Carrier detection and phenotypic expression in a family with hereditary coproporphyria

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A thesis submitted in fulfilment of the requirements for the Degree of Masters of Science (Research)

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Statutory Declaration

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Naz AL Hafid

[Signature]

Date
11/7/08
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# Table of Contents

Statutory Declaration..............................................................................................................ii
Acknowledgements..................................................................................................................iii
Table of Contents..................................................................................................................v
List of Figures.........................................................................................................................vii
List of Tables..........................................................................................................................viii
List of Abbreviations..............................................................................................................ix
Publications Arising From This Thesis..................................................................................x

**ABSTRACT**..........................................................................................................................xi

**CHAPTER 1: DIAGNOSIS OF HEREDITARY COPROPORPHYRIA**.................................12

1.1 Introduction.......................................................................................................................12
1.2 Chemical characteristics of porphyrins and porphyrinogens........................................14
1.3 The haem biosynthetic pathway......................................................................................17
  1.3.1 Formation of pyrrole structure .................................................................................18
  1.3.2 Assembly of the tetrapyrroles ..................................................................................18
  1.3.3 Modification of the tetrapyrrole side chains ..........................................................18
  1.3.4 Oxidation of protoporphyrinogen IX to protoporphyrin IX and insertion of iron ...20
1.4 Regulation of haem biosynthesis ....................................................................................21
1.5 Molecular characteristics of CPOX .............................................................................22
1.6 Clinical manifestations....................................................................................................24
1.7 Dual porphyrias..............................................................................................................25
1.8 Environmental factors....................................................................................................26
  1.8.1 Drugs .....................................................................................................................27
  1.8.2 Endocrine factors ..................................................................................................28
  1.8.3 Other factors ........................................................................................................28
1.9 Metabolic defects............................................................................................................29
1.10 Molecular defects of HCP ..........................................................................................29
1.11 How do environmental factors provoke an acute attack? ..........................................31
1.12 Diagnosis of HCP.........................................................................................................34
1.13 Diagnostic methods.......................................................................................................35
1.14 Penetration of the acute porphyrias ..........................................................................37
1.15 Prognosis/treatment......................................................................................................38
  1.15.1 Supportive treatment ............................................................................................39
  1.15.2 Symptomatic treatment.........................................................................................39
1.16 Purpose of this project...................................................................................................41

**CHAPTER 2: MATERIALS AND METHODS**......................................................................44

2.1 Blood collection ..............................................................................................................44
2.2 Sample storage and handling .......................................................................................44
2.3 Equipments, reagents and materials.............................................................................44
2.4 DNA extraction..............................................................................................................45
  2.4.1 Materials................................................................................................................45
  2.4.2 Extraction method ..................................................................................................45
2.5 Primer design ................................................................................................................46
2.6 BLAT search results......................................................................................................48
2.7 Preparation of working primers ....................................................................................48
2.8 PCR ................................................................................................................................48
  2.8.1 Materials................................................................................................................48
  2.8.2 Method ....................................................................................................................49
2.9 GC-Rich PCR................................................................................................................50
2.9.1 Materials ................................................................. 50
2.9.2 Method ................................................................. 50
2.10 Polyacrylamide Gel Electrophoresis (PAGE) ................. 50
2.10.1 Materials ............................................................... 50
2.10.2 Reagent preparation ............................................... 51
2.10.2.1 Ammonium persulfate (APS) 10% (w/v) ............... 51
2.10.2.2 10% Acrylamide in 1xTBE .................................. 51
2.10.2.3 Tris-Borate EDTA (TBE) buffer ......................... 51
2.10.2.4 Loading dye ....................................................... 51
2.10.2.5 Ethidium bromide (2μg/mL) ............................... 52
2.10.3 Method ................................................................. 52
2.11 PCR product treatment ............................................. 52
2.11.1 Materials ............................................................... 52
2.11.2 Method ................................................................. 53
2.12 Preparation of treated PCR product for sequencing ........ 53
2.12.1 Reagents .............................................................. 53
2.12.2 Method ................................................................. 53
2.13 Sequencing .............................................................. 53
2.13.1 Equipments and reagents ....................................... 54
2.13.2 Sample preparation method .................................... 54
2.14 Sequencing analysis .................................................. 55
2.14.1 Materials ............................................................... 55
2.14.2 Method ................................................................. 55
2.15 Questionnaire .......................................................... 56
2.15.1 Designing the questionnaire .................................... 56
2.16 Denaturing HPLC (dHPLC) ....................................... 58
2.16.1 Materials ............................................................... 58
2.16.2 Sample preparation method .................................... 58
2.16.3 Optimal temperature determination ......................... 59
2.17 Qualitative analysis .................................................. 59
CHAPTER 3: RESULTS ......................................................... 60
3.1 PCR and sequencing results ....................................... 60
3.2 Denaturing HPLC (dHPLC) results ............................. 67
3.3 Descriptive statistics - frequency analysis ..................... 68
3.4 Questionnaire and statistical analysis results .................. 69
3.5 Phenotypic expressions resulting from the Q355P mutation 70
3.6 Summary of results .................................................. 85
CHAPTER 4: DISCUSSION ..................................................... 86
4.1 Locating the disease causing mutation in known HCP patients 87
4.2 Q355P Mutation ........................................................ 87
4.3 Family screening for Q355P mutation ......................... 88
4.4 Biochemical results for HCP index patients ................... 90
4.5 Revealing phenotypic expressions through questionnaire studies 91
4.6 Conclusion .............................................................. 95
APPENDIX 1 .................................................................. 98
APPENDIX 2 .................................................................. 107
APPENDIX 3 .................................................................. 116
References .................................................................... 117
List of Figures

FIGURE 1.1: THE BASIC STRUCTURE OF PORPHYRIN, A TETRAPYRROLE STRUCTURE FORMED BY FOUR PYRROLE RINGS JOINED BY METHENE BRIDGES. .......................... 15
FIGURE 1.2 A TYPICAL UV-VISIBLE ABSORPTION SPECTRUM OF A PORPHYRIN .......... 16
FIGURE 1.3: HAEM BIOSYNTHETIC PATHWAY. ENZYMES ARE IN RED FONTS AND INTERMEDIATES ARE IN BOXES. .................................................................................. 17
FIGURE 1.4: CONVERSION OF UROPORPHYRINOGEN III TO COPROPORPHYRINOGEN III THROUGH THE SEQUENTIAL REMOVAL OF THE FOUR CARBOXYLIC GROUPS OF THE ACETIC ACID SIDE CHAINS. ............................................................... 19
FIGURE 1.5: CONVERSION OF COPROPORPHYRINOGEN III TO PROTOPORPHYRINOGEN IX. 20
FIGURE 1.6: THE HUMAN CPOX GENE AND LOCATIONS OF MUTATIONS CAUSING HCP.. 31
FIGURE 1.7: FLOWCHART SHOWING THE SEQUENCE OF ACTIVITIES WHEN DRUGS ARE TAKEN AND THEIR EFFECT ON HEME AND CYTOCHROME P450. ....................... 32
FIGURE 3.1: CHROMATOGRAM SHOWING A NOVEL SNP DETECTED IN INDEX PATIENT 1.. 61
FIGURE 3.2: CHROMATOGRAM SHOWING THE POSITION OF THE Q355P MUTATION........ 62
FIGURE 3.3: A NORMAL CHROMATOGRAM FROM EXON 5 OF THE CPOX GENE. ....... 63
FIGURE 3.4: CHROMATOGRAM SHOWING THE POSITION OF THE SINGLE NUCLEOTIDE POLYMORPHISM IN EXON 5 OF THE CPOX GENE FOUND IN INDEX PATIENT THREE. .. 63
FIGURE 3.5: MULTIPLE SEQUENCE ALIGNMENTS ACROSS SOME MAMMALIAN SPECIES..... 64
FIGURE 3.7: CHROMATOGRAPHIC REPRESENTATION OF DHPLC RESULTS COMPARING FOUR PATIENTS WITH DIFFERENT GENOTYPES. .................................................. 68
FIGURE 3.8: A GRAPHICAL REPRESENTATION OF COMMON PORPHYRIA SYMPTOMS REPORTED BY ALL PARTICIPANTS IN THE QUESTIONNAIRE STUDY. .................. 72
FIGURE 3.9: COMMON PORPHYRIA SYMPTOMS IN ALL FEMALES WHO PARTICIPATED IN THE QUESTIONNAIRE. .................................................................................. 74
FIGURE 3.10: A GRAPHICAL REPRESENTATION OF MALES WHO EXPERIENCED COMMON PORPHYRIA SYMPTOMS ........................................................................ 75
FIGURE 3.11: A GRAPHICAL REPRESENTATION OF COMMON PORPHYRIA SYMPTOMS REPORTED BY MALES AND FEMALES WITH THE MUTATION. ................................... 76
List of Tables

TABLE 2.1: SUMMARY OF CPOX PRIMERS USED FOR PCR 47
TABLE 3.1: SUMMARY OF SEQUENCING RESULTS OBTAINED FOR ALL MEMBERS RELATED TO INDEX PATIENT 3. 66
TABLE 3.2: FREQUENCY OF MALES AND FEMALES WHO PARTICIPATED IN THIS STUDY..... 69
TABLE 3.3: FREQUENCY OF MALES AND FEMALES WITH THE Q355P MUTATION. .......... 69
TABLE 3.4: COMMON PORPHYRIA SYMPTOMS REPORTED BY MEMBERS WHO PARTICIPATED IN THE QUESTIONNAIRE STUDY. 71
TABLE 3.5: COMMON PORPHYRIA SYMPTOMS REPORTED BY MEMBERS WHO PARTICIPATED IN THE QUESTIONNAIRE STUDY CATEGORISED BY GENDER AND MUTATION. .......... 73
TABLE 3.6: A TABULAR REPRESENTATION OF PHENOTYPIC EXPRESSIONS REPORTED BY PATIENTS IN A FAMILY WITH HCP. 77
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIP</td>
<td>Acute Intermittent Porphyria</td>
</tr>
<tr>
<td>ALA</td>
<td>δ-Aminoluvilinic Acid</td>
</tr>
<tr>
<td>ALAS</td>
<td>δ-Aminoluvilinic Acid Synthase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>CPOX</td>
<td>Coproporphyrinogen Oxidase</td>
</tr>
<tr>
<td>DHPLC</td>
<td>Denaturing High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ESE</td>
<td>Exonic Splicing Enhancers</td>
</tr>
<tr>
<td>HCP</td>
<td>Hereditary Coproporphyria</td>
</tr>
<tr>
<td>HMBS</td>
<td>Hydroxymethylbilane synthase</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBG</td>
<td>Porphobilinogen</td>
</tr>
<tr>
<td>PBGD</td>
<td>Porphobilinogen Deaminase</td>
</tr>
<tr>
<td>PCT</td>
<td>Porphyria Cutania Tarda</td>
</tr>
<tr>
<td>PPOX</td>
<td>Protoporphyrin Oxidase</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single Stranded Conformation Polymorphism</td>
</tr>
<tr>
<td>UROD</td>
<td>Uroporphobilinogen Deaminase</td>
</tr>
<tr>
<td>VP</td>
<td>Variegate Porphyria</td>
</tr>
<tr>
<td>WFI</td>
<td>Water For Injection</td>
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</tbody>
</table>
Publications Arising From This Thesis

Poster Abstracts


Oral Presentations

Western Sydney Genetics Program (2005) – The children’s Hospital at Westmead. The Study of Penetrance And Carrier Detection In Acute Intermittent Porphyria.

ABSTRACT

Carrier Detection and phenotypic expression in a family with hereditary coproporphyria

Introduction: Hereditary coproporphyria (HCP) is an autosomal dominant disorder that results from defects in the enzyme coproporphyrinogen oxidase (CPOX). A major clinical feature is neurologic damage that leads to peripheral and autonomic neuropathies and psychiatric manifestations, accompanied occasionally by cutaneous skin lesions. HCP symptoms are usually triggered by environmental factors such as drugs and hormones. However, the penetrance is low meaning that most patients remain asymptomatic most of their lives. This makes it more difficult to diagnose asymptomatic HCP patients by solely relying on biochemical methods. The aim of this study was to genetically screen carriers in a family with HCP and design a questionnaire to identify subtle porphyria symptoms.

Methods: Mutation screening was carried out in a family of thirty members, two of whom were symptomatic for HCP. The entire CPOX gene of the proband was screened for mutations. A questionnaire was designed and completed by 26 participants to review the clinical picture and lifestyle of patients and was compared with the genetic data.

Results: A novel mutation was identified in exon 5 at c.1064A>C causing a substitution in amino acid 355 from glutamine to proline (p.Q355P). Sequencing results revealed that sixteen out of thirty members of this family were carriers of the mutation. Porphyria related symptoms were noted to be as common in males as in females with the mutation.

Conclusions: Patients with the Q355P mutation reported more symptoms than those without the mutation. Females reportedly are more likely to exhibit acute porphyria symptoms due to hormonal factors. However, it was noted that the number of symptoms reported by males with the mutation was more than that reported by females with the mutation. In this small sample cohort, these results suggest that environmental factors rather than endocrine factors play a role in the phenotypic expression of this mutation. Carriers are at risk of acute attacks; identifying them is beneficial because they can be given prior advice of preventative measures.
CHAPTER 1: DIAGNOSIS OF HEREDITARY COPROPORPHYRIA

1.1 Introduction

The porphyrias are rare and complex metabolic conditions caused by defects in the haem biosynthetic pathway. There are eight enzymes in this pathway. δ-aminolaevulinic acid synthase (ALAS) is the first. ALAS catalyses the condensation of glycine and succinyl coenzyme A to form δ-aminolaevulinic acid (ALA) (Anderson et al. 2002). In the next steps, six enzymes catalyse the formation and tetramerisation of porphobilinogen to cyclic protoporphyrin IX. The final step of the haem pathway takes place in the mitochondria where ferrochelatase catalyses the chelation of ferrous iron to form haem (Kauppinen 2005). A deficiency in any one of these enzymes causes certain porphyrins and their precursors to accumulate (Figure 1.3).

Porphyrias are classified as (1) acute hepatic porphyria, (2) chronic hepatic porphyria and (3) erythropoietic porphyria. Both erythropoietic porphyria and chronic hepatic porphyrias are associated with cutaneous photosensitivity whereas acute hepatic porphyrias are characterised by neurological symptoms and some may be accompanied by photosensitivity (Sassa 2002); (Sassa & Kappas 2000); (Anderson, Sassa, Bishop, & Desnick 2002).

The acute hepatic porphyrias include Acute Intermittent Porphyria (AIP), Variegate Porphyria (VP) and Hereditary Coproporphyria (HCP). The most common neurovisceral symptoms in acute hepatic porphyrias are colicky abdominal pain, vomiting, constipation, muscle weakness, psychiatric symptoms, and pain in the extremities. HCP and VP are occasionally accompanied by cutaneous photosensitivity which is more common in VP than in HCP (Sassa 2006).

The acute hepatic porphyrias are rarely seen before puberty, and are more common in women especially in the premenstrual phase. A common biochemical feature of the acute hepatic porphyrias during an attack is a marked increase in urinary ALA and porphobilinogen (PBG). However these metabolites are far more prominent in AIP than in HCP and VP.
The biochemical hallmark of HCP is the hyper-excretion of coproporphyrinogen III in urine and faeces. Unlike AIP, increased ALA and PBG in urine generally return to normal between attacks (Gross 2000); (Gonzalez-Arriaza 2003).

HCP is an autosomal dominant disorder caused by mutations in the CPOX gene. The CPOX gene is located on chromosome 3q11.2 and spans about 14 kb. It contains six introns and seven exons that encode the mitochondrial enzyme CPOX (Cacheux et al. 1994); (Takahashi et al. 1998). The first exon has non-analysable regions and exon seven has a large non coding region (Lamoril et al. 2001).

Mutations in the acute hepatic porphyrias are "private" meaning that each mutation belongs to a particular family only. A few exceptions including VP in South Africa (Meissner et al. 1996) and AIP in Northern Sweden (Lee & Anvret 1991) where particular mutations have been transmitted over generations from single founders. More than forty mutations have been reported in the CPOX gene so far (Human Gene Mutation Database) which includes missense, nonsense and splicin g mutations as well as insertions and deletions. Most reported mutations have been heterozygous, however, dual porphyrias as well as compound heterozygous porphyrias have also been reported previously (Poblete-Gutierrez et al. 2006); (Akagi et al. 2006) (Nordmann et al. 1990). The homozygous form of HCP is very rare and has been reported (Grandchamp, Phung, & Nordmann 1977).

When any of the acute porphyrias are suspected, screening tests are carried out for urinary ALA and PBG measurements. Current quantitative measurements of urinary ALA and PBG are carried out using the method described by Mauzerall D and Granick S (1956). There are also commercial semi quantitative kits for urinary PBG analysis (Trace PBG kit; Alpha Laboratories, Eastleigh, Hampshire, UK) (Deacon & Peters 1998). Other methods include reverse-phase high performance liquid chromatography which is used for analysis of porphyrins in urine and faeces (Lim & Peters 1984). Porphyrin measurements in blood and plasma are performed using fluorometry (Blake et al. 1992). Total faecal porphyrins can also be measured using spectrophotometric methods (Lockwood et al. 1985). Even with these methods, it has always been a challenge to identify latent carriers of porphyria. In recent years, the diagnoses of
porphyrias are being approached with molecular genetic techniques such as DNA sequencing, denaturing gel electrophoresis, SSCP (Single-Stranded Conformation Polymorphism) and denaturing HPLC. With the development and advancement in molecular genetic procedures, it has been possible to achieve great accuracy and reliability in making correct diagnoses of the porphyrias.

1.2 Chemical characteristics of porphyrins and porphyrinogens

The porphyrins are important chemicals that are widely distributed in nature and, in combination with various metallic ions, form pigments and enzymes that are vital to both animal and vegetable life. In plants the pigment is chlorophyll, a magnesium porphyrin, essential for the utilization of light energy in the synthesis of carbohydrates. The porphyrin structure may well be considered as the most important chemical configuration evolved, for without chlorophyll, an oxygen-containing atmosphere would not have developed, and the earth would have retained its primitive atmosphere of hydrogen, methane, and carbon dioxide (Wiggers 2007).

In higher animal species the respiratory pigment is an iron-porphyrin-globin compound, haemoglobin which is crucial for oxygen transport. Porphyrin is also a vital component of myoglobin (oxygen storage), cytochromes (electron transport), cytochrome oxidase (reduction of molecular oxygen), catalase (decomposition of hydrogen peroxide), peroxidases (oxidation of organic molecules with peroxide), nitric oxide synthases and so forth (Lash 2005)

The basic structure of porphyrin is four pyrrole rings joined together by methene bridges (=CH-), forming the cyclic tetrapyrrole structure (Figure 1.1).
Figure 1.1: The basic structure of porphyrin, a tetrapyrrole structure formed by four pyrrole rings joined by methene bridges.

Figure 1.1 was obtained from: http://www.wpi.edu/Academics/Depts/Chemistry/Courses/General/porphyrin.html

Porphyrinogens are the reduced form of porphyrins (Anderson, Sassa, Bishop, & Desnick 2002). Most enzymatic steps in the heme biosynthetic pathway require porphyrinogens as substrates except for protoporphyrin which is the substrate for the terminal enzyme ferrochelatase. Porphyrins have a maximum absorption spectrum of 400nm (referred to as the Soret Band) and four weaker absorption bands (referred to as Q bands) at wavelengths ranging from 500-630nm (Figure 1.2). In acidic solution, porphyrins exhibit two strong emission bands, one at 600-610 nm and the other at 640-660 nm (Anderson, Sassa, Bishop, & Desnick 2002).

