VARIEGATE PORPHYRIA

MOLECULAR ASPECTS OF VARIEGATE PORPHYRIA IN SOUTH AFRICA AND THEIR BIOCHEMICAL AND CLINICAL CONSEQUENCES

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Work of the nature described in this dissertation cannot possibly be the efforts of a single individual. The specific contributions of a number of colleagues are acknowledged below.

Section 1

Work on the identification of the R59W mutation proceeded jointly in the UCT laboratory and the laboratory of Professor H. Dailey, University of Georgia, where the initial identification of the R59W mutation was made: Professor Peter Meissner participated in this work during his sabbatical in Georgia. The subsequent work described in Chapter 3 was performed in Cape Town. The major contribution of Dr Mel Ziman, who established many of the molecular biology techniques in our laboratory at the author's request, is acknowledged.

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Section 2

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ABSTRACT

VARIEGATE PORPHYRIA

Variegate porphyria (VP) is the clinical disorder associated with a deficiency of the haem-synthesising enzyme *protoporphyrinogen oxidase* (PPO). VP is one of the commonest monogenic inherited disorders in South Africa. The clinical effects include photocutaneous sensitivity and the development of potentially life-threatening acute porphyric crises.

Section 1 of this dissertation examines the molecular basis for VP in South Africa. It is shown to be a genetically heterogeneous disorder. The identification of 10 discrete mutations is reported and their characteristic features described. One mutation, the R59W mutation, is highly prevalent and represents the original founder mutation imported from Holland in 1688.

Section 2 examines the value of routine porphyrin analyses of urine, stool and plasma in the diagnosis of VP as correlated with the presence or absence of a PPO mutation on DNA analysis. Biochemical testing is shown to be imprecise and subject to individual variation in interpretation. All children and 30% of adults carrying a PPO mutation fail to express abnormal biochemistry. Using the statistical techniques of discriminant analysis and classification tree analysis, it is shown that an elevation of stool coproporphyrin and pentacarboxylic porphyrin are the most important biochemical predictors of VP. The predictive value of qualitative porphyrin analyses is assessed and plasma fluorescence scanning is demonstrated to represent the most sensitive biochemical test for VP.

Section 3 examines the clinical expression of VP. In a study of a single kindred carrying the R59W mutation, it is shown that approximately 60% of adults are clinically silent, skin disease is present in 40% and that the probability of an acute attack is now low. The mutations present in 4 subjects with compound heterozygous VP are described and their biochemical features and clinical course described. A 10-year personal experience with the management of more than 100 episodes of the acute porphyric crisis in Groote Schuur Hospital is reviewed. Unusual presentations and complications are discussed, and the outcome of treatment reported.

The significance of these findings and their implications for the care of patients with VP and for further research are briefly discussed in Section 4. The bibliography and appendices follow.

ABBREVIATIONS

General

EBV	Ebstein-Barr virus
HPLC	high performance liquid chromatography
HD	heteroduplex
SSCP	single-stranded conformational polymorphism
TLC	thin-layer chromatography
UCT	University of Cape Town

Porphyrias

AIP	acute intermittent porphyria
CEP	congenital erythropoietic porphyria
EPP	erythropoietic protoporphyria
HCP	hereditary coproporphyria
HVP	homozygous (or compound heterozygous) variegate porphyria
PCT	porphyria cutanea tarda
VP	variegate porphyria

Diagnostic categories

VPH	Non-diagnostic result but thought to represent a high probability of variegate porphyria as defined in Chapter 7
VPL	Non-diagnostic result but thought to represent a low probability of variegate porphyria as defined in Chapter 7
NAD	No abnormality detected

Porphyrins and their precursors

ALA	5-aminolaevulinate
PBG	porphobilinogen
Uro	uroporphyrin
Copro	coproporphyrin
Proto	protoporphyrinogen
C7	heptacarboxylic porphyrin
C6	hexacarboxylic porphyrin
C5	pentacarboxylic porphyrin
C3	tricarboxylic porphyrin
Isocopro	isocoproporphyrin
Pseudo-C5	pseudopentacarboxylic porphyrin

Enzymes and other chemical compounds

FAD	flavin adenine dinucleotide
PPO	protoporphyrinogen oxidase

Statistical

NPV	negative predictive value
PPV	positive predictive value

CONTENTS

CHAPTER 1:

INTRODUCTION

In this introductory chapter, the porphyrias are briefly defined and reviewed. The history of variegate porphyria (VP), and of its recognition and characterisation in South Africa, is described. The experimental work reported in this dissertation is divided into three sections, corresponding to the molecular biology, the biochemical features and clinical aspects of VP respectively. Each section begins with a review of the pertinent literature. Thereafter the experimental or descriptive work follows, chapter by chapter according to the nature of the work. Though a brief summary of the conclusions drawn from the work of each chapter is provided, the results are not discussed in detail until the concluding chapter of each section where the results as a whole are discussed.

1.1 THE PORPHYRIAS

The porphyrias are a group of metabolic disorders associated with impaired activity of the enzymes of the haem synthetic pathway. Characteristically these disorders are accompanied by the abnormal accumulation of the intermediate metabolites on this pathway: the porphyrin precursors, δ-aminolaevulinic acid (ALA) and porphobilinogen (PBG), and the porphyrins, which are the oxidised form of the porphyrinogens produced during haem synthesis (Figure 1-1). Most of the porphyrias are inherited disorders. The exception is porphyria cutanea tarda (PCT) which is usually an acquired disease though an inherited form is also encountered. Most are inherited as autosomal dominant conditions with the exception of ALA dehydratase deficiency and congenital erythropoietic porphyria (CEP) which are recessive. The inheritance of erythropoietic protoporphyria (EPP), as a clinical disorder, is complex and is described in Chapter 15.

In two of the porphyrias, haem synthesis within the erythroid precursor cells is predominantly disturbed. In these conditions, erythrocytes and plasma porphyrins are particularly elevated and erythrocytes may fluoresce under ultraviolet light. The clinical symptoms are of photosensitivity, and these porphyrias, CEP and EPP, are traditionally classified as erythropoietic porphyrias. In the remaining disorders, haem synthesis within non-erythroid cells is predominantly affected. Since the liver is an active haem-synthesising organ and much of the overproduction of porphyrias is thought to occur here, these forms of porphyria are classified as hepatic porphyrias. The characteristic features of the porphyrias are summarised in Table 1-1.

1.2 HISTORICAL ASPECTS OF VARIEGATE PORPHYRIA

It has been known for over a century that the diseases we now know as porphyrias are associated with the excretion of unusual substances, particularly in the urine (Moore et al 1987). Following the introduction of Sulphonal as a hypnotic (Kast 1888), a series of patients were described who developed abdominal pain and neuropathy and were noted to excrete dark substances in the urine. Stokvis (1889) described an elderly woman who, following the ingestion of Sulphonal, developed a neurological crisis associated with the passage of dark-red urine from which she ultimately succumbed. Stokvis suggested that the pigment causing the discolouration of the urine was related to haematoporphyrin. A number of similar cases were described over the following years (Harley 1890, Ranking and Pardington 1890) and it

appeared that between 5 and 10% of woman treated with Sulphonal developed "haematoporphyria" (Geill 1891, Fehr 1891).

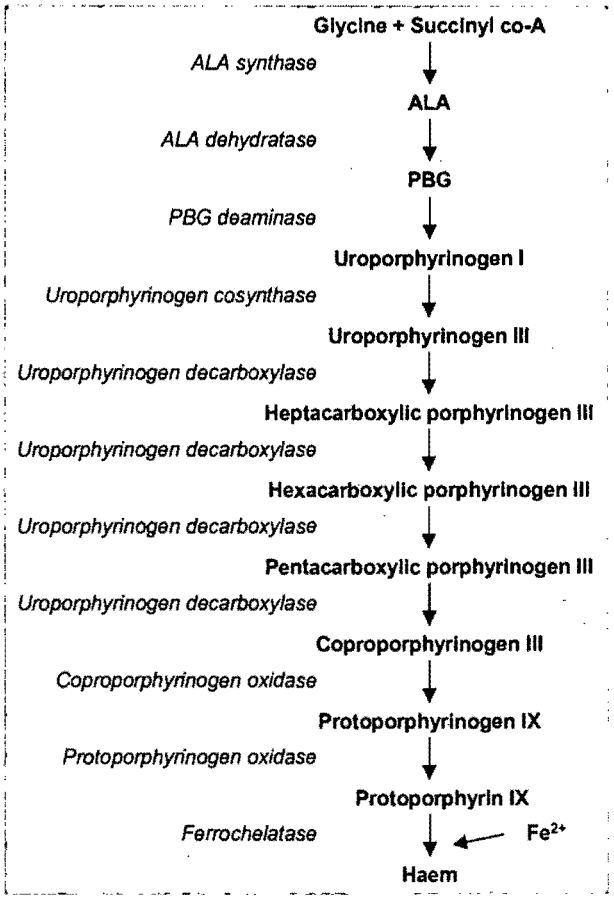


Figure 1-1. The intermediates and enzymes of the haem synthetic pathway.

Enzyme	Disorder	Inheritance	Clinical effects
ALA dehydratase	ALA dehydratase deficiency (Doss porphyria)	AR	Acute attacks
PBG deaminase	Acute intermittent porphyria	AD	Acute attacks
Uroporphyrinogen cosynthase	Congenital erythropoietic porphyria	AR	Photosensitivity
Uroporphyrinogen decarboxylase	Porphyria cutanea tarda	Sporadic/AD	Photosensitivity
Coproporphyrinogen oxidase	Hereditary coproporphyria	AD	Acute attacks, photosensitivity
Protoporphyrinogen oxidase	Variegate porphyria	AD	Acute attacks, photosensitivity
Ferrochelatase	Erythropoietic protoporphyria	AD (AR?)	Photosensitivity

Table 1-1. Summary of the porphyrias. AD=autosomal dominant, AR=autosomal recessive.

The biochemical and clinical features of variegate porphyria were first described in a patient in 1937 (Van den Bergh and Grotepass 1937). This patient, interestingly, may have been the propositus of one of the Dutch families described many years later by Te Velde et al (1989). Further case reports followed. Initially the features of VP were confused with those of PCT, acute intermittent porphyria (AIP) and hereditary coproporphyria (HCP) and a number of terms were later used to describe the disease including mixed porphyria (Watson 1960), protocoproporphyria (Waldenström 1957), porphyria cutanea tarda—resulting in confusion with the condition we know by that name today (Calvert and Rimington 1953, MacGregor et al 1952, Wells and Rimington 1953, Holti et al 1958)—and porphyria cutanea tarda hereditaria (Cormane et al 1971, Levene 1968, Magnus 1968, Rimington et al 1967, Tio 1958).

The first published description of acute porphyria in South Africa was written by two medical students, Lennox Eales and Jack Chait, who described a patient in the medical students' journal of the University of Cape Town in October 1939. The first report of a series of cases of porphyria, then known as porphyrinuria, appeared in 1951 with a description by HD Barnes of the South African Institute for Medical Research in Johannesburg. A major contribution to the understanding of porphyria in South Africa was that of Geoffrey Dean, a physician in the coastal city of Port Elizabeth. He identified a large number of affected people amongst his patients in the eastern Cape Province. Dean showed that the disease was inherited as a Mendelian dominant character, recognised that the disease could manifest with skin lesions, acute attacks or both and coined the terms *porphyria variegata* or *South African genetic porphyria* to describe it (Dean 1953, Dean and Barnes 1955).

Dean then went on to demonstrate that VP is extremely common amongst the South African population of Dutch descent, and to show that it was a disorder distinct from the genetic porphyria found in Sweden (Dean and Barnes 1959). He investigated 118 South African family groups in all. He showed that on average 50% of the children of a porphyric patient would inherit VP, that the sex distribution is equal and that patients may experience acute attacks and skin disease singly or together showing that they are features of a single disorder. He also observed that family members could be asymptomatic, yet be shown to have VP by the demonstration of abnormal urine and stool porphyrins. He commented that he had never seen a family in which both parents had VP, and thus had never seen a homozygote (Dean 1963). Furthermore, his meticulous genealogical studies suggested that almost all patients with VP were descended from a single founder. He was able to trace many of his affected families back to 5 of the 11 children of a Cornelis van Rooyen who married Jacomijntje Gerritsz, the daughter of Gerrit Jansz, in 1720. Other porphyric families were traced back to three other children of Gerrit Jansz, who had immigrated to the Cape of Good Hope from Deventer in Holland, and had married a Dutch orphan named Adriaantjie Adriaanse in 1688. Adriaantjie, born in Rotterdam, arrived on the Dutch East India Company ship *China* in 1688 and was married within a month of arrival. To this day it is not known whether VP was introduced into South Africa by Gerrit Jansz himself or by his wife, Adriaantjie.

1.3 PREVALENCE OF VARIEGATE PORPHYRIA

VP has been recognised in many countries (Mustajoki 1978), but is believed to be generally uncommon everywhere but South Africa. In Finland the prevalence has been estimated at 1.3 per 100 000 (Mustajoki 1980). In Europe generally, the prevalence of clinically-overt VP is thought to be approximately one-third that of AIP or approximately 0.5 per 100 000 (Elder et al 1997). Since it is believed that many subjects with VP are clinically latent, particularly in the temperate zones where sunshine is less intense and skin disease potentially less common

(Mustajoki 1978), the prevalence of PPO mutations in Europe is likely to be considerably higher, and the disease may not be as rare as has previously been thought.

The prevalence of VP in South Africa has never been accurately determined. Dean (1963) attempted to estimate the prevalence by screening staff and patients of European descent in Port Elizabeth hospitals for VP. He found that 8 of 645 nurses (1.2%) and 4 of 608 patients in the local mental hospital (0.66%) appeared to have biochemical evidence of VP. 5647 adults were tested prior to administration of thiopentone or barbiturates in the provincial hospital and 29 positive patients were detected (0.51%). In a second hospital, 3 of 719 patients tested positive (0.42%). In the following year, the incidences in these hospitals were 0.46% and 0.42% respectively. These figures suggested that the prevalence of VP amongst the white population of the eastern Cape Province was 4 per thousand. Dean believed that it would be lower in other cities, which had received a larger proportion of recent immigrants. He suggested an overall prevalence of 3 per thousand in the white population of South Africa and a total figure of 8000±2000 for the country as a whole. No better estimate has ever been made for South Africa.

Following this brief introduction to VP, the molecular biology of this disorder is now reviewed and our findings in the South African VP population reported.

SECTION 1

THE MOLECULAR BIOLOGY OF VARIEGATE PORPHYRIA

Section 1 of this dissertation examines the molecular basis for VP in South Africa. It is shown to be a genetically heterogeneous disorder. The literature pertinent to the molecular biology of VP is reviewed in Chapter 2. In Chapter 3, the identification of the first two mutations discovered in South African patients with VP is reported, and it is shown that one mutation, the R59W mutation, is highly prevalent and represents the original founder mutation imported from Holland in 1688. In Chapter 4, a further 8 mutations identified in South African patients are reported. The significance of these findings is discussed in Chapter 5.

CHAPTER 2:

THE MOLECULAR BIOLOGY OF VARIEGATE PORPHYRIA: A REVIEW OF THE LITERATURE

In this chapter, the molecular and enzymatic defects underlying the biochemical and clinical features of VP are discussed. Following a review of the identification of protoporphyrinogen oxidase (PPO) as the primary site of the defect in VP, the identification of the DNA sequence coding for PPO, its assignment to chromosome 1 and the recognition of mutations in this gene in patients with VP are discussed. The mutations encountered in patients with homozygous or compound heterozygous VP are not dealt with here, as they are specifically discussed in Chapters 15 and 19.

2.1 THE ENZYMATIC DEFECT IN VARIEGATE PORPHYRIA

From the pattern of porphyrin excretion—largely of coproporphyrin and protoporphyrin—in VP, it seemed likely that, as in EPP, a deficiency of one of the terminal enzymes of the haem-synthetic pathway would prove to be responsible for the disorder. Reduced ferrochelatase activity had been shown to be responsible for EPP (Bonkowsky et al 1975, Bottomley et al 1975, DeGoeij et al 1975). However, some investigators reported a 50% reduction in ferrochelatase activity in both cultured fibroblasts and bone marrow cells from patients with VP, and suggested that VP and EPP shared the same enzyme defect (Becker et al 1977, Viljoen et al 1979). Equivocal ferrochelatase activities were additionally shown in circulating lymphocytes (Deybach et al 1981). However, Brenner and Bloomer (1980) convincingly demonstrated a 50% reduction in PPO activity in cultured skin fibroblasts from patients with VP, and Bloomer (1981) later found the activity of ferrochelatase in the fibroblasts of patients with VP to be normal. All subsequent experience has supported a primary role for PPO, and not ferrochelatase, deficiency in VP.

Although PPO deficiency is the primary defect in VP, the disease does not occur in isolation from the other enzymes of the haem synthetic pathway. Evidence in the acute porphyrias, AIP, HCP and VP, all suggests that reduced activity of the dysfunctional enzyme induces an increase in ALA synthase activity, probably on the basis of haem-deficiency: this reduction in haem synthase activity is greater during the acute attack than it is in remission (Strand et al 1970, Tschudy et al 1965, Nakau et al 1966, Dowdle et al 1967, McIntyre et al 1971). Bloomer also foresaw that, in addition to the accumulation of protoporphyrinogen (subsequently oxidised to protoporphyrin and excreted in bile and stool) in VP, some other factor must operate to explain the acute attack which is always accompanied by markedly elevated concentrations of the porphyrin precursors ALA and PBG, as well as those porphyrins which precede protoporphyrinogen in the pathway.

Some studies have looked at erythrocyte PBG deaminase levels in patients with VP. McColl et al (1985) and Mustajoki (1976) had shown significant reductions in PBG deaminase activity, of 20% and 10% respectively, in patients with VP. Similar reductions in PBG deaminase activity had been reported in subjects with HCP (Brodie et al 1977, Moore et al 1980). PBG deaminase deficiency did not appear to result from a co-inherited mutation since no subjects with deficient PBG deaminase activity alone were identified in these VP families, and it appeared to be a secondary phenomenon expressed by a proportion of subjects manifesting PPO deficiency. Meissner et al (1986) simultaneously measured the activities of PPO and PBG deaminase in patients with VP and normal controls as expressed in Epstein-

Barr virus-transformed lymphoblasts. They showed that the lymphoblasts of VP subjects consistently show an approximately 50% reduction in PPO activity as well as an additional 24% reduction in PBGD activity. A 28% reduction in PBG deaminase activity in erythrocytes from patients with VP was also shown. In every case, decreased PBG deaminase activity co-segregated with decreased PPO activity. In subsequent work, Meissner et al (1993) showed that PBG deaminase is allosterically inhibited by both coproporphyrinogen and protoporphyrinogen, though not by uroporphyrinogen, uroporphyrin, coproporphyrin or protoporphyrin. In a series of experiments, they showed increasing degrees of inhibition of PBG deaminase, both in lymphoblasts and in pure form, in response to the addition of coproporphyrinogen and protoporphyrinogen. Furthermore, they showed that removal of porphyrinogens from the system by column chromatography restored normal kinetic behaviour to PBG deaminase. We therefore believe that a secondary deficiency in PBG deaminase activity in response to increasing intracellular concentrations of copro-porphyrinogen and protoporphyrinogen resulting from coproporphyrinogen oxidase or PPO activity in HCP and VP may be important in the development of the acute attack.

2.2 PROTOPORPHYRINOGEN OXIDASE

Since protoporphyrinogen IX will oxidise spontaneously to protoporphyrin IX in the presence of light and oxygen, the inter-conversion was initially thought to be the result of auto-oxidation, though some investigators suggested from an early stage that the reaction might be enzyme-mediated (Porra and Falk 1961, Sano and Granick 1961, Porra and Falk 1964). Poulson and Polglase (1974) showed that the rate of auto-oxidation of protoporphyrinogen IX in yeast cells only approximates the average rate of synthesis of haem, and that yeast cells contain antioxidants which inhibit auto-oxidation. Furthermore, they reported that a yeast mitochondrial extract appeared to facilitate the conversion of protoporphyrinogen IX under conditions unfavourable for auto-oxidation.

Initially a single cell fraction appeared responsible for the two-stage process of oxidation of coproporphyrinogen, via protoporphyrinogen, to protoporphyrin, and it seemed possible that coproporphyrinogen oxidase might be responsible for both the oxidative decarboxylation of the side chain and the subsequent dehydrogenation, but stereochemical experiments using meso-tritium-labelled coproporphyrinogen III indicated that a separate, specific enzyme would be required for the conversion of protoporphyrinogen IX to protoporphyrin IX. Poulson and Polglase (1975) then partially purified an extract from yeast mitochondria which selectively oxidised protoporphyrinogen IX to protoporphyrin IX and was postulated to contain this enzyme, now labelled PPO: neither uroporphyrinogen nor coproporphyrinogen served as a substrate for this reaction. This was followed by the partial purification of mammalian PPO from rat liver mitochondria (Poulson 1976). Poulson showed that coproporphyrinogen oxidase lacked the ability to oxidise protoporphyrinogen and that the rate of oxidation of protoporphyrinogen in the presence of partially purified ferrochelatase was less than 10%. Conversely, the mitochondrial fraction with PPO activity was substantially free of coproporphyrinogen oxidase and ferrochelatase activity, thus conclusively proving the existence and importance of PPO as an enzyme in its own right. Subsequent work (Jackson et al 1976) confirmed that protoporphyrinogen, harderoporphyrinogens, isoharderoporphyrino-gens and mesoporphyrinogen serve as substrates for this enzyme. PPO activity was additionally shown in photosynthetic bacteria (Jacobs and Jacobs 1981) and plants (Jacobs and Jacobs 1983), and was subsequently purified from plant organelles and shown to participate in both haem and chlorophyll synthesis. In 1985 the kinetic properties of partially purified human liver mitochondrial PPO were described (Camadro et al 1985).

Deybach et al (1985) were able to show, using the digitonin method and subsequent fractionation of rat liver mitochondria, that PPO was closely associated with the inner membrane fraction of the mitochondria. Chemical treatment suggested that the enzyme was anchored within the lipid bi-layer of the inner membrane. Protoporphyrinogen appeared to have equal access to the active site of the enzyme from both sides of the inner membrane though the catalytic site faces the cytosolic side of the membrane (Ferreira 1988). Furthermore, co-localisation of coproporphyrinogen oxidase and protoporphyrinogen oxidase was shown by experiments in which hypo-osmotic disruption of the mitochondrial membranes resulted in a dislocation of coproporphyrinogen oxidase from the intermembrane space, following which coproporphyrinogen oxidase continued to produce proto-porphyrinogen from coproporphyrinogen substrate at the normal rate, but there was an accumulation of protoporphyrinogen and retarded oxidation to protoporphyrin (Deybach et al 1985).

