us, if necessary, to obtain further blood samples from certain members of your family.
- Should you agree to participate further, we will keep you fully informed as to our progress, and we will ensure that you benefit as much as possible with as little inconvenience as we can manage.

We believe that our study will allow us to understand the mutations we identify and their effect on the expression of EPP in our South African families. Though it will not immediately translate into improvements in treatment, it will certainly enable us to provide your family with more complete diagnostic information and a better understanding of the disease.

With kind regards

Yours sincerely

Professor R J Hift MB ChB MMed(Med) PhD FCP(SA)

Porphyria Clinic; Groote Schuur Hospital and University of Cape Town.

ERYTHROPOIETIC PROTOPORPHYRIA

INTRODUCTION

Erythropoietic protoporphyria (EPP) is one of the less common forms of porphyria. There are only a few families known to us in South Africa carrying this condition. It differs from the other porphyrias in several respects.

It predominantly affects haem synthesis in red blood cells, rather than other organs such as the liver. The major enzyme defect is in red blood cells, and very high levels of protoporphyrin are seen in the red blood cells and plasma. Hence we concentrate on blood tests for diagnosis.

Unlike the other porphyrias, where the disease is only usually noted in adult life, symptoms of EPP often develop in childhood, and even in infancy.

EPP is not an acute porphyria. This means that patients with EPP will never suffer the acute attack. Patients with EPP for this reason need not follow drug precautions as all drugs are safe for them.

The skin disease of EPP differs from that of the other porphyrias and is described in more detail below.

INHERITANCE OF EPP

EPP is inherited as an autosomal dominant condition. This means that the disease is transmitted from generation to generation, with each child (irrespective of sex) having a 50% chance of inheriting the defective gene. However, it is known that even where a defective gene has been inherited, the person may quite commonly not show the disease at all. In other words, one can inherit the disease without showing any sign of it: an example of variable expression.

The story of the inheritance of EPP is very interesting. Recall that each of us inherits two genes for every characteristic: one from the mother, one from the father. It appears that those people who have inherited a gene for EPP and show the disease only do so because their second gene is a "slow" gene, which does not make as much enzyme as a "fast" gene. We call this a low-expression allele. Hence the patient shows signs of EPP because:

One gene carries a mutation, and makes either a faulty enzyme or no enzyme at all.

The second gene is slow, and does not make enough normal enzyme to compensate.

CLINICAL MANIFESTATIONS OF EPP

Skin Disease

The skin disease of EPP is very typical and is quite unlike that of other forms of porphyria. The skin becomes exquisitely sensitive to sunlight. After a certain threshold of exposure has been reached, the patient experiences very severe burning, tingling, pain and sometimes numbness in the exposed areas of the skin, something like a very severe sunburn. We call this immediate photosensitivity. Often there is nothing actually to be seen in the skin; occasionally the skin may become red and swollen. Children with EPP learn from a very early age to avoid the sun.

This contrasts with the skin disease of variegate porphyria and porphyria cutanea tarda. These patients do not have an immediate reaction to the sun. Often they are even unaware that the sun has any influence on their condition. However, they develop blistering, sores and scabs on the skin of the hands and face. Patients with EPP do not show these changes.

In summary then, patients with EPP react very quickly and badly to the sun, but are left with much less permanent damage, whereas patients with VP and PCT suffer no immediate ill effects from the sun, but go on to show signs of permanent damage.

In some cases, however, with continued sun exposure the skin of the patient with EPP may become coarse, thickened and rough. This is particularly noted over the bridge of the nose and on the knuckles.

Liver Damage

In EPP, the red blood cells produce vast amounts of a porphyrin known as protoporphyrin. This has three effects:

Its accumulation in the red blood cells (as well as the interruption in the formation of haem) may result in a mild anaemia. This is usually of no significance.

Large amounts of protoporphyrin escape into the plasma and from there into the skin. Since porphyrins are light-sensitive, this probably accounts for the severe skin reaction to sun evinced in EPP.

Large amounts of protoporphyrin pass through the liver where they are excreted into the bowel, emerging eventually in the stool. These large amounts of protoporphyrin may crystallise out in the bile (causing gallstones) and very occasionally sufficient may deposit in the liver to interfere with the normal functioning of liver cells. Such patients can eventually develop cirrhosis and liver failure. It must be stressed that this outcome is extremely rare. Indeed, recent research suggests that it is not the EPP alone which

causes the liver damage; one has to inherit other less well-understood factors as well which is why the liver problems are uncommon. It is however necessary to know of it, as patients with EPP should have check-ups from time to time to ensure that this is not happening.

HOW IS EPP DIAGNOSED?

The finding of very elevated amounts of protoporphyrin in the red blood cells is characteristic. For the sake of completeness however we normally test the urine and stool as well.

TREATMENT OF EPP

Reduce exposure to the sun

In the initial stages, this is the most effective action to take. Patients should limit their exposure to the sun to a period which they know from experience will not result in a painful reaction. They can increase the amount of time they spend outdoors by avoiding the hottest part of the day, emerging early in the morning and later in the afternoon. A sensible choice of clothing, such as closed shoes, a hat, and long sleeves will be very helpful. Clothes should be made of cotton rather than artificial fibres, as cotton is a better sunscreen. Obviously as much of the body as possible should be covered, particularly the forearms, legs and feet, and the hands if possible.

It must be stressed that conventional suntan preparations are useless. Though they may exclude the short wavelength ultraviolet light which causes sunburn, they do not block out those longer wavelengths of light which excite skin disease in porphyria. The only agents which may be of use are those containing zinc or titanium dioxide. These are often thick, greasy and opaque. Agents which may be helpful include those with micronised titanium dioxide and high SPF values, which are less opaque and more cosmetically acceptable.

Use of β carotene and canthaxanthine

Beta carotene is a substance which is converted in the body to vitamin A. It is also an important constituent of carrots and is responsible for their yellow colour. β -carotene in high doses builds up a protective layer in the skin which is extremely useful in improving the skin disease of EPP. When taken in sufficient amounts, the photosensitivity decreases.

This means that such people can tolerate increased amounts of exposure to the sun before they develop their typical skin reaction. Almost all people with EPP treated with β -carotene have been improved to some extent, often markedly so. In some cases, people have been able to live entirely normal lives with no skin reactions whatsoever. To achieve this effect, β -carotene has to be taken in large doses, sufficient to cause a yellow discolouration of the skin. If there is no discolouration then there is no improvement.

Vitamin A taken in large doses is not effective and is dangerous. β -carotene is very safe; as it is poorly converted in the body to vitamin A and there is no risk of it becoming toxic. No side effects of β -carotene are known, other than the yellowing of the skin.

A related substance, called canthaxanthine, may be combined with the β -carotene. This works just as well, but the resulting discolouration of the skin resembles more closely a natural tan rather than the yellowish colour of β -carotene alone. This combination has also been shown to be of benefit to patients with EPP. Canthaxanthine is safe. However, when taken in very large doses, it may cause some deposition of crystals in the retina of the eye. This has given rise to concern, though it has not been shown to endanger sight.

Unfortunately β -carotene is only available commercially at present in capsules and pills containing very small doses. Vitaforce, carotene A (Pharma Natura) contains only 1500 units of β -carotene (or about 3 mg per capsule). The optimum average adult daily dose of β -carotene is from 50-150 mg daily. It is not feasible to take up to 50 capsules of β -carotene per day! Canthaxanthine is not available in a commercially prepared form at present.

Arrangements can be made for canthaxanthine and β -carotene in adequate dosage to be made available to patients with EPP through private pharmacists, or directly from the suppliers. Our patients find it most practical to buy carotene in powder form in large containers, and then take it in teaspoonfuls! Contact us for details of availability.

Treatment with β -carotene

The required dose is from 50-150 mg daily. In practice, we have found that the best method is for the patient to be given the bottles of the raw ingredients themselves, to learn how much (in teaspoons) the required dose is by an initial weighing, and then to dose him or herself with the powder by the teaspoon and wash it down with juice.

We recommend a starting dose of 100 mg daily. This can be increased to 150 mg daily or reduced to 50 mg daily as necessary. It may take 2 or 3 months before a clinical result is evident. This coincides with the onset of the pigmentation. Once a response is obtained, the dose should be reduced to the minimum required for an effect.

Since the skin disease is worse during the summer months, it is advisable to start the β -carotene towards the end of winter, maintain it during summer, and then reduce it during autumn and winter again.

Treatment with beta-carotene and canthaxanthine

If using a carotene/canthaxanthine mixture, they should be mixed in the ratio 2 parts β -carotene and 3 parts canthaxanthine powder. The total dose is the same as for β -carotene on its own.

We recommend that patients on canthaxanthine undergo eye examinations at 3-6 monthly intervals to ensure that there is no deposition within the retina. Should there be, we would advise that the canthaxanthine be stopped and β -carotene used alone.

Protection against liver injury

It is highly recommended that all patients with EPP have their liver functions tested at least annually. All that is necessary is that a single blood test is taken and the liver functions analysed. Should there be any disturbance, specialist advice should be sought. Under such circumstances, additional treatment measures can be suggested which may allow this porphyrin to be safely cleared from the liver. These include the long term use of oral cholestyramine or charcoal which bind porphyrins and allow their excretion.

This and other information is available on our website:
<http://www.uct.ac.za/depts/porphyria>

A.2. Consent form

CONSENT FOR ANALYSIS AND STORAGE OF DNA SAMPLES

Study: Erythropoietic protoporphyria (EPP)

Subjects: Subjects with EPP and family members

This study has received ethics approval from the University of Cape Town (UCT),
Research Ethics Committee No.

All individuals participating in the above-mentioned study will be asked to read, complete and sign this form on receiving information relevant to the study.

Lab code		DNA sample number	
-----------------	--	--------------------------	--

CONSENT

I (name of person giving consent)
 herewith give consent for a blood sample to be taken from the following people:

	Participant	Relationship to person giving consent
Name (1)		
Name (2)		
Name (3)		
Name (4)		

I understand that this sample will be used to investigate the genetic factors involved in erythropoietic protoporphyria.

1. I acknowledge that I have been supplied with information regarding EPP and the nature of this study.
2. I have been informed that:
 - I am encouraged to ask questions regarding porphyria and the present research study
 - Information concerning the ethical aspects of the study can be obtained from the UCT Research Ethics Committee
 - I am at liberty to withdraw my consent for this research at any time, and this will not in any way affect my relationship with the UCT/GSH Porphyria Clinic or my future medical care.
3. Please initial each of the following paragraphs if you agree:

Our blood samples/DNA may be stored indefinitely for use in all research relating to EPP

.....

In addition, our blood sample/DNA may be used in all forms of research in future

.....

My doctor may provide clinical information relevant to our condition to the investigators on request.

.....

I request that results that may have relevance to myself or my family should be made known to me.

Our clinical information and laboratory results may be kept in a computer database. I understand that confidentiality will be maintained and that my information will not be disclosed to any person not associated with the specific purpose of studying EPP.

Signature of person giving consent

Date

Witnessed

Signature

Date

Contact details for witness

University of Cape Town

A.3. Subject information sheet

Code:.....

Surname:..... First Name(s):.....

Related to which known EPP family?

Contact details:

Address:.....

.....

Tel. No:..... Fax no:.....

Sex: M F

Age:..... Date of birth: Year..... Month..... Day.....

Ethnic origin (Please state ancestry of both your mother and father):.....

.....

Your doctor:.....

Address:.....

.....

Tel. No:..... Fax no:.....

Reason for Inclusion in study:

Clinically affected	*Family member	*Not directly related
---------------------	----------------	-----------------------

*State relationship to affected

For laboratory use only:

Patient laboratory code:..... Hospital no:.....

DNA no:..... Vol blood (ml):..... Other:.....

Date received: Year..... Month..... Day.....

APPENDIX B
EPP Questionnaire

Code: _____

What is your name? _____

1. Do you suffer from any undue photosensitivity? _____

Do these (or other) types of light have the most severe effect on your skin?

2. Sunlight _____

3. Fluorescent lights _____

4. Other _____

5. Can you describe the sensation you experience after exposure to light?

6. Can you give an indication of the maximum tolerance you have to light exposure? i.e. how long can you spend in the sun without experiencing any symptoms? _____

7. Does the length of exposure, type of light etc that you are sensitive to vary during the year or is the severity of symptoms constant throughout the year?

8. For what length of time do the symptoms persist? _____

9. How do you attempt to relieve the symptoms described above?

10. Do you experience any other physical symptoms after light exposure or that you think may be attributed to porphyria? _____

11. What specific precautions do you take to limit exposure to sunlight or to protect yourself when you are exposed? _____

12. Have you noticed any scarring or thickening of your skin? _____

13. In which areas is this most apparent? _____

14. At what age did you/a family member first become aware of the photosensitivity? _____

15. At what age were you diagnosed? _____

16. Can you describe the level at which you find the disease debilitating? i.e. to what extent does it affect your lifestyle? _____

1	2	3	4	5
Not affected	Minimal effects	Mildly limiting	Moderately limiting	Severely limiting

17. Have you ever suffered from gall stones? _____

18. Have you ever been investigated for a liver complaint? _____

19. Is there a previous family history of photosensitivity? _____

APPENDIX C

Diagnostic Techniques

C.1. Red blood cell porphyrin screening

Equipment and reagents:

- Desktop microcentrifuge DSCI58T
[Laboratory and Scientific Equipment Company (Lasec), Cape Town, SA]
- Ultra violet (UV) light
- Methanol (CH₃OH:MW=32.04g/mol)
(Kimix Chemical and Laboratory Supplies, Cape Town, SA)

Method:

- Whole blood collected in EDTA tubes was centrifuged at 700xg for 10 min
- Excess plasma was removed leaving only packed red blood cells
- 0.5ml of the red blood cells was mixed with 4ml methanol
- The methanol (upper) phase was viewed by UV illumination (λ; 400-410nm)

Interpretation of results

- Samples that screen positive (+) for porphyrins emit a pink fluorescence while negative (-) samples do not fluoresce

C.2. Plasma fluorescence scanning

Equipment and reagents:

- Fluorescence spectrophotometer 650-10S (Hitachi Koki Co., Ltd, Japan) equipped with a red-sensitive photomultiplier
- Linear 1200 chart recorder (Spectrum Medical Industries, Los Angeles, USA)
- 150 Xenon Power Supply (Perkin-Elmer, Acme Electric Corp., New York, UK)
- Plastic cuvettes 10x10x45mm (Greiner Labortechnik, Lasec)
- Phosphate Buffered Saline (PBS) (C.2.1)

Method (Poh-Fitzpatrick 1980):

- 100 μ l of plasma, previously separated from whole blood by centrifugation (C.1), was added to 900 μ l PBS
- The fluorescence spectrophotometer (λ_{ex} :405nm; λ_{em} :630nm; monochromator slit width: 5nm) was zeroed against PBS
- 1ml of diluted plasma sample was transferred to a cuvette for analysis
- An emission scan was performed from 580nm to 650nm (λ_{ex} :405nm) at a sensitivity of 10 and a scan speed of 60nm/min
- Profiles of the scan were recorded on a chart recorder (chart speed: 3cm/min) and the peak emission wavelength noted

C.2.1. 1X Phosphate buffered saline (pH 7.4)

Sodium chloride [NaCl:MW=53.44g/mol (BDH Chemicals Ltd, Poole, UK)]	16g
Potassium chloride (KCl:MW=74.55g/mol) (NT Laboratory Supplies, Gauteng, SA)	0.4g
Disodium hydrogen orthophosphate (Na_2HPO_4 :MW=141.96g/mol) (Associated Chemical Enterprises, Gauteng, SA)	2.3g
Potassium dihydrogen phosphate (KH_2PO_4 :MW=136.09g/mol) (Merck Chemicals, Darmstadt, Germany)	0.4g
Made up to 1000ml with dH ₂ O after adjusting to pH 7.4	

C.3. Porphyrin quantitative analysis

Equipment and reagents:

- Boiling tube (Lasec)
- Separating flask (Lasec)
- Sigma 3-12 Centrifuge (Wirscham Scientific, Cape Town, SA)
- Scintered glass funnel (Lasec)
- Measuring cylinder 25ml (Lasec)
- Thin-layer chromatography (TLC) plates
(TLC aluminium sheets 20X20cm Silica Gel-60) (Merck Chemicals)
- Microsyringe (Hamilton, Bonaduz, Switzerland)
- Fluoroscanning photodensitometer TLD 100 (Vitatron, UK)
- Integrator SP4290 (Spectra-Physics, California, USA)
- Sulphuric acid (H_2SO_4 :MW=98.07) (BDH Chemicals Ltd)
- Methanol AR (CH_3OH :MW=32.04g/mol) (Kimix Chemical and Laboratory Supplies)
- Chloroform (Kimix Chemical and Laboratory Supplies)
- Ammonia solution (NH_4OH : MW=35.05) (Kimix Chemical and Laboratory Supplies)
- Anhydrous sodium sulphate (Glauber's salt: Na_2SO_4 :MW=142.04)
(BDH Laboratory Supplies)
- Porphyrin methyl ester calibrator (equal concentrations of uroporphyrin, heptacarboxylic porphyrin, hexacarboxylic porphyrin, pentacarboxylic porphyrin, coproporphyrin and mesoporphyrin methyl esters)
(Frontier Scientific Porphyrin Products, Logan, Utah)
- Carbon tetrachloride (CCl_4 :MW=153.82g/mol)
(Riedel-de-Haën, Sigma Aldrich, Gauteng, SA)
- Dichloromethane (CH_2Cl_2 :MW=84.93g/mol) (Merck Chemicals)
- Ethyl acetate ($\text{CH}_3\text{COOC}_2\text{H}_5$:MW=88.11g/mol)
(SAARCHEM-holpro Analytic, Gauteng, SA)
- Ethyl propionate ($\text{C}_5\text{H}_{10}\text{O}_2$:MW=102.13g/mol) (Merck Chemicals)
- n-Dodecan ($\text{C}_{12}\text{H}_{26}$:MW=170.34g/mol) (Merck Chemicals)
- n-Hexadecan ($\text{C}_{16}\text{H}_{34}$:MW=226.45g/mol) (Merck Chemicals)
- Distilled water (dH_2O)

Method (Day 1978; Hift et al. 2004):

- 0.5ml of packed red blood cells was transferred to a boiling tube with 30ml 5% sulphuric acid in methanol
- The stopper was placed on the boiling tube and, after shaking, this mixture was left to esterify overnight in the dark at room temperature
- A separating flask was prepared by rinsing in a solution of methanol:chloroform::1:1 and adding 60ml 4% ammonia and 10ml chloroform in a fume hood
- After overnight esterification, the sample was centrifuged at 700xg for 20 min and the supernatant transferred to the separating flask and sealed
- This was shaken vigorously for 5 to 20 sec intervals, opening the tap periodically to release gaseous build-up
- The flask was then filled to $\pm\frac{3}{4}$ of its volume with dH₂O (\pm 150ml)
- The chloroform settled at the bottom of the flask and the mixture was stirred with a glass pipette until a clear layer was obtained
- The scintered funnel was rinsed with chloroform and placed in a measuring cylinder
- Approximately 2g of anhydrous sodium sulphate was added to the funnel
- The chloroform layer was tapped off into the scintered funnel, and the procedure repeated, with the addition of a further 10ml of chloroform to the flask
- Thin-layer chromatography was then performed on the extracted porphyrins in the eluted chloroform (all preparations were performed under semi-dark conditions)
- The volume of chloroform eluted was noted and 60 μ l was spotted onto a TLC plate using a Hamilton microsyringe approximately 1cm from the base of the plate
- Two lanes on each plate were reserved for porphyrin methyl ester calibrators
- TLC plate was run vertically in a chromatography tank containing; 10ml separating solvent (carbon tetrachloride:dichloromethane:ethyl acetate:ethyl propionate::2:2:1:1 v/v) for 40 min or until the solvent front reached the top of the plate
- Plate was left to dry in the dark and then dipped briefly in a fluorescence-enhancing solution (chloroform: n-dodecan:n-hexadecan::3:1:1 v/v) in order to enhance the fluorescence quantum yield