The water solubility of porphyrins depends on the number of carboxylic acid side chains. Porphyrins with the most carboxylic acid side chains eg. uroporphyrin (8-carboxylic porphyrin) is the most water soluble, whereas protoporphyrin (2-carboxylic porphyrin) is the least water soluble. Coproporphyrin has 4-carboxylic porphyrins, making it an intermediate, hence both water and lipid soluble. Protoporphyrin is only excreted into lipid-rich bile, while uroporphyrin is mostly excreted in urine. Coproporphyrin has intermediate properties; therefore it is excreted into both bile and urine. HCP results from a partial deficiency of the mitochondrial enzyme, coproporphyrinogen oxidase (CPOX; EC 1.3.3.3), which catalyses the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX during the biosynthesis of haem (Brodie et al. 1977) (Martasek 1998); (Anderson, Sassa, Bishop, & Desnick 2002).
Figure 1.2 A typical UV-visible absorption spectrum of a porphyrin

Figure 1.2 was obtained from the online source at:
http://chemgroups.ucdavis.edu/~smith/chime/Porph_Struct/start_here.html

Coproporphyrinogen oxidase (CPOX) is a highly conserved enzyme found in all aerobic organisms. It carries out oxidative decarboxylation in the presence of molecular oxygen without any conventional cofactors or metal cations. Lash (2005) proposed a mechanism for the function of CPOX suggesting that this enzyme uses deprotonated pyrrole units that react with oxygen at the α-positions. Subsequent molecular deprotonation of the propionate side chain forms an external vinylene moiety. This can undergo loss of carbon dioxide and hydrogen peroxide to form a vinyl grouping. This is the first proposal that takes into account the reactivity of pyrrolic structures (Lash 2005).
1.3 The haem biosynthetic pathway

The haem biosynthetic pathway consists of eight enzymes that sequentially convert glycine and succinyl CoA to haem (Figure 1.3).

There are four basic processes in the haem biosynthetic pathway.

1. Formation of pyrrole structure
2. Assembly of the tetrapyrroles.
3. Modification of the tetrapyrroles side chains through sequential enzyme processes.
4. Oxidation of protoporphyrinogen IX to protoporphyrin IX and insertion of iron.

Figure 1.3: Haem biosynthetic pathway. Enzymes are in red fonts and intermediates are in boxes.

Numbers refer to the sequential process of each enzyme in the pathway.
1.3.1 Formation of pyrrole structure

The first and rate-limiting enzyme in the pathway is δ-aminoluvilinic acid synthase (ALAS) which catalyses the condensation of glycine and succinyl-CoA to form ALA. ALA is a 5-carbon aminoketone which is formed in the mitochondria. Following its synthesis, ALA exits the mitochondria and enters the cytoplasm by an unknown mechanism (Ajioka, Phillips, & Kushner 2006), where aminolevulinate dehydratase (ALAD) catalyses the condensation reaction of two molecules of ALA forming a monopyrrole structure porphobilinogen (PBG) (Sassa 2006).

1.3.2 Assembly of the tetrapyrroles

In the cytosol, porphobilinogen deaminase (PBGD) combines four molecules of PBG to form an unstable linear tetrapyrrole, hydroxymethylbilane (HMB) (Ajioka, Phillips, & Kushner 2006), (Sassa 2006). HMB serves as the substrate for uroporphyrinogen III synthase (UROS). UROS catalyses the conversion of hydroxymethylbilane (HMB) to uroporphyrinogen III by inversion of the D-ring of HMB followed by closure of the tetrapyrrole. Spontaneous non enzymatic cyclisation of HMB can also occur forming Uroporphyrinogen I, which cannot be converted to haem (Ajioka, Phillips, & Kushner 2006).

1.3.3 Modification of the tetrapyrrole side chains

Uroporphyrinogen decarboxylase (UROD) is the fifth enzyme in the haem biosynthetic pathway. It converts uroporphyrinogen III to coproporphyrinogen III through the sequential removal of the four carboxylic groups of the acetic acid side chains. Decarboxylation of uroporphyrinogen III begins at the symmetric D-ring and proceeds in a clockwise manner (Figure 1.4) (Ajioka, Phillips, & Kushner 2006).
Figure 1.4: Conversion of uroporphyrinogen III to coproporphyrinogen III through the sequential removal of the four carboxylic groups of the acetic acid side chains.

(Diagram 1.4 was obtained from The Online Metabolic and Molecular basis of Inherited disease database (Anderson, Sassa, Bishop, & Desnick 2002).)

Coproporphyrinogen oxidase (CPOX; Figure 1.3, the sixth enzyme in the pathway) catalyses the substitution of the propionic acid groups of ring A followed by ring B of coproporphyrinogen III (Figure 1.5, positions 2 and 4) with vinyl groups yielding harderoporphyrinogen IX then protoporphyrinogen IX respectively.

There are two forms of the CPOX enzyme; oxygen-dependent and oxygen-independent. Both forms decarboxylate the A and B pyrrole ring propionates to vinyl groups. Mammals and other higher eukaryotes utilise only the oxygen dependent form which releases two molecules of CO₂ and H₂O₂. (Ajioka, Phillips, & Kushner 2006).(Lee et al. 2005) suggested that oxygen dependent CPOX catalyses an unusual metal- and cofactor-independent oxidative decarboxylation in which molecular oxygen acts as the immediate electron acceptor.
1.3.1.4 Oxidation of protoporphyrinogen IX to protoporphyrin IX and insertion of iron

Protoporphyrinogen oxidase (PPOX) is the seventh enzyme in the haem biosynthetic pathway. This enzyme catalyses the oxidation of protoporphyrinogen IX to form protoporphyrin IX. PPOX is synthesised in the cytosol and transported to the outer surface of the inner mitochondrial membrane. The final enzymatic step in the haem biosynthetic pathway is the insertion of ferrous iron into protoporphyrin IX by the enzyme ferrochelatase (FECH) to form haem. This step occurs on the inner surface of the inner mitochondrial membrane (Ajioka, Phillips, & Kushner 2006) (Sassa 2006) (Tsiftsoglou, Tsamadou, & Papadopoulou 2006).
1.4 Regulation of haem biosynthesis

Haem biosynthesis is mainly limited by the rate of ALAS formation. The major sites of haem production are the liver and bone marrow. ALA, which is the first intermediate in the haem biosynthetic pathway is exclusively committed to haem synthesis and is also a rate limiting step in the formation of haem (Anderson, Sassa, Bishop, & Desnick 2002). There are two ALAS isoenzymes; tissue-specific (ALAS1) and erythroid-specific (ALAS2) which are encoded by different genes (Anderson, Sassa, Bishop, & Desnick 2002).

Biosynthesis of heme in the liver is controlled mainly by the rate of formation of ALAS1 (Granick, Urata 1963; cited by (Sassa 2002)). Enzyme activity of ALAS1 in normal liver cells is very low compared to that in the bone marrow (Sassa & GRANICK 1970). Most of the haem produced in bone marrow is used for haemoglobin formation. Another regulatory factor is haem itself, with respect to its own biosynthesis and catabolism (Tenhunen, Marver, & Schmid 1970); (Tsiftsoglou, Tsamadou, & Papadopoulou 2006). The haem biosynthetic pathway has a negative feedback mechanism through haem for its regulation (Sinclair & GRANICK 1975). Hypothetically, the presence of free haem pools can affect haem biosynthesis and catabolism in a number of ways. Free haem in mitochondria may regulate the rate of synthesis of cytochrome oxidase. The activity of haem oxygenase may also be regulated by microsomal free haem. Another possible regulatory effect is through cytosolic free haem which may repress the rate of ALAS1 synthesis (Anderson, Sassa, Bishop, & Desnick 2002).

Compounds that destroy free haem or inhibit haem synthesis are strong inducers of hepatic ALAS1. ALA synthase is regulated by endogenous haem and glucose and can also be repressed by exogenous haem and glucose (Gross, Hoffmann, & Doss 2000). In acute hepatic porphyrias, certain drugs enhance the production of cytochrome P450 and haem synthesis and the primary enzyme deficiency can become rate limiting for haem formation, resulting in a decreased regulatory free haem concentration. This in turn, leads to the overproduction of ALAS1 (Anderson, Sassa, Bishop, & Desnick 2002). After induction of ALAS, ALA and PBG will be over produced, but their metabolic conversion into porphyrinogens is principally limited by the secondary controlling
sequence of PBG deaminase. This limiting function of hepatic PBG deaminase explains the simultaneous increase of porphyrin precursors (ALA and PBG) in all three types of dominant acute porphyrias (Gross, Hoffmann, & Doss 2000).

Haem oxygenase which is a rate limiting enzyme for haem degradation, can also affect the level of regulatory free haem in the liver (Anderson, Sassa, Bishop, & Desnick 2002); (Tenhunen, Marver, & Schmid 1970). Hence, hepatic haem concentration is maintained by a balance between ALAS1 and haem oxygenase synthesis, both of which are under the regulatory effect of haem (for reviews, see (Anderson, Sassa, Bishop, & Desnick 2002); (Sassa 2002); (Sassa & Kappas 2000). It has been previously reported that CPOX represents rate-limiting steps downstream from ALAS2 in the regulation of haem biosynthesis (Woodard & Dailey 2000) (Taketani, Furukawa, & Furuyama 2001).

Susa et al (2002) demonstrated that haem regulates its own synthesis by inhibiting the import of mitochondrial CPOX as well as ALAS2. However the feedback inhibition mechanism of mitochondrial import of CPOX is not clear. It is believed that ALAS2 has a haem regulatory motif in its pre-sequence which was shown to be involved in the feedback inhibition of haem (Lathrop & Timko 1993); however this regulatory motif was not shown in preCPOX (Susa et al. 2002). Susa and colleagues (2002) concluded that haem regulates the import of CPOX by a mechanism different to that of ALAS2 regulation.

1.5 Molecular characteristics of CPOX

The CPOX gene is located on chromosome 3q11.2 and spans about 14 kb. It contains six introns and seven exons that encode the mitochondrial enzyme CPOX (Cacheux, Martasek, Fougerousse, Delfau, Druart, Tachdjian, & Grandchamp 1994). The first exon has non-analysable regions and exon seven has a large non-coding region (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001). The mature human CPOX enzyme is comprised of 354 amino acid residues, with a putative N-terminal signal peptide of 110 residues (Delfau-Larue, Martasek, & Grandchamp 1994). Yeast CPOX is 27 amino acids shorter than the mammalian
enzyme, and does not have a leader peptide for import into the mitochondria (Taketani et al. 1994). These findings support earlier observations by Elder et al 1978 and Camadro et al 1986 that yeast CPOX enzyme is cytosolic whereas mammalian CPOX enzyme is mitochondrial. A comparison of the human protein with those from various sources demonstrated that the amino-terminal part of the CPOX enzyme is the most divergent, while most of the sequence is highly conserved (For review see (Grandchamp et al. 1996).

Taketani et al (1994) successfully expressed CPOX cDNA in E. coli excluding the putative leader sequence of CPOX and starting from the initiation codon at nucleotide position 91-93. In a separate experiment he ligated the full length cDNA (including the leader sequence) into the same vector, but observed no increase in enzyme activity in cell extracts of the transformed E. coli. He concluded that the mammalian leader sequence may interrupt the prokaryotic expression of the active CPOX activity.

The CPOX promoter region is GC-rich and contains multiple potential Sp1 elements, CACCC boxes, and GATA-1 binding sites (Delfau-Larue, Martasek, & Grandchamp 1994). Transient transfection assays of the promoter region indicated that synergistic action between a Sp1-like element, a GATA site and a CPOX Promoter Regulatory Element (CPRE) is necessary for the promoter activity in erythroid cells (Takahashi, Taketani, Akasaka, Kobayashi, Hayashi, Yamamoto, & Nagai 1998). However, in non-erythroid cells, the GATA site is not required (Takahashi, Taketani, Akasaka, Kobayashi, Hayashi, Yamamoto, & Nagai 1998). These results suggest that there is a single promoter for CPOX that is differentially regulated in erythropoietic and non-erythropoietic cells (Delfau-Larue, Martasek, & Grandchamp 1994) (Takahashi, Taketani, Akasaka, Kobayashi, Hayashi, Yamamoto, & Nagai 1998).
1.6 Clinical manifestations

All the porphyrias are dominantly inherited, and may be expressed clinically when there is a mutation in one allele of the relevant gene, although penetrance is incomplete. In the acute porphyrias the initial clinical picture usually presents with abdominal pain accompanied occasionally with disturbances in intestinal motility (e.g. diarrhoea or constipation). Another common feature of acute hepatic porphyrias is peripheral neuropathy and muscle weakness. Central nervous system involvement is common and highly variable (Anderson, Sassa, Bishop, & Desnick 2002). Neuropsychiatric symptoms include hysteria, anxiety, depression, phobias, psychosis, agitation, delirium and seizures (Gonzalez-Arriaza 2003). Seizures may arise from the neurologic manifestations of porphyria itself or may be secondary to hyponatraemia or can be due to coexisting idiopathic epilepsy (Anderson, Sassa, Bishop, & Desnick 2002); (Zaatreh 2005); (Magnussen et al. 1975). Cutaneous skin lesions are also a clinical feature of HCP but are not as common as the neurovisceral symptoms. Skin lesions accompany acute attacks in about one-third of the patients with HCP (Gonzalez-Arriaza 2003); (Palma-Carlos & Palma-Carlos 2005). Other symptoms include hyponatraemia, urinary retention, dysuria, tachycardia and hypertension (Lam et al. 2001);(Sassa 2002).

Also, a variant form of HCP known as harderoporphyria has been reported and is characterised by neonatal haemolytic anaemia occasionally accompanied by skin lesions (Nordmann et al. 1983); (Lamoril et al. 1995;Lamoril et al. 1998). All individuals described have been homoallelic or heteroallelic for the same mutation in the CPOX gene.

Disease expressions of the acute porphyrias are more common in females than males, and rarely occur before puberty (Schuurmans et al. 2001); Scriv er online page 3009; (Gonzalez-Arriaza 2003); (Andersson, Innala, & Backstrom 2003). Clinical expressions of acute porphyrias are usually linked to environmental or acquired factors such as nutritional status, drugs and hormones or their metabolites. These precipitating factors may cause acute episodes of a variety of neuropathic symptoms (Sassa 2002).

Clinically expressed HCP is generally milder and less common than clinically expressed AIP (Sassa 2002); Scriver page3032). In general, the prevalence of porphyria varies
from country to country, as do the types of porphyria (Crimlisk 1997). Approximately 80% of carriers of a gene mutation for AIP, HCP and VP remain asymptomatic and some may only have one or a few attacks throughout their lives (Anderson et al. 2005).

The clinical manifestations of the acute porphyrias are identical with the exception of HCP and VP in which cutaneous symptoms can occasionally present in some cases in addition to the neurovisceral symptoms (Palma-Carlos & Palma-Carlos 2005). Abdominal pain, peripheral neuropathy and neuropsychiatric symptoms are the classic triad of an acute attack. Symptoms may vary in the same patient during different episodes as well as among patients with the same porphyria subtype. Symptoms can be acute or chronic, mimicking many other psychiatric or medical disorders (Gonzalez-Arriaza 2003). Significant neuropathy and psychiatric symptoms do not occur in all patients with acute attacks of porphyria and porphyria is not necessarily associated with abdominal pain (ANDERSSON 2002); (Gonzalez-Arriaza 2003).

1.7 Dual porphyrias

In dual porphyrias, two types of porphyrias simultaneously present either in one individual or within one family. (Poblete-Gutierrez P et al 2006). The majority of these dual porphyrias comprise the combined deficiency of uroporphyrinogen decarboxylase (UROD) with either porphobilinogen deaminase (PBGD), coproporphyrinogen oxidase (CPOX), or protoporphyrinogen oxidase (PPOX), respectively. (Poblete-Gutierrez, Badloe, Wiederholt, Merk, & Frank 2006).

In 1975 Watson et al. reported for the first time the presence of two types of porphyria in one family (variegate porphyria and porphyria cutanea tarda; VP and PCT respectively). The clinical and biochemical picture of one of the index patients was suggestive of VP; however the brother of the index patient did not show any symptoms, but biochemical analysis revealed iso-coproporphyrin in faeces indicating PCT. Watson et al (1975) initially thought that this patient may have the acquired form of PCT, until later it was confirmed that another sibling also had iso-coproporphyrin in their faeces.
(Watson et al. 1976). He then concluded that this family had two types of porphyrias (now known as dual porphyrias).

Akagi et al (2005) reported for the first time the coexistence of ALA dehydratase (ALAD) and CPOX deficiency in a patient. The patient had a mutation in the ALAD gene (p.F12L; C36>G; previously reported by Akagi et al 1999) and another heterozygous mutation at the CPOX gene (Novel mutation p.G279R; G835>C). The brother and the mother of the patient only exhibited the ALAD mutation and no mutation was found in the father (Akagi, Inoue, Muranaka, Tahara, Taketani, Anderson, Phillips, & Sassa 2006). It was concluded that the proband had exhibited a new mutation which occurred in the CPOX gene (Akagi, Inoue, Muranaka, Tahara, Taketani, Anderson, Phillips, & Sassa 2006). Enzyme studies of the novel CPOX mutation (p.G279R) in this proband revealed the presence of 5% activity compared with the wild type. This mutation was located within the characteristic β-sheet that forms the back wall of the active site (Phillips et al. 2004), which is the most structurally conserved feature of the protein (Akagi, Inoue, Muranaka, Tahara, Taketani, Anderson, Phillips, & Sassa 2006).

1.8 Environmental factors

There is significant interaction between the primary gene defect in the haem biosynthetic pathway and environmental factors. Patients with acute hepatic porphyrias may not become symptomatic unless these subjects are exposed to certain drugs, liver damage, hormonal changes during the menstrual cycle, stress or starvation (Sassa 2006); (Anderson, Sassa, Bishop, & Desnick 2002).

Factors that play a major role in precipitating acute porphyria symptoms are the same for all acute porphyrias. In 100 acute porphyria patients, drugs and alcohol were the most usual triggering factors (noted in 54% of the patients), followed in frequency by fasting and alcohol, premenstrual manifestations, oestrogens, anticonvulsant treatment, several infections and stress (Gross, Hoffmann, & Doss 2000).
1.8.1 Drugs

Drugs are one of the most important factors that provoke acute attacks of porphyria. Once diagnosis of porphyria is established in a patient, it is easy to prevent acute attacks by avoiding harmful drugs such as barbiturates and sulphonamide antibiotics.

Attacks of acute porphyria were much more common 20-30 years ago than they are now. Phenobarbital and other barbiturates were usually implicated. At that time, these drugs were commonly used as tranquilizers and sleeping pills. Severe attacks of porphyria are now less frequent, partly because barbiturates are seldom prescribed. Thus, the decline in use of barbiturates has contributed greatly to improving the prognosis for patients with acute porphyrias. Barbiturates have been largely replaced by benzodiazepines, which are much less hazardous in porphyria. Commonly used benzodiazepines include chlordiazepoxide (Librium), diazepam (Valium), triazolam (Halcion) and oxazepam (Serax). It may be that benzodiazepines are harmful in porphyria if large doses are taken; however, small or occasional doses seem to pose little risk in most patients (American Porphyria Foundation at http://www.porphyriafoundation.com/).