The purification and characterization of PPO from various sources followed somewhat later, including mammalian PPO from the mouse (Dailey and Karr 1987, Ferreira and Dailey 1988, Proulx and Dailey 1992) and cattle (Siepker et al 1987), yeast (Camadro et al 1994), *Rhodopseudomonas sphaeroides* (Jacobs and Jacobs 1981) barley and soybean (Jacobs and Jacobs 1987, Jacobs et al 1989); *Desulfovibrio gigas* (Klemm and Barton 1987); spinach (Matringe et al 1992); *Arabidopsis thaliana* (Narita et al 1996), tobacco (Lermontova et al 1997) and potato plant (Johnston et al 1998). The molecular weights for the oxygen-dependent protoporphyrinogen oxidases lie within the range 51000-57000 kDa and it appears that most of these protoporphyrinogen oxidases exist either as monomers as in *Bacillus subtilis* (Dailey et al 1994a), *Saccharomyces cerevisiae* (Camadro et al 1994), and bovine enzymes (Siepker et al 1987), or homodimers as in mouse (Dailey et al 1995), *Myxococcus xanthus* (Dailey and Dailey 1996a) and human PPOs (Nishimura et al 1995, Dailey and Dailey 1996b). All protoporphyrinogen oxidases are relatively specific for their natural substrate protoporphyrinogen IX though most will oxidise the non-physiological dicarboxylic mesoporphyrinogen IX to a limited extent. *B. subtilis* PPO can additionally use utilise coproporphyrinogen III for as a substrate (Dailey et al 1994). Evidence from spectral analysis and gene/protein sequence information shows that protoporphyrinogen oxidases are flavoproteins. The flavin cofactors consist principally of flavin adenine dinucleotide (FAD) and are generally non-covalently bound to the apoprotein (Dailey and Dailey, 1996a).

Bilirubin has been shown to function as a weak competitive inhibitor of PPO with a relatively high K_i (Ferreira and Dailey 1988). A class of herbicide, the phenyl ethers, have subsequently been shown to be potent inhibitors of both plant and mammalian PPO (Matringe et al 1989, Camadro et al 1991). The prototype for this inhibition is acifluorfen. Acifluorfen methyl is a more potent inhibitor and a number of other substituted diphenyl ethers (DPE) have been shown to be inhibitory as well (Camadro et al 1991). This effect has been shown in maize etioplasts and in potato, yeast and mouse mitochondria. The inhibition reported in those experiments was uniformly of competitive type with the exception of the yeast mitochondrial enzyme in which mixed-type inhibition was shown. (Camadro et al (1991). Our own work demonstrated that acifluorfen functions as a competitive inhibitor of PPO in homogenates of human liver and placenta, mouse liver and pig placenta, but interestingly, mixed-type inhibition was shown for pig liver (Hift et al 1994).

Mechanisms

The conversion of protoporphyrinogen IX to protoporphyrin IX involves a six-electron oxidation during which the methylene bridges in protoporphyrinogen-IX are converted into methenyl bridges. The exact nature of the catalytic mechanism employed by PPO is unknown

and more than one mechanism may operate, especially in prokaryotes since these can survive under both aerobic and anaerobic conditions. The reaction consumes three molecules of molecular oxygen and generates three H_2O_2 molecules (Dailey et al 1994, Dailey and Dailey 1996a, Dailey and Dailey 1996b). Since PPO contains no redox-active metals and only one FAD molecule, it is postulated that the reaction mechanism involves three two-electron oxidation reactions. Two possible mechanisms may explain this. In the first, PPO would bind the porphyrinogen substrate and carry out the complete reaction without release of the macrocycles until completion. In the second, PPO might catalyse three independent oxidation reactions with the release of tetrahydro and dihydro- intermediates; a model similar to that of the decarboxylations catalysed stepwise by uroporphyrinogen decarboxylase (Dailey and Dailey 1997).

2.3 IDENTIFICATION OF THE PROTOPORPHYRINOGEN OXIDASE GENE

Protoporphyrinogen oxidase was the last enzyme of the haem-synthetic pathway to be identified, purified and characterised from sources such as mouse (Dailey and Karr 1987, Proulx and Dailey 1992), cattle (Siepker et al 1987) and yeast (Camadro et al 1994). Attempts to purify human PPO in our own laboratory and elsewhere proved unsuccessful: its hydrophobicity, lability; loss of the flavin cofactor during purification and the difficulty of the assay for PPO activity (Appendix 1) all militated against success.

Complementary DNA (cDNA) sequences for bacterial and yeast PPO became available from 1994, when Dailey et al (1994) recognised that an unidentified cloned segment of the *Bacillus subtilis* chromosome labeled *HemY* (Hansson and Hederstedt 1992), occurring in close proximity to the segments coding for uroporphyrinogen decarboxylase and ferrochelatase activity, appeared to share sequences with some fragmentary sequences they had identified in mammalian tissue and believed to belong to the gene for mammalian PPO. They successfully cloned and expressed this sequence in *Escherichia coli,* and showed that the expressed protein indeed had protoporphyrinogen oxidase activity and therefore represented *B subtilis* PPO. This group has subsequently cloned the PPO sequence of *Myxococcus xanthus* (Dailey and Dailey 1996b), and have published important work on the structure and function of both prokaryotic and eukaryotic PPO. They have published the gene sequence for murine PPO, and have shown that PPO has significant sequence similarities to mammalian monoamine oxidases and plant phytoene desaturases. They propose that an extended region beyond the dinucleotide binding motif represents a signature motif for a superfamily of FAD-containing enzymes comprising protoporphyrinogen oxidases, animal monoamine oxidases and plant phytoene desaturases (Dailey and Dailey 1998); this region of similarity does not appear in any other proteins in the current gene-sequence databases.

The gene for human PPO

In 1995 Nishimura et al (1995) reported the cloning of a human cDNA for PPO, using a process of in-vivo complementation with a *hemG* mutant of *E. coli*. The cDNA was obtained from a human placental cDNA library and was shown to overcome the slow growth of a strain of *E. coli* deficient in PPO activity. Its identity as PPO was further confirmed by demonstrating enhanced PPO activity when the sequence was expressed in monkey kidney *cos-1* cells. The deduced amino acid sequence of the cDNA displayed a high degree of similarity to that for the *hemY* gene in *B. subtilis*, which encodes the bacterial PPO. They determined that the amino-terminal amino acid sequence could be configured as a βαβ dinucleotide binding fold, a common structural feature within FAD- or NAD-binding domains

(Proulx and Dailey 1992). This was supported by the demonstration of the characteristic motif *gxgxxg*, an arrangement of glycine residues typical of the dinucleotide-binding domains of many flavin-containing proteins. The length and mass of the human protein product were estimated to be similar to that of the *B. subtilis* enzyme at 477 amino acids and 51 kDa.

Both ferrochelatase and coproporphyrinogen oxidase are synthesised as precursors with a pre-sequence which targets importation into mitochondria, a common feature of mitochondrial proteins. Human PPO does not appear to contain such an amino-terminal pre-sequence. It has however been shown (Allison and Schatz 1986) that such a sequence is not always necessary for mitochondrial targeting since the targeting function may depend on a balance between basic, hydrophobic and hydroxylated amino acids.

Roberts et al (1995a) amplified a 4.5 kb genomic DNA fragment from a normal individual using sense and antisense primers located in the 5' and 3' untranslated regions of the cDNA for PPO as reported by Nishimura et al (1995). This fragment was partially sequenced after subcloning and shown to contain the entire 1431 bp coding sequence of human PPO, divided into 12 exons. The deduced amino acid sequence was identical to that reported by Nishimura (1995).

Taketani et al (1995) showed by Southern blotting of human genomic DNA that there is a single copy of the PPO gene The gene was found to have thirteen exons and to span approximately 8 kb, and exons appeared to encode functional protein domains. Two major transcriptional initiation sites were found in a region with sequence motifs characteristic of a promoter. It appeared that the same transcripts for PPO are present in both erythroid and non-erythroid cells.

Dailey and Dailey (1996a) isolated normal human placental PPO cDNA by complementation of a PPO-deficient strain of *E. coli*. They recovered a clone which was shown to express PPO activity. The expressed PPO protein was then purified from the culture medium; this was facilitated by the addition of a 6-histidine leader tag attached to the amino terminus. The human placental PPO cDNA obtained by complementation was 1.8 kb in length, with 1431 nucleotides coding for a protein comprising 477 amino acids. They calculated a molecular weight of approximately 51,000 kDa. Sephacryl S-300 column chromatography in the presence of 0.2% octyl glucoside indicated that the holoenzyme has a molecular weight of 100,000, with a calculated pI of 8.0. No membrane-spanning regions (Milpetz 1995) or membrane-targeting sequences were shown. The calculated extinction coefficient was 48,000 at 275 nm, and a putative dinucleotide binding motif (Wierenga et al 1986) is present near the amino terminal end. Ultraviolet/visible absorption showed the presence of flavin which was non-covalently bound and easily extracted with acid or SDS. Its pH-dependent fluorescence identified it as FAD. Sephadex G 200 gel filtration indicated that PPO is a dimer, and spectroscopic quantitation of the extracted flavin indicated the presence of a single molecule of FAD per PPO dimer. Metal analysis by plasma emission indicated the absence of any redox-active metal. The expressed enzyme utilised protoporphyrinogen IX as a substrate with a K_m of 1.7 mM and a K_{cat} of 10.5/min. Expressed PPO could utilise both mesoporphyrinogen IX and deuteroporphyrinogen IX as substrates: though K_m values were similar to those for protoporphyrinogen IX, the K_{cat} values were 100-fold lower.

Puy et al (1996a) subsequently sequenced the entire PPO gene in 50 unrelated French controls. They demonstrated that there are 13 exons located within a 5.5 kb region which includes a sequence 660 bp upstream from the initiation of the translation site. They characterised 12 introns with relatively short sequences ranging from 84 bp to 507 bp. They were able to demonstrate that the difference in the size of the gene reported by Taketani et al (1995) and Roberts et al (1995a) was explained by an incorrect approximation of the size of introns 4, 7 and 9. Nucleotides were more or less evenly distributed along the PPO gene sequence: 21% A, 28% C, 27% G, 25% C.

Assignment to chromosome 1q22-23

The gene for PPO had originally been assigned to chromosome 14q32. Bissbort et al (1988) reported close linkage between the gene locus for VP and the α1-antitrypsin gene, which was known to reside on chromosome 14. However, in a collaborative study with the University of Stellenbosch, we demonstrated that the assignment of the VP gene to chromosome 14 was incorrect (Warnich et al 1996a). Using lymphoblast PPO data to assign an accurate diagnosis in 79 individuals from 16 families, we demonstrated no evidence for linkage between VP and 5 microsatellite markers on chromosome 14q, closely related to the α1-antitrypsin locus. Subsequently, using fluorescent *in situ* hybridisation, the human PPO gene has been localised to chromosome 1q23 (Roberts et al 1995a) and to chromosome 1q22 by Taketani et al (1995). Further confirmation that the PPO gene resides on chromosome 1 was provided by Roberts et al since they showed a significant linkage between the VP phenotype in a sample of British patients with VP and microsatellite markers on chromosome 1. No linkage could be shown to markers in the alpha-1 antitrypsin region of chromosome 14, though the investigators suggested that the possibility that a locus associated with the α1-antitrypsin gene on chromosome 14 might modify the expression of VP had not been excluded.

2.4 MUTATIONS WITHIN THE PPO GENE

In 1996, a landmark was reached with the publication of the first papers identifying mutations in the PPO gene accounting for VP. Deybach et al (1996) described two PPO mutations in four French patients with VP. The Cape Town group reported two mutations, one of which appeared to account for the high prevalence of VP in South Africa (Meissner et al 1996); this was confirmed by an independent report by Warnich et al (1996b).

Mutations in South African patients with VP

In 1996 we identified two mutations carried on the maternal and paternal alleles respectively of a compound heterozygote who is described in detail in chapter 16. The identification of these mutations is described in the chapters which follow and is therefore not discussed further here. Working independently of the UCT laboratory, a South African group based at the University of Stellenbosch reported 3 missense mutations in the PPO gene (Warnich et al 1996b). Using single-stranded conformational polymorphism/heteroduplex (SSCP/HD) analysis to screen all 13 exons of the PPOX gene, including the splice site sequences, they studied a group of 17 unrelated South African VP patients and 10 normal controls and demonstrated abnormal mobility in exons 1, 2, 3 and 6. These were further investigated by sequencing. The exon 3 and 6 abnormalities represented the R59W and R168C mutations reported from Cape Town (Meissner et al 1996). The remaining patient showed an abnormality in exon 2. Direct sequencing revealed a $336^{A \rightarrow C}$ transversion corresponding to an H20P mutation. This mutation was absent in 80 normal chromosomes. Computer simulation suggests that in all cases, amphiphatic patterns within the wild-type protein were altered. Amphiphatic regions are helices described by a non-polar and an apolar face (Cooper and Krawczak 1984) and are thought to play an important part in the maintenance of protein structure. Two additional point mutations were identified in exon 1: 26 C/A and 150 C/G. Since these were present in both controls and patients, they were believed to represent neutral polymorphisms. Allele frequencies were as follows; 26C 0.65/26A 0.35, and 150C 0.95/150G 0.05. There was some association between haplotypes and mutations; the R59W and H20P mutations were associated with haplotype 26C/150C, while the R168C mutation was associated with haplotype 26A/ C150.

Mutations in European and American patients with VP

Deybach et al (1996) described two PPO mutations in four French patients by the direct sequencing of the PCR products of two overlapping fragments covering the entire PPO cDNA coding sequence. In the first patient, a heterozygous insertion (1022insG) was identified. This insertion results in a frameshift producing a premature TGA stop codon 31 codons downstream and occurs immediately after a stretch of six G repeats; it may therefore result from strand slippage during DNA replication. The resulting frame shift and premature stop codon are likely to result in the synthesis of a truncated protein with no catalytic activity. In the remaining 3 patients, two of whom were known to be related, direct cDNA sequencing revealed a G to C transversion at nucleotide 971 resulting in a glycine to arginine substitution at position 232 (G232R) in exon 7 of the amino acid sequence. Since this mutation creates a new *AcyI* site, the investigators were able to analyse 48 unrelated normal subjects by restriction digest assay; none were found to carry this mutation; No other abnormalities were found in the PPO coding sequences in the patients. In a pedigree of 14 members of the family of two related subjects, co-segregation of the mutation with reduced lymphocyte PPO activity was shown. They further identified an intragenic biallelic polymorphism; a G to A transition at position 1188 (1188 G/A). This transition occurs in exon 9 and leads to an arginine to histidine substitution (R304H) which creates a *BssSI* restriction site. Allele frequencies were calculated in 54 normal unrelated French subjects using *BssSI* restriction analysis; the allele frequencies were G: 0.56; A: 0.44. This mutation, in which a neutral glycine residue which is strictly conserved in human, mouse, yeast and *Bacillus subtilis* is replaced by a positively charged arginine residue, may possibly result in some destabilisation of the protein, but does not appear to be pathogenic in its own right.

Puy et al (1996a) demonstrated 5 intragenic polymorphic sites amongst 50 control subjects. Two of these were exonic: a -414 A/G polymorphism in exon 1 (which has a 5' untranslated sequence) and a 3101 G/A polymorphism in exon 9, which corresponds to the R304H substitution described by Deybach et al (1996). The other three polymorphisms are intronic; a 821 G/A dimorphism in intron 4; 1909 A/C in intron 6 and 1996 T/C in intron 6.

Lam et al (1997) described a 43 year-old female of German ancestry with VP in whom heteroduplex analysis revealed abnormalities in exon 3. Her father showed a simple heteroduplex in exon 3 whereas the proband showed a more complex heteroduplex. She was shown on sequencing to have a 2 bp insertion (165insAG) which results in a frame shift and premature termination codon (TAG) 8 bp downstream from the insertion. Her father carried a polymorphism consisting of a C to G transversion at the -47 position of intron 2, adjacent to exon 3. This polymorphism was studied in the family and in 20 unrelated unaffected individuals and the following allele frequencies were found: C: 0.72/G: 0.18. The G allele had a low calculated polymorphism information content (PIC) value suggesting that it is not in itself responsible for the VP phenotype though the authors suggest that it might contribute to low expression of this allele, which would aggravate the effects of a second, more severe mutation. Pedigree studies indicated that the insertion was present on the same allele as the polymorphism although it was absent in the father. Paternity was verified using microsatellite markers and the insertion thus represents a *de novo* mutation. It is postulated that the enzyme deficiency associated with this mutation results from nonsense-mediated mRNA decay (Cooper 1993). Alternatively, dominant-negative interference by small amounts of the mutant polypeptide with wild-type PPO (Maquat 1991) may occur, since PPO is known to function as a homodimer (Dailey and Dailey 1996a).

Frank et al (1998a) investigated a proband with VP and three clinically unaffected family members. Heteroduplex analysis, automated sequencing and allele-specific hybridisation indicated a G to T transversion in exon 5 at position 396 of the PPO cDNA. This results in the

substitution of glutamic acid by a nonsense codon at position 133 (E133X). The mutation was shown in the proband and a clinically unaffected son. Both are shown to be heterozygous. This transversion occurs in a region of the PPO gene which is strictly conserved in human, mouse and yeast, suggesting that this glutamic acid residue is important for PPO function. Such nonsense mutations are likely to result in dramatically reduced amounts of cytoplasmic mRNA, and thus a significant decrease in protein concentration (Cooper 1993, Maquat 1996).

The same group have subsequently reported a number of other mutations, and have repeatedly pointed out the incomplete penetrance of the VP-associated mutations they have identified. In most cases, clinically unaffected relatives bearing the same mutations have been identified. The clinical expression of mutations in the PPO gene in families of VP is thus highly variable, underlining the incomplete penetrance of the disease. Returning to a patient with homozygous VP first reported in 1990 (Norris et al 1990), Frank et al (1998b) proved that she is a compound heterozygote. A heteroduplex was shown in exon 6 in the proband and her father and a G to A transition at nucleotide 505 of the PPO cDNA was shown. This leads to a substitution of glycine by glutamic acid at position 169 (G169E) in the paternal allele. A second heteroduplex was noted in exon 10 in the proband and mother, and was shown to be associated with a G to A transition at nucleotide 1071 of the PPO cDNA resulting in a glycine to arginine substitution at position 358 (G358R). The G169E mutation causes an amino acid change from a neutral glycine residue to a negatively charged glutamic acid residue while the G358R mutation results in a change from a neutral glycine to a positively charged arginine. Neither of these mutations were demonstrated by heteroduplex analysis in 50 unrelated control individuals, suggesting that they are not common polymorphisms, but are likely to be pathogenic.

This group have subsequently reported a missense mutation ($1348^{T\rightarrow C}$) in a 32 year-old female of Italian, German, Scottish, Irish and French ancestry (Frank et al 1998c), resulting in a serine to proline substitution at position 450 (S450P). Heteroduplex analysis of exon 13 in 15 control subjects showed no heteroduplex formation. Heterozygosity was confirmed by allele-specific oligonucleotide hybridisation. This mutation results in a change from a polar serine residue, which is conserved in human, mouse and *B. subtilis*, to a non-polar proline residue. Subsequently two apparently unrelated probands were found to carry a further missense mutation (Frank et al 1998d). The first proband was a 57 year old female of German ancestry, the second an unrelated 38 year-old female of Armenian and North American ancestry. Heteroduplex analysis followed by automated sequencing of exon 6 revealed a G to A transition at nucleotide position 502 of the PPO cDNA, leading to the substitution of arginine by histidine at position 168 (R168H). This mutation was not present in 50 unrelated controls, but was present in the first proband's clinically unaffected mother and daughter. This mutation has also been reported in a Dutch patient (De Rooij et al 1997) and in patients from France and Britain (Whatley et al 1999). None of these patients are known to be related. Though it is possible that all carry an ancestral allele bearing the R168H mutation, it seems more likely that the 168 position represents a mutational hot spot since the R168H gene consists of a G to A transition at a suspected hypermutable CpG dinucleotide. Such dinucleotides have been shown to represent hot spots for mutations in vertebrate genomes (Cooper and Krawczak 1990); and 35% of all single base-pair substitutions causing human genetic diseases have been found to occur within CpG dinucleotides (Cooper and Youssoufian 1988). We have identified a R168C mutation in a South African family (Meissner et al 1996) (Chapter 3) providing further evidence that this codon is particularly mutation-prone.

In a further paper, Frank et al (1999) described two unrelated patients of Lebanese extraction and of Irish and Polish origin respectively. Abnormal heteroduplex formation was shown in exon 2 and sequence analysis revealed an A to T transversion at nucleotide position

1 of the PPO cDNA—the first base of the initiating methionine. This corresponds to a missense mutation with a conversion from methionine to leucine (M1L). In the second proband, a T to C transition was shown at nucleotide position 2 of the PPO cDNA, resulting in substitution of methionine by threonine (M1T). Both mutations resulted in loss of a restriction site for *Hsp*92II on the mutant alleles. Restriction analysis discriminates between wild type and mutant alleles, but does not discriminate between the M1IL and M1T mutations. Heteroduplex analysis of exon 2 in 70 unrelated control individuals showed no abnormality. These are the first missense mutations affecting the initiation methionine codon of the PPO gene. This class of mutation offers some interesting possibilities. Several examples of mutations in the translation initiation codon (AUG) have been reported previously in diseases other than VP (Cooper 1993). Though an AUG codon is thought to be an absolute requirement for the initiation of mRNA translation in eukaryotic ribosomes, some exceptions have been reported. These include the inefficient but detectable initiation of translation at ACG or CUG codons, particularly in viral mRNA. The authors speculate that in transcripts containing the M1L or the M1T mutation, translation may either begin downstream or not at all. There are no ATG triplets upstream of the first residue of the PPO cDNA, but 4 are present within the first 500 base pairs downstream. The first 3 are not in frame and none are flanked by purines at positions –3 and +4 with respect to the A residue of the ATG triplet, which is a favourable arrangement for alternative translation initiation. The first in-frame ATG codon is at nucleotides 486 to 488 but lacks a purine at the –3 position. Even if transcription did start at the site, the mature PPO polypeptide would lack 162 amino acids from the N terminus, including the entire putative dinucleotide-binding motif for the FAD cofactor. It is therefore highly unlikely that such a protein would be functional.

De Rooij et al (1997) investigated 22 Dutch families with VP. Five were found to carry the R59W mutation which is highly prevalent in South Africa (Chapter 3). A number of new mutations were discovered in the remaining families. Two missense mutations were shown in exon 9; L291P and H333L. A further missense mutation was noted in exon 6—the same R168H mutation reported by Frank et al (1998d), and occurring in the same codon as the R168C mutation reported by us (Meissner et al 1996) and Warnich et al (1996b). Three additional families carried insertions in exons 8, 11 and 12 respectively. All resulted in frame shifts introducing a premature stop codon.

Kauppinen et al (1997) found that a single point mutation (R152V) accounts for 60% of all VP families in Finland. An extensive pedigree analysis failed to reveal a common ancestor during the previous two centuries, suggesting that this mutation had occurred several generations previously. Expression studies confirmed a dramatic decrease in PPO activity for this mutation. A further 2 mutations were identified in a patient with compound heterozygous VP. An I12T mutation occurred in an evolutionary conserved region of exon 2 and severely affected PPO activity. The second mutation, a P256R substitution in exon 7, appear to result in a less complete reduction in activity.

The Anglo-French experience

Whatley et al (1999) studied a large number of patients with VP from both England and France. This study is particularly informative in that the large number of mutations identified allowed some general conclusions to be drawn. In total, 108 unrelated patients were studied and in only four did the investigators fail to identify a mutation. A total of 66 mutations were identified, bringing the number of VP mutations reported to at that point to 79, with a further 9 mutations having been identified only in the homozygous state. Of these 66 mutations, 60 had not previously been described. 54 mutations were detectable by abnormalities on either heteroduplex (HD) analysis or denaturing gradient gel electrophoresis (DGGE); thus, overall, these techniques used in combination would be expected to identify approximately 77% of new mutations in a typical VP population. Direct automated DNA sequencing is the most sensitive method, detecting 66 mutations. Mutations could not be identified in 4 patients and the sensitivity of direct automated sequencing is therefore 94%. Complete deletion of the PPO gene could be excluded in the four subjects in whom the mutation was not identified since they were shown to be heterozygous for known intragenic single nucleotide polymorphisms. The authors concluded that the causative mutations must lie outside those regions of the gene which they had sequenced, or that the mutations comprised partial deletions or insertions not detectable by PCR-based methods. Though earlier reports had suggested a clustering of mutations in exon 6 (Frank et al 1998d), the 66 mutations were evenly distributed throughout the coding region of the PPO gene, and no exon had more than 10 or fewer than 2 mutations with the exception of exon 1, the untranslated region of the gene, where no mutations were detected, despite the suggestion by Kotze et al (1998) that exon 1 is particularly mutation-prone.