- After drying, the plate was read on a fluoroscanning photodensitometer using two interference filters (incident filter: 399nm; fluorescence filter: 620nm)
- Individual species of porphyrin esters were identified by direct comparison of the retention times with those of the calibrators
- The amount of fluorescence in each spot (the area under the curve for each peak) was calculated and recorded by the integrator and compared directly with those of the calibrators to give quantitative results
- Results were recorded in a customised porphyrin spreadsheet (Lotus 123)/database (UCT Porphyria database CDbase 4+) to calculate the individual concentrations of the individual porphyrins in the sample

University of Cape Town

C.4. Determination of free and zinc-chelated protoporphyrin IX concentrations in EPP blood samples

Equipment and reagents:

- Glass tube (Lasec)
- Glass bottle (Lasec)
- Vortex-Genie2 G560-E mixer (Scientific Industries Inc., New York, USA)
- Fluorescence spectrophotometer 650-10S (Hitachi Koki Co., Ltd)
- Linear 1200 chart recorder (Spectrum Medical Industries Inc.)
- 150 Xenon Power Supply (Perkin-Elmer, Acme Electric Corp.)
- Quartz cuvette 10mm light path (Optiglass Ltd, Hainault, UK)
- Saline [0.9% NaCl: MW=53.44g/mol (BDH Chemicals Ltd)]
- Acetone (SAARCHEM-holpro Analytic)
- dH₂O

Method (Modified from Hart and Piomelli 1981):

- All sample preparations were performed under semi-dark conditions
- 20µl of whole, packed blood was diluted 10X with saline in a glass tube
- A solution of acetone:dH₂O::80:20 was prepared and stored in a glass bottle to prevent evaporation
- 5ml of this mixture was added to the blood sample and vortexed for 30 sec
- Centrifugation at 700xg for 1 min followed
- The fluorescence spectrophotometer (λ_{ex} :420nm; λ_{em} :632nm) was zeroed against acetone:dH₂O solution
- 1ml of the supernatant was transferred to a cuvette for spectrofluorescent analysis
- An emission scan was performed from 400nm to 700nm (λ_{ex} :420nm) at a sensitivity of 10 and a scan speed of 60nm/min
- A profile of the scan was recorded on a chart recorder (chart speed: 3cm/min) and the peak emission wavelength recorded

Note: Zinc protoporphyrin maximum peak emission reading: 588nm
Free protoporphyrin maximum peak emission reading: 632nm

APPENDIX D

DNA Extraction and Purification

D.1. Genomic DNA isolation

Equipment and reagents:

- Microfuge tubes (Greiner, Lasec)
- Vortex-Genie2 G560-E mixer (Scientific Industries Inc.)
- Force 14 microfuge (Denver Instruments, Lasec)
- Techni driblock BD-2D heating block (Lasec)
- Wizard[®] Genomic DNA purification kit (Promega, Madison WI, USA)
- Isopropanol (Riedel-de-Haën, Sigma Aldrich)
- Ethanol (Riedel-de-Haën, Sigma Aldrich)
- Gene Quant II, RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK)

Method:

- Blood samples were placed at room temperature for 30 min prior to DNA extraction, and mixed thoroughly by inverting the tube
- 900 μ l Cell Lysis solution was aliquoted into a 1.5ml microfuge tube followed by addition of 300 μ l whole blood
- After capping, the tube was inverted 6 times and incubated at room temperature for 10 min with another 4 inversions to mix at 5 min
- Centrifugation at 14000xg followed for 1 min at room temperature
- The supernatant was discarded leaving 10-20 μ l of liquid and a visible white pellet (if the pellet contained visible RBCs the previous 3 steps were repeated)
- The pellet was completely resuspended in solution by vortexing and 300 μ l Nuclei Lysis solution was added followed by gentle agitation (samples containing aggregated cells were incubated at 37°C for \pm 60 min)
- 100 μ l Protein Precipitation solution was added followed by vortexing for 20 sec
- Centrifugation at 14000xg followed for 3.5 min at room temperature
- The supernatant was added to a microfuge tube containing 300 μ l isopropanol and mixed gently by inversion until a white, threadlike mass was formed

- Centrifugation at 14000xg followed for 3.5 min at room temperature
- The supernatant was discarded and pellet washed with 300 μ l 70% ethanol
- Centrifugation at 14000xg was repeated for 5 min at room temperature
- Ethanol was decanted and the pellet air-dried for 30 min
- 50 μ l DNA Rehydration solution was added to the pellet followed by incubation for 60 min at 65°C
- DNA concentration and purity was determined using the GeneQuant spectrophotometer using DNA Rehydration solution as a reference

Protocol modifications:

- Longer centrifugation times (3.5 min) were employed to pellet the DNA in order to prevent this pellet from being dislodged when decanting the supernatant.
- In order to obtain a white pellet free of red blood cells, cell lysis solution incubation was repeated for the majority of samples, in spite of prompt handling.

D.2. DNA purification (Isoamyl alcohol:chloroform “clean-up”)

Equipment and reagents:

- Vortex-Genie2 G560-E mixer (Scientific Industries Inc.)
- Refrigerated microcentrifuge (Sorvall Instruments, DuPont, USA)
- Gene Quant II, RNA/DNA Calculator (Pharmacia Biotech)
- Isoamyl alcohol (Merck Chemicals)
- Chloroform (Kimix Chemical and Laboratory Supplies)
- 3M Sodium acetate (pH 5.2)
- Ethanol (Riedel-de-Haën, Sigma Aldrich)
- dH₂O (Bodene Pty Ltd, Port Elizabeth, SA)

Method:

- 100µl of previously extracted, stored DNA was used for purification
- An equal volume of chilled (-20°C) isoamylalcohol:chloroform::1:49 (v/v) was added and vortexed to mix
- Centrifugation at 14000xg for 10 min at 4°C followed
- The upper phase was then removed and the previous two steps repeated until a clear upper phase was obtained, the volume of which was noted
- 2.5 x upper phase volume of chilled (-20°C) 100% ethanol and 1/10 x upper phase volume of 3M sodium acetate (pH 5.2) were added and placed at -70°C for 30 min
- DNA was pelleted by centrifugation at 14000xg for 10 min at 4°C
- The supernatant was decanted and the pellet washed with 100µl chilled (-20°C) 70% ethanol
- Centrifugation was repeated and the supernatant removed with a pipette
- The pellet was air-dried and resuspended in 50µl dH₂O
- The DNA was quantified and the purity determined by a spectrophotometric reading of each sample using dH₂O as a reference
- Working solutions (10ng/µl) were stored at 4°C and stock DNA solutions were stored at -70°C

APPENDIX E

Analysis of the FECH gene

E.1. Primer selection for fragment amplification

Criteria for primer selection:

- Primer length (18bp to 25bp)
- GC-content (48% to 55%)
- Annealing temperature (50°C to 60°C)
- In addition to using Primer3, primer melting temperatures were determined by Oligo Calculator (<http://www.pitt.edu/~rsup/OligoCalc.html>)
- Criteria for the selection of primers for the amplification of *FECH* exon 1 were far less stringent, as this is a highly GC-rich region, and primers could not be identified that met the above criteria

E.2. Polymerase chain reaction (PCR)

Equipment and reagents:

- Robocycler[®] Gradient 40 Thermal Cycler (Stratagene[®], California, USA)
- Thermophilic DNA polymerase 10X buffer
[10mM Tris-HCl pH 9, 50mM KCl and 0.1% Triton[®] X-100 (Promega)]
- MgCl₂ 25mM (Promega)
- Deoxynucleotide triphosphates (dNTPs) (E.2.1)
- *Taq* Polymerase (Promega)
- Forward and Reverse primers
(IDT-Integrated DNA Technologies Inc., Coralville IA, USA)
- dH₂O (Bodene Pty Ltd)
- Mineral oil (Sigma Chemical Company, St Louis MO, USA)

Method:

- PCR amplification of all *FECH* exons was performed on a Robocycler Gradient 40 Thermal cycler in a total reaction volume of 50 μ l
- Each reaction included 1X buffer, 2.5mM MgCl₂, 50 μ M dNTPs each, 1 Unit *Taq* Polymerase and 25pmol of each primer
- 10ng template DNA (genomic) was routinely included in the above reaction mixture, however up to 50ng was required for certain samples
- A negative control that included 1 μ l dH₂O instead of DNA was set up for each amplification to confirm that the PCR reagents were not contaminated
- All samples were overlaid with a single drop of mineral oil to prevent evaporation

E.2.1 Deoxynucleotide triphosphates (2.5mM)

dATP, ddCTP, dGTP and dTTP (100mM each)	(Promega)	25 μ l
dH ₂ O (Bodene Pty Ltd)		900 μ l

For each PCR reaction, 1 μ l was used giving a final concentration of 50 μ M

APPENDIX F

Gel Electrophoresis

F.1. Non-denaturing polyacrylamide gel electrophoresis and fragment visualisation

Equipment and reagents:

- Hoefer vertical slab gel electrophoresis unit; SE600 Series (Amersham Pharmacia Biotechnology Inc., California, USA)
- Gel documentation apparatus (gel-doc: BTS-20M UViband vs 99) (UVItec Ltd, Cambridge, UK)
- Video copy processor, model P90E(B) (UVItec Ltd)
- K65HM High-density synthetic paper (Whitehead Scientific, Cape Town, SA)
- Non-denaturing polyacrylamide gel (6%) (F.1.1)
- 1X TBE buffer (F.1.2.1)
- 6X loading dye (F.1.2.3)
- Ethidium bromide (EtBr) solution (F.1.2.4)
- 100bp DNA Ladder molecular weight marker (F.1.3a)

Method:

- Samples were prepared by adding 6X loading dye in the ratio 2:1 to 5 μ l of PCR product and loaded onto the gel
- A 100bp DNA Ladder molecular weight marker was included on the gel
- Electrophoresis was performed at 250V for 90 min in 1X TBE buffer
- Gels were stained for 10 min in EtBr solution and photographed on a gel documentation apparatus

F.1.1. Non-denaturing polyacrylamide gel (6%)

Equipment and reagents:

- Hoefer vertical slab gel electrophoresis unit; SE600 Series including 20 bay spacer comb (1.5mm), vertical slab gel glass plates, clamps, cams and spacers (1.5mm) (Amersham Pharmacia Biotechnology Inc.)
- PS1200 DC power supply (Amersham Pharmacia Biotechnology Inc.)
- 10X TBE (F.1.2.1)
- Acrylamide:bisacrylamide::29:1 (F.1.2.2)
- 10% Ammonium persulfate (APS) (Promega)
- TEMED (Merck Chemicals)

Method:

- Two glass plates, separated by spacers, were clamped together and placed in a gel casting stand utilising two cams
- 4ml 10X TBE and 8ml acrylamide:bisacrylamide solution were combined and the solution made up to 40ml with dH₂O
- 400 μ l APS and 40 μ l TEMED were added and the solution rapidly mixed and poured between the pre-cast plates, avoiding the formation of bubbles in the gel
- The comb was inserted and the gel allowed to polymerise for 45 min

F.1.2. Solutions

F.1.2.1 10X TBE (pH 8.0)

Tris (C ₄ H ₁₁ NO ₃ :MW=121.134) (Merck Chemicals)	108g
Boric Acid (Merck Chemicals)	55g
EDTA (Merck Chemicals)	9.3g
Made up to 1000ml with dH ₂ O	

For 1X TBE: 1/10 dilution was made of the 10X TBE

For 0.6X TBE: 0.6/10 dilution was made of the 10X TBE

F.1.2.2. Acrylamide:bisacrylamide::29:1 solution

Acrylamide (Whitehead Scientific)	29g
Bisacrylamide (Whitehead Scientific)	1g
Made up to 100ml with dH ₂ O	

F.1.2.3. Loading dye (6X)

Bromophenol Blue (0.25% w/v) (Associated Chemical Enterprises)	0.25g
Sucrose (40% w/v) (BDH Laboratory Supplies)	40g
0.5M EDTA, pH 8 (BDH Laboratory Supplies)	4ml
Made up to 100ml with dH ₂ O	

F.1.2.4. Ethidium bromide solution (0.5ng/μl)

EtBr (1mg/ml) (Roche Diagnostics Pty Ltd, Randburg, SA)	100μl
dH ₂ O (Bodene Pty Ltd)	200ml

F.1.2.5. Sucrose solution (40%)

Sucrose (BDH Laboratory Supplies)	4g
Made up to 10ml with dH ₂ O	

F.1.3. Molecular weight markers

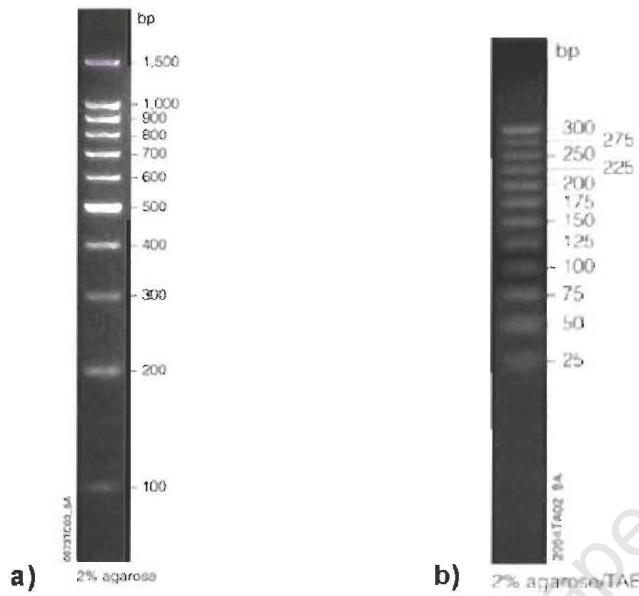


Figure F1. a) 100bp DNA Ladder and b) 25bp Step DNA Ladder (Promega)

A 1/5 dilution of each DNA Ladder stock solution was made in dH₂O (Bodene Pty Ltd)
2µl of the dilution was then mixed with 3µl loading dye (F.1.2.3)

APPENDIX G

Investigation of the IVS3-48T/C polymorphism

G.1. Restriction endonuclease analysis

Equipment and reagents:

- Water bath (Memmert GmbH+Co., KG, Schwabach, Germany)
- Techni driblock BD-2D heating block (Lasec)
- 10X restriction endonuclease buffer H
(Roche Diagnostics Corp., Mannheim, Germany)
- *I*ta I restriction endonuclease (10U/ μ l) (Roche Diagnostics Corp.)
- dH₂O (Bodene Pty Ltd.)

Method:

- The restriction endonuclease analysis reaction mixture included 2 μ l 10X restriction endonuclease buffer H, 1U of *I*ta I and 5-10 μ l PCR product as required
- This was made up to a final volume of 20 μ l with dH₂O
- Samples were incubated in a water bath at 37°C for 2.5 h

Note: The above procedure was followed for all other restriction enzymes utilised in this study but with the relevant buffer and conditions as outlined in Table 4.3 on page 53.

G.2. Silver staining and visualisation

Equipment and reagents:

- Gel documentation apparatus (gel-doc: BTS-20M UViband vs 99) (UVItec Ltd)
- Video copy processor, model P90E(B) (UVItec Ltd)
- K65HM High-density synthetic paper (Whitehead Scientific)
- UV/white light converter screen (UVItec Ltd)
- Silver staining solution I (G.2.1.1)
- Silver staining solution II (G.2.1.2)

Method:

- The non-denaturing polyacrylamide gel used for the separation of restriction fragments (Appendix F.1) was rinsed thoroughly with dH₂O
- Solution I was applied to the gel for 10 min followed by another rinse with dH₂O
- Solution II was then applied until bold, distinct bands could be observed (±5 min)
- A final rinse of the gel in dH₂O completed the staining procedure
- Gels were photographed on a gel documentation apparatus using a UV/white light converter screen for visualisation of dye/silver-stained gels

G.2.1. Silver staining solutions

(Prepared immediately before staining)

G.2.1.1. Silver staining solution I

Silver Nitrate (AgNO₃:MW = 169.87g/mol) (Merck Chemicals) 1g
Made up to 1000ml with dH₂O

G.2.1.2. Silver staining solution II

Sodium Hydroxide (NaOH:MW = 40g/mol) (Merck Chemicals) 15g
Dissolved in dH₂O 800ml
Followed by the addition of;
15% Formaldehyde (BDH Laboratory Supplies) 10ml
Made up to 1000ml with dH₂O

G.3. IVS3-48C polymorphism screening

G.3.1. Selection of restriction endonuclease *I*ta I and a positive control sample

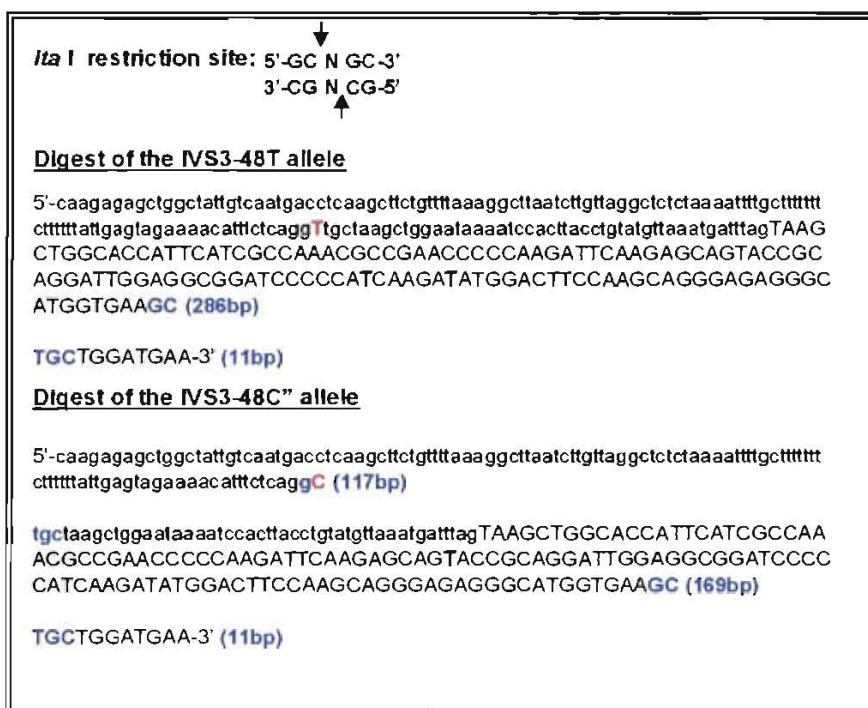


Figure G1. Expected fragment sizes for respective IVS3-48T and IVS3-48C alleles after *I*ta I digestion