In recent years, there has been increased use of recreational drugs. These are major precipitating factors in acute porphyrias (Hift & Meissner 2005). Alcohol is also another important precipitating factor causing acute attacks in porphyria patients. Ethanol consumption and its effect on the liver and its precursors have been well documented (Louis et al. 1993; Sinclair et al. 1991; Thunell et al. 1992). Smoking is also thought to be associated with acute porphyrionic attacks. A study (Lip et al. 1991) found an association between cigarette smoking and repeated attacks of porphyria in a 144 AIP patients in Britain. Therefore, avoiding cigarette smoking as well as alcohol consumption may aid in reducing porphyrionic attacks.
1.8.2 Endocrine factors

There is little in the literature regarding triggering of symptoms in HCP by endocrine factors. AIP is widely reported to be triggered by endocrine factors. Clinical manifestations are frequently noted at pregnancy and menstruation. This suggests the hormone-related factors worsen the disease, which may explain the incomplete penetrance of the acute hepatic porphyrias. (Anderson, Sassa, Bishop, & Desnick 2002)

Endogenous sex hormones are important determinants of disease activity as they might compromise haem biosynthesis by inducing ALA synthase and subsequently triggering cyclical attacks (GRANICK 1966). Progesterone has also been implicated in the increase of haem catabolism (Anderson, Sassa, Bishop, & Desnick 2002). A study (Schuurmans, Schneider-Yin, Rufenacht, Schnyder, Minder, Puy, Deybach, & Minder 2001) confirmed that the disease expression in the acute porphyrias was strongly enhanced by female gender. Acute attacks rarely start before puberty and its frequency and severity decline in women after menopause.

Andersson et. al (2003) demonstrated that there was a high frequency of miscarriage amongst symptomatic women with AIP. Their study revealed that the use of oral contraceptives was more common than expected. In 25% of the women who manifest AIP, their symptoms were attributed to the use of oral contraceptives.

1.8.3 Other factors

Stress in all its variable forms can lead to a manifestation of acute hepatic porphyria. Stress may result from strong emotions, excessive alcohol intake, surgery, intercurrent illness and social problems (Sassa 2002)((Gross, Hoffmann, & Doss 2000). Reduced caloric intake and/or fasting is also a very important precipitating factor in the acute hepatic porphyrias (Gross, Hoffmann, & Doss 2000); (Sassa 2002).
1.9 Metabolic defects

The main biochemical feature of HCP is the increased excretion of coproporphyrinogen III in urine and faeces. Increased δ-aminoluvilinic acid (ALA) and porphobilinogen (PBG) in urine are noted in all acute hepatic porphyrias, however, unlike acute intermittent porphyria (AIP) these findings return to normal between attacks (Gross, Hoffmann, & Doss 2000) (Gonzalez-Arriaza 2003).

An increase in urinary ALA alone will indicate patients with lead intoxication or ALAD deficiency porphyria (Kauppinen 2005); (Anderson, Sassa, Bishop, & Desnick 2002). HCP can be distinguished from VP by the presence of increased faecal coproporphyrin (especially isomer III) whereas a marked increase in faecal protoporphyrin is noted in VP (Sassa 2006). Harderoporphyria is characterised by an increase in faecal harderoporphyrins in as well as coproporphyrin (Nordmann, Grandchamp, de Verneuil, Phung, Cartigny, & Fontaine 1983); (Lamoril, Martasek, Deybach, Da, V, Grandchamp, & Nordmann 1995; Lamoril, Puy, Gouya, Rosipal, Da, V, Grandchamp, Foint, Bader-Meunier, Dommergues, Deybach, & Nordmann 1998). Increased faecal protoporphyrin can also be present in 20% of AIP patients. This does not differentiate AlP from VP and further diagnostic tests such as enzymatic profiles or genetic studies need to be done for correct diagnoses (Kauppinen 2005).

1.10 Molecular defects of HCP

The molecular genetics of HCP is very heterogenous. So far over 40 CPOX mutations have been reported to date (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001); (Gross, Hoffmann, & Doss 2000); (Rosipal et al. 1999); (Schreiber et al. 1997); (Sassa et al. 1997); (Martasek 1998) (Akagi, Inoue, Muranaka, Tahara, Taketani, Anderson, Phillips, & Sassa 2006). These mutations include missense, nonsense, splice-site defects as well as insertions and deletions. Most CPOX mutations are family specific and are without any hotspots or genotype/phenotype correlations (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001).
The first mutation identified in the CPOX gene was found in a previously diagnosed patient with homozygous HCP (Martasek, Nordmann, & Grandchamp 1994). This patient was born to first-cousin parents. Mutation analysis revealed a homozygous point mutation C to T, resulting in an arginine-to-tryptophan substitution at amino acid 331 (Figure 1.6). Expression studies of this mutation demonstrated that this substitution resulted in the synthesis of an unstable protein with a residual catalytic activity (Martasek, Nordmann, & Grandchamp 1994).

The heterogeneity of HCP is true for most mutations with the exception of the K404E mutation (encoded by exon 6; Figure 1.6) found in three unrelated families causing the variant form of HCP, harderoporphyria (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001; Lamoril, Martasek, Deybach, Da, V, Grandchamp, & Nordmann 1995; Lamoril, Puy, Gouya, Rosipal, Da, V, Grandchamp, Foint, Bader-Meunier, Dommergues, Deybach, & Nordmann 1998; Schmitt et al. 2005). This is the only mutation found to date causing harderoporphyria. Two studies suggest that the region encoded by exon 6 is essential for the oxidative decarboxylation of harderoporphyrin (Schmitt, Gouya, Malonova, Lamoril, Camadro, Flamme, Rose, Lyoumi, Da Silva, Boileau, Grandchamp, Beaumont, Deybach, & Puy 2005; Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001).

To-Figuera et al (2005) reported an 11 year old boy with HCP who exhibited severe clinical expression. His symptoms comprised severe psychomotor retardation, spastic motor impairment and epilepsy. He was admitted to the emergency department with severe abdominal pain, tachycardia and dark urine. Since such severe symptoms do not present before puberty it was initially thought that this patient was either homozygous or compound heterozygous. Further investigations by the investigators revealed that this patient was heterozygous for the novel mutation V135A (404T>C, exon1). Biochemical analysis did not support the presence of a dual porphyria. An incidental association of HCP with epilepsy, with a worsening of the symptoms due to the treatment with anti-epileptic porphyrinogenic drugs was a possible explanation for this case (To-Figuera et al. 2005). However, epileptic seizures have already been described as part of the clinical presentation of HCP in children. (Casali et al. 1984). Since this mutation is located at the N-terminal of the protein, another possibility suggested by To-Figuera et al (2005)
for the deleterious effect, is the interference of the V135A mutation with the mitochondrial processing of the CPOX precursor.

Figure 1.6: The human CPOX gene and locations of mutations causing HCP. (Courtesy of Dr. K. H. Astrin.) Figure was obtained from the Online Metabolic and Molecular Bases of Inherited Disease (Anderson, Sassa, Bishop, & Desnick 2002).

1.11 How do environmental factors provoke an acute attack?

Haem synthesis has multiple regulatory mechanisms which can be interrupted by environmental factors. Under normal enzyme activities, haem homeostasis can be achieved through one of the regulatory mechanisms (i.e. concentration of free haem pool, haem oxygenase, ALAS1). However, if there is a deficiency in one of the enzymes in the haem biosynthetic pathway, haem homeostasis cannot be achieved under the influence of environmental factors. Factors that induce ALAS1 activity, increase haem oxygenase or cytochrome P450 production or catabolism can trigger porphyric attacks if the enzyme activity cannot meet the body’s demand for haem production (Figure 1.7).
Figure 1.7: Flowchart showing the sequence of activities when drugs are taken and their effect on heme and cytochrome P450.

When drugs are ingested (1), the liver uses cytochrome P450 enzymes to clear the drug metabolites from the body as a detoxification mechanism. Cytochrome P450 is haem dependent, therefore when demand on cytochrome P450 increases (2), free haem pool concentration decreases (3). This in turn, induces ALAS1 activity in liver to produce more haem through the haem biosynthetic pathway (4). The free haem produced is used for the production of cytochrome P450 (5) in response to the body’s demand. This mechanism becomes problematic in porphyria patients (step 4 of the above flow chart) as it leads to the accumulation of porphyrins and their precursors.

Drugs, alcohol, smoking as well as gonadal and pituitary hormones induce the production of cytochrome P450 enzymes (Anderson, Sassa, Bishop, & Desnick 2002); (Sassa 2002); (Gross, Hoffmann, & Doss 2000); (Anderson, Freddara, & Kappas 1982); (Sassa, Bradlow, & Kappas 1979); (Waxman 1988). Cytochrome P450 is a major drug- and steroid-metabolising enzyme. It is a collection of structurally related haemoprotein mono-oxygenase enzymes that hydroxylate a large number of steroid hormones, fatty acids, drugs, carcinogens and environmental chemicals (Ortiz de Montellano & Costa 1986); (Guengerich 1987); (Porter & Coon 1991).

Cytochrome P450 is haem dependent, hence when the demand for cytochrome P450 increases, haem production is increased through induction of ALAS1 activity in the liver (See reviews (Anderson, Sassa, Bishop, & Desnick 2002); (Sassa 2002); (Gross, Hoffmann, & Doss 2000). Increased ALAS1 activity induces haem biosynthesis. If however, one of the enzymes has 50% or less activity, it cannot catalyse the reaction.
with the same rate as the ALAS1 synthesis. Hence, haem production is reduced leading to the accumulation of ALA, PBG and other porphyrin precursors. Inability of the liver to clear drugs from the system due to insufficient cytochrome P450 leads to liver damage. It is not yet clear what causes the acute neurological symptoms of the hepatic porphyrias. However, one of the two putative theories is that the accumulation of porphyrin precursors especially ALA may cause the neurological and psychiatric symptoms. The second theory is the possible effect of the lack of haem in neurons. Bonkovsky (1993) discusses the possible effects that a decreased level of haem in the nervous system and/or the liver can have on the efficiency of hemoprotein synthesis and subsequently causing a decrease in the ATP supply leading to neuronal dysfunction.

It has been proposed that ALA is involved in the neurological dysfunctions presented by patients with acute porphyrias. Emanuelli et al. (2003) demonstrated through their study that ALA dose-dependently inhibited glutamate uptake by astrocyte cultures. The inhibitory effect was irreversible. The finding that ALA significantly increased astrocyte lipoperoxidation in astrocytes suggests that the inhibitory effect of ALA might be related to an oxidative damage of the transporter. It has also been proposed (Emanuelli et al. 2000) that ALA-induced convulsions may result from the inhibition of glutamate uptake by glial cells (GLT-1 transport) which can contribute to the overstimulation of glutamate receptors. Evidence presented by Emanuelli et al. (2003) supports the possible involvement of oxidative damage and reduced glutamate uptake in the neurological effects of ALA which may be relevant to the treatment of porphyria patients.

The acute porphyrias have been considered as conditions of permanent systemic overload of oxidative stress, with long-term effects on hepatic and renal tissue, and with instances of a periodic overload of free radicals giving rise to acute neurologic involvement (Thunell et al. 1995). Experimental studies (Demasi et al. 1996) suggest that the neuropsychiatric manifestations of the acute porphyrias may result from ALA-induced oxidative stress in the brain, accompanied by alterations in iron metabolism and GABAergic receptor damage. Another possible theory is a decrease of haem in the nervous system and/or the liver which could lead to an inability of cells in the nervous
system to synthesise adequate amounts of hemoproteins, causing a deficiency in the ATP supply and neuronal dysfunction (Bonkovsky 1993).

A study by (Marks 1985) suggests that some drugs, when provided to experimental systems can increase haem synthesis by promoting the destruction of cytochrome P450 enzymes. Mechanism-based destruction of cytochrome P450 by some drugs (eg Griseofulvin) can also lead to formation of N-alkylated protoporphyrins such as N-methyl protoporphyrin which is a potent inhibitor of ferrochelatase. Increased destruction of cytochrome P450 and inhibition of ferrochelatase can further limit haem synthesis (Marks et al. 1985).

There has been little study on nutritional status as a contributor to acute attacks of porphyria mainly due to the difficulty in obtaining accurate dietary histories (Anderson, Sassa, Bishop, & Desnick 2002). Reduced caloric intake, usually with an attempt to reduce weight, can trigger acute attacks of porphyrias (Anderson, Sassa, Bishop, & Desnick 2002). Starvation in animals enhances the inducing effect of chemicals on ALAS1 (Tschudy et al. 1964). Dietary intake of carbohydrates can reduce cytochrome P450 enzymes in normal animals (Anderson & Kappas 1991). Therefore the demand on hepatic haem synthesis is decreased during increased carbohydrate intake.

It has also been shown that starvation in animals can induce haem oxygenase (Thaler & Dawber 1977), which decreases free haem pools through haem catabolism. This then contributes to induction of ALAS1 activity (Anderson, Sassa, Bishop, & Desnick 2002). Intercurrent illnesses, infection and surgery may provoke symptoms through up-regulating haem oxygenase gene activity, leading to excessive haem catabolism, hence, induced ALAS1 (Sassa 2002).

1.12 Diagnosis of HCP

The main pathological feature of HCP is the increase in urinary and faecal coproporphyrin III accompanied by a mild elevation of urinary ALA and PBG. This may also be noted in some asymptomatic HCP patients (Anderson, Sassa, Bishop, & Desnick 2002). However, an increase in the ratio of coproporphyrin III to
coproporphyrin I in faeces appears to be a better indicator of asymptomatic HCP, at least in adults (Blake, McManus, Cronin, & Ratnaike 1992); (Gross et al. 2002); (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001); (Gonzalez-Arriaza 2003). The explanation for the sensitivity of this ratio for the diagnosis of HCP is based on the different routes of excretion of the two isomers of coproporphyrin. Coproporphyrin I is mainly excreted through the biliary system, whereas coproporphyrin III is excreted primarily from the kidney nephrons (Day and Eales 1980). In contrast to other metabolite measurements, the resulting inverse ratio of coproporphyrin III: I (usually <1) in the faeces is very sensitive to any increased hepatic production of the coproporphyrinogen III isomer (Allen et al. 2005).

In the variant form of HCP, harderporphryria, diagnosis is based on the marked increase in faecal harderporphyrin as well as coproporphyrin accompanied by clinical symptoms of jaundice, early onset of severe chronic haemolytic anaemia, hepatosplenomegalay and skin photosensitivity (Nordmann, Grandchamp, de Verneuil, Phung, Cartigny, & Fontaine 1983); (Lamoril, Puy, Gouya, Rosipal, Da, V, Grandchamp, Foint, Bader-Meunier, Dommergues, Deybach, & Nordmann 1998). In a study by Schmitt et al 2005, they showed for the first time that harderporphryric patients exhibit iron overload due to dyserythropoiesis.

Misdiagnosis of HCP in patients with other conditions is very common. In order to avoid incorrect diagnosis, it is important to note that a slight increase in urinary coproporphyrin can also result from conditions other than porphyria such as liver dysfunction. Hence, the slight increase in urinary porphyrin may be clinically insignificant (Anderson, Sassa, Bishop, & Desnick 2002). The use and development of highly sensitive assays for HCP diagnosis in combination with taking into account the clinical picture (if present) is important to make a correct and precise diagnosis.

1.13 Diagnostic methods

Early diagnosis and information about precipitating factors reduces the morbidity and mortality of patients with acute porphyrias. The complexity of the diagnostic process
makes analysis in specialised laboratories necessary. Infrequent handling of the
diagnostic procedures in laboratories lacking in knowledge, experience and technical
competence is repeatedly the reason for harmful under-diagnosis and over-diagnosis.

Initial diagnostic methods involve screening for urinary ALA and PBG whenever any of
the acute porphyrias are suspected. Most specialised laboratories use the methods
described by Mauzerall D and Granick S (1956) for measurements of urinary ALA and
PBG. Less specialised laboratories may use commercial semi quantitative kits for
urinary PBG such as the Trace PBG kit from Alpha Laboratories, Eastleigh, Hampshire,
UK. Analyses of urinary and faecal porphyrins are performed using reverse-phase
HPLC (Lim & Peters 1984). Blood and plasma porphyrin measurements are carried out
using fluorometry (Blake D 1992)

Total faecal porphyrins are best analysed using spectrophotometric methods
(Lockwood, Poulos, Rossi, & Curnow 1985). However, a better diagnostic marker for
HCP in clinically overt and latent adult patients is the faecal coproporphyrin isomer III:
Coproporphyrin I (CIII:I) ratio determined by HPLC (Blake, McManus, Cronin, &

Kondo et al (2004) revealed that as many as 71% of acute hepatic porphyria cases were
initially diagnosed as non porphyria, and later revised or corrected to porphyria,
indicating the difficulty of diagnosing porphyria in the absence of specific laboratory
testing for porphyrins and their precursors in urine, stool, plasma and erythrocyte
samples.

Reliable enzymatic assays for CPOX activity are not widely available (Anderson, Sassa,
Bishop, & Desnick 2002). Spectrophotometric methods to measure CPOX activity have
been described ( del Batlle AM FAU - Benson, Benson, & Rimington); (Poulson &
Polglase 1974), however, these assays are not sensitive to accurately measure CPOX
activity in small amounts of tissues such as human lymphocytes and cultured fibroblasts
(Anderson, Sassa, Bishop, & Desnick 2002). Another major setback to this method is
the poor reproducibility of the assay due to the degradation of protoporphyrinogen or
protoporphyrin during extraction. A more sensitive radiochemical assay has been
described (Elder & Evans 1978). This method is based on measuring the rate of
production of $^{14}$CO$_2$ from the radiolabeled substrate, $[^{14}$C] coproporphyrinogen III. A major problem that was encountered in this method was the difficulty to chemically synthesise the labelled substrate. Grandchamp and Nordmann (1977) further modified this method by enzymatically synthesising $[^{14}$C] coproporphyrin III from [4-$^{14}$C] ALA. Both methods are very sensitive and highly specific for the substrate. Another enzymatic method for CPOX activity by means of fluorometric assay has been reported (Labbe, Camadro, & Chambon 1985). In this method, the enzymatic activity is measured in the presence of a large excess of protoporphyrinogen oxidase. The excess protoporphyrinogen oxidase is supplied by yeast mitochondrial membranes isolated from commercial baker’s yeast.

Gene diagnosis of the carrier state in all types of acute porphyrias is of incomparable versatility and accuracy. However, despite great achievements in the molecular biology of porphyric disease, genomic procedures cannot replace biochemical methods in monitoring the activity and progress of the disease or the effects of therapy (Thunell et al. 2000).

In recent years, the diagnoses of porphyrias is being approached with molecular techniques such as DNA sequencing (Sassa, Kondo, Taketani, Nomura, Furuyama, Akagi, Nagai, Terajima, Galbraith, & Fujita 1997); (Schreiber, Zhang, Senz, & Jamani 1997); denaturing gel electrophoresis (Petersen et al. 2000); SSCP (To-Figueras, Badenas, Enriquez, Segura, Alvarez, Mila, Lecha, & Herrero 2005) and denaturing HPLC (Lam, Poon, Tong, Lo, Lai, Choi, Tiu, Chan, & Shek 2001). With the availability and advancements in current genomic procedures, mutation screening is highly recommended before puberty where a family history of porphyria is known, to correctly identify porphyric patients before their symptoms arise (Thunell, Harper, Brock, & Petersen 2000); (Kauppinen 2005).

1.14 Penetrance of the acute porphyrias

The low penetrance of the genetic defects in hereditary acute and mixed porphyrias and the extremely variable severity of disease manifestations make it difficult to establish
preventative measures. Therefore the identification of carriers in such uncommon disorders is very important to prevent acute attacks (Lecha 2006).

Due to the rarity of the acute porphyrias and their low penetrance, it has been difficult to carry out genotype-phenotype correlation studies on a large scale. The phenotype of the acute porphyrias varies even within families. Common polymorphisms in human metabolism can affect penetrance (Kauppinen 2005). Other factors that may play a role in the phenotypic expression are genes related to tissue specific uptake and excretion of porphyrins and their precursors from the liver and kidneys (Fraunberg et al. 2005). Since increasing numbers of patients with porphyrias are diagnosed by mutation detection, more precise penetrance rates, genotype-phenotype correlations, and predictive guidelines related to biochemical analyses can be established (Kauppinen 2005).