The types of mutation encountered were variable, comprising small insertions or deletions introducing a frame shift and a premature stop codon (36%), missense mutations, including one codon deletion and three that altered the initiation codon (38%), a change in invariant nucleotide at a splice site (14%) and a change to a premature stop codon (11%). In one patient a base change adjacent to the splice acceptor site of exon 8 apparently creates an additional splice acceptor site used in preference to the normal site, since sequencing of this product showed a mixture of similar quantities of two cDNA species; one from the normal allele and one showing the addition of 8 base pairs to the 5' end of exon 8.

There was considerable allelic heterogeneity. Five of the 66 mutations were present in more than three families, accounting for 26% of the families in total. Furthermore, two mutations (L15F and G435X) were associated with at least two different haplotypes indicating that neither is likely to have been inherited from a single common ancestor. For the other three mutations, common ancestry could not be excluded. All other mutations were present in three or fewer families, and 47 mutations were restricted to a single family only. The disease appear to be more heterogeneous in France than the UK. Only two mutations were found in both countries, but neither was common. Both occur at potentially hypermutable sites: a CpG dinucleotide on the antisense strand (Cooper and Krawczak 1993)—the R168H mutation described in two other populations (De Rooij et al 1997, Frank et al 1998d)—and a polyG tract (745delG). VP shows less allelic heterogeneity in western Europe than that shown by other acute porphyrias. In France and the UK, all the mutations of the PBG deaminase gene in 166 unrelated patients with AIP have a prevalence of less than 5% with the exception of a single mutation in the UK with a prevalence of 10% (Puy et al 1997, Whatley et al 1999). HCP is also heterogeneous in France and the UK. Yet in VP, 5 of the mutations have prevalences of 7-12%, and haplotype analysis suggests that at least two of these are likely to have arisen on more than one occasion. No "founder" gene was found as is

present in South Africa, and to a lesser extent in Finland (Kauppinen et al 1997). Though polymorphisms are similar in France and the UK, there was an almost complete separation of mutations between the two countries, which suggests a relatively recent origin for the mutations in these families. The R59W mutation is not present in any British or French patient.

No significant correlation could be shown between the type of mutation—missense, nonsense, splice site or frameshift—and a specific clinical presentation, such as photosensitivity or the acute attack. Furthermore, the frequencies of each type of presentation are similar in France, the UK and South Africa. This suggests that that allelic heterogeneity does not substantially alter the pattern of clinical expression of the disease, and that VP in South Africa is clinically representative of the disease elsewhere. The genotype does not appear to be a significant determinant of clinical severity.

Homozygous VP

The mutations present in a number of patients with homozygous or compound heterozygous VP have now been described. Our own experience with four such patients is reviewed in chapter 16. Accordingly, the literature pertinent to this interesting and unusual clinical syndrome is not discussed here, but in Chapters 15 and 19.

CHAPTER 3:

THE R59W MUTATION AND THE HIGH PREVALENCE OF VARIEGATE PORPHYRIA IN SOUTH AFRICA

Published experience with the mutations identified in patients with VP was reviewed in the preceding chapter. In this chapter, our identification of the first two mutations in South African patients with VP is described. The identification of eight further mutations in South African patients is reported in Chapter 4 and the significance of these results is discussed in Chapter 5.

3.1 INTRODUCTION

In a collaborative project, the UCT porphyria group and Prof Dailey's group at the University of Georgia, USA, already had extensive experience and some success with the cloning of the PPO gene (Dailey et al 1994). Following the publication of the cDNA sequence for human PPO by Nishimura et al (1995), we were able to initiate an immediate search for the mutation underlying the high prevalence of VP in the South African population. This experimental work is described in this chapter.

The study proceeded in four stages. In the first study, two mutations were identified in a young South African compound heterozygote and her immediate family. These mutations were then sought in a small sample of apparently unrelated South African families with VP. One of these mutations was demonstrated in all four. In the third study, this mutation was shown to co-segregate with biochemical and clinical evidence of VP in a single kindred. The mutation was then shown to be present in the majority of a sample of South African families with VP.

3.2 OBJECTIVES

- To identify mutations in the PPO gene in South African patients with VP.
- To identify the putative founder mutation underlying the high prevalence of VP in South Africa.

3.3 SUBJECTS

Detection and identification of the R59W and R169C mutations

The initial subject of the investigation was LO, a child with phenotypically severe VP who was suspected to be either a VP homozygote or compound heterozygote. She is described in detail in Chapter 16. Neither parent had experienced any symptoms of VP, and there was no family history of VP.

Screening for the R59W and R168C mutations in a sample of South African VP families

Eight subjects with biochemically-proven VP from four apparently unrelated families were studied. One of these families was known to belong to the large kindred whose origins have

been traced back to the original Dutch immigrant couple postulated by Dean (1963) to represent the founder of the South African VP families. Three normal individuals served as controls.

Correlation with the VP phenotype in a single family

All members of one of these families, a large well-characterised kindred previously studied by our laboratory, were screened for the mutation (Figure 3-1). For the first two generations the diagnosis was based on the reporting of unequivocal clinical evidence of VP. From 1966 onwards, which includes some members of generation II and all members of generations III and IV, the diagnosis was based on the demonstration of classic VP porphyrin excretion patterns.

Determination of the proportion of South African families who carry the R59W mutation

A further 45 individuals from 27 well-characterised South African families with VP were tested. All had unequivocal evidence of VP by biochemical testing.

3.4 METHODS

Biochemical diagnosis of VP

Urine, stool and plasma were analysed by quantitative fluorometric porphyrin analysis using the methods described in Appendix 1 and the diagnostic criteria established in Chapter 7.

Kinetic studies on EBV-transformed lymphoblasts

Lymphocytes derived from peripheral blood were transformed to lymphoblasts by co-culture with Epstein-Barr virus as described in Appendix 2. Approximately 100 ml of culture medium containing 3-4 million cells per ml were washed twice in Hanks balanced salt solution. Lymphoblast viability was determined by Trypan blue exclusion. PPO was liberated by sonication into 100 ml of 100 mM Tris/HCl, 3 mM DTT, 1 mM EDTA, 0.1% Tween 20, pH 8. Aliquots of the sonicate were assayed for PPO activity using a fluorometric detection process as described by Meissner et al (1986). The method is described in detail in Appendix 3.

Screening for the R59W mutation

The mutation was first identified by manual sequencing of reverse-transcribed DNA as described below, and this method was used for diagnosis until the genomic DNA sequence for human PPO had been established (and was kindly shared with us by Prof George Elder, Cardiff, UK). The R59W mutation abolishes an *AvaI* restriction enzyme cutting site, and the R59W mutation was subsequently diagnosed by direct sequencing or by *AvaI* restriction digest, as stated.

DNA sequence analysis

Total RNA was extracted from Epstein-Barr virus-transformed lymphoblast cultures as described in Appendix 4.1. The RNA was reverse-transcribed and amplified by PCR (RT-PCR) as described in Appendix 4.2-3. Four sets of primers, both forward and reverse, were

	LO	DO	ULN
Urine precursors (umol/10 mmol creatinine)			
ALA	16	24	<45
PBG	9	7	<16
Urine (nmol/10 mmol creatinine)			
Uroporphyrin	31	5	<20
7-COOH Porphyrin	0	0	<1.5
6-COOH Porphyrin	0	0	
5-COOH Porphyrin	0	3	
Coproporphyrin	101	73	<240
Stool (nmol/g dry weight)			
Uroporphyrin	5	4	<1.7
7-COOH Porphyrin	0	0	
6-COOH Porphyrin	0	0	
Pseudo 5-COOH Porphyrin	0	0	
5-COOH Porphyrin	13	19	
Isocoproporphyrin	0	0	
Coproporphyrin	67	384	<50
3-COOH Porphyrin	0	0	
Protoporphyrin	698	1241	<200
Plasma (nmol/l)			
Uroporphyrin	2	0	<2.5
7-COOH Porphyrin	0	0	
6-COOH Porphyrin	0	0	
5-COOH Porphyrin	0	0	
PU	0	0	
Coproporphyrin	26	0	<1.0
Protoporphyrin	46	0	<4.5
RBC (nmol/l)			
Uroporphyrin	0	0	
7-COOH Porphyrin	0	0	
6-COOH Porphyrin	0	0	
5-COOH Porphyrin	0	0	
Coproporphyrin	0	106	<80
Protoporphyrin	4406	1301	<800

Table 3-1. Biochemical results in the proband (L0) and her mother (D0). (ULN=*upper limit of the normal range*).

chosen in order to generate four fragments covering the entire published cDNA sequence. These primers were as follows:

PF1	5'	CTACCTATTGTGGGTTTCCG
PR1	5'	CTTGGTCAGCTCCCTCAG
PF2	5'	CTGCATGCCCTACCCACTG
PR2	5'	TTCAAGGCCTGAGGCAACA
PF3	5'	ACTTCGTGGAGGTCTAGAGA
PR3	5'	CCGTCCTGCTCAGGGAAAGCAAC
PF4	5'	GGAATCGTGTATGACTCA
PR4	5'	TTTTCATGAATGAGAGTTGGGGATC

The specificity of the PCR product was assessed on a 6% acrylamide gel (Appendix 4.4). Thereafter the product was extracted from MS8 agarose using a Qiaex II gel extraction kit (Appendix 4.5) prior to sequencing. The PCR-generated fragments were then sequenced manually using the Sequenase PCR product sequencing kit (Appendix 4.6). Sequencing was performed on all four DNA fragments in both a forward and a reverse direction.

AvaI restriction digest

Genomic DNA was isolated from blood (Parzer and Mannholter 1991, Appendix 4.8) and amplified by PCR using the following primers:

- forward primer 5' GAATA TGCCT CTTCC CCTCC CC
- reverse primer 5' CACAA CCTCT CCTAG ACATC CC

This generates a 252 base-pair fragment which includes exon 3. This was digested with the *AvaI* restriction enzyme (Promega), The products of digestion were fractionated on a 6% polyacrylamide gel and visualised with ethidium bromide under ultraviolet light. All methods are described fully in Appendix 4.

3.5 RESULTS

Note that the sequencing and restriction analysis gels are shown at the end of this chapter. Negative rather than positive images are reproduced here, since the detail is more easily visible.

Porphyrin chemistry

Both LO and her mother (DO) showed the classic biochemical profile of VP (Table 3-1), whereas her father (BO) was biochemically normal.

PPO activity

A severe reduction in activity to less than 10% of the control value was demonstrated in the proband. Her mother showed a 50% reduction in activity in keeping with heterozygous VP. PPO activity in her father was reduced by approximately 25%, which is significantly less than the 50% reduction shown in a large group of heterozygous South African patients with VP (Meissner et al 1986).

Mutations in Proband LO and her parents

Two deviations from the sequence published by Nishimura et al (1995) were identified in the proband at the University of Georgia: a C to T transition in codon 59 (Figure 3-2, end of chapter), resulting in a change in amino acid sequence from arginine to tryptophan (R59W

substitution), and a C to T transition in codon 168, resulting in a change in amino acid sequence from arginine to cysteine (R168C substitution). Direct sequencing of the four PCR-generated fragments derived from PPO cDNA confirmed the presence of both the R59W and the R168C mutations in the proband (LO), the R59W mutation in her mother (DO) and the R168C mutation in her father (BO) (Table 3-2). Neither of these mutations, nor any other abnormality, were identified in 4 control cDNA samples prepared from normal individuals. In all cases, all four fragments, spanning the entire cDNA for PPO, were sequenced, and no other deviations from the published sequence were shown.

	LO (proband)	DO (mother)	BO (father)
Exon	3 and 6	3	6
Sequence change	C452/T452 and C779/T779	C452/T452	C779/T779
Amino acid substitution	R59W and R168C	R59W	R168C

Table 3-2. Mutations detected in proband and her parents.

Screening of a further 8 South African subjects with VP

All 8 subjects studied by sequence analysis of cDNA were shown to carry the R59W mutation. Neither the R168C mutation nor any other mutation were shown in these subjects. Neither mutation was present in the controls.

Correlation with the VP phenotype in a single family

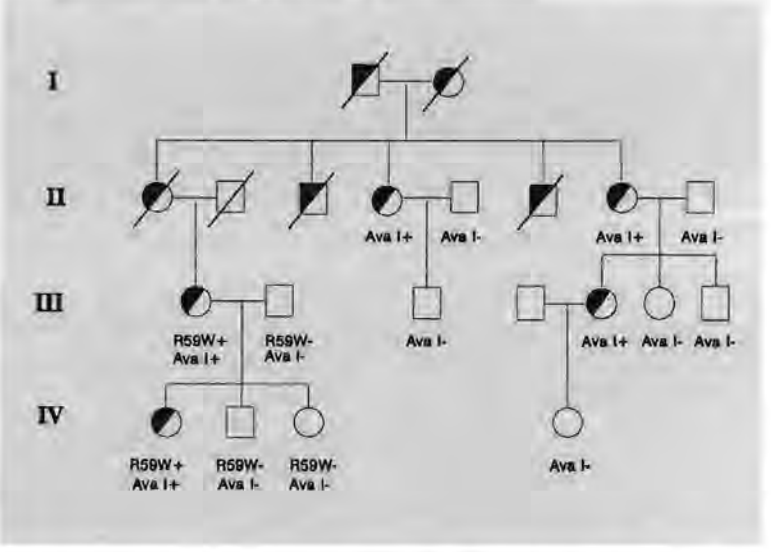


Figure 3-1. Screening for the presence of the R59W mutation in a single kindred. Positive and negative direct sequencing results are indicated asR59W + and R59W —. Positive and negative AvaI restriction digest assay results are indicated as AvaI + and AvaI —. Half-shading indicates the presence of VP confirmed biochemically (generations II-IV) or clinically (generation I, some members of generation II). A diagonal slash indicates that the subject is deceased.

Informative members of the family are shown in Fig 3-1. The R59W mutation was demonstrable in every member of the pedigree with a diagnosis of VP by a positive *AvaI* restriction analysis (Figure 3-3, end of chapter) or by direct manual sequencing of fragment 2.

Where both sequencing and restriction analysis were performed, the results agreed. Conversely, the mutation was not shown in any subject in whom there was no clinical or biochemical evidence of VP.

Screening of 34 South African families

With the addition of the 27 families to the four families studied and the families of the mother and father of the proband LO, data were available on 46 subjects from 33 apparently unrelated families (Table 3-3). The R59W mutation was present in 43 subjects whereas a further two subjects, a mother and son, carried neither the R59W nor the R168C mutations. They were subsequently shown to carry an H20P mutation (Chapter 4).

Mutation	Families
R59W	31 (94%)
R168C	1 (3%)
H20P	1 (3%)
Total	33 (100%)

Table 3-3. Contribution of the R59W mutation to the prevalence of VP in South Africa.

3.6 CONCLUSIONS IN BRIEF

We have shown that our initial proband, LO, is a compound heterozygote carrying two missense mutations: the R59W substitution in exon 3 and the R168C substitution in exon 6. These mutations are inherited from her mother and father respectively. We have further shown that the R59W mutation co-segregates with VP and is present in 94% of a sample of South African families with VP, at least one of which is known to belong to Dean's large kindred. We therefore conclude that the R59W mutation is overwhelmingly present in the South African VP population and represents the South African founder mutation. The other mutations identified in the South African VP population are described in the chapter which follows. These results are then discussed together in Chapter 5.

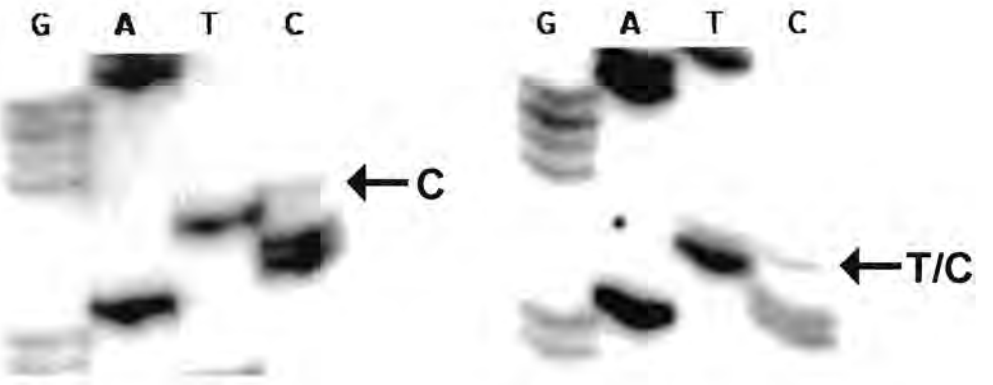


Figure 3-2. Direct sequence determination reveals the R59W missense mutation. Shown on the left is a portion of the cloned sequence from a normal subject. The sequence reads GGGGCTCCA. Shown on the right is the sequence from a R59W heterozygote. The sequence reads GGGGC/TTCCA. The altered base is indicated by the arrows.

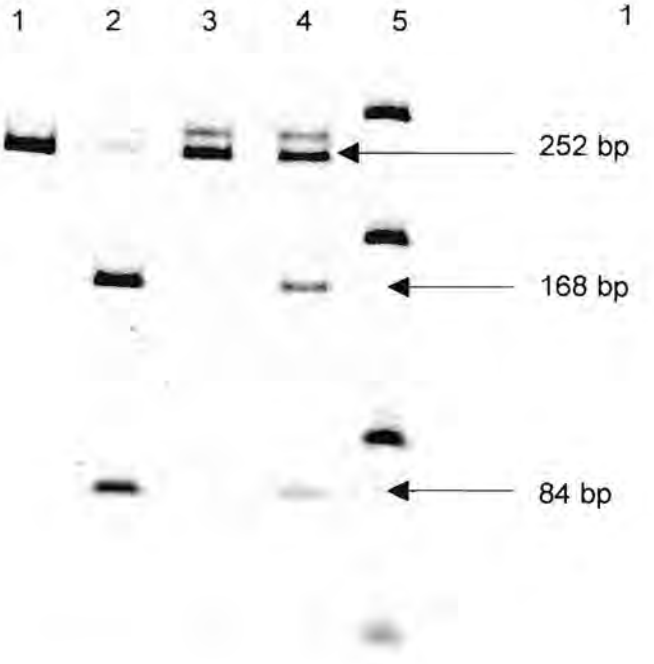


Figure 3-3. Restriction digest assay of a fragment corresponding to exon 3 in a subject with the R59W mutation. The lanes are as follows: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=size markers. In the wild-type allele, *AvaI* cuts the 252 bp fragment into two smaller fragments of 168 bp and 84 bp. In the mutant allele, the *AvaI* cutting site is abolished. Since the patient is heterozygous, both digested and undigested fragments are seen.

CHAPTER 4:

OTHER PROTOPORPHYRINOGEN OXIDASE MUTATIONS IN THE SOUTH AFRICAN VP POPULATION

The identification of two mutations in a young patient with compound heterozygous VP was described in Chapter 3, where it was shown that one of these mutations, the R59W mutation, has a very high prevalence amongst South African families with VP. One of the 33 families tested however had yet another mutation. This and those which we have subsequently encountered are described in this chapter.

4.1 INTRODUCTION

In the five years from 1996 to 2000, approximately 250 new patients with VP have been identified by biochemical testing in the UCT laboratory. Wherever possible, these subjects have been screened for the R59W mutation. Nearly all have been found, as expected, to carry the R59W defect, thus confirming the high prevalence of this mutation among the South African VP population. In a few instances however, the R59W mutation has not been found in subjects who have unequivocal biochemical evidence of VP. In such cases we have searched for other mutations. These studies are described in this chapter.

4.2 OBJECTIVES

- To identify mutations in the PPO gene in R59W-negative South African subjects with VP.

4.3 METHODS

Diagnosis of VP

A primary diagnosis of VP was confirmed by the thin-layer chromatographic separation and quantitation of porphyrins in urine, stool and plasma, supported or replaced in some later patients by the demonstration of a characteristic plasma fluorescence peak at 625 nm on plasma fluoroscanning, a technique which is discussed in Section 2. These methods are fully described in Appendix 1.

Clinical study

Wherever possible, subjects were interviewed personally. A full history was taken, with particular attention to those symptoms indicating an acute attack, the age of onset and the presence of skin disease. As part of a full examination, the presence of skin disease and of residual neuropathy, which might indicate a previous acute attack, were sought. In many cases, subjects were established patients of the UCT porphyria clinic; these patients were thus well-known to us and their course adequately documented. Where patients were not resident in Cape Town and could not travel to Cape Town for assessment, clinical details were derived from the referring doctors or from the patients telephonically.

Identification of mutation

The first mutation other than R59W, the R168C mutation, was identified by sequencing of four PCR fragments as described in Chapter 3. For the detection of subsequent mutations, a standard, sequential approach was established as follows.

Aval restriction analysis for R59W

A DNA sample was initially screened by *Aval* restriction analysis to exclude the R59W mutation. If this was negative, the biochemical evidence for a diagnosis of VP was reviewed and if necessary further samples were tested biochemically to confirm that VP was indeed present and that a further search for an unknown mutation was justified. Samples which tested negative for the known mutations were then screened for unknown mutations as follows.

Single-stranded conformational polymorphism/heteroduplex (SSCP/HD) analysis

A set of standard primers, chosen to include all exons of the PPO gene and their flanking regions, were employed (Table 4-1). Primers were designed such that the PCR product did not exceed 400 base pairs since larger products increased the possibility of a false-positive result on SSCP/HD analysis. All PCR products were analysed on a 6% acrylamide gel before SSCP/HD analysis, and only products that were absolutely pure (running as a single band on the gel) were utilised for the analysis (Appendix 4). In three cases however, the R59W, 537delAT and 569delG,$570^{T\rightarrow A}$ mutations, the mutant PCR products ran as double bands on 6% acrylamide; this finding alerted us to the presence of a mutation in that exon. Such double bands were never visible on agarose gels.

Simultaneous SSCP/HD analysis was performed on a 1xMDE gel in 0.6% TBE in both the presence and absence of glycerol. Two control samples drawn from normal subjects were included on each gel as negative controls to reduce the chances of inadvertent recognition of a non-significant polymorphism. The mobility of each product was assessed after silver staining. If a particular product ran aberrantly, the corresponding exon was again amplified by PCR and the SSCP/HD analysis was repeated. If the same result was obtained, DNA corresponding to that exon was submitted for automated sequencing in both the forward and the reverse direction at the core sequencing facility of the University of Stellenbosch, who utilise a Big Dye terminator cycle sequencing kit.

Once an apparent mutation has been identified, the following steps were undertaken in an attempt to validate its causal role in the porphyria.

- The PCR and sequence analysis were repeated to exclude PCR errors.
- A suitable restriction enzyme cutting site was sought and a restriction assay established; a positive result with the restriction assay in the subject served as further confirmation that a mutation was present.
- Approximately 50 normal controls were tested for the mutation to exclude the possibility of a common polymorphism.
- Family members were tested biochemically and by restriction assay to demonstrate co-segregation of the mutation with VP.
- Where a suitable restriction enzyme cutting site could not be found, SSCP/HD analysis or direct sequencing were used to screen controls and family members for the mutation.