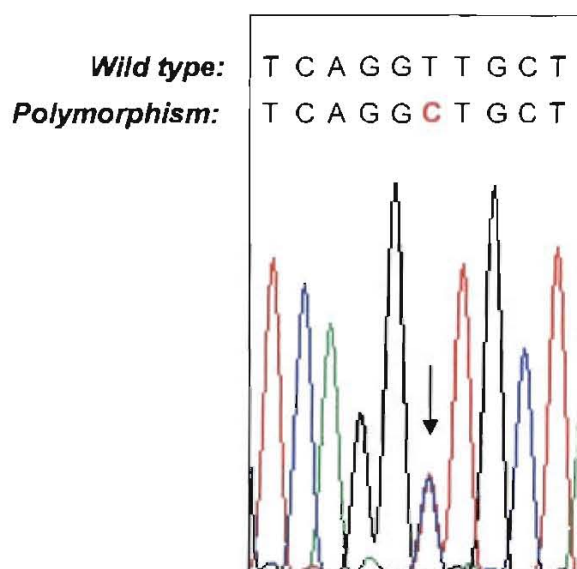


Figure G2. Direct sequencing (forward) of amplification fragment 4A (EPP2.1) indicating heterozygosity for the IVS3-48T/C polymorphism. Sample subsequently used as a positive control (EPP2.1) for screening of the cohorts

APPENDIX H

SSCP Analysis

H.1. MDE-PAGE

Equipment and reagents:

- Hoefer vertical slab gel electrophoresis unit; SE600 Series (Amersham Pharmacia Biotechnology Inc.)
- Gel documentation apparatus (gel-doc: BTS-20M UViband vs 99) (UVItec Ltd.)
- Video copy processor, model P90E(B) (UVItec Ltd.)
- K65HM High-density synthetic paper (Whitehead Scientific)
- Techni drieblock BD-2D (Lasec)
- 2X MDE[®] polyacrylamide gel (H.1.1)
- SSCP loading dye (H.1.2)
- 0.6X TBE buffer (Appendix F.1.2.1)

Method (Orita et al. 1989):

- 5 μ l of each sample was prepared with SSCP loading dye in a 1:1 ratio
- These were denatured at 95°C for 5 min and snap-cooled on ice
- A total volume of 10 μ l was loaded for each sample
- Gels were run at room temperature overnight in 0.6X TBE
- Voltage and running times varied according to the fragment size (Table 4.2)

H.1.1. 2X MDE polyacrylamide gel

Equipment and reagents:

- Hoefer vertical slab gel electrophoresis unit; SE600 Series including 20 bay spacer comb (1.5mm), vertical slab gel glass plates, clamps, cams and spacers (1.5mm) (Amersham Pharmacia Biotechnology Inc.)
- 2X MDE[®] gel solution (Cambrex Bio Science Rockland Inc., Rockland ME, USA)
- 10X TBE (Appendix F.1.2.1)
- Glycerol (*Optional) (BDH Laboratory Supplies)
- 10% Ammonium persulfate (APS) (Promega)
- TEMED (Merck Chemicals)

Method:

- Two glass plates, separated by spacers, were clamped together and placed in a gel casting stand utilising two cams
- 15ml TBE, 1.8ml MDE gel solution and 3ml glycerol were combined and the solution made up to 30ml with dH₂O (*3ml glycerol replaced with 3ml dH₂O if glycerol excluded from gel mixture)
- A gel plug of 5ml MDE gel solution, 20 μ l APS and 20 μ l TEMED was poured rapidly between the plates to seal them
- 175 μ l APS and 17.5 μ l TEMED were added to the remaining 30ml MDE gel solution
- This was rapidly mixed and poured between the pre-cast plates avoiding the formation of bubbles in the gel
- The comb was inserted and the gel allowed to polymerise for 60 min

H.1.2. SSCP loading dye

Formamide (95%) (BDH Laboratory Supplies)	9.5ml
1N NaOH (10mM) (BDH Laboratory Supplies)	100 μ l
0.5M EDTA pH 8 (20mM) (BDH Laboratory Supplies)	400 μ l
Bromophenol Blue (0.25% w/v) (Associated Chemical Enterprises)	0.002g
Xylene Cyanol (0.25% w/v) (Sigma Chemical Company)	0.002g
Made up to 10ml with dH ₂ O and stored at 4°C in the dark	

University of Cape Town

APPENDIX I

DNA purification for sequencing

I.1. DNA purification from PCR product

Equipment and reagents:

- Vortex-Genie2 G560-E mixer (Scientific Industries Inc.)
- Force 14 microfuge (Denver Instruments, Lasec)
- 1.5ml microfuge tubes (Greiner, Lasec)
- GFX™ PCR DNA and gel band purification kit (Amersham Biosciences Ltd.)
- dH₂O (Bodene Pty Ltd)

Method:

- A GFX column was placed into a collection tube and 1ml capture buffer added
- PCR amplification products for the relevant fragment (100ng template DNA used) were pooled and 90µl was added to the column
- The solution of DNA and capture buffer was mixed without disturbing the filter
- Centrifugation at 14000xg for 1 min followed and the collection tube emptied
- 1ml wash buffer was added to the GFX column and centrifugation was repeated as above discarding the flow-through
- The above step was repeated and the GFX column placed in a microfuge tube
- 50µl dH₂O was applied directly to the top of the glass fiber matrix in the GFX column and incubation at room temperature for 5 min followed
- Centrifugation at 14000xg for 3 min recovered the purified DNA

I.2. Agarose gel electrophoresis and DNA purification from agarose

I.2.1. Agarose gel electrophoresis

Equipment and reagents:

- Horizontal mini-gel system (Mgu-202T) (CBS Scientific Co., Inc., Del Mar, USA)
- Electric power supply (EPS-250 series II) (CBS Scientific Co., Inc.)
- Gel documentation apparatus (gel-doc: BTS-20M UViband vs 99) (UVIttec Ltd)
- Video copy processor, model P90E(B) (UVIttec Ltd.)
- K65HM High-density synthetic paper (Whitehead Scientific)
- Electronic UV Transilluminator (UltraLum, California, USA)
- Sterile surgical blades; 2.5M-Rads, No. 3 (B & M Scientific, Cape Town, SA)
- 1.5ml microfuge tubes (Greiner, Lasec)
- Agarose gel (2%) (I.2)
- 1X TBE buffer (Appendix F.1.2.1)
- 40% Sucrose (Appendix F.1.2.5)
- Ethidium bromide (EtBr) solution (Appendix F.1.2.4)
- 100bp DNA Ladder molecular weight marker (Appendix F.1.3a)
- 25bp Step DNA Ladder (Appendix F.1.3b)

Method:

- 10 μ l sucrose was mixed with 40 μ l of each PCR amplification product and loaded onto the gel (H.2) with two molecular weight markers and a negative control
- Electrophoresis was performed at 120V for 4 h in 1X TBE buffer followed by 10 min at 30V to resolve the bands
- Gels were photographed on a gel documentation apparatus and the relevant bands excised using a sterile surgical blade on an Electronic UV Transilluminator

1.2.2. Agarose gel (2%)

Equipment and reagents:

- Magnetic stirrer (Lasec)
- Conical flask (Lasec)
- Microwave (Defy, SA)
- Horizontal mini-gel system casting tray (CBS Scientific Company Inc.)
- 12 well comb (CBS Scientific Company Inc.)
- Metaphor[®] agarose (FMC BioProducts, Maine, USA)
- 1X TBE (Appendix F.1.2.1)
- Ethidium Bromide (1mg/ml) (Roche Diagnostics Pty Ltd.)

Method:

- 4g agarose was added to 200ml 1X TBE in a conical flask while stirring on a magnetic stirrer to avoid clumping
- The flask was heated in a microwave for 2 min (after 1 min the solution was swirled gently to avoid boiling over)
- Once dissolved the agarose solution was cooled slightly, followed by the addition of 100 μ l EtBr
- Agarose solution was poured into a horizontal casting tray and the comb inserted
- The gel was allowed to set at room temperature for 60 min
- Once set, the comb was removed and the gel placed in the tank and immersed in 1X TBE

I.2.3. DNA purification from agarose

Equipment and reagents:

- Weighing balance (M-120) (Denver Instrument Co., Colorado, USA)
- Fine Balance (X-1810) (Denver Instrument Co., Colorado, USA)
- Techni driblock BD-2D heating block (Lasec)
- Force 14 microfuge (Denver Instruments, Lasec)
- Vortex-Genie2 G560-E mixer (Scientific Industries Inc.)
- 1.5ml microfuge tubes (Greiner, Lasec)
- GFX™ PCR DNA and gel band purification kit (Amersham Biosciences Ltd.)
- dH₂O (Bodene Pty Ltd)

Method:

- The excised gel fragment was weighed in a microfuge tube
- For each 10mg of gel slice, 10µl of capture buffer was added
- This was mixed vigorously by vortexing and incubated at 65°C for 15 min
- Centrifugation at 14000xg for 30 sec followed and the supernatant was transferred to a GFX column in a collection tube (I.1)
- After incubating at room temperature for 2 min centrifugation was repeated at 14000xg for 30 sec and the collection tube emptied
- 500µl wash buffer was added to the GFX column and centrifugation repeated
- The collection tube containing the wash buffer flow-through was discarded and the GFX column transferred to a microfuge tube
- 50µl dH₂O was applied directly to the top of the glass fiber matrix in the GFX column followed by incubation at room temperature for 5 min
- Centrifugation at 14000xg 2 min recovered the purified DNA

I.3. DNA purification from acrylamide

Equipment and reagents:

- Techni driblock BD-2D heating block (Lasec)
- Force 14 microfuge (Denver Instruments, Lasec)
- Vortex-Genie2 G560-E mixer (Scientific Industries Inc.)
- Syringe barrel (Promex, Bergvleit, SA)
- Siliconised glass wool (Lasec) plug ($\pm 1.5\text{mm}$)
- Qiaex II gel extraction kit (Qiagen GmbH, Hilden, Germany)
- Diffusion buffer (I.3.1)
- dH_2O (Bodene Pty Ltd.)

Method:

- The excised gel fragment was weighed in a microfuge tube
- One volume diffusion buffer was added for every 1 volume gel slice followed by incubation at 50°C for 30 min
- Centrifugation at 14000g for 1 min followed and the supernatant was retained
- Remaining polyacrylamide gel in the supernatant was removed using a filter (a syringe containing a glass wool plug)
- The approximate volume of the recovered supernatant was determined and 3 volumes Buffer QX1 added (the colour of the supernatant was yellow)
- QIAEX II was vortexed for 30 sec to resuspend and $10\mu\text{l}$ was added to the mixture
- This was incubated at room temperature for 10 min vortexing every 2 min
- Centrifugation followed at 14000g for 30 sec
- The pellet was washed twice with $500\mu\text{l}$ Buffer PE, repeating the above centrifugation in between washes, and then air-dried for 30 min
- $20\mu\text{l}$ dH_2O was applied and the pellet resuspended by vortexing
- Incubation at room temperature for 5 min followed
- Centrifugation was repeated at 14000g for 1 min and the supernatant transferred to a microfuge tube

I.3.1. Diffusion buffer

Ammonium acetate (0.5M) (BDH Laboratory Supplies)	2.972g
Magnesium acetate (10mM) (Merck Chemicals)	0.12g
10% Sodium Dodecyl Sulphate (0.1%) (BDH Laboratory Supplies)	500 μ l
0.5M EDTA pH 8 (BDH Laboratory Supplies)	100 μ l
Made up to 50ml dH ₂ O	

University of Cape Town

APPENDIX J

Haplotype Analysis

J.1. Marker genotyping

Equipment and reagents:

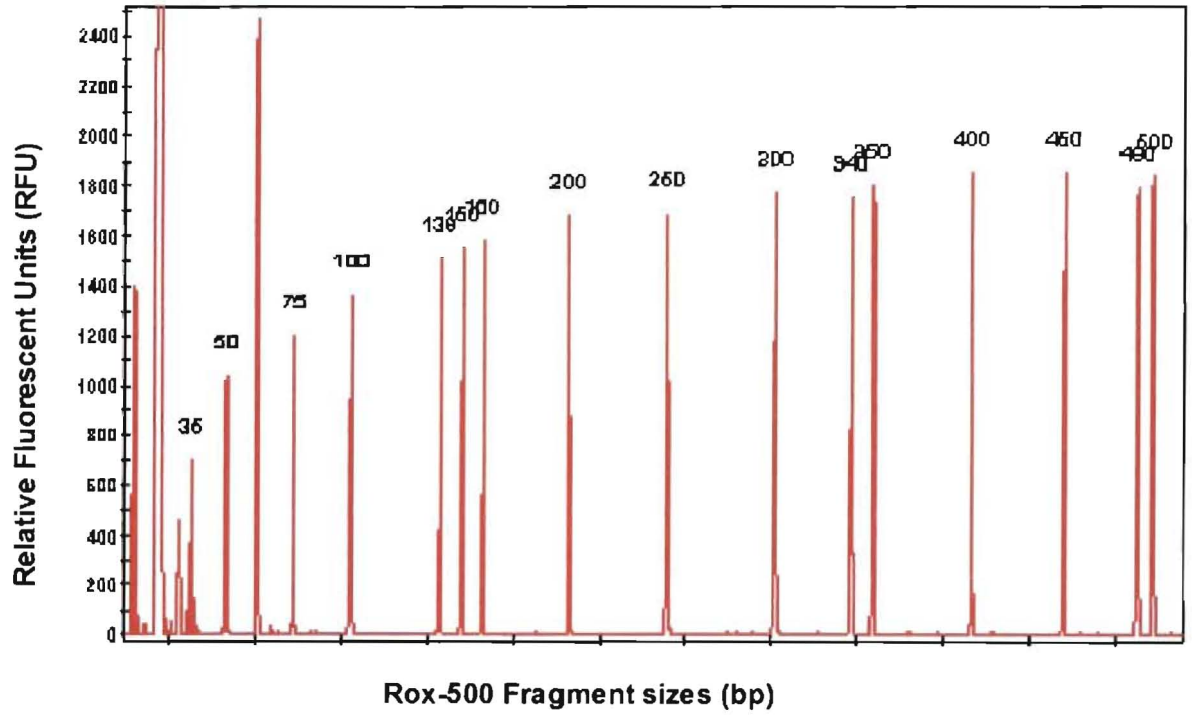
- ABI PRISM[®] 3100 automated sequencer
(PE Applied Biosystems, Foster City, CA, USA)
- Hi-Dye formamide (PE Applied Biosystems)
- Rox-500 (PE Applied Biosystems) size standard (J.2)
- 96-well plate (PE Applied Biosystems)
- Hybaid Touchdown Thermal cycler (Hybaid Ltd, Teddington, UK)
- ABI PRISM[®] GeneMapper[™] software vs 3.0 (PE Applied Biosystems)

Method (Wenz et al. 1998):

- Amplified PCR products were diluted to either 1/10, 1/20 or 1/40 depending on PCR product intensity on PAGE
- 8 μ l of Hi-Dye formamide and 0.25 μ l of Rox-500 size standard was aliquoted into the appropriate wells on a 96-well plate
- Samples were denatured at 95°C for 2 min and cooled immediately on ice
- 2 μ l of each diluted sample was then added to the respective wells
- Samples were loaded onto the ABI PRISM 3100 automated sequencer and run with filter set D and the appropriate run and analysis modules
- The ABI PRISM GeneMapper software vs 3.0 (PE Applied Biosystems) was used for analysis of samples

J.2. ROX-500 size standard

(PE Applied Biosystems)



APPENDIX K Family Pedigrees

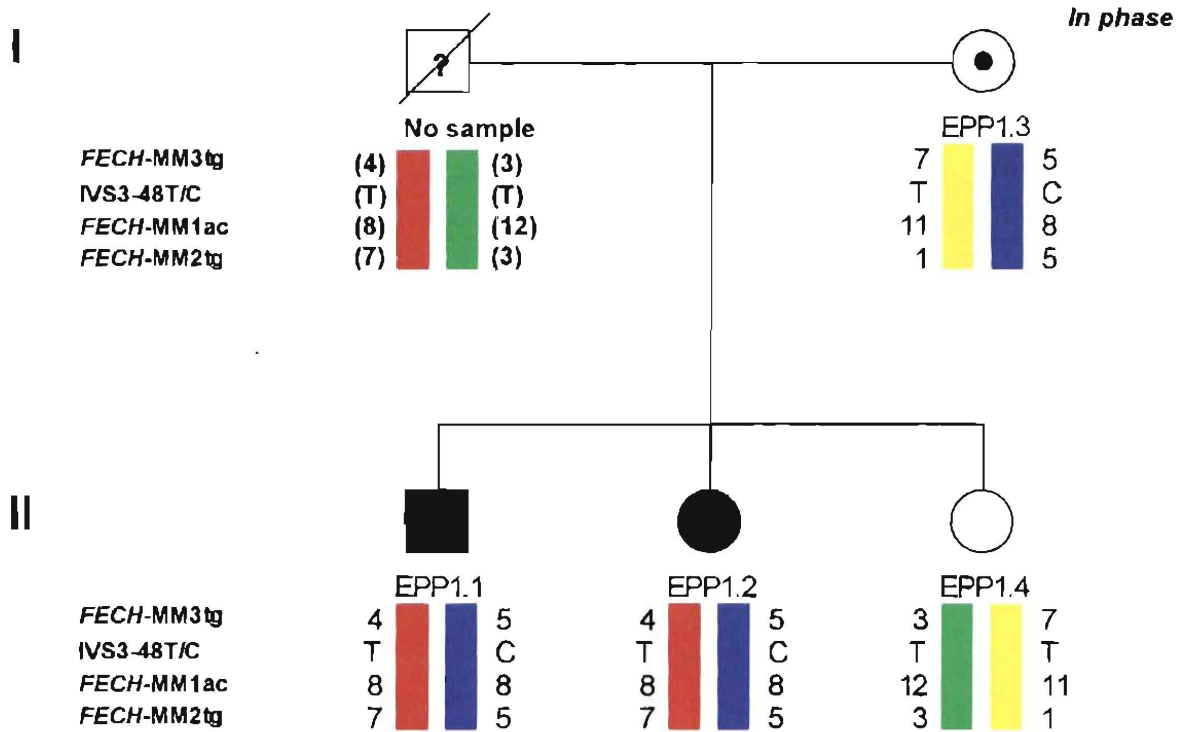


Figure K1: Pedigree of Family EPP1 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. Inferred genotypes are given in brackets. A line through a symbol indicates a deceased individual. '?' = phenotype unknown.