Gouya et al (2002) have demonstrated that in erythropoietic protoporphyrias the coinheritance of a ferrochelatase gene defect and of a wild-type low-expressed ferrochelatase allele is generally involved in the clinical expression of erythropoietic porphyria. In a later study by Goya et al 2004, they confirmed that the wild-type low-expressed allele is usually operative in the mechanism of variable penetrance in erythropoietic porphyria, but this is not the case in AIP and VP. However, the CPOX mRNA determinations strongly suggest that normal CPOX alleles with low-expression are present, but whether this low-expression of the wild-type allele could modulate the penetrance of a CPOX gene defect in HCP families remains to be ascertained (Gouya et al. 2004).

1.15 Prognosis/treatment

In the past, acute porphyrias were considered as inherited metabolic disorders with poor prognosis. However, currently we have a better understanding of these disorders and better diagnostic methods. With correct diagnosis, appropriate treatments can be provided to patients with acute hepatic porphyrias. Although appropriate treatments are
available, the prevention of clinical manifestations in asymptomatic carriers is the most important means of treatment of these disorders (Sassa 2006); (Anderson, Sassa, Bishop, & Desnick 2002). As an initial line of action, all precipitating factors should be identified (if possible) and avoided.

1.15.1 Supportive treatment

The most effective treatment for porphyria attacks is the administration of haem or haem arginate. Haem arginate can interrupt the progress of neurological symptoms that are characteristic of the acute porphyrias (ANDERSSON 2002). It addresses the underlying pathophysiology by suppressing ALAS1 activity (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005). Haem arginate is administered intravenously at a moderate dose of 3-4 mg/kg of body weight per day (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005).

Deybach et al. (2001) have confirmed the efficiency haem-arginate in the treatment of acute porphyrias. They suggested that haem-arginate might be partly catabolised or directly eliminated without the involvement of the haem oxygenase pathway.

Intravenous administration of carbohydrate (at least 300g/day) is considered as another effective therapy (Anderson, Sassa, Bishop, & Desnick 2002); (Sassa 2006) and has been the standard treatment for many decades (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005). In all four types of acute porphyrias, glucose administration on its own reduces urinary ALA, PBG and porphyrin excretion by about 50% to 90% (Gross, Hoffmann, & Doss 2000).

1.15.2 Symptomatic treatment

Symptoms such as pain, nausea and vomiting need appropriate treatment. Narcotic analgesic drugs such as meperidine or morphine are usually used for pain (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005); (Sassa 2006);
Other treatments (used as needed) are small to moderate doses of phenothiazine used for the management of nausea, vomiting, anxiety and restlessness symptoms (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005). Cholestyramine may be useful in HCP cases with photosensitivity and liver dysfunction (Hunter et al. 1971).

Management between attacks comprises adequate nutritional intake as well as avoidance of porphyrinogenic drugs and chemicals. Patients need education about the disorder and to identify the precipitating factors to help avoid future acute attacks.

Fatality rates from acute attacks of porphyria before 1970 ranged between 10%-52% (Kostrzewska & Gregor 1999). Since then, prognosis of acute porphyrias has improved dramatically. These improvements may be attributed to advancements in diagnosis, better treatments (availability of haemin after 1971), and improved preventative measures through recognition of harmful drugs (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005).

In the past, haem therapy was recommended only in cases where there was no response to intravenous glucose administration for several days (Anderson, Sassa, Bishop, & Desnick 2002). In a study by Jeans et al (1996), 12 of 86 patients who received a diagnosis after 1971 died after hospitalisation for acute attacks. Eleven of these patients received haemin after their attack had progressed to advanced stages and a great deal of damage had already taken place.

Current therapeutic recommendations for acute porphyrias recognise the benefits of the immediate use of haem therapy and urge physicians to initiate treatment with haem as soon as diagnosis is confirmed (Anderson, Bloomer, Bonkovsky, Kushner, Pierach, Pimstone, & Desnick 2005). Schuurmans et al (2001) emphasises the importance of providing comprehensive counselling to patients and asymptomatic gene carriers as well as effective treatments to reduce the frequency and severity of the acute attacks.
1.16 Purpose of this project

Currently porphyria patients are diagnosed in NSW using biochemical methods (such as fluorometry and spectrophotometry). If any of the acute porphyrias (AIP, VP or HCP) are suspected, an initial urinary screening test for ALA and PBG is carried out. PBG is a more accurate marker for the acute porphyrias, whereas screening for urinary ALA is carried out for completeness of the investigation procedures for the acute porphyrias. Screening of PBG is carried out using the resin method described by Mauzerall and Granick S (1956).

To date, a routine molecular genetic method has not been established in NSW. Most genetic methods so far have been used as part of research studies and are time consuming and labour intensive. However, with recent advances in molecular techniques, genetic screening has become possible.

In this study, the main aim was to establish a routine genetic screening method for the acute hepatic porphyrias. Due to the availability of HCP patients who volunteered to participate in this study, we designed a cascade model for routine genetic screening of HCP. This may aid in correctly diagnosing HCP patients especially asymptomatic carriers.

Genetic screening will also aid in detecting more mutations including novel as well as previously reported mutations. Once the method for HCP is established, it can be used for all other porphyrias. As genetic screening will increase the number of mutations detected in porphyrionic patients, it may also aid in carrying out genotype-phenotype correlations. However this has been difficult to achieve on a large scale due to the low penetrance and rarity of the acute porphyrias.

The porphyrias are rare inherited metabolic disorders exhibiting a low penetrance, thus, there is great variability in phenotypic expression even within families. Variability in phenotypic expression can be identified through questionnaires. Published standard
questionnaires aimed at identifying these phenotypic expressions in the acute porphyrias are lacking. A study by Lecha et. al. (2006) has been performed about genotype-phenotype correlations, where a questionnaire was used; however, they were not made available to other researchers. Another study (Fraunberg, Pischik, Udd, & Kauppinen 2005), obtained information concerning only acute attacks from hospital records and/or by interviewing patients.

A standard questionnaire aimed at identifying phenotypic expression in the acute porphyrias is a necessary step towards improving genotype-phenotype correlation studies. Making these questionnaires available to the public will aid in standardising the questionnaire. This is an essential step towards carrying out more precise correlation studies on a large scale.

There is also lack of knowledge in regards to genotype-phenotype correlations in the acute porphyrias. This study is aimed at addressing these issues by (1) initially setting up a routine genetic screening method for HCP patients; (2) designing a questionnaire to identify phenotypic expressions caused by mutations in the CPOX gene; (3) to identifying possible subtle clinical expressions experienced by patients considered as "latent carriers"; (4) looking at the correlation if any, between the disease causing mutation and the clinical symptoms within a family.

The Specific aims of this project were:

- To set up a routine genetic screening method to correctly diagnose porphyria patients.
- Set up a cascade model for routine screening of the porphyrias, and establish the method based on HCP due to the availability of HCP patients.
- To identify the disease causing mutations in 3 unrelated HCP patients.
- To screen the relatives of the index patients to identify latent carriers.
- To design a frequency and severity of symptoms questionnaire for the acute hepatic porphyrias.
• To look at the phenotypic spectrum of the disease causing mutations in HCP patients.

• To look at the variability of clinical expression in terms of frequency and severity within each family.

• To make this questionnaire available to the public in an attempt to make this as a first step towards standardising a questionnaire for the porphyrias.

• To look at genotype-phenotype correlations in this small sample cohort.

• To identify the trend of the phenotypic expressions caused by mutations in the CPOX gene.
CHAPTER 2: MATERIALS AND METHODS

2.1 Blood collection

Patients volunteered to take part in this study and had their blood collected at private pathology laboratories. Samples were then referred to the NSW Porphyria Reference laboratory at Royal Prince Alfred Hospital (RPAH). Blood samples were collected in 5ml EDTA tubes.

2.2 Sample storage and handling

Whole EDTA blood samples were frozen at -20°C until needed. DNA extracted from whole blood samples were kept refrigerated at 4°C until all analyses were completed. DNA samples were then kept at -20°C for long term storage and archiving.

2.3 Equipments, reagents and materials

All equipment and materials used were from The Molecular Genetics laboratory at the Children’s Hospital at Westmead (CHW), except for the DNA extraction procedure which was carried out in The Biochemical Genetics Department (CHW). All pipetting was carried out with air displacement pipettes using pre-sterilised aerosol resistant tips (ART) unless otherwise stated.
2.4 DNA extraction

2.4.1 Materials

QIAamp DNA Blood Mini Kit (QIAGEN Pty Ltd, Germany) (Cat no. 51104)
Ethanol (Ajax Finechem, Australia)
GeneQuant spectrophotometer (Pharmacia, UK-Cambridge)

2.4.2 Extraction method

DNA extraction was carried out using the QIAamp DNA Blood Mini Kit. This kit was selected because it was suitable for using fresh as well as frozen whole blood samples. Extraction was performed using the Blood and Body Fluid Spin Protocol as per manufacturer’s instructions (see pages 22-24 in kit manual). All steps were followed as described in the manual, with the exception of the optional step 9 which was not performed. For the very last step (step 10- see kit manual on page 24) DNA was reconstituted in 200μl AE buffer (elution buffer) instead of distilled water. DNA was then refrigerated at 4°C. The expected DNA yield using this kit was 3-12μg of DNA for every 200μl of whole blood.

DNA measurements were performed by carrying out a 1/50 dilution of the extracted DNA with sterile water (WFI; Water For Injection), then reading the absorbance at 260nm. DNA concentrations were then calculated by multiplying the absorbance value by 50 (1 absorbance unit = 50ng/μl). This value is then multiplied by the dilution factor (in this case 50) to give the final DNA concentration in ng/μl.
2.5 Primer design

Primers were designed specifically to cover all exonic and intronic regions of the CPOX gene and 50bp flanking sequence on either side of the CPOX gene sequence. Primers were designed using the ExonPrimer release version 0.9 found at the following website (Strom 2004). http://ihg.gsf.de/ihg/ExonPrimer.html.

Primers were designed such that they had similar melting temperatures. This helped set up one PCR thermal profile using the same optimal temperature for all the exons/introns to be screened. This method was used successfully for all exons except for exon 1 which could not be amplified by this methodology. A GC-Rich kit was used to amplify exon 1 (2.9 GC-Rich PCR for exon 1).

Table 2.1 represents a summary of the primers designed for CPOX gene. Primers were designed such that it covered all exonic and intronic regions of the gene. Capital letters represents exonic regions while small letters represents intronic regions. The black fonts were all generated by the ExonPrimer website. The red font represents a manual addition of base pairs in order to make the primer more specific for that region of the gene.
<table>
<thead>
<tr>
<th>EXON</th>
<th>PRIMER SEQUENCE (5'-3')</th>
<th>MELTING TEMPERATURE (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Forward – TCGAAAGGTTCCTTTCGCC</td>
<td>62.53</td>
</tr>
<tr>
<td>1a</td>
<td>Reverse - TGCACATGCCCGAAGGC</td>
<td>65.58</td>
</tr>
<tr>
<td>1b</td>
<td>Forward - AGAGGGCGGCCCCCTGGGTGGGACAG</td>
<td>64.9</td>
</tr>
<tr>
<td>1b</td>
<td>Reverse - GCAACCTGGAACCTGACCCTTT</td>
<td>65.75</td>
</tr>
<tr>
<td>2</td>
<td>Forward - ACCATCTTCATGCAAGTGTGCTTTT</td>
<td>65.17</td>
</tr>
<tr>
<td>2</td>
<td>Reverse - aaataaaaCTTGTGGGCCAAATAAGGT</td>
<td>62.27</td>
</tr>
<tr>
<td>3</td>
<td>Forward - CAGCTTTTGAAAAATTGACTTGGAAAT</td>
<td>62.84</td>
</tr>
<tr>
<td>3</td>
<td>Reverse - GCCTTTACATTGCCCTCTGAAAGAC</td>
<td>65.32</td>
</tr>
<tr>
<td>4</td>
<td>Forward - GCCTAGGCCCTTACTGCTTCAGAG</td>
<td>64.84</td>
</tr>
<tr>
<td>4</td>
<td>Reverse - CCCACTCCAGATTTAAAAAGCTCTCTC</td>
<td>64.58</td>
</tr>
<tr>
<td>5</td>
<td>Forward - TGCTGTAACCTGAAAGGCTCAGATT</td>
<td>65.24</td>
</tr>
<tr>
<td>5</td>
<td>Reverse - ATCTGACACAACAACACCGCTATT</td>
<td>64.97</td>
</tr>
<tr>
<td>6</td>
<td>Forward - GGGAGAACCACCACCCTTTATAGT</td>
<td>66.28</td>
</tr>
<tr>
<td>6</td>
<td>Reverse - GCAGTGTTAACTTTATATTTGTGGG</td>
<td>62.97</td>
</tr>
<tr>
<td>7a</td>
<td>Forward - AAAGGTCAGTTGCTACAGGCACA</td>
<td>65.03</td>
</tr>
<tr>
<td>7a</td>
<td>Reverse - ACAAAACATGGTCCCACAAATAAATC</td>
<td>63.54</td>
</tr>
<tr>
<td>7b</td>
<td>Forward - ATGAATGGTGAGATGGCAGATTGTC</td>
<td>65.54</td>
</tr>
<tr>
<td>7b</td>
<td>Reverse - TGTATGAAATGGCCTCAAGTTCTCA</td>
<td>64.99</td>
</tr>
<tr>
<td>7c</td>
<td>Forward - TGAATGTGAAGGCTTTATATCCCTGAA</td>
<td>62.17</td>
</tr>
<tr>
<td>7c</td>
<td>Reverse - ggccctaccccttaatgggttc</td>
<td>65.93</td>
</tr>
</tbody>
</table>

Newly designed primers were be checked against the human genomic DNA sequence in order to make sure that the primer a matching sequence on the desired gene. This was done using the BLAT search feature available at the Human Genome Browser website at http://genome.ucsc.edu/. (Genome Bioinformatics Group 2004).
2.6 BLAT search results

The BLAT feature, confirmed that the designed primer sequences did have a matching sequence on the genomic DNA and on the gene of interest. This feature also aids in rating the specificity of the primer for the desired region of the gene. When a primer returns too many sequence matches on the genomic DNA (either due to high GC content or short primer sequence), it can cause multiple non-specific binding to regions of the DNA.

All primers matched except for exon 1b forward. Exon 1b (forward) was manually modified by adding eight base pairs from the 5' end (as indicated by the red font in table 2.1). The modified primer was then checked against the genomic DNA returning a match for the desired region of the CPOX gene. Once primers were checked against the genomic sequences, and their matching sequence was confirmed using the BLAT search, they were ordered from Sigma-Genosys, Australia.

2.7 Preparation of working primers

Stock primers were diluted 1/10 giving a concentration of 10μM in WFI and made up to 200μl total volume. The diluted primers were then used for PCR and sequencing. Both Stock and diluted primers were kept frozen at -20°C until use.

2.8 PCR

2.8.1 Materials

All PCR materials were from Applied Biosystems-Roche (NJ- US) unless otherwise stated.

10x PCR buffer II
MgCl solution (25mM)
Ampli Taq Gold™ (250 units, 5U/μl)
Deoxynucleotides (dNTPs) (2mM of each dATP, dTTP, dGTP, dCTP) (Bioline Pty Ltd, Australia).

WFI (Water For Injection; Astrazeneca)

Primers (10µM forward and reverse; Sigma-Genossys, Australia) (table 2.1 for primer sequence)

2.8.2 Method

Initially three index patients from unrelated families were screened. The PCR cocktail consisted of 2.5µl 10x buffer II, 2.5µl MgCl, 2.5µl dNTPs, 14µl WFI. 22µl of the cocktail was added to each new PCR tube containing 1µl forward primer, 1µl reverse primer and 1µl DNA sample from the index patient. Each PCR tube contained primers for a specific fragment of the CPOX gene. The total volume of each PCR tube was 25µl.

In the case of screening the rest of the family members, only fragments of interest were amplified. At this point, forward and reverse primers for exon 5 were included in the PCR cocktail before aliquoting into multiple new PCR tubes, then the volume was made up to 25µl by adding 1µl DNA. PCR tubes were then placed in a thermal cycler (Mastercycler; Eppendorf, Germany) using a pre-programmed cycle of 94°C for 5min, {94°C for 30sec; 57°C for 30sec; 72°C for 30sec} x 35 cycles; 72°C for 5min then held at 4°C until samples were removed.

This method was used to amplify all exons of the CPOX gene except for exon 1 which could not be amplified. Exon 1 has a high GC content, which makes it difficult if not impossible to amplify that region of the DNA using the standard PCR reagents and thermal profiles. To amplify exon 1, a GC-Rich PCR kit had to be used with thermal profiles different to that mentioned above.
2.9 GC-Rich PCR

2.9.1 Materials

GC Rich resolution solution (Roche Diagnostics, Germany)
5x Reaction buffer (Roche Diagnostics, Germany)
Deoxynucleotides (dNTPs) (2mM of each dATP, dTTP, dGTP, dCTP) (Bioline Pty Ltd, Australia).
WFI (Water For Injection; Astrazeneca)
Enzyme mix 2U/µl (Roche Diagnostics, Germany)
10µM forward and reverse primers (Sigma-Genosys, Australia)

2.9.2 Method

The GC rich PCR reaction was prepared using 4µl GC rich resolution solution, 4µl of 5x reaction buffer, 2µl of dNTPs, 0.4µl enzyme, 1µl forward primer, 1µl reverse primer, 6.6µl WFI and 1µl DNA. Samples were run on the Eppendorf Mastercycler (Germany) using a pre-programmed cycle. The program consisted of 95°C for 12 minutes, then 40 cycles of 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 30 seconds. Once the 40 cycles were completed, there was 5 minutes at 72°C then held at 4°C.

2.10 Polyacrylamide Gel Electrophoresis (PAGE)

2.10.1 Materials

Ammonium persulfate (APS) 10% (w/v) (Sigma-Aldrich, Germany)
Temed (Astral Scientific, Australia)
Acrylamide/Bisacrylamide 29:1 (40%) (Amresco, USA)
Tris-Borate EDTA (TBE) buffer (1x solution: 0.089M Tris base, 0.089M borate, 0.002M EDTA) (Amresco, USA)
Loading Dye (Pharmacia, Sweden)
HypperLadder V, quantitative marker (Bioline Pty Ltd, Australia)
Ethidium Bromide (2µg/mL; anhydrous Mol. Wt. 394.3) (Sigma-Aldrich, Germany)
Mini Protean II Cell and associated gel apparatus (Bio-rad Laboratories, Australia)
Electrophoresis documentation and analysis system 120 (Eastman Kodak, USA)

2.10.2 Reagent preparation

2.10.2.1 Ammonium persulfate (APS) 10% (w/v)

Weighed 1g of ammonium persulfate then made up to 10ml with water and mixed. This APS is stable for two weeks at 4°C.

2.10.2.2 10% Acrylamide in 1xTBE

Combine 125mL Acrylamide/Bisacrylamide 29:1 (40%) with 125mL 4x TBE then make up to 500mL with water.

2.10.2.3 Tris-Borate EDTA (TBE) buffer

10x Ready Pack was made up to 1L with water to make 10x solution. 250mL (10x solution) was made up to 1L with water to make 4x solution.