Exon		Primer	Size (bp)
Exon 1.	DF_1	5'CCGCCAATCCAGATGTAGG	365
	DR_1	5'AACTAAGTGTGCACGGATGG	
Exon 2.	DF_2	5'TCTGCCTGTCCATATCGC	167
	DR_2	5'ATTAAATGAAGCTCCCTC	
Exon 3.	DF_3	5'GAATATGCCTCTTCCCCTCCCC	252
	DR_3	5'CACAACCTCTCCTAGACATCCC	
Exon 4.	SF_4	5'CCTCTTCTGAGGGCATGTGG	180
	SR_4	5'GAGGGCACAGTAAAAGGAGC	
Exon 5.	SF_5	5'GAGGTATGTCAGGAGCTTCC	247
	SR_5	5'GATTTGAACAGGGAGCTCTG	
Exon 6.	DF_6	5'TATCCCACCCTCATTCCCTACCA	317
	DR_6	5'ATTGAATAGCACCCCTTGTCC	
Exon 7.	SF_7	5'TGTGAGCCACTGCATCCAG	272
	SR_7	5'CAGGTTCATTACACTCCAGG	
Exon 8.	SF_8	5'CTCATCAAATTCTCAGGTTCTGG	197
	SR_8	5'TGTGGTCCTGCTGACCCAGG	
Exon 9.	SF_9	5'CCTTCTGAGTCAGGCCTCTGC	202
	SR_9	5'GGATTACAGGTGTGAGCCACCA	
Exon 10.	SF_{10}	5'AGAGCCCTTTCCTTCTGACGCATG	223
	SR_{10}	5'TGGCCTTGCCTACAATGGAGCAC	
Exon 11.	SF_{11}	5'GTGGCATTTCCAGAGGGCTCC	257
	SR_{11}	5'GGAGAGCTGAGGGAAGTTTATCC	
Exon 12-13	SF_{12-13}	5'CTGGATCCTCTCCTCTCTTC	333
	SR_{12-13}	5'TAGAACAGCCAGACCAAGCC	

Table 4-1. Exon specific primers used for the PCR generation of DNA fragments used for SSCP/HD analysis and direct sequencing.

Restriction analysis

Genomic DNA was extracted from peripheral whole blood by the method of Parzer and Mannholter (1991). Using the appropriate exon-specific primers (Table 4-1), the relevant exon was amplified. Approximately 1 μg of PCR product was digested with the appropriate restriction cutting enzyme (Table 4-2), using a sufficient quantity to allow for complete digestion, at the temperature and for the time recommended by the manufacturers of the enzyme. The products were then electrophoresed on a 6% polyacrylamide gel and visualised by ethidium bromide staining. Pre-and post-digestion products for both patient and a control were always included in the gel. The technique is fully described in Appendix 4-13.

4.4 RESULTS

The SSCP, heteroduplex and restriction analysis gels and automated sequencing printouts, are collected at the end of this chapter. In the case of the gels, the negative rather than the positive image is shown, since the detail reproduces more clearly.

Including the R59W mutation, ten mutations have been identified. Seven are associated with the VP phenotype in heterozygotes; three were identified in compound heterozygotes in association with the R59W mutation. Clinically evident VP has not been seen in association with these three mutations in heterozygotes, but the compound heterozygotes generally show with a more severe phenotype. The mutations we have identified in the South African population are summarised in Table 4-2 and their genomic positions are indicated in Appendix 5. With the exception of the R59W mutation, none has been identified in more than one family.

Exon	Mutation	Restriction enzyme	Clinical expression
2	H20P	None	Heterozygote: typical VP
	L15F	*EaeI*	Heterozygote: typical VP
3	R59W	*AvaI*	Heterozygote: typical VP
5	R138P	None	R59W compound heterozygote : phenotypically mild HVP
6	537 delAT	*MvaI*	Heterozygote: typical VP
	R168C	*BsaJI*	R59W compound heterozygote : phenotypically severe HVP
7	c769delG; 770T>A	*HpaII*	Heterozygote: typical VP
8	V290M	*BsiYI*	Heterozygote: typical VP
10	Y348C	*MaeIII*	R59W compound heterozygote: phenotypically severe HVP
11	Q375X	*MaeIII*	Heterozygote: typical VP

Table 4-2. Summary of the mutations, suitable restriction enzymes for diagnosis and clinical presentation identified in South African patients with VP. (HVP=homozygous VP.)

R168C: (Family Olivier)

The proband is the young female compound heterozygote described in Chapter 3 who carries both the R59W mutation and thus, the R168C mutation.

DNA analysis

The mutation was initially identified by direct manual sequencing of a fragment amplified by PCR from a cDNA clone (Chapter 3) and has subsequently been shown on automated sequencing (Figure 4-4). Exon 6 contains two *BsaJI* cutting sites, one of which is abolished by the R168C mutation (Figure 4-5).

Family studies

The R168C mutation has thus far been identified only in the proband and her father. The father belongs to a small Afrikaans-speaking family but believes that his family is originally

of German extraction. He has few living relatives, none of whom will agree to be tested. Neither he nor any of his relatives have experienced symptoms suggestive of VP.

H20P: (Family De Reuck)

The proband is a woman of European extraction in whom VP was first diagnosed clinically and biochemically by solvent extraction (stool coproporphyrin 394 μg/g, stool protoporphyrin 887 μg/g) in 1971 at the age of 43. Subsequently her son, now aged 40, was also confirmed to have VP on biochemical testing. Both have typical cutaneous VP, but have never experienced acute symptoms. These two subjects formed part of the initial test group of South African VP patients studied both by ourselves and by Warnich et al (1996) and were found to be R59W-negative. The mutation was first identified by Warnich et al and was subsequently confirmed in our laboratory as described here.

DNA analysis

PCR of all thirteen exons and subsequent SSCP/HD analysis and sequencing were performed as described above. The primer employed for exon 2 is shown in Table 4-1 and produces a 167 bp fragment.

Simultaneous SSCP/HD analysis revealed an aberrant mobility shift in exon 2 in the proband (not shown). No abnormalities were detected in the remaining exons. Direct sequencing revealed an A to C transversion resulting in a histidine to proline substitution (H20P). No suitable restriction enzyme is available for this mutation.

Family studies

Carriage of the H20P mutation was confirmed in the proband's son by direct sequencing of the exon 2 PCR product. One of his three daughters has inherited the H20P mutation whereas the remaining 2 children are negative. All are still prepubertal.

L15F: (Family Middleton)

The proband is a 44 year-old female of British extraction. She demonstrated typical photocutaneous sensitivity and had experienced acute symptoms prior to diagnosis. Biochemical analysis in the UCT laboratory demonstrated VP as shown in Table 4-3 (Subject A) and she has a fluorescence peak at a 625 nm on plasma fluoroscanning.

DNA analysis

PCR of all exons and subsequent SSCP/heteroduplex analysis and sequencing were performed as described above.

The proband was negative for the R59W and R168C mutations. On SSCP/HD analysis, an abnormal mobility shift was noted in exon 2 (Figure 4-6). All other exons appeared normal. Sequencing, both forward and reverse, revealed a G to C transversion resulting in the substitution of phenylanaline for leucine: L15F (Figure 4.7). This mutation abolishes an *Eae1* restriction site (Figure 4-8). Fifty control individuals were screened for the L15F mutation by *Eae1* analysis. All were negative.

Subject		A	B	C	D	E	F	G
Urine precursors (umol/10 mmol creatinine)								
ALA	<45	182	31	65	49	16	106	—
PBG	<16	38	18	151	36	8	106	—
Urine (nmol/10 mmol creatinine)								
Uro	<20	72	79	142	0	12	1464	16
C7	<1.5	19	30	0	0	18	570	5
C6		3	12	0	0	0	178	0
C5		232	33	258	0	6	1296	6
Copro	<240	1585	63	1175	91	177	4538	195
Stool (nmol/g dry weight)								
Uro	<1.7	0	0	0	0	2	33	0
C7		0	0	0	0	0	73	0
C6		0	0	0	0	0	120	0
pseudo C5		87	94	15	22	23	376	30
C5		43	24	10	0	2	161	15
Isocopro		0	0	0	0	0	114	0
Copro	<50	224	146	272	109	69	1212	274
C3		0	0	0	0	0	634	0
Proto	<200	1029	664	576	371	432	1804	409

Table 4-3. Biochemical values in the probands described in this chapter.

Family studies

Eae1 restriction analysis of nine other family members revealed four further subjects positive for this mutation: the proband's father, brother and twin sons, all of whom are adult. With the exception of the proband, none show evidence of VP either biochemically or on plasma fluoroscanning, and all are asymptomatic.

537 delAT (Family Henderson)

The proband is a mixed-race female who first presented with photocutaneous symptoms suggestive of VP at the age of 29 and was shown on biochemical testing to have VP (Table 4-3, Subject B).

DNA analysis

Extraction of genomic DNA, amplification by PCR and screening of all exons followed by sequencing were as described above. An aberrant SSCP mobility shift and a heteroduplex (Figure 4-9) were clearly visible in the PCR product of exon 6 in the proband. Sequence analysis revealed a 2 bp deletion following nucleotide position 537 (537 delAT), numbering from the A of the initiating methionine codon of the PO cDNA sequence reported by Nishimura et al (Figure 4-10). No abnormality was found in the other exons by SSCP/HD

analysis. This mutation creates an *Mva1* cutting site (Figure 4-11), and restriction analysis was subsequently used to screen for the mutation. An additional 48 unrelated control individuals drawn from the same racial group all tested negative for this mutation.

Family studies

31 family members were screened by *Mva1* restriction analysis (Figure 4-1). Seventeen tested positive for the mutation. Of these 17 subjects, 13 showed positive biochemical evidence for VP on TLC analysis of urine, stool and plasma. A further four subjects had negative stool biochemistry; however, subsequent plasma fluoroscanning has shown a small peak in each, including a 13 year-old boy. Thus in all four subjects in whom an initial biochemical diagnosis of "silent VP" was made; plasma fluoroscanning has revealed an abnormality consistent with VP. The mutation co-segregated with both clinical and biochemical evidence of VP, and was present in all subjects demonstrating positive biochemistry or a fluorescence peak. All 31 individuals were examined clinically; only three (all carrying the mutation and showing positive biochemistry) had clinical evidence of disease. None had had acute symptoms. Two of these, the proband and a female cousin, both manifested a somewhat severe form of skin disease but both suffered from severe acne vulgaris, which may have accentuated the skin damage.

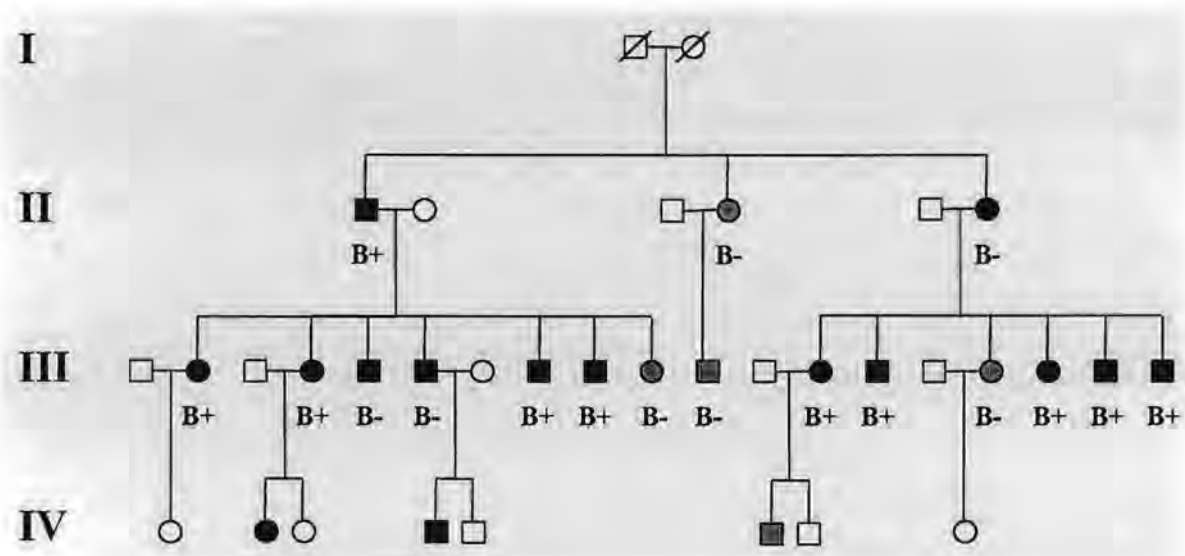


Figure 4-1. Abridged pedigree of a family carrying the 537delAT mutation. Mva1 restriction analysis was used to determine the presence or absence of the mutation. The presence of the mutation is indicated by a solid symbol, absence by a hatched symbol: an open symbol indicates that the subject was not tested. Positive biochemical evidence of VP is indicated by B+ and of normal porphyrin chemistry by B-. No subject in generation IV was tested biochemically since all are prepubertal.

Thus, of 31 family members tested, 55% carry the mutation; 76% of these show abnormal stool chemistry whereas 100% show either abnormal stool chemistry or a positive plasma fluorescence scan. Only 18% of those carrying the mutation are clinically affected (Fig 4-2).

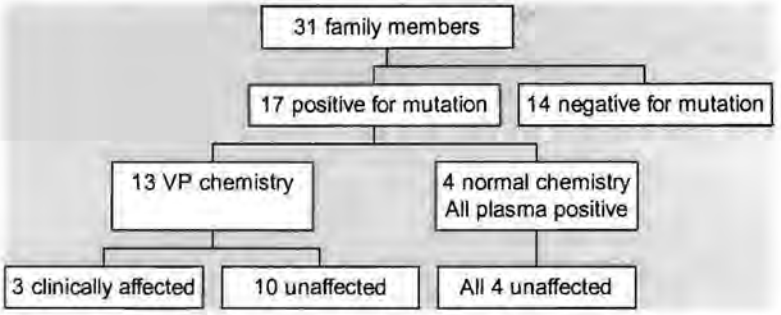


Figure 4-2. Association of positive biochemical and clinical expression of the 537delAT mutation in a single family.

4.5 C769delG; $770^{T\rightarrow A}$: (Family Breda)

The initial subject is a male of mixed race who first presented at the age of 12 with skin disease. Biochemical analysis is shown in Table 4-3 (Subject C) and is compatible with VP, though the marked elevation in erythrocyte protoporphyrin level is notable. Subsequently a diagnosis of VP was made in a sister, in whom the initial DNA studies were performed, and his mother.

DNA analysis

PCR of all exons, screening by SSCP/HD and sequencing were performed as described above. An aberrant mobility shift as well as a heteroduplex (Figure 4-12) were noted in the PCR products of exon 7 derived from the proband's sister. Direct sequencing revealed a deletion at position 769 followed by a T to A transversion (C769delG;$770^{T\rightarrow A}$) (Figure 4-13). An *HpaII* cutting site is abolished by the deletion and was subsequently used to screen for its presence (Figure 4-14). Fifty controls were screened by restriction analysis and were negative.

Family studies

Restriction analysis and direct sequencing of exon 7 of the proband's mother and brother confirm the presence of the mutation. His father is negative by restriction assay. All four subjects carrying this mutation show strongly positive stool biochemistry.

V290M: (Family Dunga)

The proband is a 30 year-old woman of African ancestry. She is not known to have any European ancestry. She presented with a problem of severe abdominal pain which was shown to due to a typical porphyric acute attack. Urine analysis confirmed elevated ALA, PBG and urine porphyrin concentrations. Since VP had not previously been encountered in the black South African population, a diagnosis of AIP was initially made. Her symptoms settled completely following treatment with haem arginate. At a subsequent consultation it was noted that her skin was abnormal with scarring and blistering in sun-exposed areas. Full biochemical analysis was performed and revealed features typical of VP (Table 4-3, subject D).

DNA analysis

Amplification of the 13 exons by PCR, screening by SSCP/HD and sequencing were performed as described above. The proband tested negative for the R59W mutation by restriction analysis. An aberrant mobility shift of a fragment corresponding to exon 8 was detected on SSCP (Figure 4-15) and sequence analysis of exon 8 (Figure 4-16) showed a G to

A transition resulting in a valine to methionine substitution in codon 290 (V290M). This mutation abolishes a *BsiYI* restriction enzyme cutting site (Figure 4-17), which was subsequently used for screening. Fifty control individuals drawn from the African population were negative for this mutation.

Family studies

A sister is positive for the V290M mutation and has a small peak at 625nm on plasma fluoroscanning but has normal biochemistry. A brother has normal biochemistry but a small peak at 625nm on plasma fluoroscanning. He has not yet supplied a DNA sample.

Y348C: (Family Van der Merwe)

The proband is a seven-year-old white female of Afrikaner descent. She presented with photosensitivity shortly after birth. She has a severe clinical phenotype compatible with the homozygous VP syndrome and is described in detail in Chapter 16. Her biochemical results are typical of VP (Table 4-3, subject E). She additionally shows an elevated red cell protoporphyrin.

DNA analysis

She and her family were screened for the R59W mutation by *AvaI* restriction analysis. PCR of all exons, screening by SSCP/HD analysis and direct automated sequencing were performed as described above. The proband tested positive for the R59W mutation by *AvaI* restriction analysis. Additionally, an aberrant mobility shift was noted in exon 10 on SSCP (Figure 4-18). All exons other than 3 and 10 were normal on SSCP/HD analysis. Sequencing of the PCR fragment containing exon 10 revealed an A to G transition in codon 348 (Figure 4-19) resulting in a substitution of cysteine for tyrosine (Y348C). This mutation creates an additional *MaeIII* restriction site which was subsequently employed for screening (Figure 4-20). Thus the proband is heterozygous for both the R59W and Y348C mutations. Fifty control subjects tested negative for the Y348C mutation by *MaeIII* restriction assay.

Family studies

The proband's mother tests positive for the R59W mutation and has the biochemical profile of VP, though she is clinically unaffected. The proband's father tests negative for the R59W mutation, but positive for the Y348C mutation. Biochemical porphyrin analysis is within normal limits but he shows a small peak at 625 nm on plasma fluoroscanning. A brother and a half-sister are also positive for Y348C; the brother has a positive biochemical profile (though an unexpectedly normal plasma fluorescence scan) while the half-sister (aged 12), is currently negative by both biochemistry and fluoroscanning.

The proband's paternal grandparents and both paternal aunts are negative for the Y348C mutation. Haplotype analysis using five randomly selected polymorphic microsatellite markers was performed by the Department of Human Genetics, University of Cape Town and was consistent with the grandparents being the biological parents of the father. The Y348C mutation would therefore appear to be a *de novo* mutation in the father. These results are summarized in Figure 4-20.

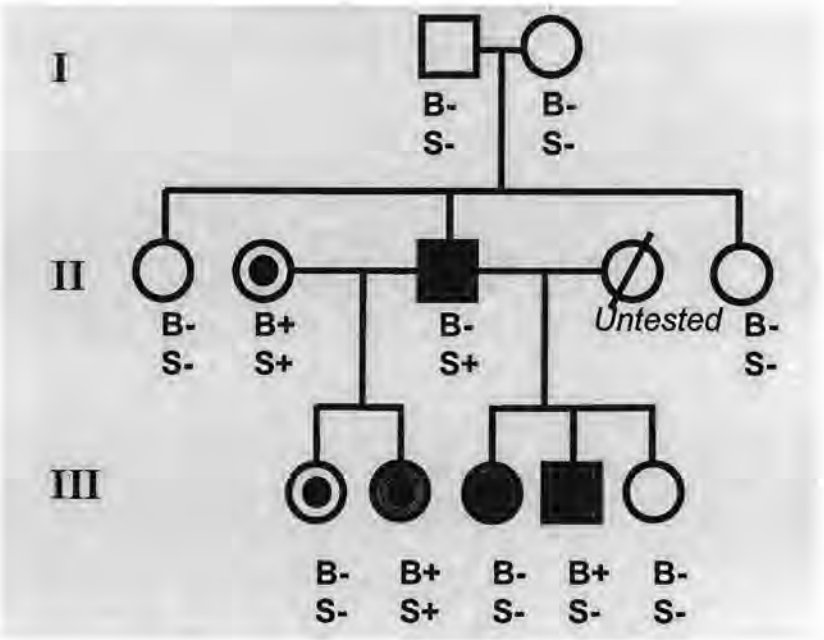


Figure 4-3. Abridged pedigree illustrating 4 generations of a family carrying both the R59W and the Y348C mutations. The presence of the R59W mutation is to noted by a central black circle and of the Y348C mutation by grey shading. Open figures indicate that no mutation was identified. B+ and B- indicate positive and negative evidence for VP on stool biochemistry respectively; S+and S- indicate positive and negative plasma fluorescence scanning respectively.

R138P: (Family Du Pont)

The proband (DdP) is a 26 year-old mixed-race female. She presented at the age of 19 with symptoms suggestive of an acute attack of porphyria. A diagnosis of VP was made biochemically (Table 4-3, subject F). She has experienced two milder acute attacks subsequently. She has an unusually severe degree of skin disease with chronic blistering of the hands and face, hypertrichosis and photomutilation of the hands with progressive shortening of the fingers and deformities of the finger joints. Her general health has otherwise been good and she has two healthy children.

DNA analysis

Confirmation of the presence or absence of the R59W mutation was by *AvaI* restriction assay. Amplification by PCR of all exons, screening by SSCP/HD and sequencing were performed as described above. The proband tested positive for the R59W mutation by *AvaI* restriction analysis. An aberrant mobility shift and a heteroduplex were additionally noted in exon 5 on SSCP/HD analysis (Figure 4-21). All other exons were normal. Direct automated sequencing of the PCR product of exon 5 (Figure 4-22) in the proband shows a G to C transversion in codon 138 resulting in an arginine to proline substitution (R138P). No suitable restriction enzyme is available for this mutation. Fifty normal control subjects from the mixed-race population were screened by SSCP/HD analysis for this mutation and were negative.

Family studies

A 25 year-old sister (AN) was also known to have clinical VP. She had typical VP skin disease with abnormal photosensitivity, blistering and scarring of a degree felt to be in keeping with typical heterozygous VP. She had never suffered an acute attack. Biochemical

analysis is in keeping with VP. She screened positive for both the R59W and R138P mutations. Proband DdP has two prepubertal sons, both of whom are positive for the R59W mutation. Sister AN has a 13 year-old daughter who is positive for the R138P mutation. No child has biochemical evidence of VP. No further family members have come forward for testing.

Q375X: (Family Dodd)

The proband is a 37 year-old white woman with a short history of blistering on the backs of the hands. Biochemical testing confirmed a diagnosis of VP (Table 4-3, subject G). She had recently entered the country from Canada, were she was born.

DNA analysis

Amplification of the 13 exons by PCR, screening by SSCP/HD and sequencing were performed as described above. SSCP analysis revealed aberrant mobility of a DNA fragment corresponding to exon 11 (Figure 4-23). Direct automated sequencing of exon 11 (Figure 4-24) showed a C to T transition in codon of 375. This results in the replacement of a codon coding for glutamine with a nonsense codon (Q375X). The Q375X mutation abolishes an *MaeIII* cutting site (Figure 4-25).

Family studies

This patient has subsequently returned to Canada, and no further information is available on the family.

4.6 CONCLUSIONS IN BRIEF

Eight further mutations associated with VP identified in South Africa have been described. VP in South Africa is therefore, as in Western Europe, genetically heterogeneous. Three of these mutations were first identified in association with the R59W mutation in compound heterozygotes. The studies described in chapters 3 and 4 are now discussed in the chapter which follows.