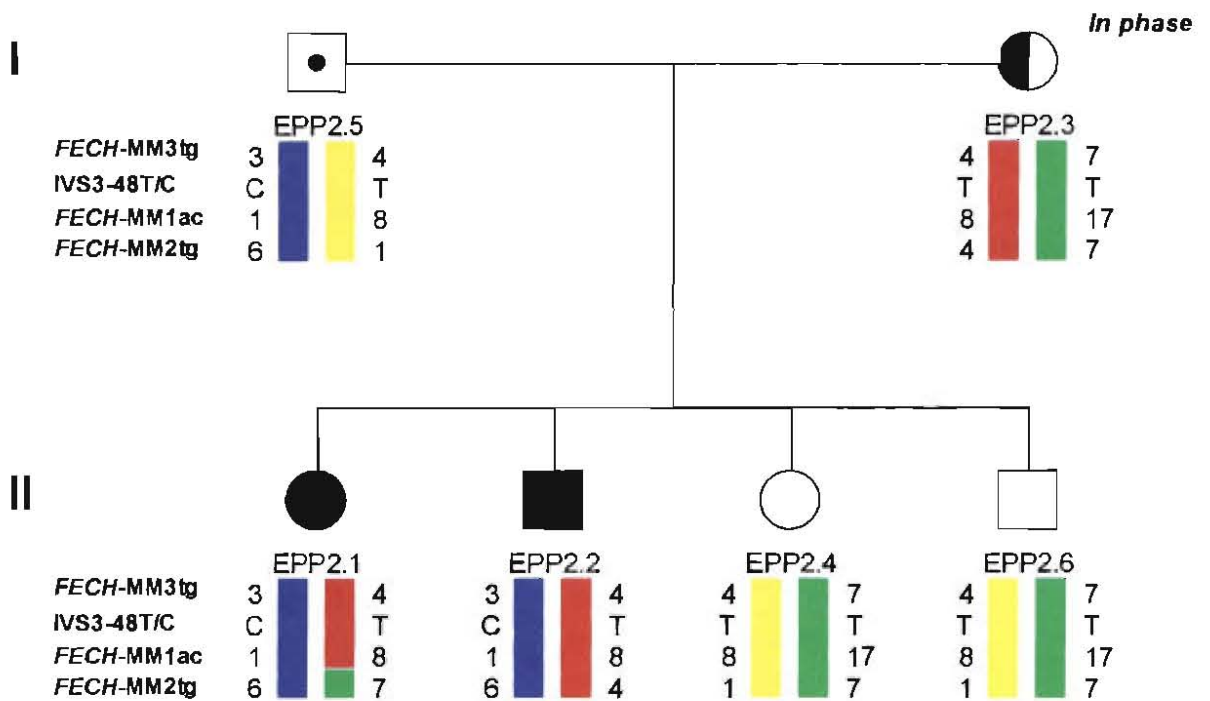


Figure K2: Pedigree of Family EPP2 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively.

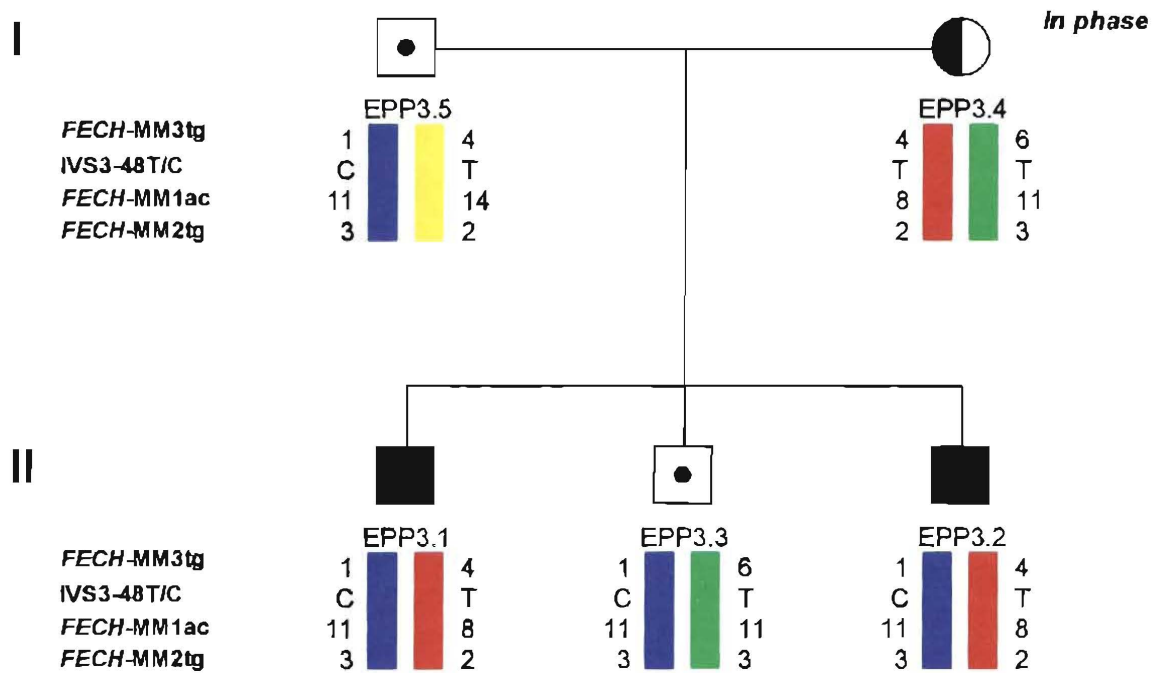


Figure K3: Pedigree of Family EPP3 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively.

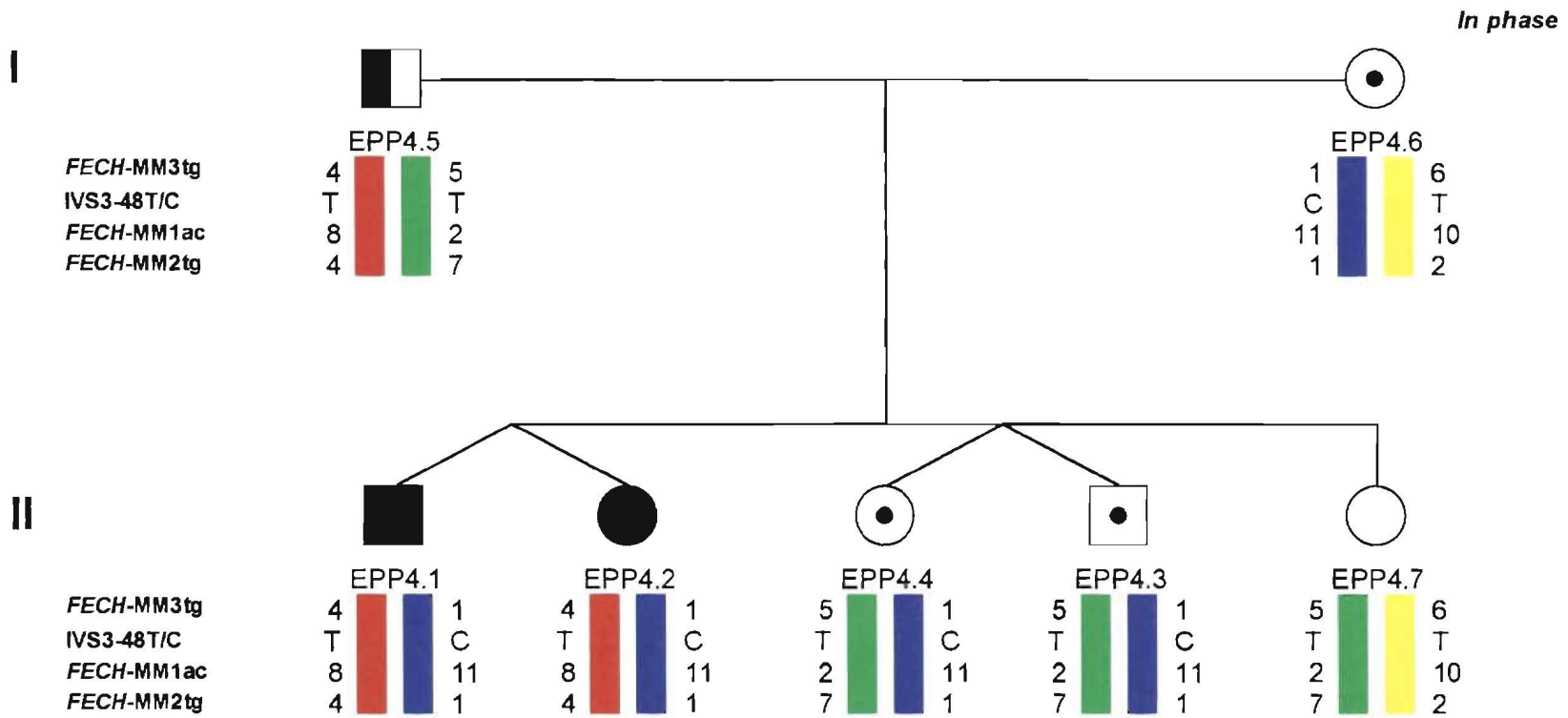


Figure K4: Pedigree of Family EPP4 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. Lines connecting two siblings indicate twins.

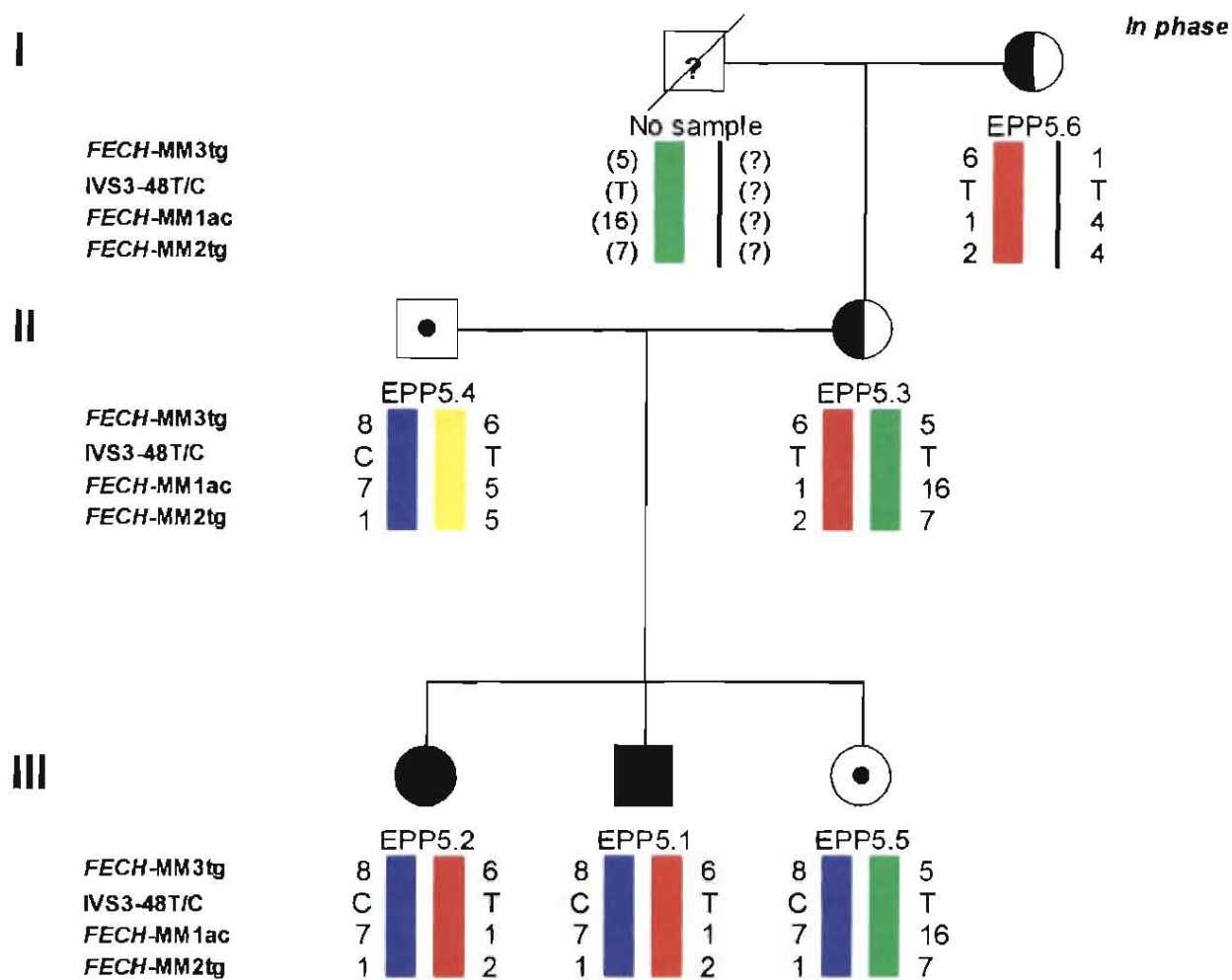


Figure K5: Pedigree of Family EPP5 showing haplotype segregation of both the allele associated with the primary mutation: 356_362delTTCAAGA (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. Inferred genotypes are given in brackets. A line through a symbol indicates a deceased individual. '?' = phenotype unknown. (?) = genotype unknown.

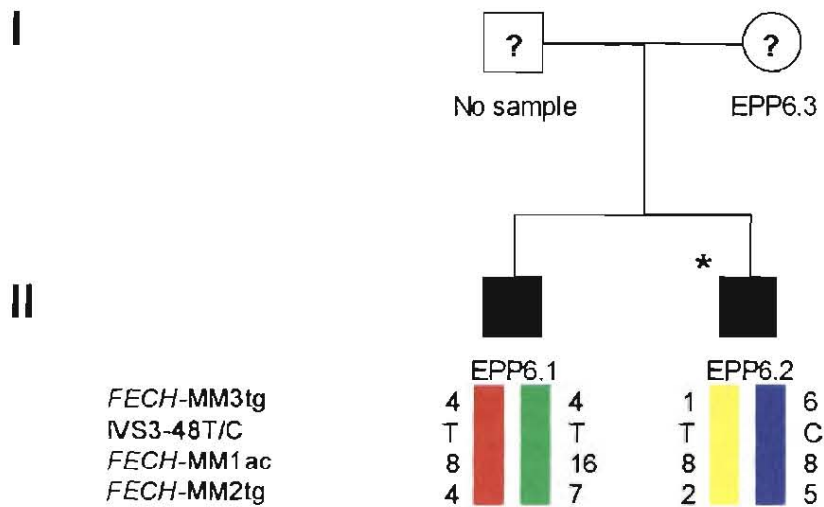


Figure K6: Pedigree of Family EPP6 showing genotyping results for individuals EPP6.1 and EPP6.2 and possible haplotypes for alleles associated with the primary: 757_761delAGAAG (red bar) and secondary: IVS3-48C (blue bar) mutations, respectively. Information is insufficient to determine haplotype phase. Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. '?' = phenotype unknown. * = Diagnosis uncertain.

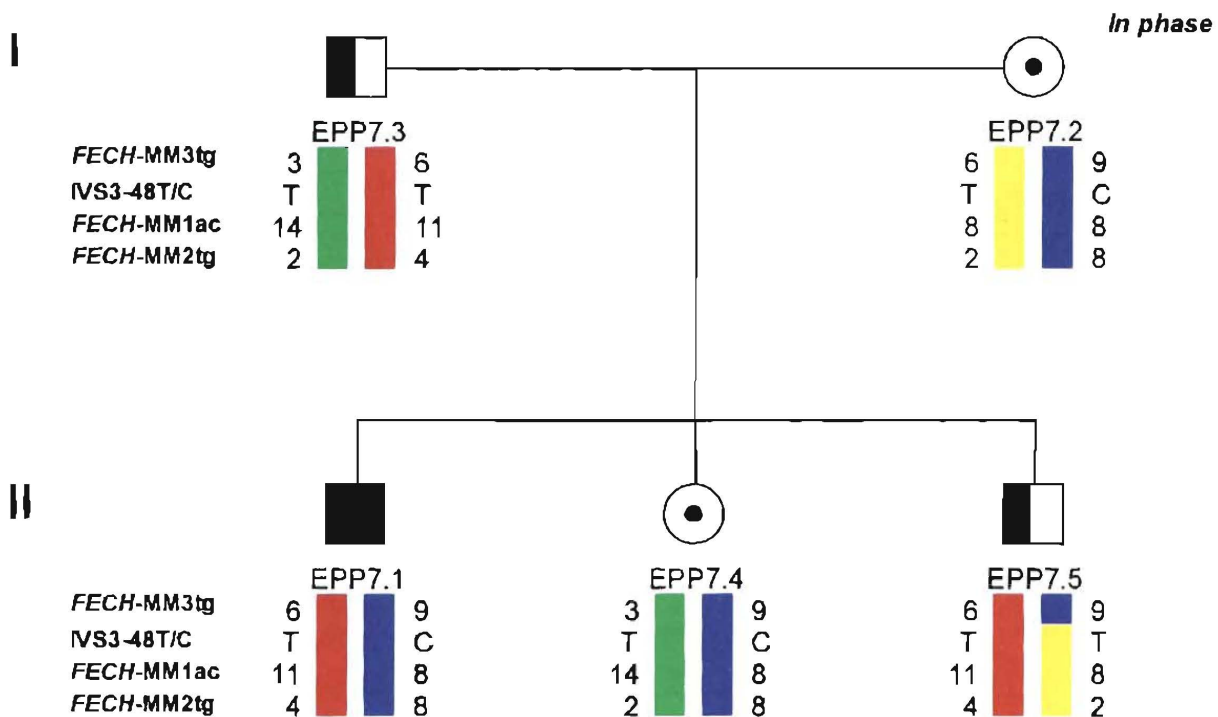


Figure K7: Pedigree of Family EPP7 showing haplotype segregation of both the allele associated with the primary mutation: IVS7+1A (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively.

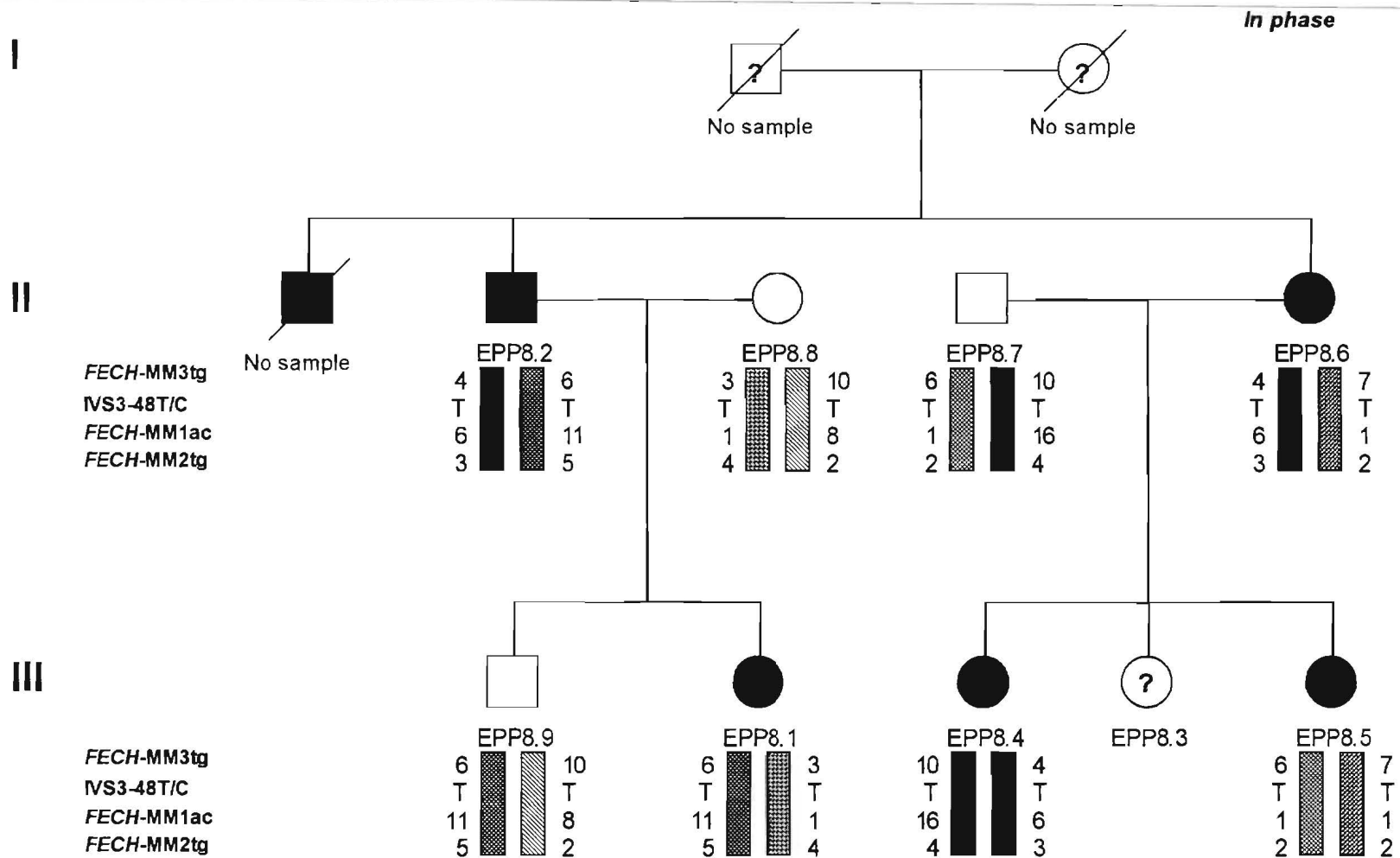


Figure K8: Pedigree of Family EPP8 showing haplotype segregation (different patterns indicate distinct haplotypes). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. A line through a symbol indicates a deceased individual. '?' = phenotype unknown.