2.10.2.4 Loading dye

Weighed 2g of Ficoll 400 and placed in a 15mL falcon tube. One mL 0.5M EDTA (pH 8.0) was used to wash down 2mg of bromphenol blue (sigma-Aldrich, Germany) into the falcon tube. Also, 2mg of Xylene Cyanol (Sigma-Aldrich, Germany) was weighed and washed into the falcon tube with the same manner. 500μl 20% dodecyl sulfate was added then the solution was made up to 10mL with water. Solution was mixed gently by inversion. When ficoll was completely dissolved, water was added to refill to 10mL and mixed by inversion.
2.10.2.5 *Ethidium bromide* (2μg/mL)

200μl stock ethidium bromide (5μg/mL) was made up with water to 500mL to get a final concentration of 2μg/mL.

2.10.3 Method

The electrophoresis gel was made with 14mL of 10% PAG, 140μl of 10% APS and 20μl Temed. This solution was briefly mixed then transferred into the plates. 3μl of the PCR product was mixed with 3μl of the loading dye, the mixture was then loaded into a predetermined well. Once all samples were loaded, the lid was placed on the tank and run for 25min at 200V. At the end of the run, the gels were removed and stained in ethidium bromide solution for 2min. Ethidium bromide was then poured back into its storage bottle for later use and the gels were rinsed in tap water and viewed under UV light. A digital photograph was taken using Eastman Kodak documentation system.

2.11 PCR product treatment

Prior to sequencing of PCR products, they were treated with Shrimp Alkaline Phosphatase (SAP) to degrade any residual PCR primers and dNTPs that could interfere with the DNA sequencing reaction.

2.11.1 Materials

Exonuclease I (*ExoI*) (20U/μl) (Roche Diagnostics, Germany)

Shrimp Alkaline Phosphatase (Roche Diagnostics, Germany)
2.11.2 Method

0.5\mu l of ExoI and 1.0\mu l of SAP were added to a fresh PCR tube for each sample. 7.5\mu l of the PCR product was added, mixed and placed in a thermal cycler (GeneAmp PCR System 2400; Perkin Elmer, Australia) on the SapExo pre-programmed file. The thermal profile for this program was 37°C for 59min, 80°C for 15min and held at 4°C.

2.12 Preparation of treated PCR product for sequencing

2.12.1 Reagents

10\mu M forward and reverse primers (Sigma-Genosys, Australia )
WFI (Water For Injection; Astrazeneca)
SAP treated PCR samples

2.12.2 Method

Duplicate samples were prepared for each exon, one containing the forward primer and the second sample containing the reverse primer. 1\mu l of primer (10\mu M) and 3\mu l of WFI was added to the SAP treated PCR product mixed and stored at 4°C until transported to SUPAMAC laboratory at Royal Prince Alfred Hospital via the RPAH courier system.

2.13 Sequencing

Sequencing of samples was carried out by staff at the SUPAMAC laboratory at RPAH. A request form and an identification number were assigned to each sample to be sequenced.
2.13.1 Equipments and reagents

ABI PRISM® 3730XL DNA analyser; 50 cm 96 Capillary array (Applied Biosystems)
BigDye Terminator® v3.1 Cycle Sequencing kit (Applied Biosystems,)
BigDye Terminator mix (thermostable DNA polymerase, dNTPs, ddNTPs and buffer)
Spin Columns (Sephadex G-50) (Sigma-Aldrich, Germany)

2.13.2 Sample preparation method

The BigDye Terminator® v3.1 was diluted 1/8 and 4μl of the diluted Dye was added to 8μl of the treated PCR products. This mixture was then placed in the thermal cycler. The thermal cycling profile consisted of 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Prior to injecting the cycled mixture into the sequencing analyser, the samples were cleaned from any excess BigDye Terminator mix. This step was carried out by spinning the samples in a plate of spin columns.

The ABI PRISM® 3730 DNA analyser can automatically analyse multiple runs of 96 samples. The plates containing processed samples were placed on the deck of the instrument and the run commenced. The autoloader transferred the samples to the loading bar in the electrophoresis chamber. The samples are electro-kinetically injected into thin, glass capillaries that are filled with a stationary polymer. Electrophoresis began when voltage was applied across all capillaries. The DNA fragments migrated through the polymer with shorter fragments moving faster than longer fragments. When the samples reached the end of their capillaries, they were carried across a transparent cuvette by moving polymer that flows over the ends of the capillaries (called sheath flow). A CCD camera captured the fragments as they moved through the path of the laser beam and fluoresced. The CCD camera converted this information into electronic information that was transferred to the computer workstation for processing by the 3730 Data Collection Software. The data was displayed as an electropherogram which plotted relative dye concentration against time for each of the dyes used to label the DNA fragments. Each peak in the electropherogram represented a single fragment (Supamac Staff 2007). (http://www.supamac.com/content/index.php)
2.14 Sequencing analysis

2.14.1 Materials

Chromas 2.23 Software (Technelysium Pty Ltd, Australia)

- Blast (NCBI – National centre for Biotechnology Information, a feature included in the Chromas Software)
  

2.14.2 Method

Initially, the sequence was viewed using the Chromas software. The chromatogram was scanned for dye blobs or non specific interferences. A Blast search was performed through the Chromas software to match the patient’s sample sequence with the draft human genome sequence (NCBI reference sequence). Any mismatches were flagged indicating a mutation or a polymorphism.

Sequence variations that were found through the Blast search were then assessed for polymorphisms or pathogenicity. These variations were searched against the HGMD or literature to see whether they have been previously reported or not. If the variation identified has not been previously reported, further investigations were carried out to determine its pathogenicity.

The Human Gene Mutation Database (HGMD) represents collated published gene lesions responsible for human inherited disease. This database embodies an up-to-date and comprehensive reference source to the spectrum of inherited human gene lesions (for more details visit website http://www.hgmd.cf.ac.uk/ac/index.php).

HGMD does not usually include mutations lacking obvious phenotypic consequences although a few such variants have been included where they could conceivably have some clinical effect (e.g. albumins, butyrylcholinesterases).

The predicted effect of the mutation on the protein was performed using the PolyPhen prediction website at http://genetics.bwh.harvard.edu/(pph/)
PolyPhen (Polymorphism Phenotyping) is a tool which predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. Conservation analysis at the protein level was also performed using PolyPhen (Sunyaev & Adzhubey 2006).

The Exonic Splicing Enhancer (ESE)finder (release version 2) accessed at (http://rulai.cshl.edu/tools/ESE2/) was used to determine whether the mutation identified lies at the specific site at which splicing of an intron takes place during the processing of precursor messenger RNA into mature messenger RNA. The abolishment of the splicing site results in one or more introns remaining in mature mRNA and may lead to the production of aberrant proteins (Cartegni et al. 2003).

Conservation analysis at the nucleotide level was performed using the UCSC Genome Bioinformatics site at http://genome.ucsc.edu/ (Genome Bioinformatics Group 2003 270 /id). Last release version accessed was v.128.

2.15 Questionnaire

A questionnaire was necessary to study the genotype-phenotype correlation. Literature searches and searches performed on the internet for a standard questionnaire that was specific for porphyria were not found. A questionnaire was then designed in order to carry out the genotype-phenotype correlation studies.

2.15.1 Designing the questionnaire

Intensive literature searches on questionnaires were performed. Standard general health questionnaires such as SF-12 and SF-36 (Bowling 1997); (- Watson EK FAU - Firm et al.) were reviewed. A paper on how to write and analyse a questionnaire was also reviewed (Williams 2003) Experts (Prof. Bruce Lord-from CHW) in regards to designing questionnaires were consulted. A statistician (Prof. Jenny Peat-CHW) was
consulted to ensure that the questionnaire could be converted to useful statistical data for analysis. The questionnaire was designed such that it would not take more than 20 min to complete, taking into consideration that it had to be very simple and easy to read and understand by any person without a science background. The questionnaire was designed to cover all acute hepatic porphyrias and not only HCP.

Two versions were created for the questionnaire, one for adults over the age of 16, the other for children between 6 years of age up to 16 years of age. The questionnaire consisted of six categories. The first 5 categories were regarding common porphyria symptoms which included abdominal symptoms, peripheral neuropathies, skin symptoms and psychological symptoms. The final two categories included questions regarding life style which might help us understand the effect of, or recognise environmental factors that can play a role in the expression and severity of the phenotype. These included questions regarding medications, smoking, and diet and alcohol consumption. The alcohol questionnaire, which was at the end of the “adult” version, was obtained from the Alcohol Use Disorders Identification Test (Babor et al. 1992).

Questions were designed such that it covered one year recall periods, while some questions were designed to cover life time recall periods. Most questions had two parts, the first one relating to frequency and the second relating to the severity of the symptoms. Frequency questions were placed into 5 categories, and severity questions were placed into 4 categories. The life style category involved mostly yes or no answers. See appendix for a sample of the questionnaire.

A template of the information sheet for participants was modified and submitted along with the questionnaires. Initially, participants were contacted by telephone and were given a code number (which was their DNA number) as a mean of identification ensuring the privacy of patients. They were also notified that within a few days of this phone call, they will receive a parcel containing the questionnaire, information sheet as well as the consent forms. They were advised to send the completed consent forms (if willing to participate) first, then send the completed questionnaire with their identification number. Questionnaires, information sheets and consent forms were all sent out by mail to each participant. Participants were provided with two pre-stamped
envelopes to return their completed consent forms and their completed questionnaire separately. A checklist sheet was also created for the participants stating how many questionnaire and consent forms and which versions they should expect in their parcel.

2.16 Denaturing HPLC (dHPLC)

I attempted to apply dHPLC to screen for the family with HCP based on the index patient. This method was chosen because it has the most sensitive physical mutation screening method that requires the least amount of sample preparation (Premstaller & Oefner 2003); (Xiao & Oefner 2001).

2.16.1 Materials

PCR products
Helix System comprising Autosampler (proStar 520), Oven (ProStar 310), Pumps A and B (ProStar 210) and computer with Star Software v.6.2 (Varian Inc.)
Helix analysis columns (3.0m ID x 50mm length, packed with C18 alkylated silica)
Varian Buffer Pak A (100mM TEAA, pH 7.0, 0.1mM EDTA)
Varian Buffer Pak B (100mM TEAA, pH 7.0, 0.1mM EDTA 25% acetonitrile)

2.16.2 Sample preparation method

PCR products need to be denatured and slowly re-annealed to form heteroduplexes. A pre-programmed thermal profile was specifically used for this purpose on the Hybaid (UK-Middlesex) thermal cycler. The thermal profile is set to 95°C for 10 minutes; 95°C for 1 minute (-1°C per cycle for 20 minutes).
2.16.3 Optimal temperature determination

DHPLC is a temperature-dependent method, so optimal temperatures needed to be determined in order to achieve good chromatographic separation. This was done by submitting the sequence for CPOX exon 5 via the internet to http://insertion.stanford.edu/melt. This website allows you to calculate the melting temperature of the entire sequence you submit and a recommended temperature.

The dHPLC procedure was performed by laboratory staff from the Molecular Genetics Department at CHW. Samples were run at temperatures ranging from 50 - 61°C, in order to obtain the optimum chromatographic separation. Multiple cluster analyses were performed such as samples with mutation and polymorphism, samples with polymorphism or mutation only. Cluster analysis was performed to detect a pattern in the chromatographic separation between samples and be able to distinguish samples with mutations and without mutations.

2.17 Qualitative analysis

Raw data collected from questionnaires were entered into the SPSS v13.0 statistical software. All sequencing results were also included. Due to the small sample size, when performed qualitative analysis, categories for the frequency had to be recoded. Category 1 was coded as “0” and categories 2 to 5 were combined as one category and renamed as “1 or more times a year” where it was coded as “1”. Questions that were left blank were given the code “0”. Males were coded as “0” and females were coded as “1”. Children between 6 and sixteen were coded as “0”, and patients that were 16 years or over were coded as “1”. Questions that involved “Yes” and “No” answers were coded as “1” and “0” respectively.

SPSS software (version 13) was used to perform frequency analysis of the completed questionnaires as well as “cross tabs”. Cross tabs, compares two or more variables at the same time. Cross tab analysis was performed such that it compared gender, mutation status, frequency of symptoms and severity at the same time.
CHAPTER 3: RESULTS

3.1 PCR and sequencing results

HCP is an autosomal dominant disorder resulting from mutations in the CPOX gene. Most mutations are private making the disorder highly heterogenous. In this study, we attempted to identify the disease causing mutation in three unrelated, previously diagnosed HCP patients followed by screening the rest of the family members once the mutation was identified.

Index patient 1 had normal urinary porphyrins and PBG; however, total faecal porphyrins were about 8 fold higher than the reference range (table A & B, appendix 3). Faecal coproporphyrin was elevated and a lower percentage of faecal protoporphyrin was reported. Diagnosis of HCP was based on these results. Faecal isomer CIII: I ratio was not performed as results were convincing that patient has HCP. This is based on the fact that the only coproporphyrin was increased in faeces.

The diagnosis of HCP for index patient 2 was made by another laboratory based on the biochemical results obtained (table A & B, appendix 3). Patient 2 had slightly elevated urinary porphyrins and coproporphyrins. Faecal porphyrin was markedly elevated. Faecal coproporphyrin and protoporphyrin were both markedly equally increased. Faecal coproporphyrin isomer III:I ratio was fifteen times higher than the reference range. The diagnosis of HCP for index patient 2 was based on the marked increase in the faecal isomer CIII: I ratio which if greater than 5.0 generally exclude VP. Plasma porphyrin was not performed to further confirm VP.

Index patient 3 presented at the age of 26 with bullous lesions in sun exposed areas of the skin. Biochemical analysis (table A & B, appendix 3) revealed increased total porphyrin in urine, normal urinary PBG, increased total faecal porphyrin with increased coproporphyrin species (99%). Plasma and blood porphyrins were also elevated. The diagnosis of HCP was based on clinical symptoms and further supported by the above mentioned biochemical results.
Initially, screening of the entire CPOX gene was performed on the three known unrelated index patients. DNA samples from all three HCP probands were amplified for the entire CPOX gene including 100bp flanking the CPOX gene from the 5' prime and the 3' prime end. Index patient 1 showed a mutation in exon 1 (Figure 3.1). This mutation causes a substitution in amino acid 134 from Proline to Histidine (p. P134H). Based on PolyPhen analysis, this mutation is predicted to be "benign". This mutation has not been previously reported, and could possibly be a novel single nucleotide polymorphism (SNP). The disease causing mutation was not found in this patient; hence, no further screening was done on other members of this family.

Figure 3.1: Chromatogram showing a novel SNP detected in index patient 1.
The SNP indicated by the arrow is a benign mutation causing a change in amino acid 134 from Proline to Histidine. This SNP is caused by a C>A transversion at nucleotide c.401.

No mutation or SNP was found in index patient two. However, index patient three showed a mutation in exon 5. The mutation in exon 5 is caused by an A>C transversion at nucleotide c.1064 (Figure 3.2). This mutation causes a glutamine to proline substitution at amino acid 355 (p.Q355P). The change from an aromatic amino acid to a hydrophobic one could possibly affect the structure of the protein. For comparison, a normal chromatogram from patient 11 is also included (Figure 3.3). Also, a SNP was detected in exon 5 in index patient 3. This SNP is an A>G transversion at nucleotide c.990 causing no change in amino acid (Figure 3.4). This is a previously reported SNP (HGMD).
Figure 3.2: Chromatogram showing the position of the Q355P mutation

A chromatogram showing the heterozygous p.Q355P mutation found in index patient three as indicated by the arrow.

I have not submitted the Q355P mutation to the HGMD database as the phenotypic expressions are variable, and the effect of this mutation on the structure and function of the protein needs to be studied.

To be included, there must be a convincing association of the polymorphism with the phenotype. These polymorphisms are currently identified in the database by an addition to the phenotypic description. These additions are limited to "association", "association with" and "increased" or "lower" "risk", depending on how the polymorphism was reported.
Figure 3.3: A normal chromatogram from exon 5 of the CPOX gene.
A normal chromatographic pattern obtained from exon 5 of the CPOX gene of patient 11.
Arrow indicates the position of the wild type allele at c.1064.

Figure 3.4: Chromatogram showing the position of the single nucleotide polymorphism in exon 5 of the CPOX gene found in index patient three.
The polymorphism as indicated by the arrow is an A>G transversion at nucleotide c.990 causing no change in amino acid.
Amino acid conservation analysis showed that the glutamine at position 355 is relatively conserved across mammalian species except for rat and mouse (Figure 3.5). PolyPhen analysis predicted this mutation to be “probably damaging” to the protein based on alignment structure. However, it did not recognise the type of effect it could have on the protein. Results obtained from the ESE (Exonic Splicing Enhancer) finder website indicated that the p.Q355P mutation is not a splice site mutation. Based on PolyPhen prediction and conservation analysis, we screened twenty-nine members related to index patient three for the mutation p.Q355P in exon 5 of the CPOX gene.

Figure 3.5: Multiple sequence alignments across some mammalian species. Arrow showing the position of amino acid 355 in the CPOX protein sequence.
Sequencing analysis of the rest of the family members revealed that 15 out of 29 patients had the Q355P mutation (Table 3.1). All patients had the SNP (A>G at c.990) except for patient 20. Nine out of twenty nine patients were homozygous for this polymorphism. A second polymorphism was detected in patients 2, 5 and 7 (Table 3.1) at nucleotide c.1054C>T (Figure 3.6). This C>T transversion causes a benign change in amino acid 352 from arginine to cystine. This is a previously reported SNP and is not a disease causing mutation (HGMD).

![Chromatogram of second polymorphism in CPOX gene](image.png)

**Figure 3.6: Chromatographic representation of the second polymorphism in exon 5 of the CPOX gene.**

Chromatogram obtained from DNA sample of patient 2, shows the second polymorphism at c.1054C>T as indicated by the arrow. This SNP causes a benign change in amino acid 352 from arginine to cystine.
Table 3.1: Summary of sequencing results obtained for all members related to index patient 3.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Patient</th>
<th>Gender</th>
<th>Age(^\text{a}) (years)</th>
<th>Mutation</th>
<th>Poly 1 at c.990</th>
<th>Poly 2 at c.1054</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3(^f)</td>
<td>M</td>
<td>88</td>
<td>N</td>
<td>Y</td>
<td>N</td>
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<td>6</td>
<td>F</td>
<td>61</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>M</td>
<td>70</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
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<td>8</td>
<td>F</td>
<td>69</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>F</td>
<td>61</td>
<td>N</td>
<td>Y (Homo)</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>M</td>
<td>87</td>
<td>N</td>
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<td>N</td>
</tr>
<tr>
<td>1</td>
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<td>Y</td>
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<td>N</td>
</tr>
<tr>
<td>2</td>
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</tr>
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<td>40</td>
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<td>Y</td>
<td>N</td>
</tr>
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<td>37</td>
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<td>Y (Homo)</td>
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<td>N</td>
</tr>
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<td>11</td>
<td>F</td>
<td>18</td>
<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
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<td>N</td>
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<td>N</td>
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<td>F</td>
<td>5</td>
<td>N</td>
<td>Y</td>
<td>N</td>
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<td>19</td>
<td>F</td>
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<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>M</td>
<td>11</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>F</td>
<td>13</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>M</td>
<td>9</td>
<td>Y</td>
<td>Y (Homo)</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>24(^f)</td>
<td>M</td>
<td>3</td>
<td>Y</td>
<td>Y (Homo)</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>25(^f)</td>
<td>M</td>
<td>5</td>
<td>Y</td>
<td>Y (Homo)</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>F</td>
<td>15</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>M</td>
<td>8</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>M</td>
<td>13</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
</tbody>
</table>

\(^{a}\) The age of the patients at time of the questionnaires.

\(^{*}\) Index patient 3.

\(^{\#}\) Excluded from questionnaire study.

\(^{f}\) Deceased.

Patient 3 is the father of index patient, porphyria is inherited from mother’s side.