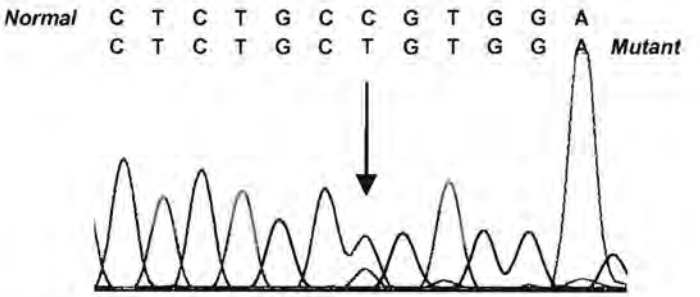


Figure 4-4. The R168C mutation identified on automatic sequencing. The site of the C to T transition is arrowed.

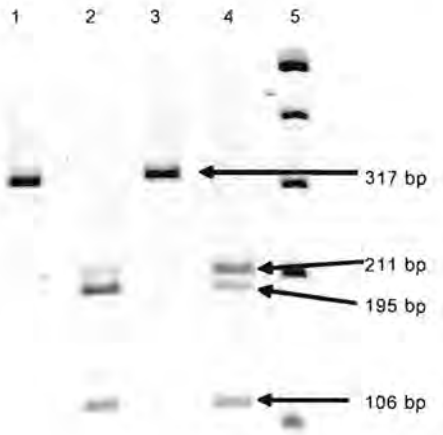


Figure 4-5. Restriction analysis of a fragment corresponding to exon 6 in a subject with the R168C mutation. One of 2 BsaJ1 cutting sites is abolished. The mutation is disclosed by the presence of 3 rather than 2 fragments owing to partial digestion. (A third fragment resulting from normal digestion is, at size 16 bp, invisible). (Lanes: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=size markers.)

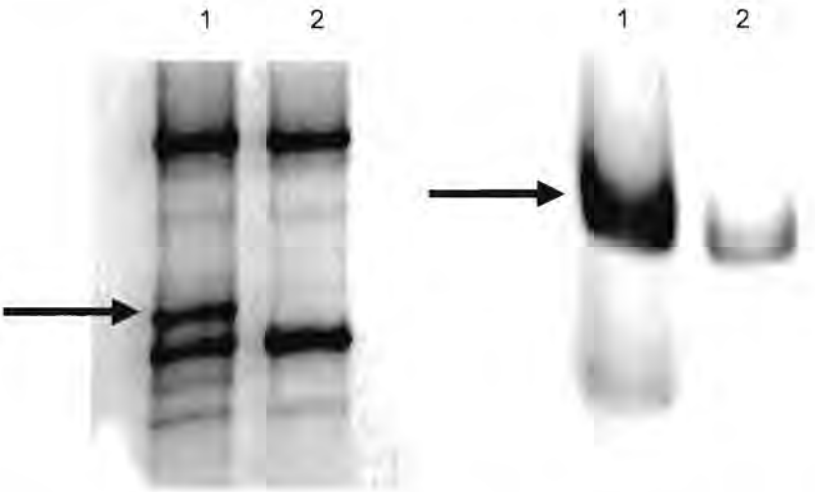


Figure 4-6. SSCP (left) and HD analysis (right) of a fragment corresponding to exon 2 in a subject with the L15F mutation. The abnormal bands are arrowed. Lanes: 1=Patient, 2=Control.

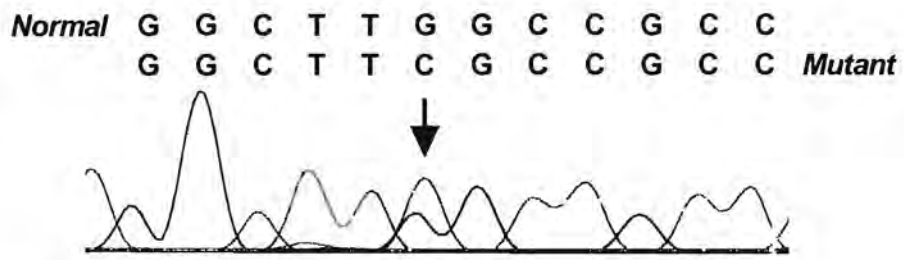


Figure 4-7. The L15F mutation revealed by automatic sequencing. The G to C transversion is arrowed.

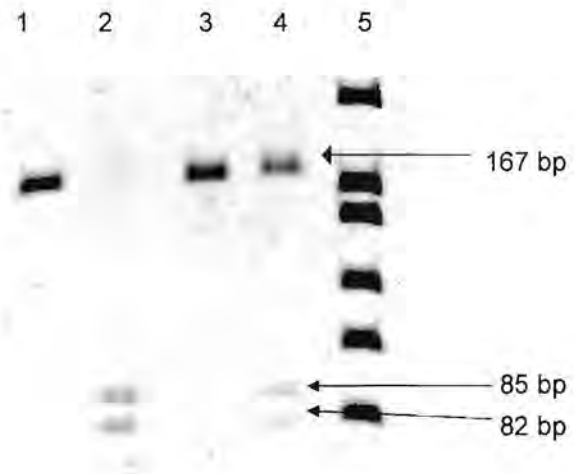


Figure 4-8. Restriction analysis of a fragment corresponding to exon 2 in a subject with the L15F mutation. An *Eae1*cutting site is abolished. Since the patient is a heterozygote, bands corresponding to both digested and undigested PCR fragments are present. (Lanes: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=size markers.)

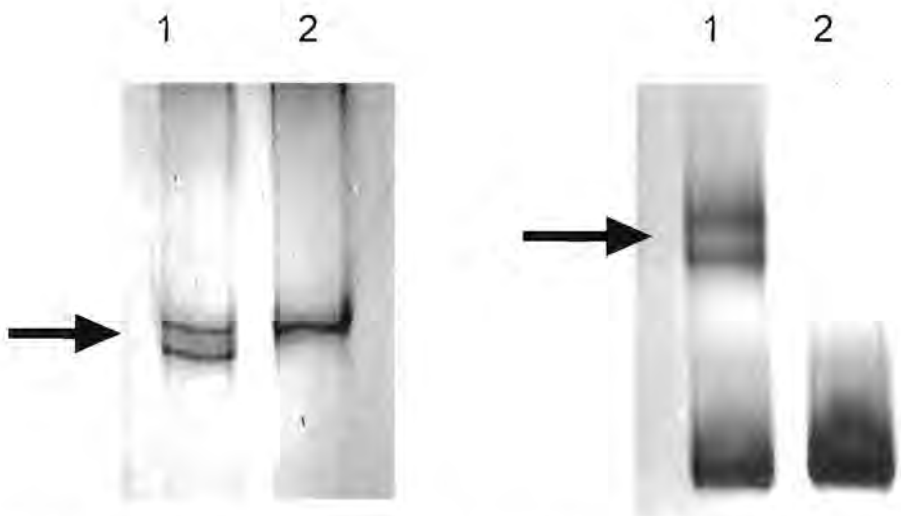


Figure 4-9. SSCP (left) and HD analysis (right) of a fragment corresponding to exon 6 in a subject with the 537 delAT mutation. Lanes: 1=patient, 2=control.

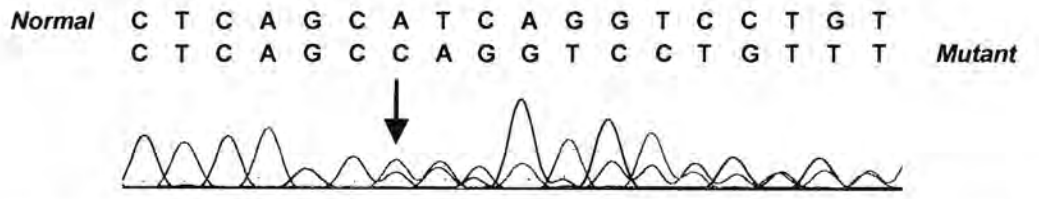


Figure 4-10. The 537delAT mutation shown by automatic sequencing. Following the deletion, the sequence is seen to be 2 bases out of phase.

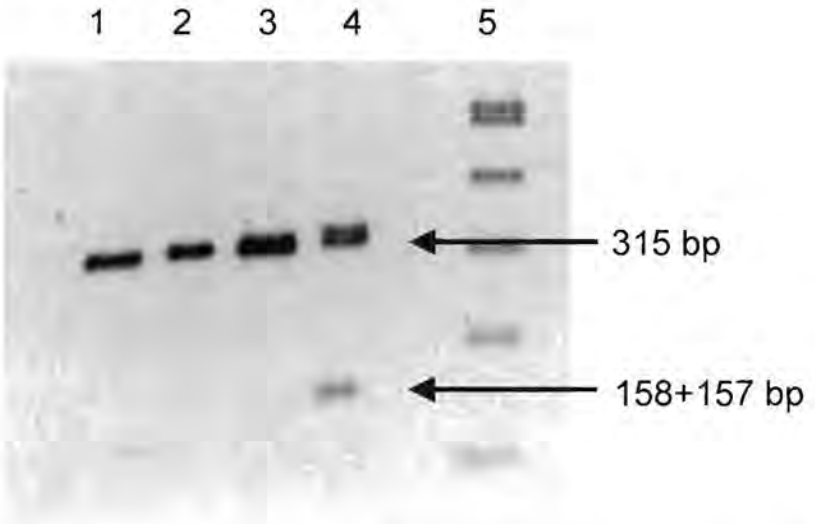


Figure 4-11. Restriction analysis of a fragment corresponding to exon 6 in a subject with the 537 delAT deletion. An *MvaI* cutting site is created. The subject is heterozygous and therefore both digested and undigested fragments are seen. (Lanes: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=size markers.)

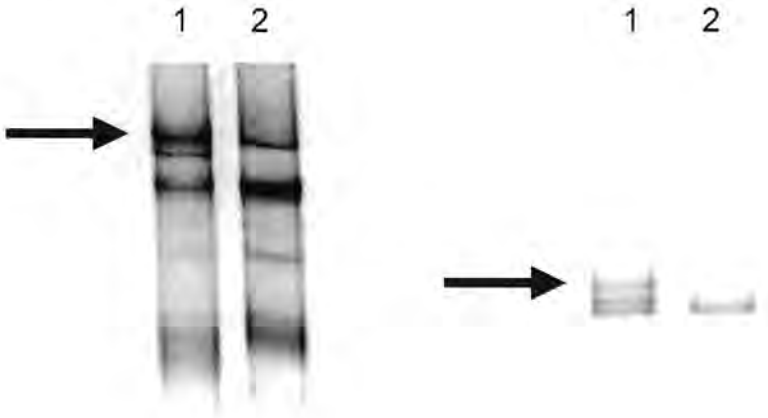


Figure 4-12. SSCP (left) and HD analysis (right) of a fragment corresponding to exon 7 in a subject with the C769delG;770$^{T\rightarrow A}$ mutation. Lanes: 1=patient, 2=control.

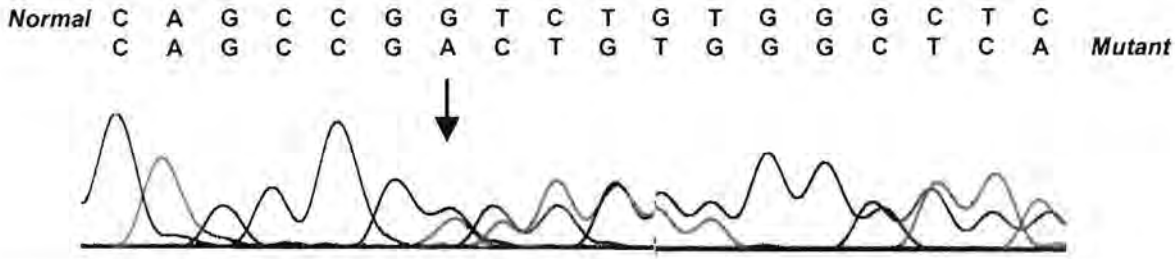


Figure 4-13. The C769delG;770$^{T\rightarrow A}$ mutation shown by automatic sequencing. A *G* base is deleted at position 769 (arrowed); the following *T* is replaced by an *A*, all subsequent bases are out of phase by 1 base.

Figure 4-14. Restriction assay of a fragment corresponding to exon 7 in a subject with the C769delG;770$^{T\rightarrow A}$ mutation. An *HpaII* cutting site is abolished. The subject is heterozygous and therefore both digested and undigested fragments are seen. (Lanes: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=Size markers.)

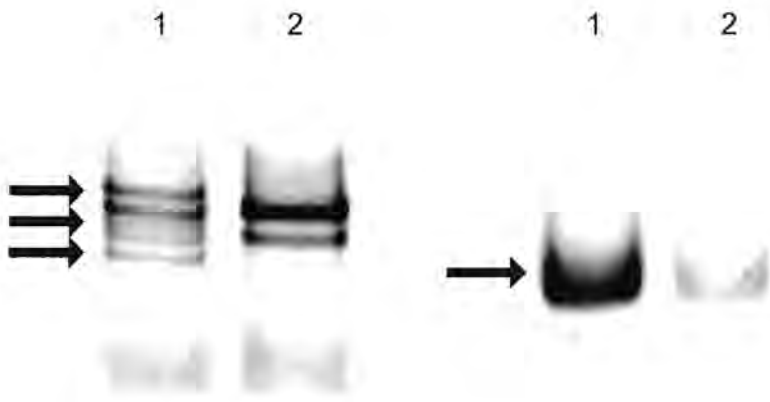


Figure 4-15. SSCP (left) and HD analysis (right) of a fragment corresponding to exon 8 in a subject with the V290M mutation. Lanes: 1=patient, 2=control.

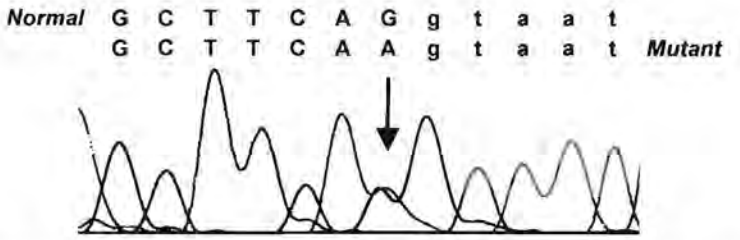


Figure 4-16. The V290M mutation revealed by automatic sequencing. The G to A transition is arrowed. This codon spans exons 8 and 9: the normal and abnormal sequences read *GGT* and *AGT* respectively.

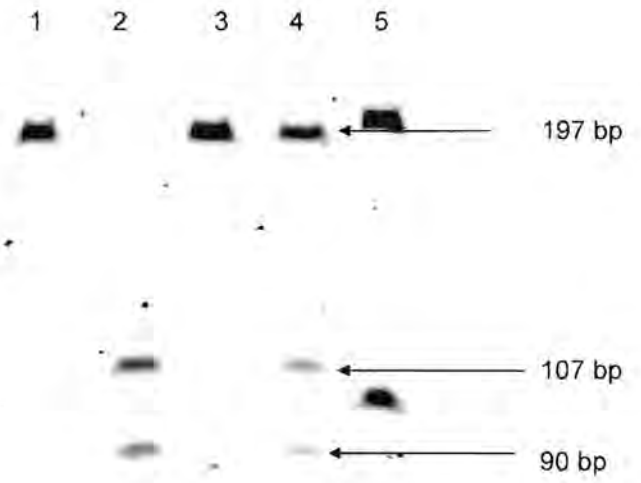


Figure 4-17. Restriction analysis of a fragment corresponding to exon 8 in a subject with the V290M mutation. A *BsiYI* cutting site is abolished. The subject is a heterozygote and both digested and undigested fragments are therefore visible. (Lanes: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=size markers.)

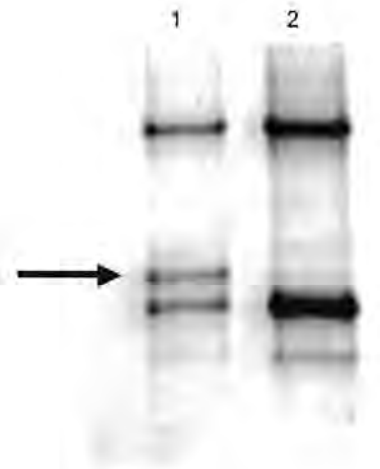


Figure 4-18. SSCP analysis of a fragment corresponding to exon 10 in a subject with the Y348C mutation. Lanes: 1=patient, 2=control.

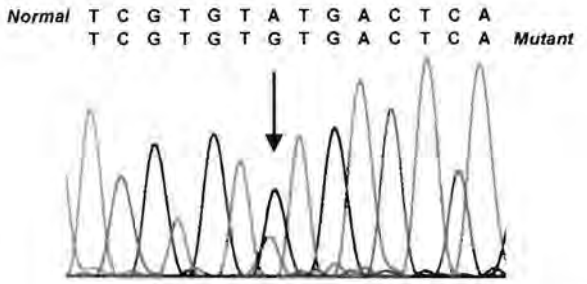


Figure 4-19. The Y348C mutation as shown by direct automatic sequencing. The position of the A to G transition is arrowed.

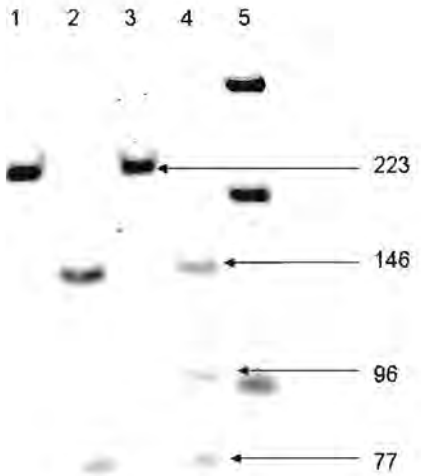


Figure 4-20. Restriction assay of a fragment corresponding to exon 10 in a subject with the Y348C mutation. An additional *MaeIII* cutting site is established, leading to the appearance of additional bands at 96 bp and 50 bp (not visible in this figure). (Lanes: 1=control pre-digestion, 2=control post-digestion, 3=patient pre-digestion, 4=patient post-digestion, 5=Size markers.)

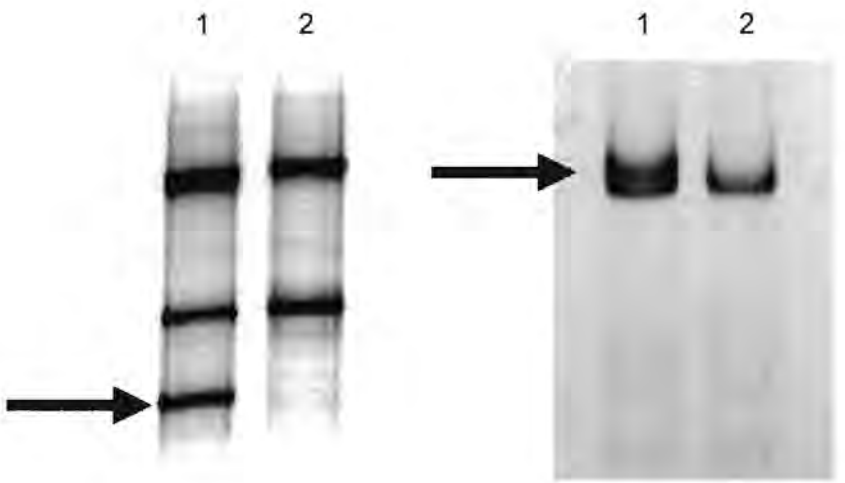


Figure 4-21. SSCP (left) and HD analysis (right) of a fragment corresponding to exon 5 in a subject with the R138P mutation. Lanes: 1=patient, 2=control.

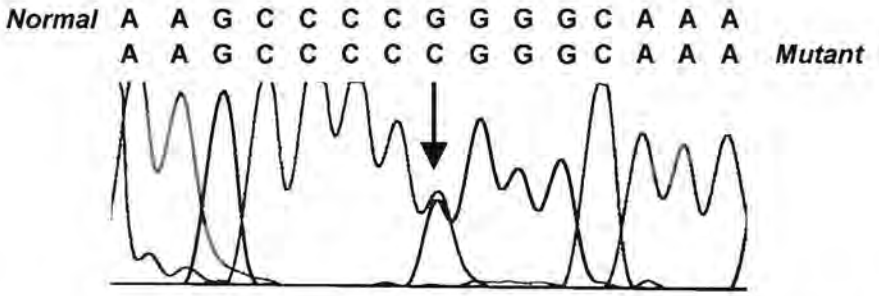


Figure 4-22. The R138P mutation shown by automated sequencing. The position of the G to C transversion is arrowed.

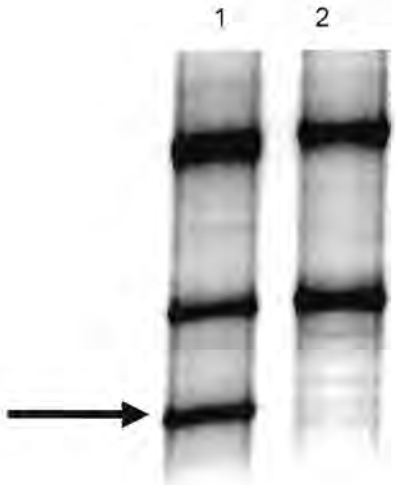


Figure 4-23. SSCP analysis of a fragment corresponding to exon 11 in a subject with the Q375X mutation. Lanes: 1=patient, 2=control.

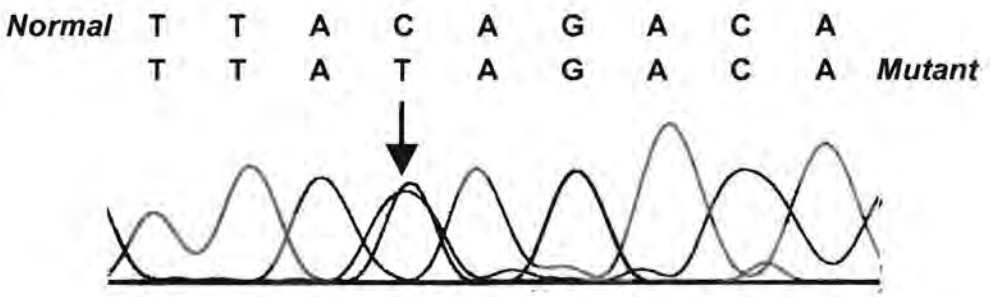


Figure 4-24. The Q375X mutation as shown by automated sequencing. The position of the C to T transition is arrowed.

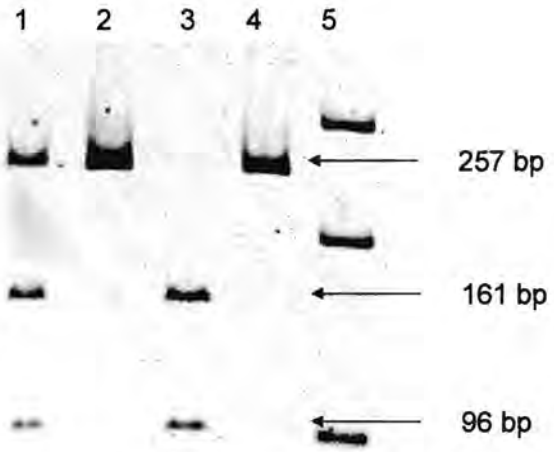


Figure 4-25. Restriction analysis of a fragment corresponding to exon 11 in a subject with the Q375X mutation. An *MaeIII* cutting site is abolished. (Lanes: 1=patient post-digestion, 2=patient pre-digestion, 3=control post-digestion, 4=control pre-digestion, 5=size markers.)