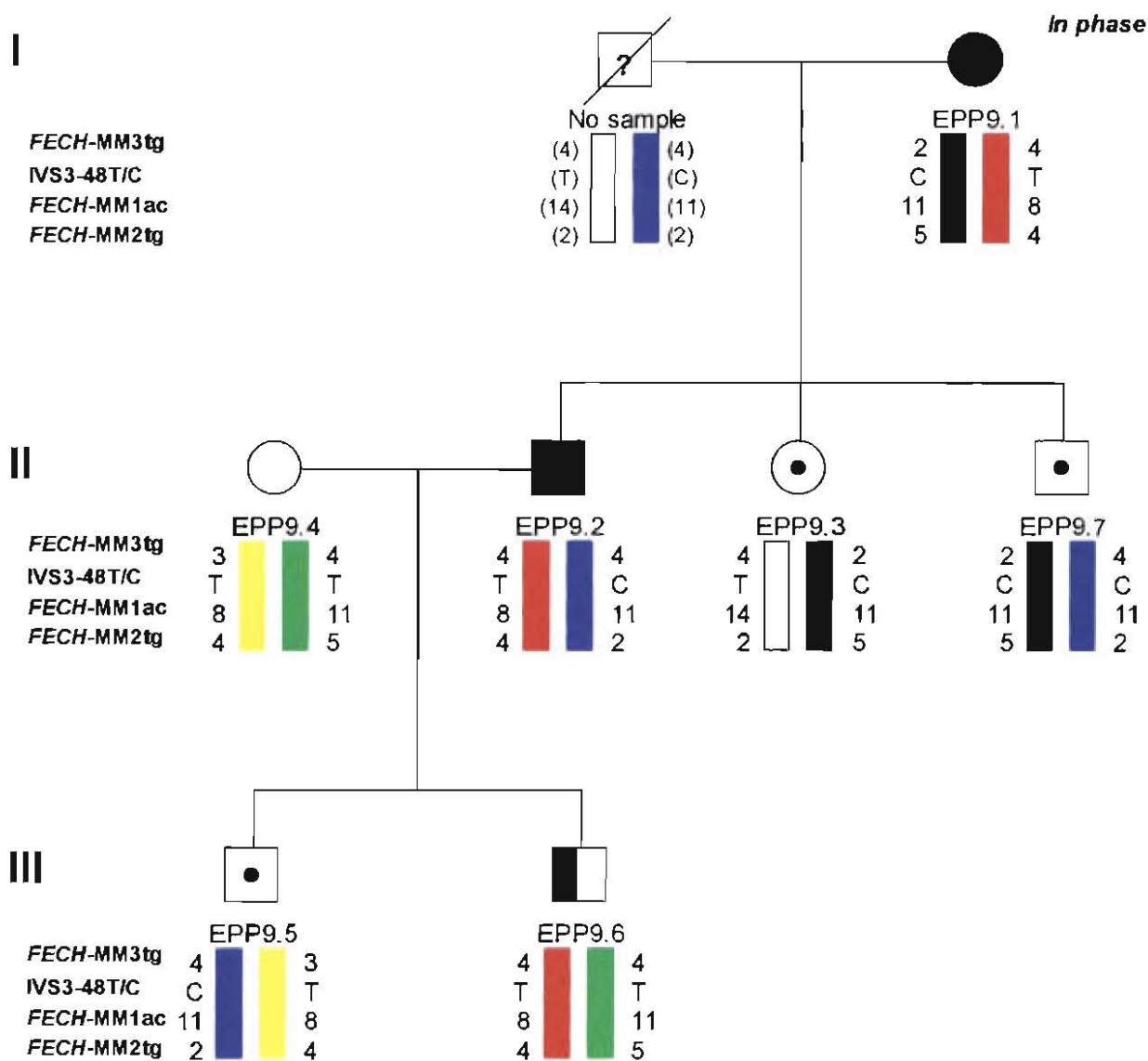


Figure K9: Pedigree of Family EPP9 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). A distinct allele containing the IVS3-48C polymorphism is indicated by a black bar. Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. Inferred genotypes are given in brackets. A line through a symbol indicates a deceased individual. '?' = phenotype unknown.

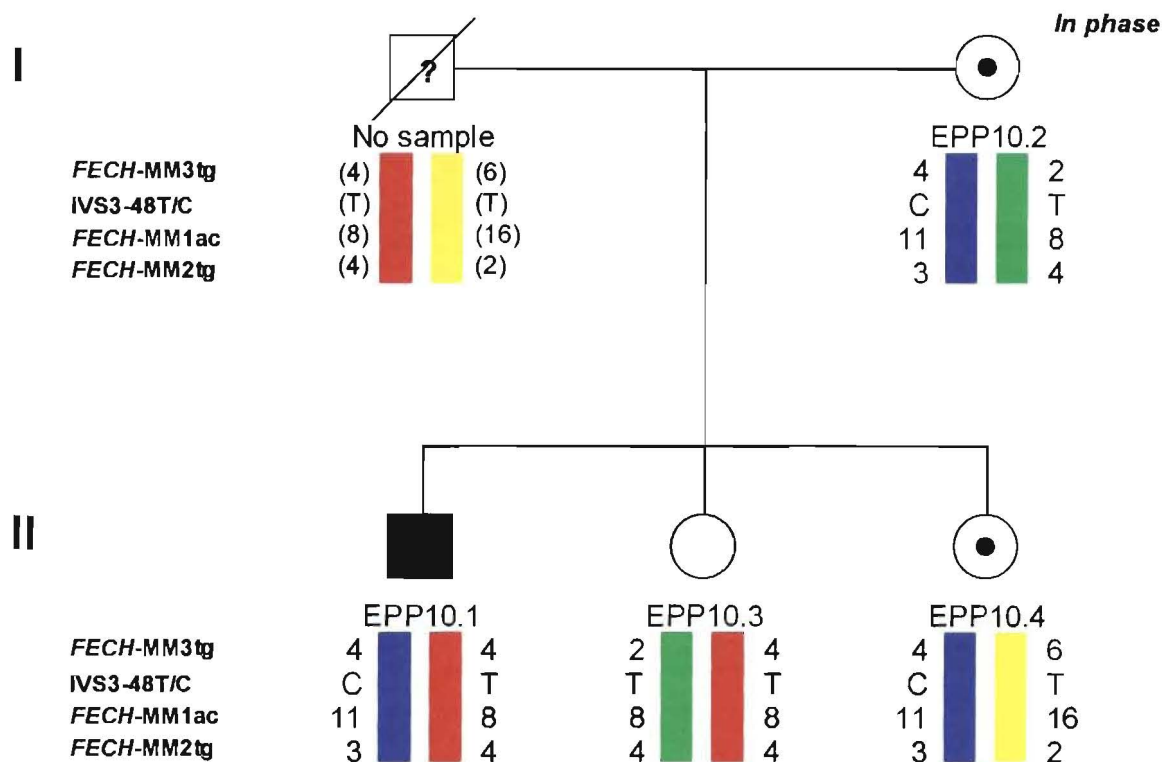


Figure K10: Pedigree of Family EPP10 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. Inferred genotypes are given in brackets. A line through a symbol indicates a deceased individual. '?' = phenotype unknown.

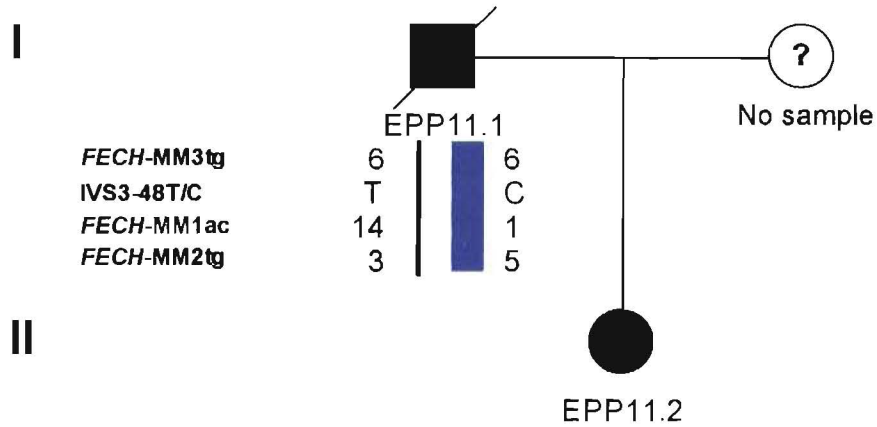


Figure K11: Pedigree of Family EPP11 showing genotyping results for individual EPP11.1 and one possible haplotype for the allele associated with the secondary mutation: IVS3-48C (blue bar). No primary mutation was identified for this individual and information is insufficient to determine haplotype phase. Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. A line through a symbol indicates a deceased individual. '?' = phenotype unknown.

In phase

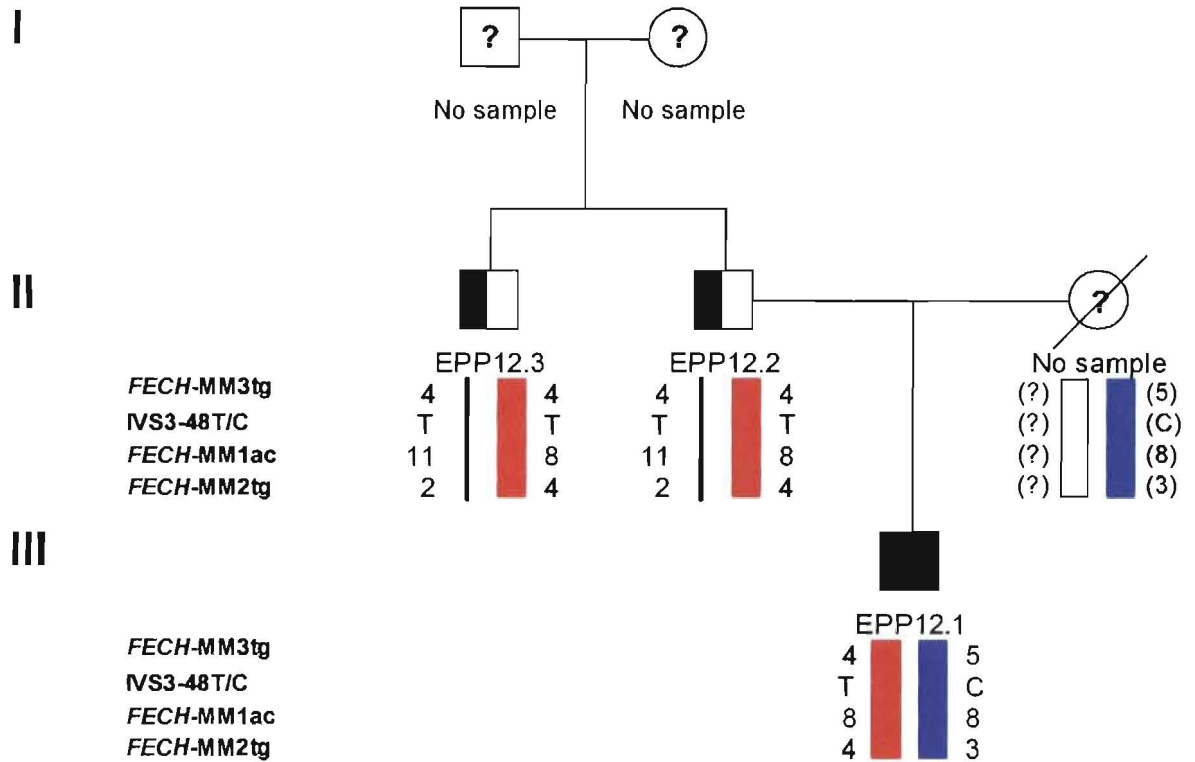


Figure K12: Pedigree of Family EPP12 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. Inferred genotypes are given in brackets. A line through a symbol indicates a deceased individual. '?' = phenotype unknown. (?) = genotype unknown.

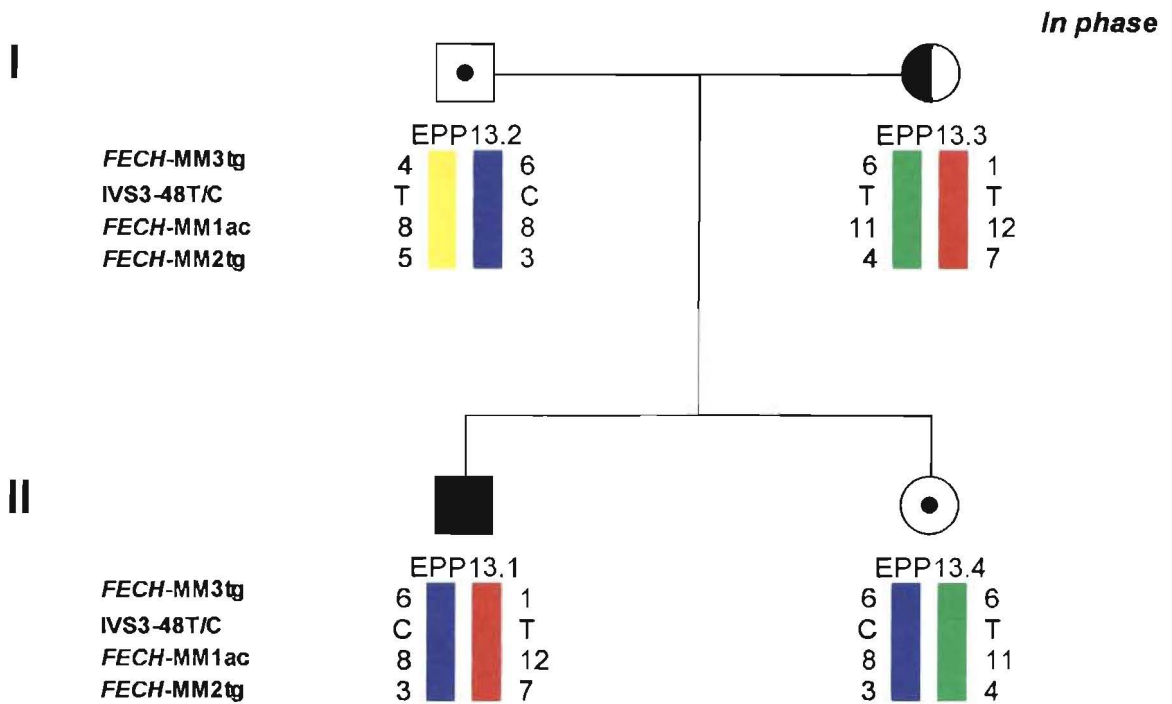


Figure K13: Pedigree of Family EPP13 showing haplotype segregation of both the allele associated with the primary mutation: IVS3+2G (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively.

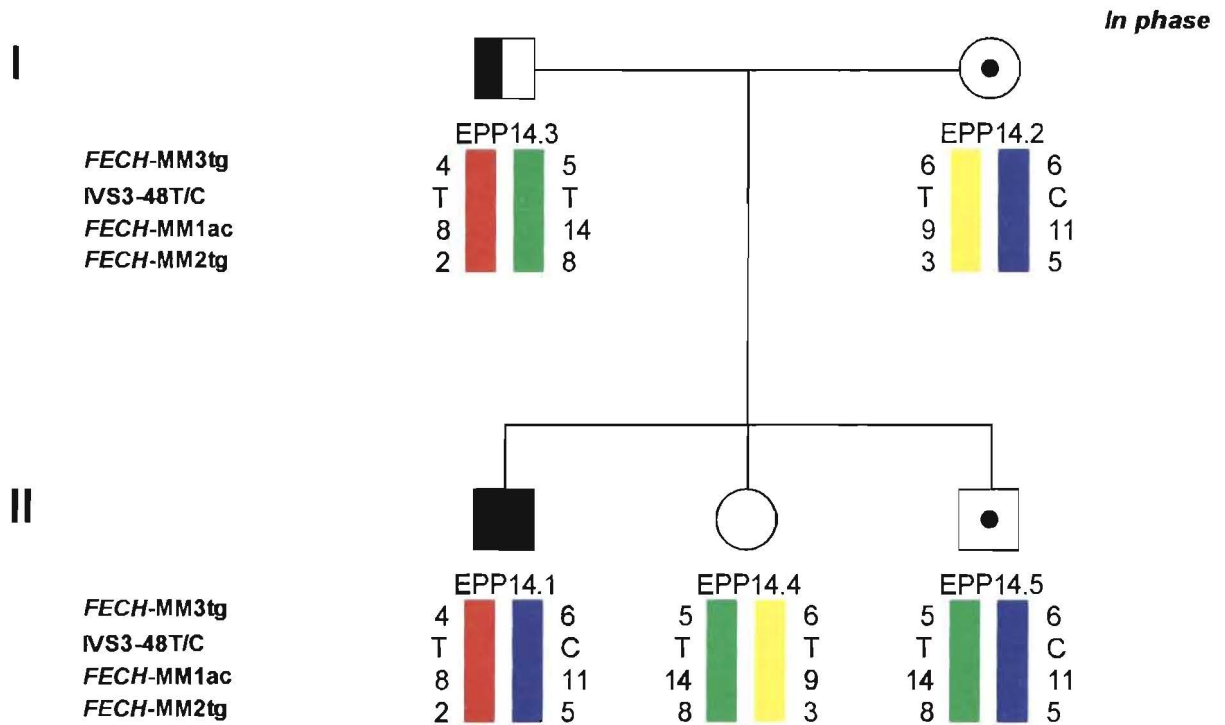


Figure K14: Pedigree of Family EPP14 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively.