Generation 1 related biologically to mother are 6 people. From generation 1, 50% have the mutation, 71% in generation 2 and 50% in generation 3. Number of subjects in generation 3 was almost double that in generation 1 and 2. Generation 1 and 2 had the same number of subjects. This table shows that there are no major differences in the mode of inheritance between the generations. This mutation is inherited at 50% through each generation.
3.2 Denaturing HPLC (dHPLC) results

A dHPLC method was designed using samples from this family in an attempt to set up a routine screening method for HCP. The attempt to screen for the Q355P mutation using dHPLC as a screening method failed. A distinctive pattern could not be obtained from samples with the mutation as compared to samples without the mutation (Figure 3.7).

A temperature gradient ranging from 50 - 61°C did not seem to resolve this problem. Comparing patients that were positive for the mutation against those who were negative did not highlight any differences in the peaks obtained from these samples. Multiple other comparisons were performed resulting in no differences between the peaks. The dHPLC method did not deliver its purpose of mutation detection in this study; the possible reasons will be further discussed in the discussion. The method was discontinued and preceded with re-sequencing the samples for confirmation.
Figure 3.7: Chromatographic representation of dHPLC results comparing four patients with different genotypes.

Each colour represents a patient’s sample that was processed for dHPLC. The x-axis represents the elution time, the y-axis represents the voltage. At the top left hand side, is a box representing the legend, the legend is colour coded and each colour corresponds to one patient. Each peak represents a patients’ result and is colour coded to correspond to the details in the legend. Four samples were compared at the same time in order to pick out a distinctive pattern based on different genotype characteristics. The purple peak represents patient 17 who had no mutation but was homozygous for polymorphism 1. The red peak represents patient 26 who has the mutation and is homozygous for polymorphism 1. The green peak is patient 14 who is normal and is heterozygous for polymorphism 1. Patient 15 is represented by the orange peak. He has the mutation and is homozygous for polymorphism 1.

3.3 Descriptive statistics - frequency analysis

Male to female ratio was 1:1 for genotype studies, however, only fourteen females and twelve males were included for phenotype studies. Children under the age of 6 years were not included in the questionnaire study; however they were screened for the Q355P mutation. Patients were grouped according to their age into three separate groups (Table 3.2). The mean age for males was 33 years, ranging from 3-88 years and for females the mean age was 30 years with a range of 5-69 years.
Table 3.2: Frequency of males and females who participated in this study.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Frequency of Males</th>
<th>Frequency of Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child &lt;6 years</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>6≤ child ≥16</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Adult &gt;16 years</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

The frequency of males and females who participated in this study as categorised into three different age groups.

Table 3.3 illustrates the frequency of males and females with the mutation as categorised into three different age groups. The ratio of males: females with the mutation are 1:1. All females with the mutation were included in the questionnaire study, whereas two males who had the mutation were under the age of 6 years, hence they were excluded from the questionnaire.

Table 3.3: Frequency of males and females with the Q355P mutation.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Males with mutation</th>
<th>Females with mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child &lt;6 years</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>6≤ child ≥16</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Adult &gt;16 years</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

3.4 Questionnaire and statistical analysis results

Thirty patients participated in this study. Four patients were excluded from the questionnaire studies as one patient was deceased, and the other three were under the age of 6. Twenty six participants returned their completed consent forms and questionnaires. Of these twenty six participants, fourteen were females and twelve were males. The small size of the cohort prevented a significant correlation being made. However, a summary of the observations based on genetic results and completed questionnaires is reported in section 3.5 (Table 3.6).
90% of the patients reported at least one abdominal symptom. Pain in the upper body and lower limbs was reported by 30% of the patients, while muscle weakness was reported by 17%. Partial paralysis was reported by three patients, two of whom had the Q355P mutation. Skin symptoms reported in this family were blistering of the skin in sun exposed areas (13%) and wearing away of the skin in sun exposed areas (10%).

Very few patients experienced the psychological symptoms commonly associated with HCP. Anxiety was experienced by 13% of the patients, while paranoia was experienced by 10%. Two patients suffered depression which lasted 2-3 months, one of whom also suffered prolonged anxiety and decreased appetite. However, the other patient experienced audio and visual hallucination as well as lack of orientation with time and space. This patient also described his abdominal pain as “sharp stabby pain” which occurred one to three times a year. Epileptic fits were reported by three patients, however only one patient reported to be on anti-epileptic medications.

Six categories of medications were reported by this family. These included medications for blood pressure (10%), lowering cholesterol (10%), anti-coagulants (7%), asthma (13%), pain (mainly muscle)/migraine (10%) and over the counter drugs (13%). Most patients who were over the age of 16 years, reported that they rarely drink alcohol (once or twice a month), and never exceed more than six drinks on one occasion.

In this study, 2 females and 2 males who have the mutation had not reached puberty at the time of the questionnaire (patients 4, 19, 23 and 28, respectively; table3.1). These patients did not report any porphyria symptoms.

3.5 Phenotypic expressions resulting from the Q355P mutation

The results presented here are based on my observations of the completed questionnaires and sequencing results. The frequency and severity of symptoms in the questionnaire were taken into consideration so that the criteria for inclusion as “true” porphyria symptoms were such that patients must have experienced the reported symptom one or more times a year with at least moderate to severe intensity. Symptoms
that were rated as moderate or severe but infrequent (i.e. 1-3 times a year) were included in our analysis. This pattern was noted frequently in patients with the mutation. However, if patients reported a particular symptom as slight and infrequent, they were not included in the analysis for that particular symptom regardless of mutation status.

Table 3.4 summarises the common porphyria symptoms reported by the participants in this study. Based on the above mentioned criteria, we observed that more females experienced porphyria symptoms than males. The most common symptom reported by females was constipation, while males reported colicky abdominal pain. These data were based on observations of patients who experienced moderate to severe porphyria symptoms one or more times a year. However, one female (patient 9, Table 3.1) was excluded from the muscle pain and weakness category from the questionnaire as she suffers from fibromyalgia.

Table 3.4: Common porphyria symptoms reported by members who participated in the questionnaire study.

<table>
<thead>
<tr>
<th>Common Porphyria Symptoms</th>
<th>Males n=12</th>
<th>Females n=14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colicky abdominal pain</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Vomiting</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Nausea</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Constipation</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Upper body pain</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Pain in arms</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Pain in lower legs</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Muscle Weakness</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 3.8 illustrates the main symptoms reported in this study by all 26 patients who participated in this study. It was observed that the number of females who reported the porphyria symptoms slightly exceeded those of the males. However, due to the small sample size, the significance of this observation could not be stated. These results are further categorised such that females with the mutation and without the mutation can be compared to males with and without the mutation (Table 3.5).
Figure 3.8: A graphical representation of common porphyria symptoms reported by all participants in the questionnaire study.

Table 3.5 illustrates that most porphyria symptoms were reported by participants who had the mutation. However, constipation was observed more in females without the mutation. When comparing females with the mutation against those without the mutation, we observed that most symptoms were reported by females with the mutation except for constipation and vomiting. These differences are not significant due to the small sample size. Only one male without the mutation (patient 10, Table 3.1) complained of frequently experiencing severe constipation and moderate pain in the upper body. His symptoms could possibly be age related.
Table 3.5: Common porphyria symptoms reported by members who participated in the questionnaire study categorised by gender and mutation.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Male with mutation n=6</th>
<th>Male without Mutation n=6</th>
<th>Female with Mutation n=8</th>
<th>Female without mutation n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal pain</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Vomiting</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nausea</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Constipation</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Upper body pain</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pain in arms</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Pain in lower legs</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Muscle weakness</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Females with the mutation who reported common porphyria symptoms against females without the mutation are represented in Figure 3.9. Only one female (patient 16) without the mutation experienced pain in the lower legs and muscle weakness in the lower limbs. Patient 16 also reported frequent and severe abdominal symptoms as well as upper body pain. Patient 9, a female without the mutation suffers from fibromyalgia. Due to her condition, she was excluded from the pain in the upper and lower limbs' category from the questionnaire.
Figure 3.9: Common porphyria symptoms in all females who participated in the questionnaire.

Figure 3.10 illustrates the symptoms reported by males with the mutation against those without the mutation. Three males without the mutation reported one or two of the abdominal symptoms, one of whom reported upper body pain as well. Most of the porphyria symptoms were reported by most males with the mutation. Two males with the mutation are under the age of 10, hence too early to show any symptoms.
Porphyria Symptoms in Males

Figure 3.10: A graphical representation of males who experienced common porphyria symptoms.

Figure 3.11 illustrates that porphyria symptoms were reported by females with the mutation more than males with the mutation. As mentioned earlier, the sample size is too small to state how significant the difference is between males and females. Colicky abdominal pain was the most reported symptom amongst males and females with the mutation.
Figure 3.11: A graphical representation of common porphyria symptoms reported by males and females with the mutation.
Table 3.6: A tabular representation of phenotypic expressions reported by patients in a family with HCP.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Gender</th>
<th>Age</th>
<th>Mutation</th>
<th>Abdominal Symptoms</th>
<th>Peripheral neuropathy</th>
<th>Skin symptoms</th>
<th>Psychological symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>38</td>
<td>Y</td>
<td>Mod. &amp; frequent abdominal pain; severe nausea &amp; constipation</td>
<td>Severe pain in upper body; mod. pain in arms</td>
<td>Blisters and scarring of skin in sun exposed areas of the skin</td>
<td>Non</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>16</td>
<td>N</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>88</td>
<td>N</td>
<td>Deceased</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>12</td>
<td>N</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>46</td>
<td>N</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>61</td>
<td>Y</td>
<td>Mod. Abdominal pain and nausea</td>
<td>Mod. Pain in the upper body, arms &amp; lower legs; mod. weakness in the lower limbs</td>
<td>Blisters &amp; wearing away of skin in sun exposed areas; fragile skin</td>
<td>Non</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>70</td>
<td>N</td>
<td>Mod. vomiting &amp; nausea</td>
<td>Non</td>
<td>Blisters &amp; wearing away of skin in sun exposed areas; fragile skin</td>
<td>Non</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>69</td>
<td>Y</td>
<td>Mod. Abdominal pain &amp; constipation</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>61</td>
<td>N</td>
<td>Mod. Abdominal pain; severe constipation</td>
<td>Severe pain in the upper body; mod. Pain in the arms and lower legs; severe muscle weakness in the lower limbs</td>
<td>Blisters in sun exposed areas</td>
<td>Non</td>
</tr>
<tr>
<td>Patient #</td>
<td>Gender</td>
<td>Age</td>
<td>Mutation</td>
<td>Abdominal Symptoms</td>
<td>Peripheral neuropathy</td>
<td>Skin symptoms</td>
<td>Psychological symptoms</td>
</tr>
<tr>
<td>-----------</td>
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<td>----------</td>
<td>-------------------------------------</td>
<td>----------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>87</td>
<td>N</td>
<td>Severe constipation</td>
<td>Mod. Upper body pain</td>
<td>Blistering of skin in sun exposed areas; fragile skin</td>
<td>Audio hallucinations</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>18</td>
<td>N</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>21</td>
<td>N</td>
<td>Mod. Vomiting &amp; constipation</td>
<td>Severe pain in upper body (left side of the chest)</td>
<td>Non</td>
<td>anxiety due to social stress; paranoia</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>42</td>
<td>Y</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>5</td>
<td>N</td>
<td>Excluded</td>
<td>Excluded</td>
<td>Excluded</td>
<td>Excluded</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>15</td>
<td>Y</td>
<td>Mod. Abdominal pain &amp; diarrhoea; severe nausea; severe &quot;sharp stabby pain&quot; in the abdomen</td>
<td>Mod. Pain in the upper body</td>
<td>Non</td>
<td>Visual and audio hallucinations; lack of orientation with respect to time and space</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>18</td>
<td>N</td>
<td>Mod. Abdominal pain, vomiting and nausea; severe diarrhoea &amp; constipation</td>
<td>Mod. Pain in the upper body, arms and lower legs; mod. Muscle weakness in the lower limbs; partial paralysis of the limbs</td>
<td>Non</td>
<td>Anxiety; paranoia</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>44</td>
<td>N</td>
<td>Severe abdominal pain</td>
<td>Non</td>
<td>Blistering of skin in sun exposed areas</td>
<td>Non</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>67</td>
<td>Y</td>
<td>Mod. constipation</td>
<td>Mixed sensory/motor neuropathy of the feet and lower limbs</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>Patient #</td>
<td>Gender</td>
<td>Age</td>
<td>Mutation</td>
<td>Abdominal Symptoms</td>
<td>Peripheral neuropathy</td>
<td>Skin symptoms</td>
<td>Psychological symptoms</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>-----</td>
<td>----------</td>
<td>-------------------</td>
<td>----------------------</td>
<td>---------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>10</td>
<td>N</td>
<td>Severe constipation</td>
<td>Mod. Pain in the upper body &amp; arms</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>11</td>
<td>N</td>
<td>Non</td>
<td>Non</td>
<td>Wearing away of skin in sun exposed areas; fragile skin</td>
<td>Non</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>40</td>
<td>Y</td>
<td>Mod abdominal pain, vomiting and nausea</td>
<td>Mod. Pain in the upper body, arms and lower legs; mod. Muscle weakness in the lower limbs</td>
<td>Blisters in sun exposed areas; excessive hair growth in sun exposed areas</td>
<td>Non</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>13</td>
<td>Y</td>
<td>Mod. Abdominal pain &amp; diarrhoea; severe vomiting &amp; nausea; extremely severe stomach migraines</td>
<td>Mod. Pain in the upper body, arms &amp; lower legs; Severe weakness in the lower limbs; partial paralysis of the limbs</td>
<td>Non</td>
<td>anxiety; lack of orientation with respect to time and place</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>9</td>
<td>Y</td>
<td>Non</td>
<td>Non</td>
<td>Blisters and wearing away of skin in sun exposed areas; fragile skin</td>
<td>Non</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>3</td>
<td>Y</td>
<td>Excluded</td>
<td>Excluded</td>
<td>Excluded</td>
<td>Excluded</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>5</td>
<td>Y</td>
<td>Excluded</td>
<td>Excluded</td>
<td>Excluded</td>
<td>Excluded</td>
</tr>
<tr>
<td>Patient #</td>
<td>Gender</td>
<td>Age</td>
<td>Mutation</td>
<td>Abdominal Symptoms</td>
<td>Peripheral neuropathy</td>
<td>Skin symptoms</td>
<td>Psychological symptoms</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
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<td>----------</td>
<td>--------------------</td>
<td>----------------------</td>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>37</td>
<td>Y</td>
<td>Mod. Vomiting &amp; constipation; severe abdominal pain &amp; diarrhoea</td>
<td>Mod. Pain in the arms; mod muscle weakness in the lower limbs; partial paralysis of the limbs</td>
<td>Blisters, scarring &amp; wearing away of skin in sun exposed areas; fragile skin</td>
<td>Depression; paranoia</td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>15</td>
<td>Y</td>
<td>Mod. Nausea</td>
<td>Mod. &amp; Frequent pain in lower legs</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>8</td>
<td>Y</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Anxiety</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>13</td>
<td>Y</td>
<td>Mod.diarrhoea</td>
<td>Mod. Pain in lower legs</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>30</td>
<td>F</td>
<td>39</td>
<td>Y</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
<td>Non</td>
</tr>
</tbody>
</table>

Summary of symptoms observed from completed questionnaire for each individual who participated in this study as well as their mutation status. The age (in years) listed here is the age of the patient at time of questionnaire. This table only covers the symptoms reported by these patients; however it does not cover other information regarding medication intake and the life style category.
Patient 1 is the index patient who presented at the age of 26 with bullous lesions in sun exposed areas of the skin. Biochemical analysis revealed increased total porphyrin in urine, normal urinary PBG, increased total faecal porphyrin with increased coproporphyrin species. Plasma and blood porphyrins were also elevated. The diagnosis of HCP was based on clinical symptoms and further supported by the above mentioned biochemical analysis. Mutation analysis of the index patient, revealed a novel mutation in exon 5 of the CPOX gene (p.Q355P mutation). Current symptoms reported by her in the questionnaire were abdominal symptoms, peripheral neuropathy which included severe pain in the upper body as well as moderate pain in the arms (Table 3.6). She still suffers from skin symptoms which include blistering and scarring of skin in sun exposed areas, however, she did not suffer from any psychological symptoms. She also suffers from uncontrolled epilepsy and takes Neurotonin medication to control her condition.

Patient 6 had a hysterectomy at the age of 30. Symptoms that were experienced frequently (7-10 times a year) by her were moderate pain in the upper body, arms and lower legs. She also suffers less frequently (1-3 times a year) from moderate muscle weakness in the lower limbs (Table 3.6). Most of the medications consumed by patient 6 were mainly for muscle pain, migraine and high blood pressure.

Patient 8 has the p.Q355P mutation and is on Lipitor medication to lower her cholesterol. Other than experiencing frequent (7-10 times a year) colicky abdominal pain and constipation, she did not suffer from any of the other porphyria symptoms. Patient 9 did not have the mutation, however she complained from moderate abdominal pain and severe constipation. Her peripheral neuropathy symptoms were striking (Table 3.6) especially for the fact that she did not have the mutation. However, she claimed that she suffers from fibromyalgia, a chronic condition which is characterised by diffuse or specific muscle, joint, or bone pain, fatigue, and a wide range of other symptoms. People with fibromyalgia may be genetically predisposed (2007).

According to The American College of Rheumatology criteria (Whinfield, Smith, & Smith 2007), it affects more females than males, with a ratio of 9:1. Since the peripheral symptoms reported by patient 9 were due to fibromyalgia, she was excluded from this section of the questionnaire.
Patient 15 is a male who has the mutation and is going through puberty at the age of 15. He reported severe abdominal symptoms described as sharp “stabby” pain, moderate peripheral neuropathy as well as psychological symptoms. His psychological symptoms included visual and audio hallucinations as well as lack of orientation with respect to time and space. This patient has not been tested for biochemical analysis to make a diagnosis at the time of attacks, however, his symptoms reported in the questionnaire and the presence of the Q355P mutation is highly suggestive of being an affected HCP patient. As he was under the age of 16 at the time of completing the questionnaire, he was given the “parent/guardian” questionnaire form to be completed by his parents. This version of the questionnaire does not cover questions about consumption of alcohol and smoking. It is therefore not clear whether this patient’s symptoms are triggered by the consumption of porphyrinogenic substances such as alcohol.

Patient 16 is an 18 year old female who suffers from moderate to severe abdominal symptoms as well as moderate peripheral neuropathy. It is interesting to note that this patient does not have the Q355P mutation, yet suffers from the above mentioned porphyria symptoms. It was mentioned earlier that, fibromyalgia, affects more females than males and may have a genetic predisposition. A family history with fibromyalgia may explain the moderate – severe peripheral neuropathies experienced by females without the p.Q355P mutation. These symptoms are very general and non-specific and may well be due to conditions other than porphyria.

Patient 18 is the mother of patient 1, who is also a known HCP patient. Her symptoms were triggered at the age of 59 by a cholesterol lowering medication (Lipitor). The main symptoms of patient 1 were mixed sensory/motor neuropathy in her lower limbs and feet. Her biochemical analysis showed a marked increase in total urinary porphyrin. Urinary coproporphyrin was elevated; also, a marked increase in faecal porphyrin and coproporphyrin was noted. A year after starting Lipitor, she was diagnosed with peripheral neuropathy and ceased taking Lipitor. However this did not reverse her symptoms. She is currently on Ezitrol to lower her cholesterol, and seems to be more tolerant to it. Other medications taken by patient 18 were for blood pressure and anti-coagulants. The biochemical diagnosis is further supported by mutation analysis.
Interestingly, patient 21, who is the brother of the index patient (patient 1), experienced porphyria symptoms only recently after starting Lipitor medication. He had similar symptoms to his mother (patient 18) however of a milder form. Lipitor, is a porphyrinogenic medication, and since patient 21 has the p.Q355P mutation, he was advised to stop the Lipitor medication immediately to prevent further progress of the motor/ sensory neuropathy as was the case in patient 18 (his mother).