CHAPTER 5:

DISCUSSION: THE MOLECULAR BIOLOGY OF VARIEGATE PORPHYRIA IN SOUTH AFRICA

The identification of ten mutations in South African patients with VP has been described in the preceding chapters. One of these, the R59W mutation, has been shown to be responsible for most South African families with VP. These results are discussed in this chapter.

5.1 R59W: THE SOUTH AFRICAN FOUNDER MUTATION

In view of her severe phenotype and her markedly reduced PPO activity, we believed that the female child who served as the subject of our first study would prove to have homozygous VP, and more specifically, was likely to be a compound heterozygote. This was further supported by the findings of unequally reduced PPO activity in her parents. These predictions were borne out by the finding of both an R59W and a R168C missense mutation in the proband. We believed that one of these mutations was likely to represent the South African founder mutation. Neither the mother nor the father had a family history of VP, and thus no link to the large family documented by Dean (1963). Our demonstration, in the next phase of our study, that the R59W mutation alone was present in a sample of four South African families with VP suggested strongly that this mutation indeed represents the South African founder mutation, particularly since one of these families was indeed known to belong to the family studied by Dean. Further proof for this was provided by our subsequent demonstration that the R59W mutation was present in 31 of 33 families. South Africa is therefore exceptional in that a single mutation is responsible for the overwhelming majority of cases of VP, amounting to approximately 94% of all families with VP. Corroborative evidence has been provided by De Rooij et al (1997) who have demonstrated by haplotype analysis that a sample of South African patients with VP share a common haplotype with Dutch families carrying the R59W mutation. Unfortunately none of these families could be traced back far enough genealogically to demonstrate a common ancestor with the Dutch immigrants who married at the Cape in 1688.

Functional significance of the R59W mutation

Two lines of evidence suggest that the R59W mutation is pathogenic and accounts for the clinical disease in our patients. In the first place, we have demonstrated co-segregation of the R59W defect with the clinical and biochemical expression of VP in a single kindred (Figure 3-2). Co-segregation has subsequently been shown in a larger kindred, our study of which is described in Chapter 17. Secondly, in an experiment performed by the Dailey group at the University of Georgia (Meissner et al 1996), and therefore not described in detail here, the R59W mutant was recreated by site-directed mutagenesis. After modification, the cDNA was introduced into the pTrcHis B vector which expressed the mutant protein, which was then purified to homogeneity. A kinetic assay demonstrated a nearly undetectable K_{cat}, though relatively invariant K_m (Table 5-1). This indicates severe loss of functionality, which would accord with the hypothesis that the R59W mutation is responsible for the VP phenotype in these families. Certainly, the demonstration of a 50% reduction in lymphoblast PPO activity in all patients with heterozygous VP (Meissner et al 1986) is in keeping with compete or near-complete inactivation of the enzyme produced by the mutant allele.

	K_{cat} (min^{-1})	K_m (μM)
Wild type	10.5	1.7
R59W	<0.2	1.1

Table 5-1. Functional activity of expressed site-directed mutation and wild-type PPO after Meissner et al (1995).

The R59W mutation occurs in a CpG trinucleotide, known to confer a high probability of mutation, particularly with a C to T transversion (Cooper and Krawczak 1990). It falls within the 60 bp flanking region of the putative FAD dinucleotide-binding motif of PPO (Nishimura et al 1995, Dailey and Dailey 1996a), an extended region conserved in a large superfamily of flavoproteins of which PPO is a member. This appears to account for the severe loss of catalytic activity of the R59W mutant enzyme. Dailey and Dailey (1997) have subsequently shown that the R59W mutant is less stable than the normal enzyme and is proteolytically degraded by the expressing cells, with consequently both lower enzyme yields and decreased stability during storage. The UV/visible spectrum showed minimal absorption in the 300-500 nm range, reflecting a reduced FAD content. Since crude cell extracts from cells producing R59W also have much lower PPO activity, it appears that this mutation leads to an inability to bind FAD *in vivo*, rather than the FAD merely being lost as a consequence of purification. The expressed enzyme exhibits a normal K_m for protoporphyrinogen, but has a greatly decreased K_{cat} as would be expected. We believe that the R59W mutation, by disrupting the dinucleotide-binding motif coding for the cofactor binding site, results in reduced affinity for FAD and ultimately in reduced catalytic activity. This is a situation similar to that found in pyridoxine-responsive x-linked sideroblastic anaemia, where the mutant enzyme 5 ALA synthase has decreased affinity for its cofactor, pyridoxal 5'- phosphate. In a manner analogous to the supplementary pyridoxine given to elevate subcellular levels of this cofactor in x-linked sideroblastic anaemia, Dailey and Dailey suggest that riboflavin supplements might be useful in the treatment of R59W-positive VP.

Figure 5-1. Derived amino acid sequence for the first 100 amino acids of human PPO and aligned PPO sequences from mouse, plant (*Arabidopsis thalania*, yeast (*Saccharomyces cerevisiae*) and bacteria (*Mycobacterium tuberculosis*, *Bacillus subtilis*, *Myxococcus xanthus* and *Propionibacterium freudenreichii*). The putative dinucleotide binding motif is indicated by the first shaded box; the extended sequence shared by the flavoprotein superfamily extends for approximately 60 residues beyond this. The site of the R59W mutation is shown by the second shaded box.

Mutations and haplotypes in the PPO gene in South African families of VP

Confirmation of our findings has come from the University of Stellenbosch who have independently reported the R59W and R168C mutations (Warnich et al 1996b). They initially showed that the R59W mutation was present in 15 of 17 South African subjects with VP and then demonstrated that the R59W mutation appeared to co-segregate with the disease in subjects drawn from 43 families. Furthermore, 80 normal chromosomes were screened for the R59W mutation and were negative indicating that the R59W mutation is not merely a common polymorphism in the South African population.

Since codon 59 contains a mutation-prone CpG trinucleotide, there is a a possibility that the R59W mutation might have arisen independently in the South African population on more than one occasion. Warnich et al (1996b) have shown that the R59W mutation was associated with only one of four potential haplotypes defined by two diallelic polymorphisms in exon 1 which suggests that this is not so. However, since those alleles associated with the R59W mutation are also the more common alleles in the normal population for each of the polymorphisms, this is not totally conclusive. They have subsequently used linkage disequilibrium and haplotype analysis to characterise the haplotypes associated with the R59W mutation further (Groenewald et al 1998). Their original 15 nuclear families with the R59W mutation were extended to include 132 members, of whom 58 were affected. These families were unrelated to the second degree. Using 15 microsatellite markers spanning a region of approximately 21cM around the PPO gene, they identified disease-associated haplotypes from alleles that were transmitted from affected parent to affected offspring. Their observed combinations limits the location of the PPO gene to a 2.4 cM region between markers D1S2705 and D1S2707; the highest resolution genetic mapping of this gene yet.

Their results suggest that there are two distinct groups of haplotypes, differing in flanking markers; however both appeared to descend from the same founder, as they share the same core haplotype. Additionally, a highly significant linkage disequilibrium score is shown for a continuous area of 10 cM; these results are in agreement with data from other populations in which the historical age of the founder effect is estimated to be 12 generations, as it is for VP in South Africa (310 years: approximately 12 generations). One of Warnich et al's 15 families however appeared to have a completely different haplotype; which may indicate that a small percentage of South African families with the R59W mutation do indeed represent either recurrent mutations at the CpG hot spot, or a second, more recent, importation of the gene.

R168C

This site is evolutionarily conserved in man, mouse and yeast. The mutation has been expressed in *E. coli* by Dailey and Dailey (1997) and has been shown to have reduced enzyme activity, with a lower K_{cat} but unaltered K_m for protoporphyrinogen. Though the K_{cat} is not stated in this paper, it would appear that the R168C mutation is not associated with a complete loss of function. The evidence for this is twofold: firstly, the measured PPO activity in lymphoblasts derived from the proband's father is reduced by only 30%, rather than the 50% expected in a heterozygote carrying an allele associated with complete loss of function as is seen with the R59W mutation. Secondly, there is evidence to suggest that mutant alleles associated with complete loss of function are always balanced by alleles coding for some residual function in compound heterozygotes (Roberts et al 1998). This is discussed further in Chapters 15 and 19. Indeed, current work using the expressed protein resulting from site-directed mutagenesis in our laboratory has recently confirmed that the R168C mutation is associated with some residual activity (M Maneli, personal communication). The R168C mutation occurs at a hypermutable CpG site and appears to be a frequent site of mutation since a related R168H mutation has been reported on 3 occasions from apparently unrelated

patients (Frank et al 1998d, De Rooij et al 1997, Whatley et al 1999), and it is one of the two mutations found in both the United Kingdom and France (Whatley et al 1999). The R168H mutation would appear to be a more severe mutation than the R168C since it is associated with phenotypically expressed VP, whereas we have not identified the R168C mutation in association with clinically expressed VP, suggesting that a cysteine residue in position 168 may be less damaging to the protein then a histidine residue.

H20P

This mutation occurs in a region of PPO which can be configured as part of the of the α helix of a typical FAD-binding domain of a flavoprotein and as such would be expected to result in a loss of catalytic activity. This area may also be constructed as an α-amphiphatic region; a helix described by a polar and an apolar face (Eisenberg 1984), which is considered important in the maintenance of protein structure. An unusually high frequency of mutations associated with the replacement of the wild-type amino acid by proline has been reported by Whatley et al (1999). 35% of their missense mutations represent a substitution by proline, with most of these being leucine to proline mutations. A similar serine to proline mutation (S450P) has also been reported elsewhere by Frank et al (1998c), and we have identified two further such mutations—this and the R138P mutation described later. This is an unusually high frequency for this type of mutation. Proline is known to decrease protein flexibility (Tian et al 1998) and to interrupt α-helical regions; which may indicate that the PPO protein is very sensitive to small structural changes.

L15F

The L15F mutation is situated in the putative FAD-binding domain which is likely to account for its functional effect. The leucine in this position is highly conserved in both plant and animal PPO species. This mutation has been identified in 7 families from the United Kingdom (Whatley et al 1999) and is therefore one of the commoner mutations in Europe. Intragenic polymorphism analysis in the UK has shown that this mutation is associated with at least two different haplotypes suggesting that it has risen independently on more than one occasion. It is likely that the L15F mutation has been imported into South Africa from Britain, though in the absence of haplotype analysis, this cannot be established with certainty. Yet it appears to be an uncommon mutation in South Africa, and to this point has been identified in only one family.

537 delAT

The family in whom this mutation was found is of mixed racial ancestry, and the gene may have been of European, Asian or indigenous origin; interestingly, this mutation has subsequently been reported from the United Kingdom (Whatley et al 1999). This mutation clearly co-segregated with biochemical evidence for VP in this kindred. The 537 delAT mutation results in a frame shift and produces a premature TGA stop codon 11 codons downstream. It is therefore likely to result in the synthesis of a truncated protein with reduced or absent catalytic activity. Nonsense or frameshift mutations may result in a reduction in mRNA levels and several possible reasons for this have been advanced: reduced transcription rate, reduced efficiency of mRNA processing, decreased transport to the cytoplasm or reduced mRNA stability (Cooper 1993).

In this family, plasma fluoroscanning was a more sensitive screening test for the inheritance of the in VP allele than was conventional porphyrin analysis. Whereas biochemical analysis was positive in 13 of 17 subjects, plasma fluoroscanning was positive in

all. The sensitivity of plasma fluoroscanning is discussed in Chapters 13 and 14. It therefore appears that this mutation is accompanied by a high level of phenotypic expression as revealed by porphyrin abnormalities. In terms of clinical expression however, only 3 of 17 subjects carrying the mutation (17.6%) had clinical evidence of VP. This was restricted to skin disease and no patient had suffered acute symptoms. The rate of clinical expression is therefore less than that shown for R59W-positive VP (Chapter 16) though the difference is not statistically significant ($p=0.16$, χ^2).

c769DELG; $770^{T\rightarrow A}$

The C769delG;$770^{T\rightarrow A}$ mutation introduces a premature stop codon 16 codons downstream. This is likely to be associated with the formation of unstable mRNA and the production of a truncated and nonfunctional protein. The unusually severe presentation in the original proband and the presence of elevated erythrocyte protoporphyrin concentrations suggested that he might be a compound heterozygote. Screening of all other exons by SSCP/HD analysis has produced no evidence of any other mutation in the proband and his sister . In the experience of Whatley et al (1999), a combination of DGGE and heteroduplex analysis has a sensitivity of 77% in detecting new PPO mutations. The authors suggest therefore that a screening approach using DGGE cannot be applied to the whole PPO gene and that heteroduplex analysis alone may lack sensitivity as has been shown in AIP (Tchernitchko et al 1999). They recommend a combination of heteroduplex analysis and DGGE with direct automated sequencing of exons and their flanking regions. A small possibility hence remains that we may have failed to detect a second mutation, though this would appear unlikely.

V290M

Though the valine at position 290 is not evolutionarily conserved, the V290M mutation results in the substitution of an aliphatic, hydrophobic amino acid by methionine, which contains a sulphur atom in a thioether linkage. There thus exists a possibility of conformational change with concomitant loss of activity. Clinically it is a significant mutation, resulting in both photocutaneous sensitivity and the acute attack. A related V290L mutation has been described from a European family (Whatley et al 1999).

This is the first African family yet described in South Africa with VP, and the V20M mutation is the first reported VP mutation in a black subject. VP would appear to be rare in black populations, with individual cases described in a Caracao negroid woman (Van der Sar and Den Ouden 1981), an African-American male (Hughes and Davis 1983) and an 11 year-old Nigerian girl who presented with both skin disease and acute symptoms, and finally died of respiratory failure (Durosinmi et al 1991). The first two patients are however of unspecified ancestry, though the third would appear to be of pure African stock. The reason for the infrequency with which VP is identified in African populations is unknown. Though there is evidence that melanin may protect against photosensitisation (Westerhof et al 1981), a dark skin alone does not, in our experience with PCT in African people and with VP in those of mixed race, mask the cutaneous symptoms of porphyria. It therefore appears unlikely that the under-representation of black people with VP is due only to shortcomings in recognition and diagnosis. It is however surprising that, with more than 90 mutations associated with VP having been reported throughout the world, mainly in people of European extraction, the African population does not appear similarly prone to mutations in the PPO gene.

Y348C

The Y348C mutation results in a tyrosine to cysteine substitution, that is from an aromatic to a sulphur-containing amino acid, which may alter the secondary structure of the protein with consequent reduction in activity. This tyrosine is present in both human and mouse, though not in yeast or bacteria. Since this mutation is found heteroallelic to the R59W mutation, which codes for a mutant protein with essentially no residual activity, it is likely that the Y348C allele is associated with at least some residual activity. The observation that no heterozygote has experienced clinical symptoms is therefore not unexpected, and the Y348C and R168C mutations are similar in this regard. An interesting observation is that the proband's half-brother, who carries the Y348C mutation, appears to express VP biochemically, suggesting that this mutation is in itself sufficient in the heterozygous state to result in biochemical expression of VP. We have recently produced objective evidence for this in our laboratory: the expressed protein, following site-directed mutagenesis, has been shown to have some catalytic activity; furthermore, the activity would appear to be intermediate between that of the R168C mutant protein and the normal protein (M Maneli, personal communication).

R138P

The R138P mutation results in an arginine to proline substitution, representing in a change from a basic to a non-polar amino acid with an aliphatic hydrocarbon R group. This arginine is conserved in both human and mouse, though not in yeast or bacteria. The R138P mutation is a further instance of a proline subsitution; an unusually high incidence of substitutions with which has been noted in the PPO gene (Whatley 1999). As with the R168C and Y348C mutations, this mutation was first identified in a compound heterozygote. We have not studied enough family members to allow us to comment on the clinical effects of this mutation. However, the clinical evidence would suggest that it is likely to be a mild mutation, with relatively well preserved activity, since even in the compound heterozygous state, the proband and her sister are not as severely affected as are our R59W/R168C and R59W/Y348C compound heterozygotes.

An interesting finding is the discrepancy in clinical severity between these two sisters, both of whom are compound heterozygotes. The proband is clearly more severely affected than a typical R59W heterozygote, in keeping with our observations in compound heterozygous VP. Her sister would be expected to be similarly affected, and yet appears not to be so. The clinical effect of the co-inheritance of these two mutations is therefore variable. This is examined in more detail in Chapters 16 and 19.

Q375X

The Q375X mutation is a nonsense mutation which, by introducing a premature stop codon, is likely is to abolish PPO activity, primarily by accelerating mRNA decay (Culbertson 1999). It is interesting that this mutation was not reported in the large series from United Kingdom and France (Whatley et al 1999) since we believe that this Canadian patient is likely to be of English, or perhaps French, descent.

5.2 CONCLUSIONS

We have thus identified 10 mutations associated with VP in South Africa. Eight of these are novel whereas one (L15F) had been described in the United Kingdom and a second, apparently limited to South Africa, had been reported by Warnich et al (1996b). The 537 delAT mutation has subsequently been reported in Europe by Whatley et al (1999). Seven are missense mutations, one a nonsense substitution and two are deletions. The two deletions were both found in mixed-race families, one missense mutation in an African family and the remaining mutations in white families. The common R59W mutation is present in both white and mixed-race families. Three of the missense mutations were only identified in association with the R59W mutation; none has been shown in association with clinical symptoms in the heterozygous state, though one subject with the Y348C mutation had had biochemically-expressed VP. Preliminary evidence (M Maneli, personal communication) suggests that this mutation is indeed associated with a fair degree of residual activity. Our experience, though smaller, is similar to that of Whatley et al, with the demonstration that VP is genetically a heterogeneous disease associated with a variety of mutation types spread across the entire gene.

5.3 SIGNIFICANCE OF OUR FINDINGS FOR THE DIAGNOSIS OF VP IN SOUTH AFRICA

The most important difference between the South African and European findings is our demonstration of the extraordinarily high prevalence of a single mutation within this background of genetic heterogeneity. We have shown that a single mutation accounts for 94% of all cases of VP in South Africa. This has important implications for diagnosis. Work to be described in Chapter 10 has shown that conventional biochemical porphyrin analysis is relatively insensitive for the diagnosis of VP. Furthermore, intermediate results are difficult to interpret and lead to a reduction in specificity, the techniques are labour-intensive and time-consuming, and experience has shown that many candidates for testing find providing a stool sample distasteful and a disincentive to diagnosis. Though plasma fluoroscanning, discussed in Chapters 13 and 14 has many advantages, it will still fail to detect some adults and all children carrying a gene for VP.

A DNA-based test for porphyria offers many advantages: it may be performed at any stage of life, including antenatally, it requires only a blood test and relatively simple techniques. It is 100% sensitive and specific for the mutation for which one is testing. There are however two major drawbacks. Firstly, it tests only for a specific mutation and not for disease; where several mutations occur within a population, testing will be inaccurate. Secondly, DNA testing reveals only the presence or absence of the potential to develop clinical disease, but says nothing about the severity of clinical expression.

Any single DNA-based test is of no use for the diagnosis of AIP in Europe, where facilities for DNA testing have been available for several years, since more than 70 mutations are now described (Grandchamp et al 1998). Even in Scandinavia, where a single mutation is particularly prevalent as a result of a founder effect (Lee and Anvret 1991), DNA testing is made more difficult by the range of mutations within the population. To some extent this problem may be overcome by offering a batch of tests covering all the more frequent mutations known to exist in the population or by performing a complete SSCP/HD analysis with exon-specific primers on all samples (A Brock, personal communication). The same

problems will apply to the diagnosis of VP in Europe, with in excess of 70 mutations described (Whatley et al 1999).

The situation in South Africa is different. Our results suggest that screening patients suspected of having VP by *Aval* restriction analysis would detect approximately 94% of all cases of VP. This would appear to be a reasonable approach provided that the following cautionary warnings are born in mind. Firstly, the absence of the R59W mutation does not exclude VP entirely, since there remains a small probability that another mutation may be present. Secondly, proving the presence of the R59W mutation does not in any way confirm that a patient's symptoms are due to VP. Since South African laboratories other than our own have offered DNA testing for the R59W mutation, we have begun to identify errors in diagnosis and management arising from a failure to understand these points.

A rational protocol for the diagnosis of VP in South Africa

We have therefore set out the following protocol for the diagnosis of VP in South Africa.

1. For the screening of an asymptomatic subject with a family history of VP

Since there appear to be a considerable number of people in South Africa who erroneously believe they have VP has a result of previous inadequate biochemical testing , it is prudent to test the family member believed to have VP first. If VP is confirmed biochemically or by plasma fluorescence scanning, then he or she can be tested for the the presence of the R59W mutation. A positive result for R59W indicates that DNA testing is suitable for further family screening (Figure 5-2).

Alternatively, the subject presenting him or herself for screening on the grounds of a family history may be tested for the R59W mutation directly. If positive, the inheritance of VP is confirmed and all family members are appropriately tested for the R59W mutation. If the R59W result is negative, then it is again imperative to prove the presence of VP biochemically in whichever member of the family was believed to have originally tested positive biochemically. If VP is proven, he or she should be tested for the R59W mutation. However, if on review no evidence for VP can be found, then further testing is unlikely to be useful.

In view of the ease and simplicity of plasma fluorescence scanning (Chapter 13), this should additionally be carried out on every subject presenting for testing on whatever grounds.

Where a subject reports symptoms suggestive of VP

Two approaches are reasonable (Figure 5-3). Our own preference is to test the subject biochemically first, based on the premise that all patients in whom VP is symptomatic will show positive biochemistry. If positive, the diagnosis is confirmed and some assessment of the biochemical activity of the disease made. The subject is thereafter tested for the R59W mutation, so that the result can be used subsequently for family screening.

An alternative approach is to test for the R59W mutation directly. If positive and the patient's symptoms characteristic of VP, it is reasonably likely that the symptoms are indeed due to VP and he or she can be managed accordingly. If the R59W mutation result is negative, the patient's symptoms must be re-evaluated. If highly atypical, it is improbable that VP is present and further testing is unlikely to be helpful, therefore another diagnosis should be sought. If the clinical picture is indeed suggestive of porphyria, then it is essential to test the patient biochemically so as not to overlook a diagnosis of a porphyria other than VP or of VP due to a mutation other than R59W.

Assessing the significance of symptoms in a patient with VP

It is essential to test patients with symptoms biochemically to prove that their symptoms are due to porphyria, even where they have been proven to be R59W-positive. This is still neglected in South Africa, where some patients with any complaint having a vague resemblance to the symptoms of the acute attack are assumed to be having such an attack. Not infrequently, subsequent investigation in our laboratory has shown conclusively that the assumption was unjustified.

These recommendations form the basis of our recommendations to South African doctors and the public as published in our informative publications. The guidelines we issue to the medical profession for the diagnosis of VP in South Africa are reproduced in Appendix 6.