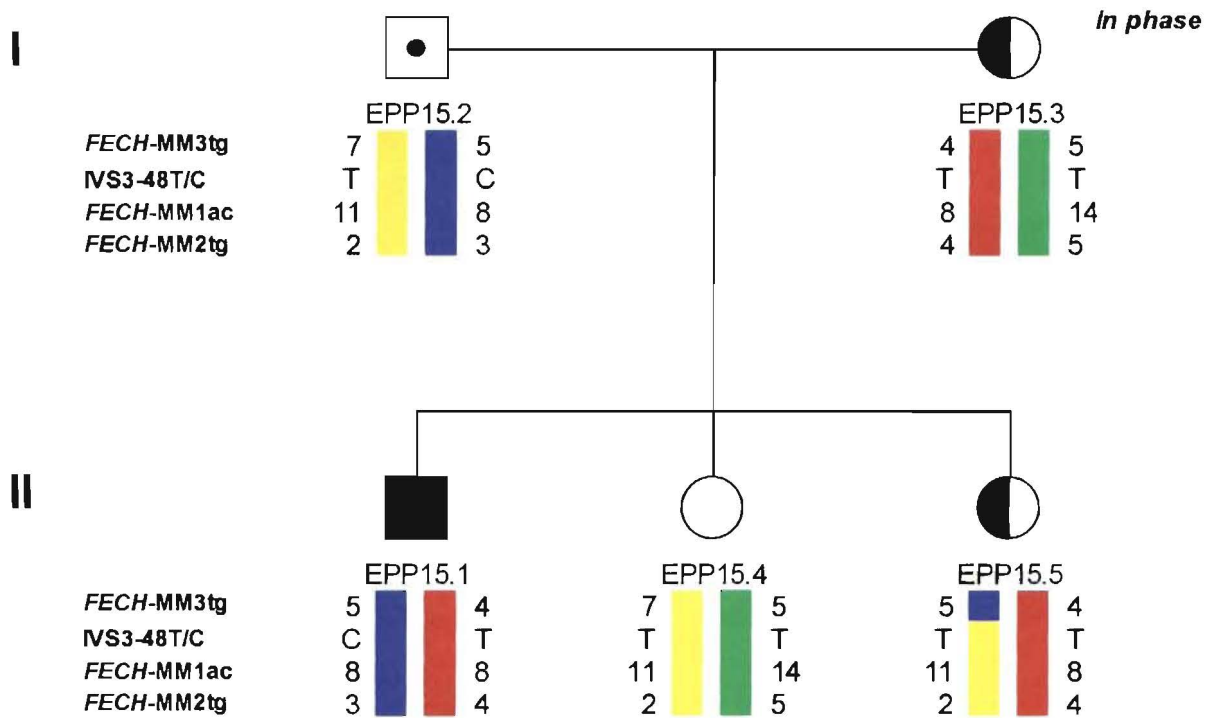


Figure K15: Pedigree of Family EPP15 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively.

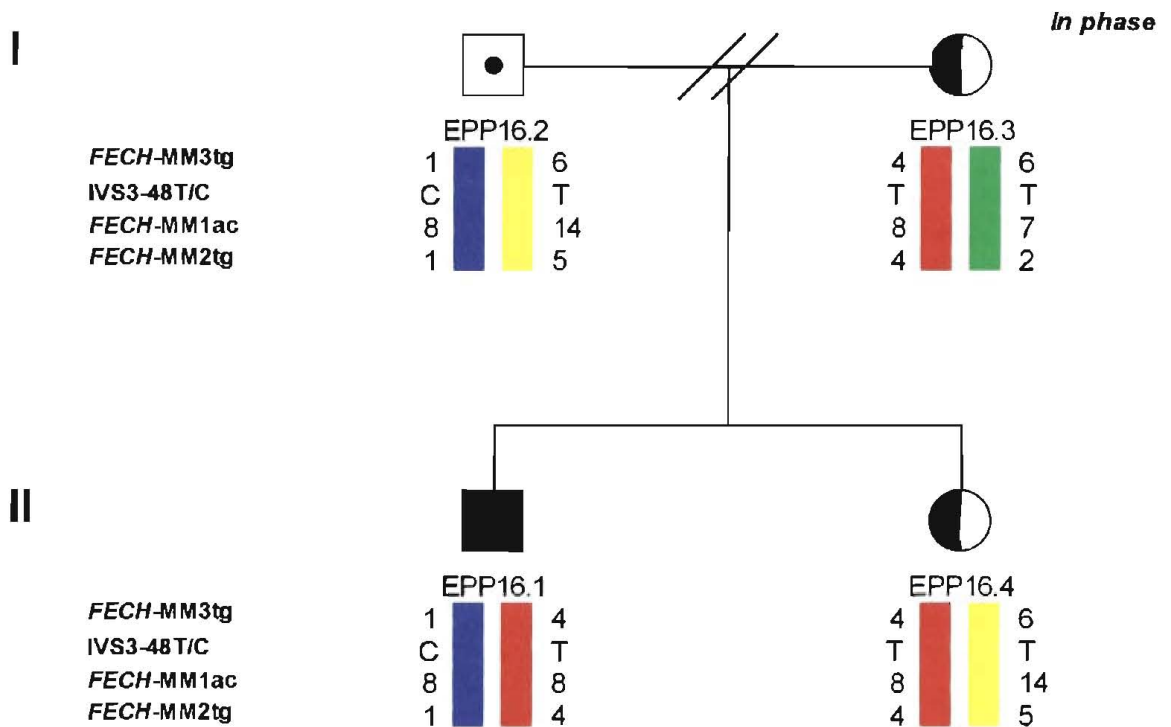
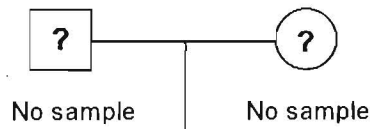


Figure K16: Pedigree of Family EPP16 showing haplotype segregation of both the allele associated with the primary mutation: 757_761delAGAAG (red bar) and the allele associated with the secondary mutation: IVS3-48C (blue bar). Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. Primary and secondary mutation carriers are indicated by symbols that are half-filled or contain a dot, respectively. // = divorced.

I



II

FECH-MM3tg
IVS3-48T/C
FECH-MM1ac
FECH-MM2tg

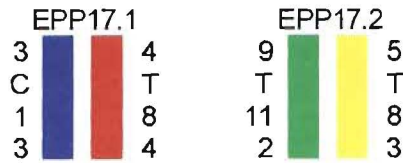


Figure K17: Pedigree of Family EPP17 showing genotyping results for individuals EPP17.1 and EPP17.2 and possible haplotypes for alleles associated with the primary: 757_761delAGAAG (red bar) and secondary: IVS3-48C (blue bar) mutations, respectively. Information is insufficient to determine haplotype phase. Squares represent males and circles represent females. Symptomatic individuals are represented by filled symbols while clear symbols represent asymptomatic individuals. '?' = phenotype unknown.

References

University of Cape Town

References

- Abitbol M, Bernex F, Puy H, Jouault H, Deybach JC, Guenet JL, Montagutelli X (2005) A mouse model provides evidence that genetic background modulates anemia and liver injury in erythropoietic protoporphyria. *Am J Physiol Gastrointest Liver Physiol* 288:G1208-16
- Al-Karadaghi S, Hansson M, Nikonov S, Jonsson B, Hederstedt L (1997) Crystal structure of ferrochelatase: the terminal enzyme in heme biosynthesis. *Structure* 5:1501-10
- Alemzadeh R, Feehan T (2004) Variable effects of beta-carotene therapy in a child with erythropoietic protoporphyria. *Eur J Pediatr*
- Alloisio N, Morle L, Marechal J, Roux AF, Ducluzeau MT, Guetarni D, Pothier B, Baklouti F, Ghanem A, Kastally R, et al. (1991) Sp alpha V/41: a common spectrin polymorphism at the alpha IV-alpha V domain junction. Relevance to the expression level of hereditary elliptocytosis due to alpha-spectrin variants located in trans. *J Clin Invest* 87:2169-77
- Anderson KE, Bloomer JR, Bonkovsky HL, Kushner JP, Pierach CA, Pimstone NR, Desnick RJ (2005) Recommendations for the diagnosis and treatment of the acute porphyrias. *Ann Intern Med* 142:439-50
- Andrew TL, Riley PG (1990) Regulation of heme biosynthesis in higher animals. In: Dailey HA (ed) *Biosynthesis of heme and chlorophylls*. McGraw-Hill Publishing Company, New York, pp 163-200
- Anstey AV (2002) Systemic photoprotection with alpha-tocopherol (vitamin E) and beta-carotene. *Clin Exp Dermatol* 27:170-6
- Aplin C, Whatley SD, Thompson P, Hoy T, Fisher P, Singer C, Lovell CR, Elder GH (2001) Late-onset erythropoietic porphyria caused by a chromosome 18q deletion in erythroid cells. *J Invest Dermatol* 117:1647-9
- Atkins AM, Nedorost ST (2003) Swollen upper extremities. *Clin Pediatr (Phila)* 42:827-9
- Baart de la Faille H, Bijlmer-Iest JC, van Hattum J, Koningsberger J, Rademakers LH, van Weelden H (1991) Erythropoietic protoporphyria: clinical aspects with emphasis on the skin. *Curr Probl Dermatol* 20:123-34
- Badminton MN, Elder GH (2005) Molecular mechanisms of dominant expression in porphyria. *J Inherit Metab Dis* 28:277-86

- Bailey GG, Needham LL (1986) Simultaneous quantification of erythrocyte zinc protoporphyrin and protoporphyrin IX by liquid chromatography. *Clin Chem* 32:2137-42
- Banchs I, Bosch A, Guimera J, Lazaro C, Puig A, Estivill X (1994) New alleles at microsatellite loci in CEPH families mainly arise from somatic mutations in the lymphoblastoid cell lines. *Hum Mutat* 3:365-72
- Baran R, Juhlin L (2002) Photoonycholysis. *Photodermatol Photoimmunol Photomed* 18:202-7
- Battersby AR (1978) The discovery of nature's biosynthetic pathways. *Experientia* 34:1-13
- Battersby AR, Fookes CJ, Matcham GW, McDonald E (1980) Biosynthesis of the pigments of life: formation of the macrocycle. *Nature* 285:17-21
- Battersby AR, McDonald E (1975) In: Smith KM (ed) *Porphyryns and metalloporphyryns*. 2nd ed. Elsevier, Amsterdam, pp 61-122
- Bishop DF, Henderson AS, Astrin KH (1990) Human delta-aminolevulinate synthase: assignment of the housekeeping gene to 3p21 and the erythroid-specific gene to the X chromosome. *Genomics* 7:207-14
- Bissell DM (1985) Peculiar purine nucleotides and liver regeneration. *Gastroenterology* 89:914-6
- Bissell DM, Schmid R (1987) Hepatic Porphyrias. In: Schiff L, Schiff ER (eds) *Diseases of the liver*. 6th ed. JB Lippincott, Philadelphia Toronto, pp 1075-1092
- Blackwood ME Jr, Rush TS III, Medlock A, Dailey HA, Spiro TG (1997) Resonance raman spectra of ferrochelatase reveal porphyrin distortion upon metal binding. *J Am Chem Soc* 119:12170-12174
- Blackwood ME, Jr., Rush TS, 3rd, Romesberg F, Schultz PG, Spiro TG (1998) Alternative modes of substrate distortion in enzyme and antibody catalyzed ferrochelation reactions. *Biochemistry* 37:779-82
- Bloks VW, Plosch T, van Goor H, Roelofsen H, Baller J, Havinga R, Verkade HJ, van Tol A, Jansen PL, Kuipers F (2001) Hypertlipidemia and atherosclerosis associated with liver disease in ferrochelatase-deficient mice. *J Lipid Res* 42:41-50
- Bloomer JR, Enriquez R (1982) Evidence that hepatic crystalline deposits in a patient with protoporphyria are composed of protoporphyrin. *Gastroenterology* 82:569-73
- Bloomer JR, Hill HD, Kools AM, Straka JG (1991) Heme synthesis in protoporphyria. *Curr Probl Dermatol* 20:135-47

- Bloomer J, Bruzzone C, Zhu L, Scarlett Y, Magness S, Brenner D (1998) Molecular defects in ferrochelatase in patients with protoporphyria requiring liver transplantation. *J Clin Invest* 102:107-14
- Bloomer J, Wang Y, Singhal A, Risheg H (2005) Molecular studies of liver disease in erythropoietic protoporphyria. *J Clin Gastroenterol* 39:S167-75
- Bloomer JR, Wang Y, Singhal A, Risheg H (2006) Biochemical abnormality in erythropoietic protoporphyria: cause and consequences. *J Pediatr Gastroenterol Nutr* 43 Suppl 1:S36-40
- Bonkovsky HL, Barnard GF (1998) Diagnosis of porphyric syndromes: a practical approach in the era of molecular biology. *Semin Liver Dis* 18:57-65
- Bonkovsky HL, Bloomer JR, Ebert PS, Mahoney MJ (1975) Heme synthetase deficiency in human protoporphyria. Demonstration of the defect in liver and cultured skin fibroblasts. *J Clin Invest* 56:1139-48
- Boulechfar S, Lamoril J, Montagutelli X, Guenet JL, Deybach JC, Nordmann Y, Dailey H, Grandchamp B, de Verneuil H (1993) Ferrochelatase structural mutant (Fechm1Pas) in the house mouse. *Genomics* 16:645-8
- Brenner DA, Frasier F (1991) Cloning of murine ferrochelatase. *Proc Natl Acad Sci USA* 88:849-53
- Brenner DA, Didier JM, Frasier F, Christensen SR, Evans GA, Dailey HA (1992) A molecular defect in human protoporphyria. *Am J Hum Genet* 50:1203-10
- Brun A, Western A, Malik Z, Sandberg S (1990) Erythropoietic protoporphyria: photodynamic transfer of protoporphyrin from intact erythrocytes to other cells. *Photochem Photobiol* 51:573-7
- Brun A, Sandberg S (1991) Mechanisms of photosensitivity in porphyric patients with special emphasis on erythropoietic protoporphyria. *J Photochem Photobiol B* 10:285-302
- Burden AE, Wu C, Dailey TA, Busch JL, Dhawan IK, Rose JP, Wang B, Dailey HA (1999) Human ferrochelatase: crystallization, characterization of the [2Fe-2S] cluster and determination that the enzyme is a homodimer. *Biochim Biophys Acta* 1435:191-7
- Camadro JM, Labbe P (1982) Kinetic studies of ferrochelatase in yeast. Zinc or iron as competing substrates. *Biochim Biophys Acta* 707:280-8
- Camadro JM, Ibrahim NG, Levere RD (1984) Kinetic studies of human liver ferrochelatase. Role of endogenous metals. *J Biol Chem* 259:5678-82

- Camadro JM, Labbe P (1988) Purification and properties of ferrochelatase from the yeast *Saccharomyces cerevisiae*. Evidence for a precursor form of the protein. *J Biol Chem* 263:11675-82
- Chakrabarti A, Tan CY (2002) Dietary fish oils as a therapeutic option in erythropoietic protoporphyria. *Clin Exp Dermatol* 27:324-7
- Chang YH, Su WH, Lee TC, Sun HF, Chen CH, Pan WH, Tsai SF, Jou YS (2005) TPMD: a database and resources of microsatellite marker genotyped in Taiwanese populations. *Nucleic Acids Res* 33:D174-7
- Chen FP, Risheg H, Liu Y, Bloomer J (2002) Ferrochelatase gene mutations in erythropoietic protoporphyria: focus on liver disease. *Cell Mol Biol (Noisy-le-grand)* 48:83-9
- Cobbold C, Roberts A, Badminton M (2006) Erythropoietic protoporphyria: A functional analysis of the leader sequence of human ferrochelatase. *Mol Genet Metab*
- Cox TC, Bawden MJ, Martin A, May BK (1991) Human erythroid 5-aminolevulinic synthase: promoter analysis and identification of an iron-responsive element in the mRNA. *Embo J* 10:1891-902
- Cox TM (1997) Erythropoietic protoporphyria. *J Inherit Metab Dis* 20:258-69
- Cox TM, Alexander GJ, Sarkany RP (1998) Protoporphyria. *Semin Liver Dis* 18:85-93
- Cripps DJ, MacEachern WN (1971) Hepatic and erythropoietic protoporphyria. Delta-aminolevulinic acid synthetase, fluorescence, and microfluorometric study. *Arch Pathol* 91:497-505
- Crouse BR, Sellers VM, Finnegan MG, Dailey HA, Johnson MK (1996) Site-directed mutagenesis and spectroscopic characterization of human ferrochelatase: identification of residues coordinating the [2Fe-2S] cluster. *Biochemistry* 35:16222-9
- Dailey HA, Fleming JE (1983) Bovine ferrochelatase. Kinetic analysis of inhibition by N-methylprotoporphyrin, manganese, and heme. *J Biol Chem* 258:11453-9
- Dailey HA (1990) Conversion of coproporphyrinogen to protoheme in higher eukaryotes and bacteria: Terminal three enzymes. In: Dailey HA (ed) *Biosynthesis of heme and chlorophylls*. McGraw-Hill Publishing Company, New York, pp 139
- Dailey HA, Sellers VM, Dailey TA (1994) Mammalian ferrochelatase. Expression and characterization of normal and two human protoporphyrinic ferrochelatases. *J Biol Chem* 269:390-5
- Dailey HA (1997) Enzymes of heme biosynthesis. *JBIC* 2: 411-417
- Dailey HA, Dailey TA, Wu CK, Medlock AE, Wang KF, Rose JP, Wang BC (2000) Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters. *Cell Mol Life Sci* 57:1909-26

- Dailey HA, Dailey TA (2003) In: Kadish KM, Smith KM, Guillard R (eds) *Ferrochelatase – the iron and cobalt pigments: Biosynthesis, structure and degradation*. Academic Press, California USA, 12:93-121
- Daly MJ, Rioux JD, Schaffner SF, Hudson TJ, Lander ES (2001) High-resolution haplotype structure in the human genome. *Nat Genet* 29:229-32
- Davenport RD (2004) Transfusion medicine illustrated. Erythropoietic protoporphyria. *Transfusion* 44:1401
- Davies R, Schuurman A, Barker CR, Clothier B, Chernova T, Higginson FM, Judah DJ, Dinsdale D, Edwards RE, Greaves P, Gant TW, Smith AG (2005) Hepatic gene expression in protoporphyric Fech mice is associated with cholestatic injury but not a marked depletion of the heme regulatory pool. *Am J Pathol* 166:1041-53
- Day RS, Pimstone NR, Eales L (1978) The diagnostic value of blood plasma porphyrin methyl ester profiles produced by quantitative TLC. *Int J Biochem* 9:897-904
- de Goeij AF, Christianse K, van Steveninck J (1975) Decreased haem synthetase activity in blood cells of patients with erythropoietic protoporphyria. *Eur J Clin Invest* 5:397-400
- De Matteis F, Gibbs AH (1975) Stimulation of the pathway of porphyrin synthesis in the liver of rats and mice by griseofulvin, 3,5-Diethoxycarbonyl-1,4-dihydrocollidine and related drugs: evidence for two basically different mechanisms. *Biochem J* 146:285-7
- De Smet C, Lurquin C, Lethe B, Martelange V, Boon T (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* 19:7327-35
- DeLeo VA, Poh-Fitzpatrick M, Mathews-Roth M, Harber LC (1976) Erythropoietic protoporphyria. 10 years experience. *Am J Med* 60:8-22
- den Dunnen JT, Antonarakis SE (2001) Nomenclature for the description of human sequence variations. *Hum Genet* 109:121-4
- Di Pierro E, Cappellini MD, Mazzucchelli R, Moriondo V, Mologni D, Poma BZ, Riva A (2005) A point mutation affecting an SP1 binding site in the promoter of the ferrochelatase gene impairs gene transcription and causes erythropoietic protoporphyria. *Exp Hematol* 33:584-91
- Do KD, Banner BF, Katz E, Szymanski IO, Bonkovsky HL (2002) Benefits of chronic plasmapheresis and intravenous heme-albumin in erythropoietic protoporphyria after orthotopic liver transplantation. *Transplantation* 73:469-72
- Doss MO, Frank M (1989) Hepatobiliary implications and complications in protoporphyria, a 20-year study. *Clin Biochem* 22:223-9