Patient 22 has the p.Q355P mutation and suffers most of the porphyria symptoms (Table 3.6). She has reached puberty only recently. She has not been diagnosed for HCP; however, her symptoms suggest that she is most likely to be an affected HCP patient. For further diagnosis and confirmation, biochemical analysis of urinary and faecal coproporphyrin should be performed.

Patient 23 is a male with the mutation who has not reached puberty and only suffers from skin symptoms. His skin symptoms are claimed to be due to eczema, however, he should be closely monitored when he reaches puberty, as his skin symptoms could possibly be due to HCP or might get worse with the combination of eczema and HCP. Once he reaches puberty, his urinary and faecal coproporphyrin should be analysed and tested for coproporphyrin isomer III: I to confirm mutation analysis.

Patient 26 clearly suffers most porphyria symptoms; however, he has not been admitted for hospitalisation for his symptoms. The symptoms reported by him in the questionnaire, along with mutation analysis results, suggests that he is suffering from HCP. However, he has not been diagnosed on clinical, or biochemical grounds.

Patient 26 did report occasional consumption of alcohol (i.e. 2-4 times per month with no more than 4 drinks on each occasion). He also reported that on rare occasions, he does consume more than 6 standard drinks. His alcohol consumption could be the environmental factor that contributes to his symptoms as the use of medications has not been reported by him. He also reported experiencing epileptic attacks; however, he does not suffer from epilepsy and does not take anti convulsant medications. This may be due to the excessive alcohol consumption on those rare occasions.
A 15 year old female (patient 27) who is suffering from moderate and frequent pain in the lower legs as well as depression and decreased appetite which resulted in decreased nutritional intake. She also suffered from anxiety and was generally not feeling well as described by her mother. Her situation improved when she started working at a bakery. Her depression resolved after two months and she regained normal appetite, and was eating more carbohydrates.

Patient 28 is under the age of 10, hence, too early to show any symptoms if he is symptomatic. It is interesting to note that patient 30, who has the mutation, did not report any of the porphyria symptoms. She does not consume any medications and rarely drinks alcohol which does not exceed more than 2 drinks each occasion. Therefore, the environmental factors of this patient might not be strong enough to trigger the porphyria symptoms.

In this study, there were four females over the age of 60 who were post menopausal, three of whom have the p.Q355P mutation (patient 6, 8 and 18). Patient 6, regardless of having had a hysterectomy at the age of 30, still experienced most of the porphyria symptoms (Table 3.6). It is possible that she had hysterectomy without oophorectomy. This could explain why she still experiences these porphyria symptoms as her ovaries continued to produce hormones. Patient 8 is on Lipitor but only experienced frequent (7-10 times a year) colicky abdominal pain and constipation. However, patient 18, reported mixed sensory/motor neuropathy in her lower limbs and feet after taking Lipitor medication for lowering her cholesterol. This indicates that some environmental factors may trigger symptoms in some patients but not all. Hence it is the combination of environmental factors involved that trigger the symptoms or may be the presence of mutations in other genes that can make these patients more susceptible to these environmental factors.
3.6 Summary of results

Three unrelated HCP probands were first screened for disease causing mutations in the CPOX gene. A novel and possibly a benign SNP (p. P134H) was found in exon 1 of the CPOX gene of proband 1. No mutation or SNP was found in proband 2.

A novel mutation (p. Q355P) in exon 5 of the CPOX gene was found in proband 3. Also a previously reported SNP causing no change in amino acids was found in exon 5 of proband 3. Mutation screening by sequencing of family members of proband 3 revealed that 15 out of 29 are carriers of this mutation. Another previously reported polymorphism (p.R352C) was detected in three members of this family (Table 3.1-patient 2, 5 and 7).

In this family, the proband and her mother are symptomatic and were diagnosed based on clinical grounds. The questionnaire helped in identifying symptoms reported by patients who have not had an acute attack to be hospitalised, yet suffer from mild form of the disorder. The most reported symptom in this family was abdominal related symptoms followed by neuropathies and skin photosensitivity.
CHAPTER 4: DISCUSSION

HCP is a rare metabolic disorder resulting from a decrease in the enzyme activity of coproporphyrinogen oxidase. HCP is an autosomal dominant disorder characterised by peripheral and autonomic neuropathies, psychiatric manifestations and occasionally cutaneous skin lesions. These clinical suspicions lead to biochemical testing and then diagnosis. The biochemical hallmark of HCP is marked increase in faecal coproporphyrin predominantly isomer III. The increased coproporphyrin III isomer is typically 10-200 times compared to normal controls (Martasek 1998). Urinary ALA and PBG are also increased during an acute attack.

An increased coproporphyrin III to coproporphyrin I ratio is a diagnostic marker for clinically overt HCP patients (Deacon & Elder 2001). This ratio may also be increased in clinically latent patients, (Blake, McManus, Cronin, & Ratnaike 1992); (Gross, Puy, Kuhnel, Meissauer, Deybach, Jacob, Martasek, Nordmann, & Doss 2002); (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001). For symptomatic patients, the coproporphyrin isomer III: I ratio is useful to exclude variegate porphyria at time of diagnosis. In patients with a family history of HCP, fractionation of the faecal porphyrin is carried out regardless of the total faecal porphyrin results. However, the isomer III:I ratio is not reliable for children under the age of 11 (Blake, McManus, Cronin, & Ratnaike 1992); (Allen, Whatley, Degg, & Barth 2005). It is not yet understood why the faecal isomer CIII: I ratio or the enzymatic assays are not reliable in children under the age of 11.

Previous studies (Allen, Whatley, Degg, & Barth 2005); (Blake, McManus, Cronin, & Ratnaike 1992); (Gross, Puy, Kuhnel, Meissauer, Deybach, Jacob, Martasek, Nordmann, & Doss 2002); (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001); (Kuhnel, Gross, & Doss 2000) indicate that the isomer III:I ratio for the detection of clinically latent HCP patients over 11 years of age is nearly 100% sensitive, however it is less useful for diagnosing patients below this age (Blake, McManus, Cronin, & Ratnaike 1992); (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001).
Implementing molecular analysis as a diagnostic tool supported by the current biochemical analysis is an important step towards minimizing equivocal diagnosis of porphyria patients. Molecular analysis can also accurately identify latent HCP patients below the age of 11. Hence, provide preventative measures for patients at a much earlier age.

4.1 Locating the disease causing mutation in known HCP patients

In this study, I attempted to set up a routine molecular screening method. PCR and sequencing of the entire CPOX gene was performed on three known unrelated index patients. Index patient 1 showed a mutation in exon 1 (see Figure 3.1). This mutation caused a substitution in amino acid 134 from Proline to Histidine (p. P134H). Based on PolyPhen analysis, this mutation is predicted to be "benign". This SNP has not been reported previously in HGMD or the literature. No mutation or SNP was found in index patient 2.

No further screening was done for index patients 1 and 2 or other members of their family due to time restraints. If time allowed further testing, repeat biochemical analysis including urine, faecal and plasma porphyrins and fractionation into individual porphyrins. If VP is excluded, then sequencing the promoter region should be performed. If VP is not excluded, then the PPOX gene should be screened for mutations. However, a SNP and a mutation were found in exon 5 of index patient 3. This is a previously reported SNP (HGMD), which is an A>G transversion at nucleotide c.990 causing no change in amino acids.

4.2 Q355P Mutation

The mutation in exon 5 of the CPOX gene found in index patient 3 causes a substitution at amino acid 355 from glutamine to proline (p.Q355P). Amino acid conservation analysis showed that this amino acid is relatively conserved across mammalian species except for rat and mouse (Figure 3.3). PolyPhen analysis predicted this mutation to be
“probably damaging” to the protein based on alignment structure. However it did not recognise the type of effect it could have on the protein. Results obtained from the ESE finder website indicated that the p.Q355P mutation is not a splice site mutation. Based on previous biochemical analysis of index patient 3 as well as PolyPhen prediction and conservation analysis of the mutation, I screened twenty nine members related to index patient three for the mutation p.Q355P in exon 5.

4.3 Family screening for Q355P mutation

Initially I attempted to implement a dHPLC method as a screening tool to screen the rest of the family members of index patient 3. The predominant application of dHPLC is that of the mutational analysis of genes. It has also been applied successfully to the mapping and cloning of genes in yeast (Steinmetz et al. 2002). To date, there are more than 100 genes that are screened for mutational analysis using dHPLC.

dHPLC appears to be more sensitive than other methods in mutation detection, as exemplified in the detection of tuberous sclerosis 1 gene (Jones et al. 1999) and breast cancer 1 gene (Wagner et al. 1999). dHPLC compares two or more chromosomes as a mixture of denatured and reannealed PCR amplicons. In the presence of a mutation in one of the two chromosomal fragments, heteroduplexes which are less thermally stable than the homoduplexes are formed resulting in one or more additional peaks appearing in the chromatogram. Different mutations yield, in most but not all instances, distinctively different peak profiles (see review Premstaller & Oefner 2003).

In this study, I could not obtain a distinctive dHPLC pattern for samples with the mutations despite trying a number of different melting temperatures (Figure 3.7). It maybe that this particular mutation causes no change or a very small change in the melting profile such that it is too subtle to be clearly differentiated from the wild type sequence. This could have resulted in similar peaks for the mutated sequence and the wild type sequence. Discussion with the Department of Molecular Genetics at the Children’s Hospital at Westmead (personal communication with Kathy Holman and Maggie Brett) indicated from their extensive experience with the FBN1 gene, that there are a number of sequence variations that only subtly alter the dHPLC melting profiles.
Therefore, dHPLC is not a suitable method for subsequent cascade testing of family members. For this reason, the use of dHPLC method was discontinued and screening the rest of the family members was performed by direct sequencing of only exon 5 of the CPOX gene.

The role for indirect screening methods such as dHPLC in HCP is probably limited for several reasons. HCP is a rare metabolic disorder that is highly heterogeneous, where most mutations are private, direct sequencing will still be necessary to identify the mutation. The small number of referrals also favours a direct sequencing approach as indirect screening methods are best suited to larger batches and thirdly the small number of exons in the CPOX gene means that there is little saving in reagents when comparing direct sequencing and mutation screening.

Sequencing of the rest of the family members detected a second polymorphism in exon 5 causing a benign change in amino acid 352 from arginine to cystine. This SNP was found in three members of this family. There was no correlation/relationship between this polymorphism and the mutation. This is a previously reported polymorphism and is not a disease causing mutation. DNA sequencing results indicated that 16 out of thirty members in this family had the p.Q355P mutation, two of whom had been previously diagnosed by biochemical analysis as well as clinical assessment. The mutation found in this family is probably the disease causing mutation, however further biochemical analysis such as faecal coproporphyrin III: I ratio should be carried out to confirm the diagnosis in asymptomatic patients. The determination of faecal coproporphyrin III: I ratio as a means of confirmatory test has many advantages over enzymatic studies. The samples are less invasive to obtain and more stable, hence easier to transport than blood samples for enzymatic activity. The isomer ratio method is also technically much easier to perform than quantifying the lymphocyte CPOX activity (Blake, McManus, Cronin, & Ratnaike 1992). Carriers of HCP under the age of 11, excrete normal or borderline levels of coproporphyrins in urine and faeces, hence, the usefulness of this method remains uncertain for children, however, it has proved to be useful for adult patients (Blake, McManus, Cronin, & Ratnaike 1992).
4.4 Biochemical results for HCP index patients

A confirmatory test based on biochemical analysis or enzymatic assay was not performed in this study for all patients due to time restraints. Faecal samples are difficult to obtain, as most patients feel reluctant about providing their own faeces especially if they are asymptomatic. It is perhaps better to request these samples after suspecting the presence of the mutation in their DNA samples if the nature of the mutation is unknown.

Mutation analysis cannot be carried out alone without biochemical or enzymatic analysis. Biochemical results obtained for index patient 1 (table A & B, appendix 3) clearly indicates a marked elevation in total faecal coproporphyrin (97%) and only 3% faecal protoporphyrin. Although Coproporphyrin isomer III: I ratio was not performed, these results are convincing that patient is suffering from HCP. Further studies including faecal isomer III: I ratio and plasma fluorescence would be useful to confirm these biochemical results. Mutation analysis of the CPOX gene revealed a mutation in exon 1 (P134H). PolyPhen analysis predicted this mutation to be "benign". However, it is not confirmed whether this mutation is truly benign or not. Further studies such as gene expression would be beneficial to confirm the nature and effect of this mutation.

Diagnosis for index patient 2 was made by another laboratory based on biochemical results (table A & B, appendix 3). However on reanalysing the biochemical results, they have not completely excluded VP. The ambiguity lies in the fact that there was almost an equal increase in faecal protoporphyrin as there was in coproporphyrin. This would indicate VP, however, the faecal coproporphyrin isomer III: I ratio was 23.3 which is consistent with HCP. Plasma was not analysed to further confirm the diagnosis. If this is to be followed further, faecal porphyrins should be reanalysed and porphyrins including protoporphyrin as well as plasma fluorescence should be performed. If reanalysis of biochemical tests still indicates that patient has HCP, then more genetic studies such as screening the promoter region of the CPOX gene would be useful to perform. Mutations present in other genes which may interact directly or indirectly with the CPOX gene may also cause disease. However, the lack of knowledge of gene-gene interaction makes it very difficult to find the cause of disease.
before their clinical symptoms become acute enough to be admitted to hospital and thus provide them with early preventative measures.

The use of questionnaires in line with mutation screening for HCP patients may be of use for identifying asymptomatic patients who have mild or subtle symptoms of HCP. Since HCP is rare, has a very low penetrance (Allen, Whatley, Degg, & Barth 2005) and variable phenotypic expression (Kauppinen 2005), genotype-phenotype correlation studies are difficult to achieve especially on a large scale. A study by Lamoril et al (2001) looked at genotype-phenotype correlations by recording mode of presentation from symptomatic HCP patients only. They found no association between the type of mutation and mode of presentation. In two families with HCP, different missense mutations gave rise, in different relatives to more than one type of presentation (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder 2001). Studies that look at the mode of presentation only across symptomatic patients might be more biased towards the severe form of the disorder and undermining the variability of expression across the milder form of the disease which could advance to the severe form if preventative measures are not undertaken.

The use of questionnaires may aid in identifying the broad spectrum of variability in phenotypic expression when used with mutation screening. This may also help in performing more precise genotype-phenotype correlation studies on a large scale. However, this can be achieved through the use of standardised questionnaires specifically designed for the porphyrias. There have been several studies (Lecha 2006); (Fraunberg, Pischik, Udd, & Kauppinen 2005) which involved the use of a questionnaire with either biochemical analysis or molecular analysis or both. However these questionnaires were not published and were used only for the particular study. Literature searches for a published questionnaire did not return any results, indicating lack of published questionnaires for the porphyrias.

I design a questionnaire for the acute hepatic porphyrias. The questionnaire had two parts; one was aimed at identifying the common acute hepatic porphyria symptoms in patients with respect to frequency and severity of the symptoms. The second part of the questionnaire attempted to identify environmental factors (including medications, smoking and alcohol) which play an important role in the variability and severity of the
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symptoms provoked by these factors. It was also considered useful to understand the patient’s medication regime to help to distinguish whether symptoms were arising from porphyria or other disorders with similar symptoms. This could help identify individual/personal exposure to environmental factors that contribute to the patient’s symptoms whether they were severe or subtle. It was hoped that these questions would aid in identifying environmental factors that provoked their symptoms in individual patients and hence provide better preventative measures for each individual.

In this study, 26 out of 30 participants were included. The four members that were excluded, three were under the age of 6 and one was deceased. All 26 participants returned their completed questionnaire. The analysis of the questionnaire revealed that there was a wide variability in phenotypic expression in terms of type of presentation and its severity in this family.

The first part of the completed questionnaire which was related to the common porphyria symptoms revealed that 90% of the patients in this family reported abdominal related symptoms irrespective of their mutation status. The non specific nature of porphyria symptoms makes it difficult to show that those with the mutation have increased frequency/severity of symptoms than those without. The next most common symptom reported was peripheral neuropathy related symptoms. This included pain in the upper body and lower limbs (reported by 30% of the patients) and muscle weakness (reported by 19% of patients). Also partial paralysis was reported by 12% of the patients, 8% of whom had the mutation. One female who did not have the mutation reported severe and frequent pain in the upper body and lower limbs as well as muscle weakness. She indicated elsewhere in the questionnaire that she took various medications for fibromyalgia. Due to her condition, she was excluded from the peripheral neuropathy category in the result analysis. Skin symptoms were reported by 38% of patients irrespective of their mutation status. Of those, 27% were males and 12% were females.

Very few patients reported psychological symptoms. Anxiety was reported by four patients, while paranoia was reported by three patients. Two patients, both of whom had the mutation reported depression (patient 15 and 27; table 3.1). Patient 15 also reported
audio and visual hallucination as well as lack of orientation with time and space. Also three patients reported epileptic fits.

In this small sample cohort, we observed that the number of females who reported the porphyria symptoms slightly exceeded those of the males with the exception of vomiting and diarrhoea. Due to the small sample size, I cannot comment on the significance of this observation.

Questionnaire results from females with the mutation were compared to results from females without the mutation. The results observed did not show a considerable difference between females with and without the mutation. However, this was not the case when comparison was made between males with and without the mutation. Most porphyria symptoms were reported mainly by males with the mutation. Out of the six females and six males without the mutation, four females and three males reported at least one porphyria symptom. Although this study included a set of control subjects and a set of positive cases, the sample size was small making it difficult to show that those with the mutation have increased frequency and/or severity of symptoms than those without especially in females. Also, due to the porphyria symptoms being so general and non-specific, the reported symptoms experienced by these patients may well be due to factors or conditions other than porphyria.

The questionnaire revealed that patients with the mutation reported more symptoms than those without. Most patients without the mutation reported only three or less symptoms except for one male (patient 10) and one female (patient 16). The observed data also suggests that there are no major differences in the phenotypic expression between males and females with the Q355P mutation. However, it was noted that the number of symptoms reported by males with the mutation was more than that reported by females with the mutation. This is contrary to the literature which states that attacks are more common in females than males.

It is well documented in the literature (GRANICK 1966); (Tschudy, Valsamis, & Magnussen 1975); (Anderson 1989), that sex hormones play a key role in the expression of the acute porphyrias. Symptoms experienced by females with acute porphyrias are most likely to decrease after menopause. However this was not the case.
in three females (patient 6, 8 and 18) from this study. Patient 1 was asymptomatic all her life and was diagnosed at the age of 61 after taking a cholesterol lowering medication which triggered her symptoms. Patient 8, who was on the same medication as patient 18, did not suffer from the extreme form of the porphyria symptoms as did patient 18. Patient 6, who had hysterectomy at the age of thirty, still experienced mild form of the porphyria symptoms, after stopping medications for muscle pain and sleep problems. However, it is probable that she had hysterectomy without oophorectomy, which explains why she still suffers from these porphyria symptoms.

A male patient (patient 21) only recently became symptomatic at the age of 40 after taking the same cholesterol lowering medication (Lipitor) as his mother (patient 1). However his symptoms were still mild and were advised to discontinue the medication to prevent his symptoms from worsening.

In this small sample cohort, these results suggest that environmental factors rather than endocrine factors play a role in the phenotypic expression of this mutation. The different phenotypic expression that was caused by Lipitor in patient 8, 18 and 21 may be explained by the presence of other SNPs that may impact on the individual’s response to drug therapy. A genetic variability in N-acetyl transferase, for example, is associated with a high incidence of peripheral neuropathy when taking isoniazid, an anti-tuberculosis drug (Nebert 1997). Another major factor contributing to quantitative phenotypic variation was expected to be heritable variation in the regulation of gene expression, which has been predicted to reside principally within cis-regulatory sequences (Doerge 2002).