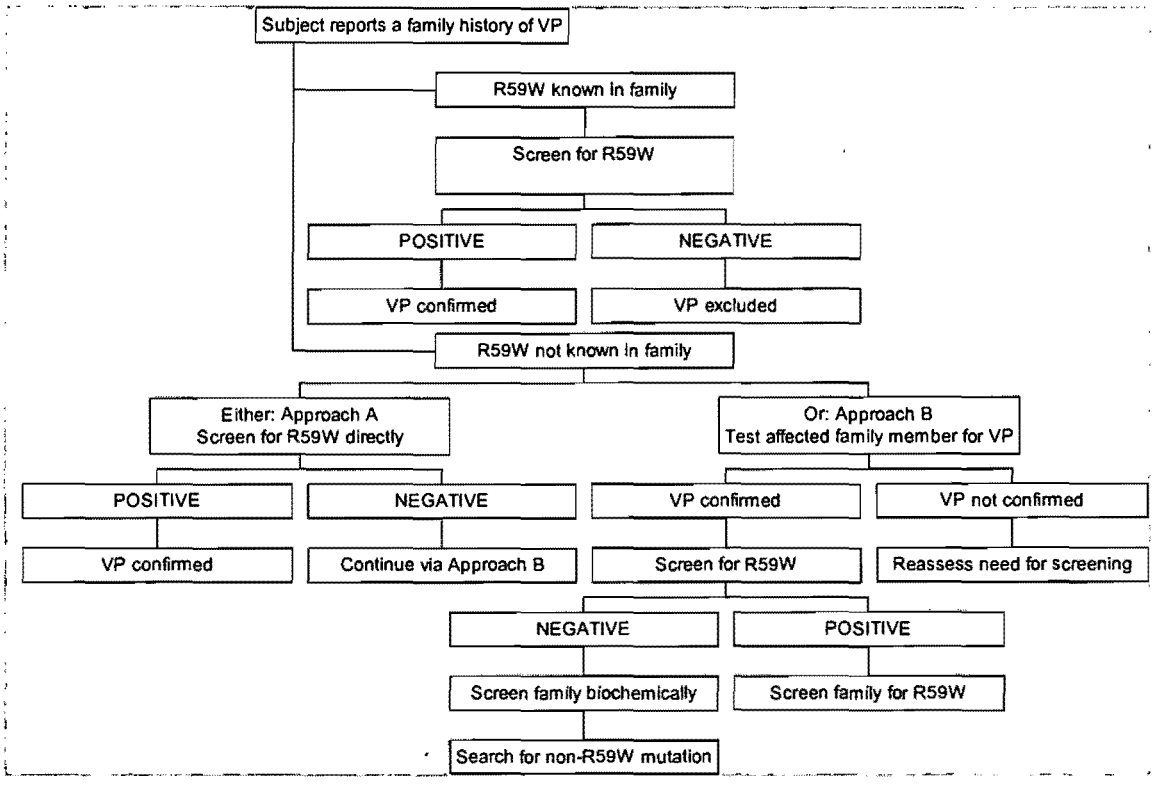


Figure 5-2. Algorithm for a diagnostic approach to a patient who reports a family history of VP in South Africa. The approach is based on the premise that 95% of all families with VP are likely to carry the R59W mutation.

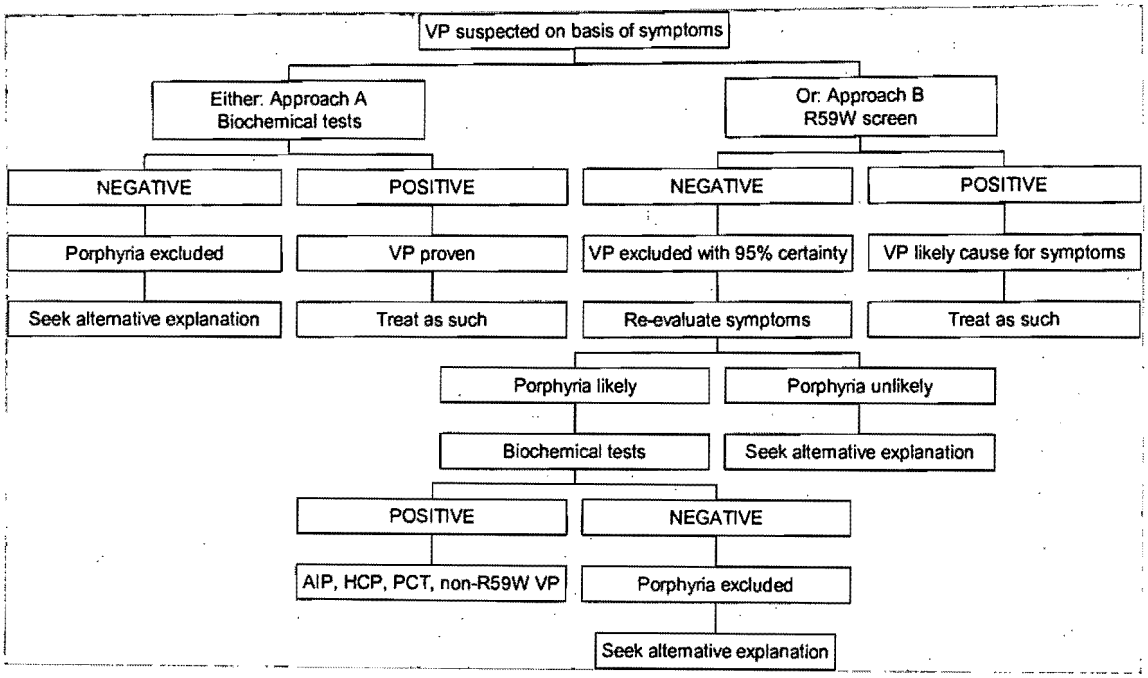


Figure 5-3. Algorithm for a diagnostic approach to a patient with symptoms suggestive of VP in South Africa. The approach is based on the premise that 94% of all families with VP are likely to carry the R59W mutation.

SECTION 2

THE BIOCHEMICAL FEATURES OF VARIEGATE PORPHYRIA

Section 1 of this dissertation examined the molecular biology of variegate porphyria, with particular reference to the mutations present in South African families. Section 2 now examines the value of routine porphyrin analyses of urine, stool and plasma in the diagnosis of VP as correlated with the presence or absence of a PPO mutation on DNA analysis. The section begins with a review of the literature. In the chapters which follow, the extent to which observers agree on the interpretation of porphyrin excretion profiles is examined, and the basis on which a diagnosis of VP is made biochemically is retrospectively analysed. Biochemical testing is shown to be imprecise and subject to individual variation in interpretation. In Chapter 10, porphyrin excretion profiles are correlated with the presence or absence of PPO mutations. It is shown that all children and approximately 30% of adults carrying a PPO mutation fail to express abnormal stool porphyrin excretion patterns. Using the statistical techniques of discriminant analysis and classification tree analysis, we show that an elevation of stool coproporphyrin and pentacarboxylic porphyrin are the most important biochemical predictors of VP. Thereafter the place of simple screening tests and of plasma fluorescence scanning in the diagnosis of VP are assessed. Though the more important conclusions to be drawn from each study appear at the end of each chapter, the results are not discussed in detail until Chapter 14, where the results of all studies are discussed as a whole.

CHAPTER 6:

THE BIOCHEMICAL FEATURES OF VARIEGATE PORPHYRIA: A REVIEW OF THE LITERATURE

6.1 RECOGNITION OF THE PORPHYRINS AND THEIR PLACE IN THE DIAGNOSIS OF PORPHYRIA

Historically, laboratory work on the porphyrias was largely confined to the biochemistry of porphyrins, and to a description of those characteristic profiles of porphyrin excretion in urine and stool which allow a diagnosis to be made. Thereafter followed the identification and eventual purification and characterisation of the enzymes of the haem synthetic pathway. In some porphyrias, particularly AIP, direct determination of enzyme activity proved more sensitive for a diagnosis than porphyrin quantitation alone. More recently, with the development of sensitive techniques in molecular biology, identification and characterisation of porphyria at the molecular level has become possible.

That the diseases we now know as porphyrias are associated with the excretion of unusual substances has been known for over a century. Following the introduction of Sulphonal as a hypnotic (Kast 1888), a series of patients were described who developed symptoms compatible with porphyria and were noted to excrete pigments in the urine (Stokvis 1889, Harley 1890, Geill 1891, Fehr 1891, Günther 1911, Günther 1922). Initially these substances were thought to be haematatoporphyrin, the compound obtained by adding concentrated sulphuric acid to dried powdered blood, washing it to remove iron and then heating it in the presence of alcohol. During the first years of the 20th century however it was shown that these substances, urinary porphyrins, were distinct from haematoporphyrin, and this led ultimately to the identification of uroporphyrin, coproporphyrin and protoporphyrin (Willstätter and Mieg 1906, Willstätter and Stoll 1913, Fischer and Zerweck 1924, Fischer and Orth 1934).

Variegate porphyria was described clinically and biochemically in 1937 (Van den Bergh and Grotepass 1937) and it was realised that, unlike AIP and CEP, the biochemical hallmark of this disease was the over-excretion of porphyrins in the stool (Barnes 1945, Barnes, 1958). Separation experiments showed that the stools of patients with VP contained large amounts of protoporphyrin and, to a lesser extent, coproporphyrin.

6.2 ORIGIN OF THE STOOL PORPHYRINS

Bile appears to contain both coproporphyrin and protoporphyrin (Aziz and Watson 1969). England et al (1962) showed that the porphyrin in stools from normal subjects was largely coproporphyrin and protoporphyrin, with small amounts of at least three other porphyrins. Two of these were identified as deuteroporphyrin and mesoporphyrin. The final porphyrin was provisionally named pemptoporphyrin. They suggested several sources for the origins of stool porphyrins. These included endogenous pigments excreted in the bile, exogenous pigments—which might be ingested with foodstuffs, derived from chlorophyll or haem pigments in meat or from bleeding within the bowel—and porphyrins synthesised by micro-organisms within the bowel. Indeed, they demonstrated diminished faecal porphyrin excretion—particularly of deuteroporphyrin and mesoporphyrin, which lie outside the direct haem synthetic pathway—in subjects following the administration of broad-spectrum

antibiotics, suggesting a bacterial origin for a considerable proportion of faecal porphyrins. Since bile contains more coproporphyrin than protoporphyrin, it appears that much of the dicarboxylic porphyrin present in stool is formed within the gut, particularly by microbial metabolism of haem derived from the diet and other sources (Elder 1980). Additionally, protoporphyrin may itself be converted to other porphyrins such as mesoporphyrin by intestinal bacteria which have the ability to reduce or remove vinyl groups. Consequently stool contains a mixture of dicarboxylic porphyrins, though protoporphyrin is usually present as the main component.

More recently it has been shown that different bacteria have varying effects on stool porphyrin excretion. Beukeveld et al (1987) observed strong fluctuations in faecal porphyrin content while determining reference values for stool porphyrins by HPLC. They discovered that suppression of aerobic flora by selective antibiotic use had no effect on faecal porphyrin excretion. Suppression of the anaerobic flora however completely inhibited the transformation of protoporphyrin to pemptoporphyrin and deuteroporphyrin, and the effect persisted for five days after stopping medication. When the flora were suppressed for long periods, the production of both protoporphyrin and coproporphyrin was decreased, and the production of deuteroporphyrin, pemptoporphyrin and mesoporphyrin was abolished. Thus, though aerobic gram-negative bacteria have little effect on porphyrin synthesis, anaerobic organisms appear variously to produce mesoporphyrin, convert porphyrins to pemptoporphyrin and deuteroporphyrin, or produce coproporphyrin and protoporphyrin. Problems with the diagnosis of porphyria resulting from variations in these faecal porphyrins were shown.

Though gastro-intestinal bleeding appears to result in an increase in the amount of porphyrin— predominantly protoporphyrin—recovered in stool, a gross excess of faecal porphyrin sufficient to complicate the diagnosis of VP is rarely observed, an observation with which both Eales and Dean concurred (Barnes 1963). The ingestion of yeast tablets has also been implicated in the production of falsely elevated stool porphyrin levels (Day 1978).

6.3 BIOCHEMICAL TESTS FOR PORPHYRIA

Screening methods

Elder (1980) and Moore and Disler (1985) have summarised appropriate methods for the screening of urine and stool for abnormal amounts of porphyrin following their concentration and partial purification by solvent extraction, using methods developed by Rimington (1958) and Eales et al (1966). Screening tests for urine have a lower limit of sensitivity of approximately 0.7-1 μmol/l, and stool porphyrin screening is sensitive enough to detect porphyrins even in some normal samples. It is therefore recommended that all positive tests should be followed by the direct measurement of porphyrin concentrations. Newer, fluorometric screening tests for porphyrins have been described (Pudek et al 1991).

Solvent extraction

For many years the mainstay of porphyrin analysis was the quantitation of porphyrins by solvent extraction techniques (Schwartz et al 1960, Fernandez et al 1966, Rimington 1971) or anion-exchange chromatography (Sobel et al 1974) followed by spectrophotometry (Rimington 1971) or fluorometry (Schwartz et al 1960). These techniques are based on the premise that porphyrins which contain acid side chains are ampholytes and are soluble in both acids and bases (Fuhrhop and Smith 1975). At the isoelectric point they are least soluble and at this pH can be transferred from aqueous solutions to organic solvents such as ether, ethyl acetate, butanol and amyl alcohol. Porphyrins can thereafter be extracted from the solvent into

dilute mineral acids. Solubility in the organic solvent is inversely proportional to the number of acid side chains.

Spectrophotometry was initially employed for the quantitative analysis of the porphyrins. This technique is relatively insensitive. In addition, urine contains compounds which themselves absorb in the Soret region, and spectrophotometry is not sensitive enough to allow sufficient dilution to reduce the influence of these confounding substances. Methods suggested to increase specificity included adsorption to calcium phosphate (Rimington & Sveinssen 1950) or preceding anion-exchange resin chromatography (Doss 1974, With and Pedersen 1977).

Fluorescence detection by examination under ultraviolet light with a peak output at 365 nm allows very low concentrations of porphyrins to be detected, particularly if a red-sensitive photomultiplier is used (Chisholm and Brown 1975, Sassa et al 1975a). Fluorometry is therefore superior to spectrophotometry in terms of a greater sensitivity and lesser susceptibility to interference by impurities. Its greater sensitivity allows interfering compounds to be neutralised by dilution (Schwartz et al 1976, Martinez and Milla 1971), though other sources of inaccuracy remain. These include quenching or enhancement of fluorescence, especially by organic solvents, and the formation of non-fluorescent metal chelates with contaminating metal ions resulting in under-reading. Fluorometry also requires very pure porphyrin standards (Chisholm and Brown 1975). The sensitivity of fluorometry allows the visualisation of extremely small quantities of porphyrin on thin-layer chromatography plates developed with kerosene or iso-octane (Fuhrhop and Smith 1975), and a sensitivity threshold of as little as 10 pM has been reported.

Chromatography

The major advantage of solvent-partition methods is that no special apparatus is required, nor are porphyrins standards necessary if spectrophotometric techniques are employed for the actual quantitation. The disadvantage is that porphyrins are poorly separated and the extracted fractions are usually mixtures (Deacon and Ledden 1998). The main component may not even be identified accurately. These factors are particularly limiting in the case of samples containing low porphyrin concentrations, such as blood (Wranne 1960, With 1976). Thin-layer chromatography for detailed analysis of porphyrin mixtures (Doss and Bode 1968, Doss 1970, Eales et al 1975, Elder 1975, With 1975, With 1977) proved superior in that it was both more sensitive and was able to resolve a porphyrin mixture into individual species, depending on the number of carboxyl groups carried on the porphyrin. In the 1970s, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) completely replaced earlier forms of chromatography including column chromatography, paper chromatography, counter-current distribution and electrophoresis.

Conversion of free acids to their ester derivatives greatly facilitates the separation of porphyrins by chromatography. Suitable substances for the esterification of urinary, faecal and tissue porphyrins include diazomethane (Carlson and Dolphin 1976), methanol with hydrochloric acid or concentrated sulphuric acid (Falk 1964, Fuhrhop and Smith 1975) and boron fluoride in methanol (Grosser and Eales 1973, Petryka and Watson 1978). Sulphuric acid-methanol 10% (v/v) has proved a convenient reagent (With 1973, Wilson et al 1978) and is currently used in the UCT porphyria laboratory. Following TLC, porphyrins can be quantitated by one of two methods. The first is by elution with chloroform-containing methanol (Doss 1974). The second, still used in the UCT laboratory, is by fluorescence scanning on a scanning densitometer equipped with a fluorescence detector and integrator (Day et al 1978a).

Day et al (1978a) standardised the procedure for esterification, extraction and quantitative TLC of porphyrins which is still used in the UCT laboratory today. They advocated development of the TLC plate in a mixture of chloroform, dodecane and hexadecane: this simple step enhances fluorescence ten-fold on silica gel TLC plates. An advantage of the introduction of TLC with fluoroscanning is that it provided a detailed permanent quantitative record of all the porphyrins present in the sample.

Several problems are recognised with quantitation of stool porphyrins by TLC. Porphyrin isomers are not easily separated by standard techniques, though this is possible, particularly for coproporphyrin series I and III, provided specific techniques are employed (Doss 1974, Sears et al 1974, Schermuly and Doss 1975, Smith 1978). The various dicarboxylic porphyrin esters are poorly resolved. Porphyrin esters may remain at the origin or migrate more slowly than uroporphyrin; this residual material may represent partially esterified porphyrin or true hydrophobic porphyrins such as X-porphyrin (Elder 1980).

Other techniques

A number of other techniques for the determination of porphyrins were described in the 1970s (Elder 1980). These included mass spectrometry, nuclear magnetic resonance spectroscopy (Smith 1975, Jackson 1977), magnetic circular dichroism (Ivanetich et al 1984), atomic absorption spectrophotometry (Bourdon et al 1972), electrophoresis (Fischl et al 1970, Lockwood and Davies 1962), second derivative spectroscopy (Jones and Sweeney 1979), gel filtration (Rimington and Belcher 1967, Bachman and Burnham 1969) and fast-atom bombardment mass spectrometry (Luo et al 1997). In many centres, TLC has been replaced by HPLC (Gray et al 1977, Jackson 1977, Petryka and Watson 1978) for the separation and measurement of porphyrins. A variety of columns have been employed and several techniques have been described. Fluorometric rather than spectrophotometric greatly increases sensitivity (Sagen and Romslo 1985), and further refinements in technique continue to be published (Lam et al 1998). An important advantage of HPLC, particularly when coupled with the use of a fluorescence detector, is that better resolution of individual porphyrins is obtained. Porphyrins are fairly easily resolved into their constituent isomers (Jackson et al 1982, Kuhnel et al 1999), and dicarboxylic porphyrin esters can be resolved into their component porphyrins. Elder (1980) concluded that the easier quantitation, better resolution and more rapid separation of porphyrins by HPLC is to some extent offset by the higher cost, but that qualitative identification by TLC is adequate for clinical purposes, particularly where large numbers of samples must be examined.

6.4 THE PRACTICAL DIAGNOSIS OF PORPHYRIA IN THE LABORATORY

A practical approach to the laboratory diagnosis of porphyria has been reviewed many times, including reviews by With (1978), Elder (1980), Moore and Disler (1985) and more recently, Bonkovsky and Barnard (1998). With the exception of enzyme-based and DNA-based diagnosis however, it should be borne in mind while reviewing the suggested biochemical methods for the diagnosis of porphyria that they will detect only those cases who express biochemical abnormalities. Elder pointed out that failure to examine appropriate samples, particularly stool, was an importance source of diagnostic error. In patients presenting with acute symptoms, the first investigation is to examine a fresh sample of urine for PBG excess using the Watson-Schwartz or Hoesch reactions. Occasional false positives may occur and therefore positive results support but do not prove a diagnosis of acute porphyria (Eales et al 1966, Pierach et al 1977). Positive results should be followed by quantitative measurement of

PBG with or without the measurement of ALA. A negative test however absolutely excludes the acute attack. This should be followed by the screening of stool for porphyrins; if positive, porphyrins should be quantitated by solvent extraction, HPLC or TLC, which will allow the differentiation of AIP, HCP and VP. The possibility that faecal blood loss may result in false-positive screening results (Eales et al 1966); is recognised, as also that haem-derivatives produced by the gut flora may also lead to a positive screening result.

In patients with skin lesions, the first step is to exclude CEP and EPP; this is easily accomplished by looking for erythrocyte fluorescence, followed if necessary by the separation and quantitation of erythrocyte and plasma porphyrins. Thereafter PCT should be distinguished from VP. Screening tests alone may not differentiate these two porphyrias, and quantitation or chromatography is important. In PCT, the earlier and more hydrophilic porphyrins predominate in urine (Doss 1974), whereas in VP, the concentration of coproporphyrin is greater than that of uroporphyrin. Examination of stool porphyrins should unequivocally separate PCT, VP and HCP.

In the non-acute phase, AIP is best diagnosed by determination of PBG deaminase activity (Sassa et al 1975b). Owing to the difficulties inherent in assaying PPO activity, Elder (1980) suggested that the most appropriate diagnostic test for VP was the biochemical measurement of faecal protoporphyrin, coproporphyrin and X-porphyrin fractions, though he recognised that obligatory latent porphyrics with normal faecal porphyrin levels are encountered and would not be detected.

Most authors, including Moore and Disler (1985), recommend a preliminary screening of samples before one proceeds to the actual determination of porphyrin concentrations. Day (1986) recommends that quantitative assays for porphyrins and precursors be preceded by rapid screening tests which have proved of great value in practice, especially in suspected acute attacks where urgent therapy is required (Eales et al 1966). A dangerous practice is the screening of urine samples alone when investigating the porphyrias. This will not differentiate the three acute porphyrias and will not exclude a wide range of non-porphyric conditions accompanied by raised urine porphyrins such as coproporphyrinuria. Other screening tests, particularly faecal screening, must be used. Rapid qualitative screening methods for the presence of elevated PBG concentrations in urine include the Watson-Schwartz and Hoesch tests. Urine and stool extracts may be screened by methods which maximise fluorescence with minimal quenching. In the standard method, using Dean's solution, porphyrins are extracted into a solution of equal volumes of diethyl ether, amyl alcohol and glacial acetic acid; stool porphyrins are additionally extracted into 1.5 M HCl. The extract is then viewed under ultraviolet light for fluorescence.

In assessing the results of chromatography, it is important to realise that the relative proportions of the individual porphyrins offer important information and this aspect is often of greater value in diagnosis than a mere comparison of porphyrin concentrations with normal values. In latent porphyria, porphyrin values may lie within normal limits, and yet distribution on chromatography may reveal the presence of the porphyria. (Moore and Disler 1985).

More recently, Bonkovsky and Barnard (1998) have reviewed the diagnosis of porphyria in the era of molecular biology They recommend the following approach to a suspected porphyria. In the presence of acute symptoms, a rapid screening test for PBG should be performed; urine should also be sent to a laboratory for confirmatory quantitative testing for ALA and PBG. In the presence of cutaneous symptoms, suggestive of HCP, VP or PCT, they recommend a urine screening test. If positive, urine should be screened for ALA and PBG and porphyrins. If elevated, the diagnosis of an acute cutaneous porphyria (HCP or VP) is established. As an alternative, ALA and PBG quantitation coupled with the measurement of plasma porphyrin concentration should be made since plasma porphyrins are always elevated in patients with active skin lesions. If total plasma porphyrin concentrations are increased,

plasma fluoroscanning should be carried out. Urine, stool and plasma porphyrins should then be measured. An assay of biliary porphyrins and porphyrinogens can help to distinguish HCP from VP (Logan et al 1991) but is not necessary for routine diagnosis. We would however disagree with the importance placed on showing an elevated ALA and PBG in the diagnosis of VP and HCP. We would recommend a more direct approach: initial plasma fluoroscanning coupled with the chromatographic quantitation of urine and stool porphyrins, which is a simpler approach to the identification of VP, HCP and PCT.