- Doss MO, Kuhnel A, Gross U (2000) Alcohol and porphyrin metabolism. *Alcohol* 35:109-25
- Eales L, Dowdle EB (1968) Clinical aspects of importance in the porphyrias. *Br J Clin Pract* 22:505-15
- Eales L, Day RS, Pimstone NR (1978) Erythrohepatic protoporphyria (EHP) in South Africa. *Ann Clin Res* 10:195-204
- Eales L, Day RS, Pimstones NR (1978) Protoporphyrin (proto)-determined hepatopathy in a South African Jewish family. *Ann Clin Res* 10:205-13
- Eichbaum QG, Dzik WH, Chung RT, Szczepiorkowski ZM (2005) Red blood cell exchange transfusion in two patients with advanced erythropoietic protoporphyria. *Transfusion* 45:208-13
- Elder GH, Smith SG, Smyth SJ (1990) Laboratory investigation of the porphyrias. *Ann Clin Biochem* 27 (Pt 5):395-412
- Elder G (2005) Normal dermal ferrochelatase activity does not protect human skin from protoporphyrin-induced photosensitivity. *J Invest Dermatol* 125:580; author reply 580-1
- Ellegren H (2004) Microsatellites: simple sequences with complex evolution. *Nat Rev Genet* 5:435-45
- Elliott W, Bishop P, Daley W, Nowicki MJ (2004) Erythropoietic protoporphyria in an adolescent female. *J Hepatol* 40:353
- Farr PM, Diffey BL, Matthews JN (1990) Inhibition of photosensitivity in erythropoietic protoporphyria with terfenadine. *Br J Dermatol* 122:809-15
- Ferreira GC, Franco R, Lloyd SG, Pereira AS, Moura I, Moura JJ, Huynh BH (1994) Mammalian ferrochelatase, a new addition to the metalloenzyme family. *J Biol Chem* 269:7062-5
- Ferreira GC (1999) Ferrochelatase. *Int J Biochem Cell Biol* 31:995-1000
- Findlay GH, Scott FP, Cripps DJ (1966) Porphyria and lipid proteinosis. A clinical, histological and biochemical comparison of 19 South African cases. *Br J Dermatol* 78:69-80
- Fischer H, Orth H (1934) *Die chemie des pyrrols*, 3 Bands. Akademische Verlagsgesellschaft MBH, Leipzig
- Fontanellas A, Mendez M, Mazurier F, Cario-Andre M, Navarro S, Ged C, Taine L, Geronimi F, Richard E, Moreau-Gaudry F, Enriquez De Salamanca R, de Vermeuil H (2001) Successful therapeutic effect in a mouse model of erythropoietic protoporphyria by partial genetic correction and fluorescence-based selection of hematopoietic cells. *Gene Ther* 8:618-26

- Franco R, Ma JG, Lu Y, Ferreira GC, Shelnut JA (2000) Porphyrin interactions with wild-type and mutant mouse ferrochelatase. *Biochemistry* 39:2517-29
- Frank M, Doss MO (1991) Severe liver disease in protoporphyria. *Curr Probl Dermatol* 20:160-7
- Fujita H (1997) Molecular mechanism of heme biosynthesis. *Tohoku J Exp Med* 183:83-99
- Fusaro RM, Runge WJ (1970) Erythropoietic protoporphyria. IV. Protection from sunlight. *Br Med J* 1:730-1
- Gant TW, Baus PR, Clothier B, Riley J, Davies R, Judah DJ, Edwards RE, George E, Greaves P, Smith AG (2003) Gene expression profiles associated with inflammation, fibrosis, and cholestasis in mouse liver after griseofulvin. *EHP Toxicogenomics* 111:37-43
- Girotti AW (1990) Photodynamic lipid peroxidation in biological systems. *Photochem Photobiol* 51:497-509
- Goldstein BD, Harber LC (1972) Erythropoietic protoporphyria: lipid peroxidation and red cell membrane damage associated with photohemolysis. *J Clin Invest* 51:892-902
- Goodwin RG, Kell WJ, Laidler P, Long CC, Whatley SD, McKinley M, Badminton MN, Burnett AK, Williams GT, Elder GH (2006) Photosensitivity and acute liver injury in myeloproliferative disorder secondary to late-onset protoporphyria caused by deletion of a ferrochelatase gene in hematopoietic cells. *Blood* 107:60-2
- Gordeuk VR, Brittenham GM, Hawkins CW, Mukhtar H, Bickers DR (1986) Iron therapy for hepatic dysfunction in erythropoietic protoporphyria. *Ann Intern Med* 105:27-31
- Gouya L, Deybach JC, Lamoril J, Da Silva V, Beaumont C, Grandchamp B, Nordmann Y (1996) Modulation of the phenotype in dominant erythropoietic protoporphyria by a low expression of the normal ferrochelatase allele. *Am J Hum Genet* 58:292-9
- Gouya L, Puy H, Lamoril J, Da Silva V, Grandchamp B, Nordmann Y, Deybach JC (1999) Inheritance in erythropoietic protoporphyria: a common wild-type ferrochelatase allelic variant with low expression accounts for clinical manifestation. *Blood* 93:2105-10
- Gouya L, Puy H, Robreau AM, Bourgeois M, Lamoril J, Da Silva V, Grandchamp B, Deybach JC (2002) The penetrance of dominant erythropoietic protoporphyria is modulated by expression of wildtype FECH. *Nat Genet* 30:27-8
- Gouya (2004) Modulation of penetrance by the wild-type allele in dominantly inherited erythropoietic protoporphyria and acute hepatic porphyrias. *Human Genetics* 114:256-262

- Gouya L, Martin-Schmitt C, Robreau AM, Austerlitz F, Da Silva V, Brun P, Simonin S, Lyoumi S, Grandchamp B, Beaumont C, Puy H, Deybach JC (2006) Contribution of a common single-nucleotide polymorphism to the genetic predisposition for erythropoietic protoporphyria. *Am J Hum Genet* 78:2-14
- Gray CH, Lim CK, Nicholson DC (1977) The differentiation of the porphyrias by means of high pressure liquid chromatography. *Clin Chim Acta* 77:167-78
- Guberman AS, Scassa ME, Giono LE, Varone CL, Canepa ET (2003) Inhibitory effect of AP-1 complex on 5-aminolevulinate synthase gene expression through sequestration of cAMP-response element protein (CRE)-binding protein (CBP) coactivator. *J Biol Chem* 278:2317-26
- Guo R, Lim CK, Peters TJ (1991) High-performance liquid chromatographic assays for protoporphyrinogen oxidase and ferrochelatase in human leucocytes. *J Chromatogr* 566:383-96
- Haeger-Aronsen B (1982) Why is the patient with lead intoxication not light sensitive? *Acta Derm Venereol Suppl (Stockh)* 100:67-71
- Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE (1991) Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. *Arch Biochem Biophys* 289:387-92
- Harber LC, Poh-Fitzpatrick MB, Walther RR, Grossman ME (1982) Cutaneous aspects of the porphyrias. *Acta Derm Venereol Suppl (Stockh)* 100:9-15
- Hart D, Piomelli S (1981) Simultaneous quantitation of zinc protoporphyrin and free protoporphyrin in erythrocytes by acetone extraction. *Clin Chem* 27:220-2
- Hayashi K (1992) PCR-SSCP: a method for detection of mutations. *Genet Anal Tech Appl* 9:73-9
- Henriksson M, Timonen K, Mustajoki P, Pihlaja H, Tenhunen R, Peltonen L, Kauppinen R (1996) Four novel mutations in the ferrochelatase gene among erythropoietic protoporphyria patients. *J Invest Dermatol* 106:346-50
- Hift RJ, Meissner PN (2005) An analysis of 112 acute porphyric attacks in Cape Town, South Africa: Evidence that acute intermittent porphyria and variegate porphyria differ in susceptibility and severity. *Medicine (Baltimore)* 84:48-60
- Hirsch RE, Lin MJ, Pulakhandam UR, Nagel RL, Sandberg S (1993) Hemoglobin oxygen affinity is increased in erythropoietic protoporphyria. *Photochem Photobiol* 57:885-8

- Holme SA, Anstey AV, Finlay AY, Elder GH, Badminton MN (2006) Erythropoietic protoporphyria in the U.K.: clinical features and effect on quality of life. *Br J Dermatol* 155:574-81
- Holme SA, Anstey AV, Finlay AY, Elder GH, Badminton MN (2006) Abstract No. 1 Epidemiology of erythropoietic protoporphyria: the U.K. experience. *Br J Dermatol* 155:866
- Honigsmann H, Gschnait F, Konrad K, Stingl G, Wolff K (1976) Mouse model for protoporphyria. III. Experimental production of chronic erythropoietic protoporphyria-like skin lesions. *J Invest Dermatol* 66:188-95
- Imoto S, Tanizawa Y, Sato Y, Kaku K, Oka Y (1996) A novel mutation in the ferrochelatase gene associated with erythropoietic protoporphyria. *Br J Haematol* 94:191-7
- Inafuku K, Takamiyagi A, Oshiro M, Kinjo T, Nakashima Y, Nonaka S (1999) Alteration of mRNA levels of delta-aminolevulinic acid synthase, ferrochelatase and heme oxygenase-1 in griseofulvin induced protoporphyria mice. *J Dermatol Sci* 19:189-98
- Jones MS, Jones OT (1969) The structural organization of haem synthesis in rat liver mitochondria. *Biochem J* 113:507-14
- Jones A, Reed R, Weyers J (1994) In: *Practical skills in biology*. Longman Group Ltd, UK, pp 165-167
- Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH, Schinkel AH (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A* 99:15649-54
- Karr SR, Dailey HA (1988) The synthesis of murine ferrochelatase in vitro and in vivo. *Biochem J* 254:799-803
- Kauppinen R (2005) Porphyrins. *Lancet* 365:241-52
- Key NS, Rank JM, Freese D, Bloomer JR, Hammerschmidt DE (1992) Hemolytic anemia in protoporphyria: possible precipitating role of liver failure and photic stress. *Am J Hematol* 39:202-7
- Kirsch RE, Meissner PN, Hift RJ (1998) Variegate porphyria. *Semin Liver Dis* 18:33-41
- Kohno H, Okuda M, Furukawa T, Tokunaga R, Taketani S (1994) Site-directed mutagenesis of human ferrochelatase: identification of histidine-263 as a binding site for metal ions. *Biochim Biophys Acta* 1209:95-100
- Komatsu H, Sajima Y, Imamura K, Masuda H, Yonei Y, Dohmori K, Kokutoh M, Ishii K, Ishii H (2000) An ultrastructural study of the liver in erythropoietic protoporphyria. *Med Electron Microsc* 33:32-8

- Komatsu H, Ishii K, Imamura K, Maruyama K, Yonei Y, Masuda H, Tsuchihashi T, Sajima Y (2000) A case of erythropoietic protoporphyria with liver cirrhosis suggesting a therapeutic value of supplementation with alpha-tocopherol. *Hepatol Res* 18:298-309
- Kosenow W, Treibs A (1953) [Light hypersensitivity and porphyriaemia.]. *Z Kinderheilkd* 73:82-92
- Krook G, Haeger-Aronsen B (1982) beta-Carotene in the treatment of erythropoietic protoporphyria. A short review. *Acta Derm Venereol Suppl (Stockh)* 100:125-9
- Labbe-Bois R (1990) The ferrochelatase from *Saccharomyces cerevisiae*. Sequence, disruption, and expression of its structural gene HEM15. *J Biol Chem* 265:7278-83
- Lamola AA, Yamane T (1974) Zinc protoporphyrin in the erythrocytes of patients with lead intoxication and iron deficiency anemia. *Science* 186:936-8
- Lamola AA, Piomelli S, Poh-Fitzpatrick MG, Yamane T, Harber LC (1975) Erythropoietic protoporphyria and lead intoxication: the molecular basis for difference in cutaneous photosensitivity. II. Different binding of erythrocyte protoporphyrin to hemoglobin. *J Clin Invest* 56:1528-35
- Lamola AA, Asher I, Muller-Eberhard U, Poh-Fitzpatrick M (1981) Fluorimetric study of the binding of protoporphyrin to haemopexin and albumin. *Biochem J* 196:693-8
- Lamola AA (1982) Fluorescence studies of protoporphyrin. Transport and clearance. *Acta Derm Venereol Suppl (Stockh)* 100:57-66
- Lamoril J, Boulechfar S, de Verneuil H, Grandchamp B, Nordmann Y, Deybach JC (1991) Human erythropoietic protoporphyria: two point mutations in the ferrochelatase gene. *Biochem Biophys Res Commun* 181:594-9
- Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409:860-921
- Lareu V, Pestoni C, Phillips C, Barros F, Syndercombe Court D, Lincoln P, Carracedo A (1998) Normal and anomalous electrophoretic behavior of polymerase chain reaction-based DNA polymorphisms in polyacrylamide gels. *Electrophoresis* 19:1566-72
- Lathrop JT, Timko MP (1993) Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. *Science* 259:522-5
- Lavallee DK (1988) Porphyrin metalation reactions in biochemistry. In: Liebman JF, Greenberg A (eds) *Mechanistic principles of enzyme activity*. VCH Publishers Inc, New York, pp 279-314
- Leboulche P and Mathews-Roth M (2005) Skin ferrochelatase levels and photosensitivity. *J Invest Dermatol* 125:580-581

- Lecerof D, Fodje M, Hansson A, Hansson M, Al-Karadaghi S (2000) Structural and mechanistic basis of porphyrin metallation by ferrochelatase. *J Mol Biol* 297:221-32
- Lecha M (2003) Erythropoietic protoporphyria. *Photodermatol Photoimmunol Photomed* 19:142-6
- Leeming JA, Kramer S (1968) Four cases of erythropoietic protoporphyria. *S Afr Med J* 42:586-9
- Leone N, Marzano A, Cerutti E, Actis GC, Marchesa PE, David E, Salizzoni M, Rizzetto M (2000) Liver transplantation for erythropoietic protoporphyria: report of a case with medium-term follow-up. *Dig Liver Dis* 32:799-802
- Lewin B (2000) In: *Genes VII*. Oxford University Press, UK, pp 678-681
- Lewontin RC (1964) The Interaction of Selection and Linkage. II. Optimum Models. *Genetics* 50:757-82
- Lim HW, Poh-Fitzpatrick MB, Gigli I (1984) Activation of the complement system in patients with porphyrias after irradiation in vivo. *J Clin Invest* 74:1961-5
- Lim HW (2005) Pathogenesis of photosensitivity in the cutaneous porphyrias. *J Invest Dermatol* 124:xvi-xvii
- Lin HF, Juo SH, Cheng R (2005) Comparison of the power between microsatellite and single-nucleotide polymorphism markers for linkage and linkage disequilibrium mapping of an electrophysiological phenotype. *BMC Genet* 6 Suppl 1:S7
- Longas MO, Poh-Fitzpatrick MB (1982) A tightly bound protein-porphyrin complex isolated from the plasma of a patient with variegate porphyria. *Clin Chim Acta* 118:219-28
- Magness ST, Tugores A, Diala ES, Brenner DA (1998) Analysis of the human ferrochelatase promoter in transgenic mice. *Blood* 92:320-8
- Magness ST, Maeda N, Brenner DA (2002) An exon 10 deletion in the mouse ferrochelatase gene has a dominant-negative effect and causes mild protoporphyria. *Blood* 100:1470-7
- Magnus IA, Jarrett A, Prankerd TA, Rimington C (1961) Erythropoietic protoporphyria. A new porphyria syndrome with solar urticaria due to protoporphyrinaemia. *Lancet* 2:448-51
- Marini JC, Levene SD, Crothers DM, Englund PT (1982) Bent helical structure in kinetoplast DNA. *Proc Natl Acad Sci U S A* 79:7664-7668
- Mathews-Roth MM, Pathak MA, Fitzpatrick TB, Harber LC, Kass EH (1970) Beta-carotene as a photoprotective agent in erythropoietic protoporphyria. *Trans Assoc Am Physicians* 83:176-84

- Mathews-Roth MM (1974) Letter: Anemia in erythropoietic protoporphyria. *Jama* 230:824
- Mathews-Roth MM (1977) Erythropoietic protoporphyria--diagnosis and treatment. *N Engl J Med* 297:98-100
- Mathews-Roth MM, Drouin GL, Duffy L (1987) Isolation of human ferrochelatase. *Arch Dermatol* 123:429-30
- Mathews-Roth MM, Rosner B (2002) Long-term treatment of erythropoietic protoporphyria with cysteine. *Photodermatol Photoimmunol Photomed* 18:307-9
- May BK, Dogra SC, Sadlon TJ, Bhasker CR, Cox TC, Bottomley SS (1995) Molecular regulation of heme biosynthesis in higher vertebrates. *Prog Nucleic Acid Res Mol Biol* 51:1-51
- McClements BM, Bingham A, Callender ME, Trimble ER (1990) Erythropoietic protoporphyria and iron therapy. *Br J Dermatol* 122:423-4
- Meerman L (2000) Erythropoietic protoporphyria. An overview with emphasis on the liver. *Scand J Gastroenterol Suppl*:79-85
- Meissner PN, Hift RJ (2005) Diagnosis of porphyria in South Africa. *CME: The SA journal of CPD* 23:412-414
- Meissner PN, Hift RJ, Kirsch RE (2001) The porphyrias. In: Arias IM, Boyer JL, Chisari FV, Fausto N, Schachter D, Shafritz DA (eds) *The liver: biology and pathobiology*. 4th ed. Lippincott Williams & Wilkins, Philadelphia, pp 311-329
- Michal G (1999) In: *Biochemical pathways: An atlas of biochemistry and molecular biology*. John Wiley & Sons Inc, New York, pp 68-69
- Milligan A, Graham-Brown RA, Sarkany I, Baker H (1988) Erythropoietic protoporphyria exacerbated by oral iron therapy. *Br J Dermatol* 119:63-6
- Moore MR, McColl KEL, Rimington C, Goldberg A (1987) In: *Disorders of porphyrin metabolism*. Plenum Press, New York, pp 21-35
- Moore MR, McColl KEL, Rimington C, Goldberg A (1987) In: *Disorders of porphyrin metabolism*. Plenum Press, New York, pp 3
- Moore MR, McColl KEL, Rimington C, Goldberg A (1987) In: *Disorders of porphyrin metabolism*. Plenum Press, New York, pp 38
- Moore MR (1990) Historical introduction to porphyrins and porphyrias. In: Dailey HA (ed) *Biosynthesis of heme and chlorophylls*. McGraw-Hill Publishing Company, New York, pp 1-4
- Morris SD, Mason NG, Elder GH, Hawk JL, Sarkany RP (2002) Ferrochelatase gene polymorphism analysis for accurate genetic counselling in erythropoietic protoporphyria. *Br J Dermatol* 147:572-4