4.6 Conclusion

From this study, I found that the use of questionnaire along with mutation analysis was very useful in identifying “asymptomatic” patients that actually suffer from subtle or mild forms of the symptoms. It also helped in identifying some of the environmental factors for some individuals, Lipitor in two cases, decreased appetite resulting in insufficient dietary intake in one patient, puberty in two cases and possibly alcohol
consumption in one patient. This indicates that some environmental factors may trigger symptoms in some patients but not all. Hence it is the combination of environmental factors involved that trigger the symptoms or may be the presence of mutations in other genes that can make these patients more susceptible to these environmental factors.

It was also concluded from the questionnaire, that there was a wide phenotypic spectrum within this family with the p.Q355P mutation. Based on this small cohort, our results suggest that there is no correlation between the genotype and severity of the clinical manifestations. This finding supports earlier studies by Lamoril et al. (2001).

The questionnaire was designed specifically for the porphyrias, covering mainly the acute hepatic porphyrias on a general basis. This questionnaire was reviewed by public health experts and by a statistician as well as the ethics committee. However, due to time restraints, a study for the validity of the questionnaire was not performed. This questionnaire was used as a preliminary tool for genotype-phenotype studies; however, one cannot assume the results to be of complete accuracy. Further studies using the current questionnaire on a larger scale would shed light on the reliability and validity of this questionnaire. Once a standardised questionnaire is established for the acute porphyrias, genotype-phenotype correlation studies on a large scale might help in expanding our knowledge about the low penetrance of this disorder.

Making this questionnaire available to porphyria specialised groups might be the first step towards establishing a standardised questionnaire for the porphyrias. Hence, achieving more accurate genotype-phenotype correlation studies on a large scale. However, more collaborative work between researchers is necessary.

This study also showed that mutation analysis as a screening tool is very accurate and reliable. This finding supports previous studies (Grob et al. 2006); (Hessels 2004); (Allen, Whatley, Degg, & Barth 2005). A study by Grob et al (2006) indicated that the levels of urinary porphyrin precursors in children are often only slightly elevated. Hence, molecular screening method is especially useful for identifying carriers below the age of 11 years, in which biochemical or enzymatic analysis can be ambiguous (Grob, Puy, Jacob, Deybach, Kremer, & Doss 2006); (Blake, McManus, Cronin, & Ratnaike 1992); (Lamoril, Puy, Whatley, Martin, Woolf, Da, V, Deybach, & Elder
2001). Once a patient is diagnosed with an acute hepatic porphyria based on clinical and biochemical grounds, molecular analysis seems to be a very useful method in confirming the diagnosis and screening the rest of the family members if a mutation is identified.

Asymptomatic carriers may not experience acute attacks that require hospitalisation; however, they may be suffering from mild porphyria symptoms that remain unrecognised. This questionnaire can recognise and identify these subtle and mild porphyria symptoms as well as identifying most of the environmental factors that can possibly provoke symptoms. It is well known that in many cases once an acute attack is triggered, it is more likely that recurrent subsequent attacks will follow. Therefore, it is necessary to avoid this initial attack in order to prevent recurrent attacks.

Understanding the nature and exact effect of this p.Q355P mutation through gene expression studies and/ or crystal structure studies would be vital. Performing enzymatic and biochemical analysis especially coproporphyrin isomer III:I ratio on all individuals with this mutation would be helpful. Using the biochemical analysis along with the evaluation of their phenotypic expression would give a better understanding on the effect of this mutation on the enzyme.
APPENDIX 1

Participant’s Questionnaire for acute hepatic porphyrias

The following questions relate to the main symptoms of porphyria. The questionnaire is divided into 7 categories (1- abdominal symptoms, 2&3- nerve/muscle pain, 4- skin symptoms, 5- psychological symptoms; 6&7- about personal life style).

This questionnaire will only take 30 minutes to complete, please read through all 7 categories and select the answers that best represent your condition.

The information that you will provide will remain confidential and will only be available to the researchers directly working on this project (Professor Peter Stewart, Dr Victor Poulos and Naz Al Hafid – Masters student).

Ref #: (Provided prior to receiving this questionnaire via a phone call).

Occupation: ____________________ Sex: M □ F □

□ P/T □ F/T

1. Abdominal symptoms

Have you ever experienced any of the following symptoms? If so, please indicate by ticking the box that best describes your condition.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>(1) less than once a year</th>
<th>(2) 1-3 times a year</th>
<th>(3) 4-6 times a year</th>
<th>(4) 7-10 times a year</th>
<th>(5) more than 10 times a year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colicky (gassy) abdominal pain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constipation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others (please specify)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
If you have experienced any of the above symptoms; how severe would you rate the symptoms? Please circle the number that best represents your symptoms.

Colicky abdominal pain

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Vomiting

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Nausea

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Diarrhoea

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Constipation

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Other

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe
2. Pain in upper and lower limbs

The following questions relate to pain in upper and lower limbs that can be associated with porphyria. Please tick the box that best represents your symptoms.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>(1) less than once a year</th>
<th>(2) 1-3 times a year</th>
<th>(3) 4-6 times a year</th>
<th>(4) 7-10 times a year</th>
<th>(5) more than 10 times a year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain in the upper body</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain in the arms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain in the lower legs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

How severe would you rate your pain? Please circle the number that best describes your pain.

Pain in the upper body

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Pain in the arms

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe

Pain in lower legs

(1) Slight  (2) Moderate  (3) Severe  (4) Extremely severe
3. **Muscle weakness**

The following questions relate to muscle weakness which can sometimes be associated with porphyria.

a) Have you ever suffered from muscle weakness in the lower limbs?

   Yes ☐ No ☐

b) If you answered yes to the above question, please specify how often you get muscle weakness?

   (1) Less than once a year
   (2) 1-3 times a year
   (3) 4-6 times a year
   (4) 7-10 times a year
   (5) More than 10 times a year

c) If you answered yes to question 3.a, please specify how severe do you rate your muscle weakness?

   (1) Slight
   (2) Moderate
   (3) Severe
   (4) Extremely severe

d) Have you ever experienced partial paralysis of the limbs?

   Yes ☐ No ☐

e) Have you ever had an epileptic fit or attack?

   Yes ☐ No ☐
4. Skin Symptoms/ photosensitivity (sensitivity to sunlight)

The following questions relate to skin symptoms and sensitivity to sunlight.

a) Have you developed blisters in sun-exposed areas of your body?
   
   Yes □  No □

b) Have you ever developed scarring of skin from blisters in sun-exposed areas?
   
   Yes □  No □

c) Have you ever developed wearing away/ damaging of your skin in sun-exposed areas?
   
   Yes □  No □

d) Do you have excessive hair growth in sun-exposed areas?
   
   Yes □  No □

e) Is your skin fragile (i.e. breaks easily)?
   
   Yes □  No □

f) How would you rate the overall health of your skin?

(1) Very poor  (2) Poor  (3) Neither good nor poor  (4) Good  (5) Very good
5. **Psychological Symptoms**
The following questions relate to psychological symptoms

- a) Did you feel depressed for long periods extending 2-3 months within the last year?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

- b) If you answered yes to the above question, how often did you feel depressed?
  
<table>
<thead>
<tr>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once a year</td>
<td>Twice a year</td>
<td>Three times a year</td>
<td>Four times a year</td>
</tr>
</tbody>
</table>

- c) Have you experienced any hallucinations in the past year?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

- d) If you answered yes to question 5.c;
  
  i. Did you experience visual hallucinations (i.e. seeing things that do not exist in the reality of your surroundings)?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

  ii. Did you experience audio hallucinations (i.e. hearing sounds or things that are not real)?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

- e) Have you experienced prolonged lack of orientation with respect to time, place and/or self within the last year?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

- f) Have you suffered from prolonged anxiety (a feeling of stress, fear and dread) within the past year?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

- g) Within the last year, have you suffered from paranoia (i.e. becoming overly suspicious and emotionally sensitive about people and situations)?
  
<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>
6. Personal Life Style
The following questions ask about your personal details and life style. Some of these questions relate to factors that increase the likelihood of experiencing porphyria symptoms. It is important to know the answers to these questions in order to be able to give accurate advice about avoiding an attack.

   a. If you are a **female**, have you experienced any of the porphyria symptoms during menstruation (period)?
      
      Yes □ No □

   b. Do you take any medication (including antibiotics) for any health problem?
      
      Yes □ No □

      If yes, please specify the following for all medications that have been taken in the last Year.

      | Name of medication | Dose of medication | Reason for taking medication |
      |--------------------|--------------------|-----------------------------|
      |                    |                    |                             |
      |                    |                    |                             |
      |                    |                    |                             |

   c. If you answered yes to the above question, did you experience any of the porphyria symptoms after taking medication/s?
      
      Yes □ No □

   d. Did you stop taking the medication?
      
      Yes □ No □

   e. Did stopping the medication help reduce or stop your symptoms?
      
      Yes □ No □
f. Were you at any stage of your life a heavy smoker?

*Note: a heavy smoker is one who smokes \( \frac{1}{2} \) a pack or more of cigarettes per day.*

Yes ☐ No ☐

g. Did you experience any of the porphyria symptoms after excessive smoking?

Yes ☐ No ☐

h. If you answered yes to the above question, did you try to cut down or quit smoking?

☐ Yes, cut down
☐ Yes, quit smoking
☐ No, did not do anything about it

i. Did quitting or cutting down smoking reduce your symptoms?

Yes ☐ No ☐

j. Have you ever been on a very strict diet?

Yes ☐ No ☐

k. If yes, did this make you ill by getting one or more of the porphyria symptoms?

Yes ☐ No ☐

l. Did stopping the diet, help reduce or stop the symptoms?

Yes ☐ No ☐
7. The following questions relate to alcohol consumption, please read the drink definitions before proceeding to the questions.

**Drink definitions:**

A standard drink is defined as:
- A single small (8 ounces; 1/2 pint) glass of beer
- A single shot/measure of liquor/spirits
- A single glass of wine

a. How often do you have a drink containing alcohol?

- □ Never
- □ Monthly or less
- □ Two to four times per month
- □ Two to three times per week
- □ Four or more times per week

b. How many standard drinks containing alcohol do you have on a typical day when you are drinking?

- □ 1 or 2
- □ 3 or 4
- □ 5 or 6
- □ 7, 8 or 9
- □ 10 or more

c. How often do you have 6 or more drinks on one occasion?

- □ Never
- □ Less than monthly
- □ Monthly
- □ Weekly
- □ Daily or almost daily

d. Did you experience any of the porphyria symptoms after drinking alcohol?

- □ Yes
- □ No

THANK YOU FOR YOUR HELP AND COOPERATION
APPENDIX 2

Parent/ Guardian Questionnaire for Acute Hepatic Porphyrias

The following questions relate to the main symptoms of porphyria. The questionnaire is divided into 6 categories (1-abdominal symptoms, 2&3-nerve/muscle pain, 4-skin symptoms, 5-psychological symptoms; 6- about personal life style).

This questionnaire will only take 25 minutes to complete, please read through all 6 categories and select the answers that best represent your child’s condition.

The information that you will provide will remain confidential and will only be available to the researchers directly working on this project (Professor Peter Stewart, Dr Victor Poulos and Naz Al Hafid – Masters student).

Ref #: (Provided prior to receiving this questionnaire via a phone call).

Occupation: ____________________________ Sex: M □ F □

□ P/T □ F/T

1. Abdominal symptoms

Has your child experienced any of the following symptoms? If so, please indicate by ticking the box that best describes your child’s condition.

<table>
<thead>
<tr>
<th>Frequency of symptoms</th>
<th>(1) less than once a year</th>
<th>(2) 1-3 times a year</th>
<th>(3) 4-6 times a year</th>
<th>(4) 7-10 times a year</th>
<th>(5) more than 10 times a year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colicky (gassy) abdominal pain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Nausea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constipation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others (please specify)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
If your child has experienced any of the above symptoms; how severe would you rate the symptoms? Please circle the number that best represents your child’s symptoms.

Colicky abdominal pain

- Slight
- Moderate
- Severe
- Extremely severe

Vomiting

- Slight
- Moderate
- Severe
- Extremely severe

Nausea

- Slight
- Moderate
- Severe
- Extremely severe

Diarrhoea

- Slight
- Moderate
- Severe
- Extremely severe

Constipation

- Slight
- Moderate
- Severe
- Extremely severe

Other

- Slight
- Moderate
- Severe
- Extremely severe
2. **Pain in upper and lower limbs**

The following questions relate to pain in upper and lower limbs that can be associated with porphyria. Please tick the box that best represents your child’s symptoms.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Frequency of symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td>Pain in the upper body</td>
<td></td>
</tr>
<tr>
<td>Pain in the arms</td>
<td></td>
</tr>
<tr>
<td>Pain in the lower legs</td>
<td></td>
</tr>
</tbody>
</table>

How severe would you rate the pain? Please circle the number that best describes your child’s pain.

**Pain in the upper body**

(1) Slight     (2) Moderate     (3) Severe     (4) Extremely severe

**Pain in the arms**

(1) Slight     (2) Moderate     (3) Severe     (4) Extremely severe

**Pain in lower legs**

(1) Slight     (2) Moderate     (3) Severe     (4) Extremely severe
3. **Muscle weakness**

The following questions relate to muscle weakness which can sometimes be associated with porphyria.

a) Has your child ever suffered from muscle weakness in the lower limbs?

- Yes [□]
- No [□]

b) If you answered yes to the above question, please specify how often did your child get muscle weakness?

- Less than 1-3 times a year [1]
- 4-6 times a year [2]
- 7-10 times a year [3]
- More than 10 times a year [4]


c) If you answered yes to question 3.a, please specify how severe do you rate your child’s muscle weakness?

- Slight [1]
- Moderate [2]
- Severe [3]
- Extremely severe [4]


d) Has your child ever experienced partial paralysis of the limbs?

- Yes [□]
- No [□]

e) Has your child ever had an epileptic fit or attack?

- Yes [□]
- No [□]
4. **Skin Symptoms/ photosensitivity (sensitivity to sunlight)**

The following questions relate to skin symptoms and sensitivity to sunlight.

**g) Has your child developed blisters in sun-exposed areas of his/her body?**

Yes ☐ No ☐

**h) Has your child ever developed scarring of skin from blisters in sun-exposed areas?**

Yes ☐ No ☐

**i) Has your child ever developed wearing away (i.e. damaging of his/her skin in sun-exposed areas)?**

Yes ☐ No ☐

**j) Does your child have excessive hair growth in sun-exposed areas?**

Yes ☐ No ☐

**k) Is your child’s skin fragile (i.e. breaks easily)?**

Yes ☐ No ☐

**l) How would you rate the overall health of your child’s skin?**

<table>
<thead>
<tr>
<th>(1) Very poor</th>
<th>(2) Poor</th>
<th>(3) Neither good nor poor</th>
<th>(4) Good</th>
<th>(5) Very good</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>
5. **Psychological Symptoms**
The following questions relate to psychological symptoms

h) Did your child feel depressed for long periods extending 2-3 months within the last year?
   - Yes
   - No

i) If you answered yes to the above question, how often did your child feel depressed? *Note: each time refers to a period of 2-3 months*

   - (1) Once a year
   - (2) Twice a year
   - (3) Three times a year
   - (4) Four times a year

j) Has your child experienced any hallucinations in the past year?
   - Yes
   - No

k) If you answered yes to question 5.c;

   iii. Did your child experience visual hallucinations (i.e. seeing things that do not exist in the reality of your child’s surroundings)?
       - Yes
       - No

   iv. Did your child experience audio hallucinations (i.e. hearing sounds or things that are not real)?
       - Yes
       - No

l) Has your child experienced prolonged lack of orientation with respect to time, place and/or self within the past year?
   - Yes
   - No

m) Has your child suffered from prolonged anxiety (a feeling of stress, fear and dread) within the past year?
   - Yes
   - No
n) Within the last year, has your child suffered from paranoia (i.e. becoming overly suspicious and emotionally sensitive about people and situations?)

Yes ☐ No ☐
6. **Personal Life Style**
The following questions ask about your child’s personal details and life style. Some of these questions relate to factors that increase the likelihood of experiencing porphyria symptoms. It is important to know the answers to these questions in order to be able to give accurate advice about avoiding an attack.

a) If your child is female, has she reached puberty?

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
</tbody>
</table>

i. If you answered yes to question 6.a, has your child experienced any of the porphyria symptoms during menstruation (period)?

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b) Does your child take any medication (including antibiotics) for any health problems?

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If yes, please specify the following for all medications that have been taken in the last Year.

<table>
<thead>
<tr>
<th>Name of medication</th>
<th>Dose of medication</th>
<th>Reason for taking medication</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>
c) If you answered yes to the above question, did your child experience any of the porphyria symptoms after taking medication/s?

Yes ☐ No ☐

d) Did your child stop taking the medication?

Yes ☐ No ☐

e) Did stopping the medication help reduce or stop your child’s symptoms?

Yes ☐ No ☐

f) Has your child ever been on a very strict diet?

Yes ☐ No ☐

g) If yes, did this make your child ill by getting one or more of the porphyria symptoms?

Yes ☐ No ☐

h) Did stopping the diet, help reduce or stop the symptoms?

Yes ☐ No ☐

THANK YOU FOR YOUR HELP AND COOPERATION
APPENDIX 3

Table A: Biochemical results of urinary porphyrins for known HCP patients.

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>PBG (μmol/mmol creatinine)</th>
<th>PORPHYRIN (nmol/mmol creatinine)</th>
<th>COPROPORPHYRIN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.43 (ref&lt;1.5)</td>
<td>30 (ref &lt;35)</td>
<td>Not available</td>
</tr>
<tr>
<td>2^</td>
<td>0.4 (ref&lt;1.5)</td>
<td>37 (ref &lt;35)</td>
<td>88 (ref 60-80)</td>
</tr>
<tr>
<td>3</td>
<td>1.05 (ref&lt;1.5)</td>
<td>142 (ref &lt;35)</td>
<td>78 (ref 60-80)</td>
</tr>
<tr>
<td>4*</td>
<td>1.42 (ref&lt;1.5)</td>
<td>99 (ref &lt;35)</td>
<td>97 (ref 60-80)</td>
</tr>
</tbody>
</table>

Table B: Faecal porphyrin results of known HCP patients.

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>Total PORPHYRIN (μmol/K/dry wt)</th>
<th>Total COPROPORPHYRIN (%)</th>
<th>PROTOPORPHYRIN N (%)</th>
<th>COPROCHIII:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1700 (ref &lt;200)</td>
<td>97 (ref 30-60)</td>
<td>3 (ref 30-60)</td>
<td>Not available</td>
</tr>
<tr>
<td>2^</td>
<td>873 (ref &lt;200)</td>
<td>48 (ref 30-60)</td>
<td>50 (ref 30-60)</td>
<td>23.0 (ref&lt;1.5)</td>
</tr>
<tr>
<td>3</td>
<td>11230 (ref &lt;200)</td>
<td>99 (ref 30-60)</td>
<td>1 (ref 30-60)</td>
<td>Not available</td>
</tr>
<tr>
<td>4*</td>
<td>4940 (ref &lt;200)</td>
<td>98 (ref 30-60)</td>
<td>2 (ref 30-60)</td>
<td>Not available</td>
</tr>
</tbody>
</table>

^ Biochemical tests were performed at another laboratory and results were obtained upon request. Could not obtain plasma and blood results.

* Mother of index patient 3, who is also a known HCP patient.
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