- Murphy GM, Hawk JLM, Corbett MF (1985) The UK erythropoietic protoporphyria register: a progress report. *Br J Dermatol* 113 (Suppl. 29): 11
- Murphy GM (2003) Diagnosis and management of the erythropoietic porphyrias. *Dermatol Ther* 16:57-64
- Najahi-Missaoui W, Dailey HA (2005) Production and characterization of erythropoietic protoporphyric heterodimeric ferrochelatases. *Blood* 106:1098-104
- Nakahashi Y, Taketani S, Okuda M, Inoue K, Tokunaga R (1990) Molecular cloning and sequence analysis of cDNA encoding human ferrochelatase. *Biochem Biophys Res Commun* 173:748-55
- Nakano H, Nakano A, Toyomaki Y, Ohashi S, Harada K, Moritsugu R, Takeda H, Kawada A, Mitsuhashi Y, Hanada K (2006) Novel Ferrochelatase Mutations in Japanese Patients with Erythropoietic Protoporphyria: High Frequency of the Splice Site Modulator IVS3-48C Polymorphism in the Japanese Population. *J Invest Dermatol*
- Nordmann Y (1992) Erythropoietic protoporphyria and hepatic complications. *J Hepatol* 16:4-6
- Nordmann Y, Puy H (2002) Human hereditary hepatic porphyrias. *Clin Chim Acta* 325:17-37
- Ohgari Y, Sawamoto M, Yamamoto M, Kohno H, Taketani S (2005) Ferrochelatase consisting of wild-type and mutated subunits from patients with a dominant-inherited disease, erythropoietic protoporphyria, is an active but unstable dimer. *Hum Mol Genet* 14:327-34
- Onaga Y, Ido A, Uto H, Hasuike S, Kusumoto K, Moriuchi A, Numata M, Nagata K, Hori T, Hayashi K, Tsubouchi H (2004) Hypermethylation of the wild-type ferrochelatase allele is closely associated with severe liver complication in a family with erythropoietic protoporphyria. *Biochem Biophys Res Commun* 321:851-8
- Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5:874-9
- Pathak MA, West JD (1982) Porphyrias: office procedures and laboratory tests for diagnosis of porphyrin abnormalities. *Acta Derm Venereol Suppl (Stockh)* 100:91-105
- Pawliuk R, Bachelot T, Wise RJ, Mathews-Roth MM, Leboulch P (1999) Long-term cure of the photosensitivity of murine erythropoietic protoporphyria by preselective gene therapy. *Nat Med* 5:768-73

- Pawliuk R, Tighe R, Wise RJ, Mathews-Roth MM, Leboulch P (2005) Prevention of murine erythropoietic protoporphyria-associated skin photosensitivity and liver disease by dermal and hepatic ferrochelatase. *J Invest Dermatol* 124:256-62
- Piomelli S, Lamola AA, Poh-Fitzpatrick MF, Seaman C, Harber LC (1975) Erythropoietic protoporphyria and lead intoxication: the molecular basis for difference in cutaneous photosensitivity. I. Different rates of disappearance of protoporphyrin from the erythrocytes, both in vivo and in vitro. *J Clin Invest* 56:1519-27
- Piomelli S (1977) Free erythrocyte porphyrins in the detection of undue absorption of Pb and of Fe deficiency. *Clin Chem* 23:264-9
- Plosch T, Bloks VW, Baller JF, Havinga R, Verkade HJ, Jansen PL, Kuipers F (2002) Mdr P-glycoproteins are not essential for biliary excretion of the hydrophobic heme precursor protoporphyrin in a griseofulvin-induced mouse model of erythropoietic protoporphyria. *Hepatology* 35:299-306
- Poh-Fitzpatrick MB, Lamola AA (1976) Direct spectrofluorometry of diluted erythrocytes and plasma: a rapid diagnostic method in primary and secondary porphyrinemias. *J Lab Clin Med* 87:362-70
- Poh-Fitzpatrick MB (1980) A plasma porphyrin fluorescence marker for variegate porphyria. *Arch Dermatol* 116:543-7
- Poh-Fitzpatrick MB (1985) Porphyrin-sensitized cutaneous photosensitivity: pathogenesis and treatment. *Clin Dermatol* 3:41-82
- Poh-Fitzpatrick MB (1989) The "priming phenomenon" in the acute phototoxicity of erythropoietic protoporphyria. *J Am Acad Dermatol* 21:311
- Poh-Fitzpatrick MB, Wang X, Anderson KE, Bloomer JR, Bolwell B, Lichtin AE (2002) Erythropoietic protoporphyria: altered phenotype after bone marrow transplantation for myelogenous leukemia in a patient heteroallelic for ferrochelatase gene mutations. *J Am Acad Dermatol* 46:861-6
- Prasad AR, Dailey HA (1995) Effect of cellular location on the function of ferrochelatase. *J Biol Chem* 270:18198-200
- Prolla TA (1998) DNA mismatch repair and cancer. *Curr Opin Cell Biol* 10:311-6
- Rademakers LH, Koningsberger JC, Sorber CW, Baart de la Faille H, Van Hattum J, Marx JJ (1993) Accumulation of iron in erythroblasts of patients with erythropoietic protoporphyria. *Eur J Clin Invest* 23:130-8
- Richard E, Mendez M, Mazurier F, Morel C, Costet P, Xia P, Fontanellas A, Geronimi F, Cario-Andre M, Taine L, Ged C, Malik P, de Verneuil H, Moreau-Gaudry F (2001) Gene therapy of a mouse model of protoporphyria with a self-inactivating erythroid-specific lentiviral vector without preselection. *Mol Ther* 4:331-8

- Richard E, Robert E, Cario-Andre M, Ged C, Geronimi F, Gerson SL, de Verneuil H, Moreau-Gaudry F (2004) Hematopoietic stem cell gene therapy of murine protoporphyria by methylguanine-DNA-methyltransferase-mediated in vivo drug selection. *Gene Ther* 11:1638-47
- Rimington C, Cripps DJ (1965) Biochemical and Fluorescence-Microscopy Screening-Tests for Erythropoietic Protoporphyria. *Lancet* 17:624-6
- Risheg H, Chen FP, Bloomer JR (2003) Genotypic determinants of phenotype in North American patients with erythropoietic protoporphyria. *Mol Genet Metab* 80:196-206
- Roberts JE, Mathews-Roth M (1993) Cysteine ameliorates photosensitivity in erythropoietic protoporphyria. *Arch Dermatol* 129:1350-1
- Roberts AG, Elder GH (2001) Alternative splicing and tissue-specific transcription of human and rodent ubiquitous 5-aminolevulinic synthase (ALAS1) genes. *Biochim Biophys Acta* 1518:95-105
- Roelandts R (1995) Photo(chemo)therapy and general management of erythropoietic protoporphyria. *Dermatology* 190:330-1
- Romeo G (1977) Enzymatic defects of hereditary porphyrias: an explanation of dominance at the molecular level. *Hum Genet* 39:261-76
- Rufenacht UB, Schneider-Yin X, Schafer BW, Taketani S, Deybach JC, Minder EI (1998) Rapid molecular diagnosis of erythropoietic protoporphyria among Swiss patients. *Clin Chem Lab Med* 36:763-5
- Rufener EA (1987) Erythropoietic protoporphyria: a study of its psychosocial aspects. *Br J Dermatol* 116:703-8
- Sambrook J, Fritsch EF, Maniatis T (1990) Standard gel electrophoresis protocol. In: *Molecular cloning: A laboratory manual*. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY
- Sarkany RP, Alexander GJ, Cox TM (1994) Recessive inheritance of erythropoietic protoporphyria with liver failure. *Lancet* 343:1394-6
- Schneider-Yin X, Gouya L, Meier-Weinand A, Deybach JC, Minder EI (2000) New insights into the pathogenesis of erythropoietic protoporphyria and their impact on patient care. *Eur J Pediatr* 159:719-25
- Schneider-Yin X, Rufenacht UB, Hergersberg M, Schnyder C, Deybach JC, Minder EI (2001) Haplotype analysis in determination of the heredity of erythropoietic protoporphyria among Swiss families. *J Invest Dermatol* 117:1521-5
- Schoenfeld N, Mamet R, Minder EI, Schneider-Yin X (2003) A "null allele" mutation is responsible for erythropoietic protoporphyria in an Israeli patient who underwent

- liver transplantation: relationships among biochemical, clinical, and genetic parameters. *Blood Cells Mol Dis* 30:298-301
- Scholnick PL, Hammaker LE, Marver HS (1972) Soluble delta-aminolevulinic acid synthetase of rat liver. II. Studies related to the mechanism of enzyme action and hemin inhibition. *J Biol Chem* 247:4132-7
- Schothorst AA, Van Steveninck J, Went LN, Suurmond D (1970) Protoporphyrin-induced photohemolysis in protoporphyria and in normal red blood cells. *Clin Chim Acta* 28:41-9
- Schothorst AA, Suurmond D, Ploem JS (1977) In vitro studies on the protoporphyrin uptake and photosensitivity of normal skin fibroblasts and fibroblasts from patients with erythropoietic protoporphyria. *J Invest Dermatol* 69:551-7
- Scott AI, Townsend CA, Okada K, Kajiwara M, Whitman PJ (1972) Biosynthesis of corrinoids. Concerning the origin of the methyl groups in vitamin B₁₂. *J Am Chem Soc* 94:8267-9
- Sellers VM, Johnson MK, Dailey HA (1996) Function of the [2Fe-2S] cluster in mammalian ferrochelatase: a possible role as a nitric oxide sensor. *Biochemistry* 35:2699-704
- Sellers VM, Wang KF, Johnson MK, Dailey HA (1998) Evidence that the fourth ligand to the [2Fe-2S] cluster in animal ferrochelatase is a cysteine. Characterization of the enzyme from *Drosophila melanogaster*. *J Biol Chem* 273:22311-6
- Sellers VM, Wu CK, Dailey TA, Dailey HA (2001) Human ferrochelatase: characterization of substrate-iron binding and proton-abstracting residues. *Biochemistry* 40:9821-7
- Sharp PA, Sugden B, Sambrook J (1973) Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* 12:3055-63
- Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW, Stone EM (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 16:325-32
- Shehade SA, Chalmers RJ, Prescott RJ (1991) Predictable and unpredictable hazards of erythropoietic protoporphyria. *Clin Exp Dermatol* 16:185-7
- Sil S, Bose T, Roy D, Chakraborti AS (2004) Protoporphyrin IX-induced structural and functional changes in human red blood cells, haemoglobin and myoglobin. *J Biosci* 29:281-91
- Slater TF, Riley PA (1966) Photosensitization and lysosomal damage. *Nature* 209:151-

- Soret JL (1883) Recherches sur l'absorption des rayons ultraviolets par diverses substances. *Arch Sci Phys Nat* 10:430-485
- Srivastava G, Borthwick IA, Maguire DJ, Elferink CJ, Bawden MJ, Mercer JF, May BK (1988) Regulation of 5-aminolevulinate synthase mRNA in different rat tissues. *J Biol Chem* 263:5202-9
- Straka JG, Bloomer JR, Kempner ES (1991) The functional size of ferrochelatase determined in situ by radiation inactivation. *J Biol Chem* 266:24637-41
- Sweeney GD, Dowdle EB, Saunders SJ, Eales L (1963) Erythropoietic Protoporphyrria. A Brief Report of the First South African Case. *S Afr J Lab Clin Med* 14:247-9
- Takeshita K, Takajo T, Hirata H, Ono M, Utsumi H (2004) In vivo oxygen radical generation in the skin of the protoporphyria model mouse with visible light exposure: an L-band ESR study. *J Invest Dermatol* 122:1463-70
- Taketani S, Tokunaga R (1981) Rat liver ferrochelatase. Purification, properties, and stimulation by fatty acids. *J Biol Chem* 256:12748-53
- Taketani S, Inazawa J, Nakahashi Y, Abe T, Tokunaga R (1992) Structure of the human ferrochelatase gene. Exon/intron gene organization and location of the gene to chromosome 18. *Eur J Biochem* 205:217-22
- Taketani S, Fujita H (1995) The ferrochelatase gene structure and molecular defects associated with erythropoietic protoporphyria. *J Bioenerg Biomembr* 27:231-8
- Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res* 30:2083-8
- Thunell S, Harper P, Brun A (2000) Porphyrins, porphyrin metabolism and porphyrias. IV. Pathophysiology of erythropoietic protoporphyria--diagnosis, care and monitoring of the patient. *Scand J Clin Lab Invest* 60:581-604
- Todd DJ, Burrows D (1992) Predictable hazards of erythropoietic protoporphyria. *Clin Exp Dermatol* 17:141
- Todd DJ, Hughes AE, Ennis KT, Ward AJ, Burrows D, Nevin NC (1993) Identification of a single base pair deletion (40 del G) in exon 1 of the ferrochelatase gene in patients with erythropoietic protoporphyria. *Hum Mol Genet* 2:1495-6
- Todd DJ (1994) Erythropoietic protoporphyria. *Br J Dermatol* 131:751-66
- Tugores A, Magness ST, Brenner DA (1994) A single promoter directs both housekeeping and erythroid preferential expression of the human ferrochelatase gene. *J Biol Chem* 269:30789-97
- Tutois S, Montagutelli X, Da Silva V, Jouault H, Rouyer-Fessard P, Leroy-Viard K, Guenet JL, Nordmann Y, Beuzard Y, Deybach JC (1991) Erythropoietic

- protoporphyrin in the house mouse. A recessive inherited ferrochelatase deficiency with anemia, photosensitivity, and liver disease. *J Clin Invest* 88:1730-6
- Upchurch DA, Shankarappa R, Mullins JI (2000) Position and degree of mismatches and the mobility of DNA heteroduplexes. *Nucleic Acids Res* 28:E69
- Van der Walt JJ, Heyl T (1971) Lipoid proteinosis and erythropoietic protoporphyria. A histological and histochemical study. *Arch Dermatol* 104:501-7
- Van Hattum J, Baart de la Faille H, Van den Berg JW, Edixhoven-Bosdijk A, Wilson JH (1986) Chenodeoxycholic acid therapy in erythrohepatic protoporphyria. *J Hepatol* 3:407-12
- Varma S, Haworth A, Keefe M, Anstey AV (2000) Delayed onset of cutaneous symptoms in erythropoietic protoporphyria. *Br J Dermatol* 143:221-3
- Wang X, Poh-Fitzpatrick M, Carriero D, Ostasiewicz L, Chen T, Taketani S, Piomelli S (1993) A novel mutation in erythropoietic protoporphyria: an aberrant ferrochelatase mRNA caused by exon skipping during RNA splicing. *Biochim Biophys Acta* 1181:198-200
- Wang X, Poh-Fitzpatrick M, Piomelli S (1994) A novel splicing mutation in the ferrochelatase gene responsible for erythropoietic protoporphyria. *Biochim Biophys Acta* 1227:25-7
- Wang X, Poh-Fitzpatrick M, Chen T, Malavade K, Carriero D, Piomelli S (1995) Systematic screening for RNA with skipped exons--splicing mutations of the ferrochelatase gene. *Biochim Biophys Acta* 1271:358-62
- Wang X (1996) Molecular characterization of a novel defect occurring de novo associated with erythropoietic protoporphyria. *Biochim Biophys Acta* 1316:149-52
- Wang X, Yang L, Kurtz L, Lichtin A, DeLeo VA, Bloomer J, Poh-Fitzpatrick MB (1999) Haplotype analysis of families with erythropoietic protoporphyria and novel mutations of the ferrochelatase gene. *J Invest Dermatol* 113:87-92
- Warnich L, Kotze MJ, Groenewald IM, Groenewald JZ, van Brakel MG, van Heerden CJ, de Villiers JN, van de Ven WJ, Schoenmakers EF, Taketani S, Retief AE (1996) Identification of three mutations and associated haplotypes in the protoporphyrinogen oxidase gene in South African families with variegate porphyria. *Hum Mol Genet* 5:981-4
- Watanabe N, Hayashi N, Kikuchi G (1983) delta-Aminolevulinic synthase isozymes in the liver and erythroid cells of chicken. *Biochem Biophys Res Commun* 113:377-83
- Watanabe N, Hayashi N, Kikuchi G (1984) Relation of the extra-sequence of the precursor form of chicken liver delta-aminolevulinic synthase to its quaternary structure and catalytic properties. *Arch Biochem Biophys* 232:118-26

- Weiss G, Houston T, Kastner S, Johrer K, Grunewald K, Brock JH (1997) Regulation of cellular iron metabolism by erythropoietin: activation of iron-regulatory protein and upregulation of transferrin receptor expression in erythroid cells. *Blood* 89:680-7
- Went LN, Klasen EC (1984) Genetic aspects of erythropoietic protoporphyria. *Ann Hum Genet* 48 (Pt 2):105-17
- Wenz H, Robertson JM, Menchen S, Oaks F, Demorest DM, Scheibler D, Rosenblum BB, Wike C, Gilbert DA, Efcavitch JW (1998) High-precision genotyping by denaturing capillary electrophoresis. *Genome Res* 8:69-80
- Whatley SD, Mason NG, Khan M, Zamiri M, Badminton MN, Missaoui WN, Dailey TA, Dailey HA, Douglas WS, Wainwright NJ, Elder GH (2004) Autosomal recessive erythropoietic protoporphyria in the United Kingdom: prevalence and relationship to liver disease. *J Med Genet* 41:e105
- Whitcombe DM, Albertson DG, Cox TM (1994) Molecular analysis of functional and nonfunctional genes for human ferrochelatase: isolation and characterization of a FECH pseudogene and its sublocalization on chromosome 3. *Genomics* 20:482-6
- Williams M, Krootjes BB, van Steveninck J, van der Zee J (1994) The pro- and antioxidant properties of protoporphyrin IX. *Biochim Biophys Acta* 1211:310-6
- Wiman A, Floderus Y, Harper P (2003) Novel mutations and phenotypic effect of the splice site modulator IVS3-48C in nine Swedish families with erythropoietic protoporphyria. *J Hum Genet* 48:70-6
- Wood LH, Whatley SD, McKenna K, Badminton MN (2006) Exonic deletions as a cause of erythropoietic protoporphyria. *Ann Clin Biochem* 43:229-32
- Wu CK, Dailey HA, Rose JP, Burden A, Sellers VM, Wang BC (2001) The 2.0 A structure of human ferrochelatase, the terminal enzyme of heme biosynthesis. *Nat Struct Biol* 8:156-60
- Yasui Y, Muranaka S, Tahara T, Shimizu R, Watanabe S, Horie Y, Nanba E, Uezato H, Takamiyagi A, Taketani S, Akagi R (2002) A new ferrochelatase mutation combined with low expression alleles in a Japanese patient with erythropoietic protoporphyria. *Clin Sci (Lond)* 102:501-6
- Yotsumoto G, Masuda H, Iguro Y, Kinjo T, Matsumoto H, Sakata R (2003) Aortic valve replacement in a patient with erythropoietic protoporphyria. *Ann Thorac Surg* 75:1003-5
- Zhou S, Zong Y, Ney PA, Nair G, Stewart CF, Sorrentino BP (2005) Increased expression of the Abcg2 transporter during erythroid maturation plays a role in decreasing cellular protoporphyrin IX levels. *Blood* 105:2571-6

Conference abstracts:

De Rooij FWM, de Baar-Heesakkers E, Minderman-Voortman GJ, Wilson JHP (1996)
Abstract: Similarity in mutations of the ferrochelatase gene between Dutch and Japanese patients with erythropoietic protoporphyria. Netherlands Journal of Medicine 48

Kaappinen R and Timonen K (2005) Abstract: Spectrum of mutations in the ferrochelatase gene identified among Finnish erythropoietic protoporphyria patients including a novel splicing defect. Porphyrins and Porphyrrias Conference, Cape Town 2005

University of Cape Town

***Note:** Referencing convention - American Journal of Human Genetics