Since family screening using biochemical methods alone will fail to detect biochemically silent porphyria, enzymatic or DNA-based tests are in theory superior. A number of molecular methods are available for the determination of unknown mutations in porphyria (Bonkovsky and Barnard 1998). These include oligonucleotide specific hybridisation, direct sequencing, single-stranded conformational polymorphisms (SSCP) analysis or denaturing gradient gel electrophoresis (DGGE). SSCP and DGGE are however not 100% sensitive; in Finnish AIP families, SSCP detected 89% of 19 mutations (Kauppinen et al 1995), and these procedures failed to identify the mutation in some European patients with VP (Whatley et al 1999). Asymmetric PCR amplification or repeat cycling may facilitate sequencing by enriching one PCR strand (Mgone et al 1994, Lee and Anvret 1991) and PCR can be used with allele-specific amplification such that a PCR product is or is not produced depending on the mutated sequence within the primer region. In the situation where a few mutations are responsible for the porphyria phenotype, it is appropriate to screen by rapid molecular methods. Examples of the few situations where this is appropriate include the R116W mutation in Dutch patients with AIP, the W198X mutation in Swedish patients with AIP, the R59W mutation in South African patients with VP and the C73R mutation in CEP. Bonkovsky and Barnard conclude however that the traditional biochemical screening methods may still be best when the specific mutation is not known or when there is genetic heterogeneity in the disease and population served.

6.5 THE LABORATORY DIAGNOSIS OF VARIEGATE PORPHYRIA

Much of the early literature around the diagnostic biochemical features of VP originated from the University of Cape Town as a result of the high prevalence of VP in South Africa and the interest of Lennox Eales and his colleagues in the disease. Large amounts of coproporphyrin and protoporphyrin are present in the stool in both the acute and the remission phase of VP (Eales 1963, Eales 1974). Though typically the predominant porphyrin is protoporphyrin, some investigators have occasionally found coproporphyrin in greater quantity than protoporphyrin in the stools of patients with VP (Rimington and Lockwood 1968, Poh-Fitzpatrick 1980). A high combined level has been found to be indicative of VP in doubtful cases (Dean 1971, Eales 1974). It was shown however that there were both qualitative and quantitative differences in porphyrin excretion patterns between the two phases. In the remission phase, small amounts of porphyrins with more than 4 carboxyl groups are excreted in the stool, whereas in the acute attack, the excretion of urinary PBG is greatly increased and is accompanied by an increase in faecal uroporphyrin and, to a lesser extent, heptacarboxylic porphyrin (C7), hexacarboxylic porphyrin (C6) and pentacarboxylic porphyrin (C5). Even in remission, patients with VP excreted as much as 10 mg of coproporphyrin daily, of which more than 75% was of the series III isomer. During the acute attack, a relative increase in coproporphyrin I occurs. Though the increased excretion of protoporphyrin is described as the most characteristic feature of VP, Sweeney (1963) has been pointed out that what is recovered from the stool as protoporphyrin is almost certainly not protoporphyrin alone.

Gray et al (1948) described a case of VP in which faecal porphyrins decreased and urinary porphyrins increased during an acute attack associated with jaundice from intercurrent viral

hepatitis. They described this as a reciprocal excretion of porphyrins in VP, high faecal and low urine porphyrins in the quiescent phase with reversal in the acute phase whereas Eales (1963, 1974) believed that it was the diversion of porphyrin from bile to urine in the presence of cholestatic hepatitis which underlay this change in the pattern of porphyrin excretion.

The initial biochemical methods for the determination of stool porphyrins established in Johannesburg (Barnes 1958) and Cape Town (Eales 1959) were based on the solvent extraction method of Holti et al (1958). This was selected in preference to the method of Schwartz et al (1960) since it is a simpler method, though it has the disadvantage of not extracting uroporphyrin as a separate species. Grosser and Eales (1973) compared solvent extraction and TLC for the differentiation of PCT and VP. The solvent extraction profile for VP was dominated by a marked increase in the protoporphyrin fraction and a lesser though considerable increase in the coproporphyrin fraction. The profile for PCT showed a lower total porphyrin concentration, and the coproporphyrin fraction constituted nearly 60% of the total. With TLC, all intermediate porphyrins were shown. The marked increase in protoporphyrin and the lesser but considerable increase in coproporphyrin were confirmed in VP. Other intermediate porphyrins were present in much smaller amounts, and uroporphyrin, though variable in amount was visible in 89%. A C3 porphyrin was noted in many chromatograms. Eriksen (1962), Doss (1969) and Chu and Chu (1962) had all observed this; and Eriksen had suggested that at least some of the C3 porphyrin represents a coproporphyrinogen precursor. Interestingly, TLC was no more efficient than solvent extraction in differentiating VP and PCT.

Also shown in VP was the presence of a characteristic hydrophobic porphyrin complex in stool termed X-porphyrin (Rimington et al 1968). The X-porphyrin fraction comprises the peptide conjugates of a dicarboxylic porphyrin along with traces of uroporphyrin, heptacarboxylic and pentacarboxylic porphyrins. Though the X-porphyrin is most markedly raised in VP, it is also increased in PCT and so cannot of itself be used as a diagnostic feature for VP (Eales et al 1975).

Eales et al (1980) stated that the definitive diagnosis of VP depends on: 1) the consistent presence of a strongly positive faecal screening test which may be accompanied by a positive urinary test; 2) the finding of a raised total faecal porphyrin consisting mainly of protoporphyrin on solvent extraction analysis; 3) the presence in the plasma of porphyrin PU, running between coproporphyrin and C5, and 4) the presence of X-porphyrin in the stool. PCT could be distinguished by the finding of a high urinary uroporphyrin and C7 and a normal or only slightly raised faecal porphyrin; the diagnosis is made beyond doubt by the demonstration of a high faecal isocoproporphyrin (Elder 1972). Day et al (1978b) had suggested the utility of TLC plasma porphyrin profiles for the diagnosis of VP. Though the erythrocyte protoporphyrin in VP is normal, plasma porphyrin profiles show elevated protoporphyrin concentrations, sometimes a slightly increased uroporphyrin and a diagnostic marker, a then unidentified porphyrin, labelled *peak unknown* (PU) which ran between the coproporphyrin and C5 markers on the TLC plate. PU has since been identified as a complex between coproporphyrin and a non-fluorescent molecule, which may be cholesterol, and is therefore quantitated against the coproporphyrin standard. A further feature said to be characteristic of VP is the presence of a so-called pseudopentacarboxylic porphyrin noted on TLC of faecal porphyrin esters, which runs immediately before the pentacarboxylic porphyrin. This has been identified as a hydroxylated dicarboxylic porphyrin (Moore and Disler 1985). Eales et al (1980) stated that TLC and quantitative fluoroscanning of porphyrin methyl esters (Day et al 1978b) is over 100 times more sensitive than previously-employed routine methods. This is due in part to the enhancement of fluorescence produced by development in a dodecane/hexadecane solvent. Diagnostic and distinctive plasma porphyrin profiles for the different porphyrias were shown, even when total plasma porphyrin amounts

were only slightly elevated. Day et al stated that all porphyrias could clearly be distinguished on the TLC plasma porphyrin profile alone with one exception: VP and HCP, which produce identical patterns. More recently, Hindmarsh et al (1999) have examined the proposition that careful separation and quantitation of plasma porphyrins by HPLC might replace urine and stool porphyrin analyses in the diagnosis of porphyria. They concluded that on the basis of their studies, plasma HPLC was insufficiently specific, though it appeared to have some utility in PCT.

Day (1986) provided the definitive description of VP in its various phases. He defined the *quiescent phase* as a phase in which excessive porphyrin concentrations are often found only in the stool and plasma: the diagnostic features are an elevated coproporphyrin and protoporphyrin, though lesser amounts of C5 and C3, together with traces of isocoproporphyrin, are almost invariably present. In the quiescent phase, the plasma is dominated by the coproporphyrin/cholesterol complex, PU, (Day et al 1978b) with lesser amounts of protoporphyrin and traces of uroporphyrin. The plasma of quiescent VP often contains a large amount of porphyrin not extracted by normal methods, but bound to the protein fraction (Day et al 1978b, Longas and Poh-Fitzpatrick 1982).

In the *acute phase*, the main abnormalities shift to the urine. Urine coproporphyrin and especially uroporphyrin are markedly increased, accompanied by a striking increase in the precursors ALA and PBG. Stool coproporphyrin levels become more elevated than is seen in the quiescent phase. There is a further increase in the plasma porphyrin levels, largely due to an increase in the uroporphyrin fraction, and plasma precursor levels are mildly increased.

Dual porphyria is the name given to a biochemical picture suggestive of PCT superimposed on quiescent VP (Day et al 1982, Martasek et al 1983), and was only recognised after the introduction of sensitive analytical methods such as TLC. Dual porphyria could be misdiagnosed as PCT in view of the elevated urinary porphyrins: this is an important reason for recommending that no case of porphyria be screened by urine analysis without stool analysis (Day 1986).

Silent variegate porphyria is defined as a phase of VP distinguished by an absence of clinical or biochemical signs of the disease other than reduced PPO activity. Though obligate gene carriers with silent VP were occasionally reported in family surveys (Mustajoki and Koskelo 1976, Fromke et al 1978, Muhlbauer et al 1982), the only reliable way to detect silent cases until recently has been by demonstrating reduced PPO activity (Meissner et al 1986). Disler et al (1982) had suggested that up to 40% of gene carriers might have silent VP. Day (1986) stated that it is important to identify these silent carriers as they were believed to be at risk of developing the acute attack if exposed to porphyrinogenic drugs; additionally, their offspring require appropriate screening and education.

For practical purposes VP does not present biochemically before puberty, and exceptions are extremely rare. Day reports uncommon cases of patients who developed cutaneous or acute symptoms in childhood. Two young boys described by Kramer (1980) developed acute and cutaneous symptoms following treatment for epilepsy with porphyrinogenic drugs, recovered following removal of the medication and were found to be heterozygous for VP (Day and Eales 1983). Day (1986) also stated that a surprisingly high incidence of prepubertal VP had been discovered in the Pretoria area of South Africa. In the light of our own experience, we believe that this should be discounted since the laboratory whose results Day quoted, using qualitative TLC alone, have in our experience substantially over-diagnosed VP.

The usual reason advanced for the excess of protoporphyrin in stools of patients with VP is that deficiency of PPO results in a reduction in the conversion of protoporphyrinogen to protoporphyrin, resulting in the accumulation and excretion of protoporphyrinogen which is then non-enzymatically oxidised to protoporphyrin. Logan et al (1991) analysed the porphyrinogen and porphyrin content of bile in 10 patients with VP and 17 control subjects.

They found a complete separation in total porphyrin levels in bile between the VP group and the control group, with a 9-fold difference between the highest value in any control subject and the lowest value in any patient with VP. In contrast, the mean faecal level of total porphyrin in the patients with VP was higher than that of the controls, but the faecal levels in VP patients overlapped those of the control group. The prominent porphyrin in the bile of normal subjects was coproporphyrin, whereas in VP the predominant porphyrins were protoporphyrin and other dicarboxylic and tricarboxylic porphyrins. They concluded that bile porphyrin concentration is a more sensitive and specific predictor of VP than is stool porphyrin concentration. They ascribe this discrepancy to the observation that, as reviewed above, a substantial proportion of the porphyrin in faeces is not of biliary origin.

Plasma fluorescence scanning

Poh-Fitzpatrick and Lamola (1976) described the determination of the fluorescence maxima of unextracted native porphyrin-protein complexes in patients' erythrocytes and plasma when diluted with phosphate-buffered saline, and showed that this method reliably differentiated patients with EPP from those with PCT or CEP. Subsequently, Poh-Fitzpatrick (1980) studied 10 patients shown biochemically to have VP. Heparinised blood was separated by centrifugation. 1 ml of plasma was diluted with 9 ml of phosphate-buffered saline, and the fluorescence emission spectra were scanned from 550 to 700 nm at a fixed excitation wavelength of 400 nm since she had shown that the fluorescence emission maximum remained the same whether excited at 400 nm or at the specific excitation wavelength maximal for each specimen. In all specimens from patients with VP, a sharply defined fluorescence emission maximum at 626 ± 1 nm was noted. Blister fluid from a patient gave the same fluorescence maximum. This emission maximum was easily distinguished from the maxima of plasma specimens from patients with PCT, CEP and AIP, all of which lie at 619 ± 1 nm and cannot be distinguished one from the other. Poh-Fitzpatrick (1980) concluded that the fluorescence emission wavelength maximum observed with unextracted porphyrin and plasma is a useful diagnostic indicator for VP. Indeed, several patients with an original diagnosis of HCP or PCT, when tested by plasma scanning, yielded a peak characteristic of VP and on subsequent biochemical analysis were shown to have been misclassified initially.

Poh-Fitzpatrick and Lamola (1976) further noted that the fluorescence maxima noted in the plasma samples of patients with VP did not correspond to those of any native porphyrin. The porphyrin present in the plasma of VP is relatively resistant to extraction by acidified ethyl acetate, a solvent which is 90% efficient in extracting porphyrins from plasma samples of patients with PCT or CEP. This phenomenon had been noted by Rimington et al (1968) who suggested the possibility of an unusual porphyrin-protein complex related to the porphyrin-peptides (X-porphyrin) of urine and stool observed in VP. Poh-Fitzpatrick (1980) reported that this plasma porphyrin is more effeciently though still incompletely extracted into perchloric acid, followed by methyl alcohol. Though it was not possible to identify the porphyrin completely, she noted that mixtures of protoporphyrin and coproporphyrin in ratios of 8:2 in perchloric acid and methyl alcohol produced spectra indistinguishable from those of the extracts from VP plasma specimens. The values also overlap with those for the tricarboxylic porphyrin harderoporphyrin, and it is possible that the characteristic fluorescence maximum reflects a mixture of porphyrins, analogous to the heterogeneous mixtures of porphyrins and peptide conjugates in the X-porphyrin fractions of urine and stool (Rimington et al 1968). When separated chromatographically, these mixtures frequently include bands suggestive of tricarboxylic and dicarboxylic porphyrins. Belcher et al (1969) have isolated porphyrins behaving chromato-graphically as tricarboxylic porphyrins and lesser amounts of dicarboxylic porphyrins in the porphyrin-peptide complexes present in the

bile of patients with VP. Porra and Falk (1964) reported that during the enzymatic conversion of coproporphyrinogen to protoporphyrinogen, porphyrin intermediaries are covalently bound to a protein from which they are unextractable by ethyl acetate/acetic acid. Though Fitzpatrick therefore suggested that a dicarboxylic or tricarboxylic porphyrin intermediary might be the source of the fluorescence maximum seen in this study; it may also be related to the coproporphyrin-peptide conjugates labelled PU described by Day et al (1978b).

Longas and Poh-Fitzpatrick (1982) subsequently analysed the porphyrins present in the plasma of a single patient with VP in more detail. They found that two major classes of porphyrin were present. The first-class was loosely bound, and the porphyrins were readily extracted from plasma with an ethyl acetate/acetic acid mixture. Uroporphyrin, harderoporphyrin, isoharderoporphyrin, protoporphyrin and a small amount of penta-carboxylic porphyrin were identified on HPLC. Coproporphyrin was not detected despite the statement by Day et al (1978b) that coproporphyrin III was the major porphyrin in the plasma of patients with VP. They then attempted to isolate and identify the second, tightly-bound, group of porphyrins, by a process which included acid hydrolysis. HPLC analysis suggested that this consisted of uroporphyrin. However, acid hydrolysis of a protoporphyrin IX standard itself resulted in extensive derivitisation yielding a substance with the chromatographic properties of uroporphyrin. The authors therefore suggested that this uroporphyrin-like entity represents the decomposition or derivitisation product of a tightly-bound dicarboxylic porphyrin. The molecule carrying the tightly-bound porphyrin appeared to be albumin. They concluded that the VP plasma porphyrin fluorescence marker is a dicarboxylic porphyrin bound to albumin. This complex is possibly formed by the reaction of porphyrinogen with sulphydryl groups as this type of reaction is known to take place *in vitro* (Sano et al, 1961). Meanwhile, loosely bound porphyrin such as protoporphyrin IX, harderoporphyrin and uroporphyrin (in a molar ration of 1.2:1:0.5) and traces of a pentacarboxylic porphyrin are also present.

The utility of plasma fluoroscanning for the diagnosis of VP has been confirmed by others. Enriquez da Salamanca et al (1993) assessed the value of plasma scanning in 75 patients with PCT, 14 with EPP, 10 with VP, 7 with AIP, 2 with HCP and 1 with CEP. All diagnoses had been established by clinical and standard biochemical criteria. In PCT, emission maxima were found at 618-622 nanometers and the intensity of this major peak depended on the activity of the porphyria; it was noted that PCT patients in remission may show normal plasma scanning in keeping with their normal plasma porphyrin concentration. Plasma from patients with EPP exhibited a pathognomonic peak at 636 nm, and plasma from VP at 626-628 nm. The typical porphyrin plasma marker of VP was found in patients both with and without cutaneous involvement, in contrast with the authors' expectation that a normal plasma fluorometric profile should be expected in people who were merely carriers of the VP defect. They also noted that the presence of plasma shifts the emission maxima of porphyrins slightly. Standard porphyrins diluted in phosphate-buffered saline showed the following maxima: 613 nm for coproporphyrin, 617 nm for uroporphyrin and 633 nm for protoporphyrin. When incubated in porphyrin-free plasma, the emission maxima shifted to 619 nm, 620 nm and 635 nm respectively which are close to the maxima noted in porphyria plasma samples. They suggest that an absent or minimal peak in the emission spectrum of PBS-diluted plasma means that the diagnosis of an active cutaneous porphyria may be rejected, and that plasma scanning has the ability to differentiate plasma samples of patients with VP and EPP from those of patients suffering from PCT, CEP or HCP.

Long et al (1993) evaluated qualitative fluorescence emission scanning of saline-diluted plasma as a method for identifying asymptomatic VP gene carriers. They screened 36 unrelated patients with VP and 136 of their asymptomatic relatives aged 15 or older. Some patients with other forms of porphyria as well as normals were also studied. Scans from all

normal subjects showed no peak, or a very small peak with an emission maximum of 619 nm or less and whose height above baseline did not exceed 3 mm at a specified sensitivity. Patients with VP showed a peak between 621 and 627 nm and the height exceeded baseline by at least 15 mm. In most patients, the fluorescence emission maximum was at 625 nm. By statistical comparisons with the expected frequency of a gene for VP in the asymptomatic relatives, they estimated that the sensitivity of plasma fluoroscanning was 0.86, with a 95% confidence interval of 0.71-0.98. They estimated therefore that the posterior risk of an adult first- or second-degree relative of having a negative plasma scan was 1 in 8 and 1 in 22 respectively. They also found that 10 of 55 asymptomatic relatives had elevated faecal porphyrin concentrations, and that all of these had had an abnormal plasma scan, as did 16 who had normal faecal porphyrin concentrations. They calculated therefore that the sensitivity of an increased faecal porphyrin concentration in the detection of asymptomatic carriers of VP is 36-38%.

Da Silva et al (1995) examined the utility of plasma fluoroscanning for the detection of silent VP. They demonstrated that all of 32 biochemically expressed VP patients had a fluorescence emission peak at 630 nanometers. However, in 56 asymptomatic carriers proven by the demonstration of a 50% reduction in PPO activity, this peak was found in only 50% of adults and in none of the children. They concluded that though plasma fluoroscanning will pick up symptomatic VP patients, it is insufficiently sensitive in the screening of asymptomatic relatives and is of no use in children aged less than 18. They recommended that the determination of PPO activity remain the diagnostic method of choice for the detection of silent carriers.

CHAPTER 7:

THE BIOCHEMICAL DIAGNOSIS OF VARIEGATE PORPHYRIA IN THE UCT LABORATORY

The experimental work which follows is based to a large extent on an analysis of the accumulated porphyrin data of the University of Cape Town porphyria laboratory. Diagnoses are in most cases based on the expert interpretation of urine and stool porphyrin profiles determined by thin-layer chromatography. Since these techniques, and the process of diagnosis, are crucial to the interpretation of the results described in the following chapters, aspects of porphyria diagnosis as currently performed in the UCT laboratory are reviewed here. Additionally, the statistical techniques of discriminant analysis and classification tree analysis are briefly described since they are used extensively in the analysis of the porphyrin data in the chapters which follow.

7.1 BIOCHEMICAL TECHNIQUES EMPLOYED IN THE UCT PORPHYRIA LABORATORY

The standard biochemical technique for the diagnosis of porphyria in the UCT laboratory is thin-layer chromatography (TLC). All samples initially undergo a screening test. Erythrocytes are screened under ultraviolet light for fluorescence as a simple test for CEP and EPP. Urine is screened for PBG by the Watson-Schwartz test. Urine and stool are treated with Dean's solution and screened for fluorescence under ultraviolet light. All methods are described in detail in Appendix 1.

7.2 THE UCT PORPHYRIA DATABASE

This database is central to the data-handling, record-storing and record-retrieval functions of the UCT porphyria service diagnostic laboratory. Complete records of all biochemical tests, routine R59W screening and plasma fluoroscanning results are contained within the database and extensive software programming in dBase language was undertaken by the author to automate most of the laboratory's data-handling operations. Thus the database software calculates the final porphyrin concentrations from the raw data obtained from the fluorescence integrator by making appropriate corrections for sample volumes and weights, dilution factors and comparisons with porphyrin standards. Once the results for a patient are stored within the database, they are reviewed and interpreted by one of three senior professionals: a professional nurse with 30 years of experience in porphyria, the principal biochemist or the principal clinician working within the porphyria service. The result is coded by biochemical diagnosis and, where appropriate, by a DNA diagnosis. This, with additional interpretative comments, is entered into the database and the results are then printed and sent out. An example of the laboratory result form is shown in Appendix 7.

The porphyria database and its associated diagnostic codes were introduced into full-time use from January 1993. In developing algorithms for the diagnosis of porphyria, the concept of *prior probability* is important; it is essential to know the proportion of samples submitted to a laboratory which might reasonably be expected to carry a particular diagnosis in terms of defining methods for their identification. Accordingly, the calculations which follow are based on results from the years 1993 to 1998 inclusive. The figures labelled *n* in Table 7-1

reflect the total number of samples during this six-year period for which a particular diagnostic code was assigned; the percentage in the adjacent column therefore reflects the prior probability of that particular diagnosis.

Diagnostic categories employed in the UCT porphyria database

The following diagnostic codes are routinely assigned to porphyria diagnostic tests in our laboratory (Table 7-1).

Code	Category	n	%
	Major categories		
1	No biochemical evidence of porphyria	2981	56.9
11	Variegate porphyria	481	9.2
17	Suggestive of variegate porphyria: confirmation needed (VPH)	114	2.2
18	Probably normal but variegate porphyria not excluded (VPL)	189	3.6
31	Porphyria cutanea tarda	241	4.6
41	Acute intermittent porphyria	287	5.5
	Minor categories		
0	No diagnosis entered	4	0.1
2	Minor abnormalities: probably normal	46	0.9
3	Tests inadequate for diagnosis	747	14.3
15	Variegate porphyria: dual phase	1	0.0
21	Variegate porphyria (dual phase) or porphyria cutanea tarda	8	0.2
32	Suggestive of porphyria cutanea tarda	18	0.3
51	Congenital erythropoietic porphyria	3	0.1
55	Erythropoietic protoporphyria	24	0.5
56	Suggestive of erythropoietic protoporphyria	5	0.1
61	Hereditary coproporphyria	6	0.1
71	Suggestive of porphyria: unclassified	2	0.0
72	Coproporphyrinuria	17	0.3
76	Abnormal porphyrin profile: unclassified	64	1.2
77	Suggestive of lead poisoning	3	0.1
	Total	5241	100

Table 7-1. Diagnostic categories used in the porphyria database and number of times each was recorded for the period 1993-1998.

It is seen that the following are the prior probabilities for samples submitted to the UCT porphyria laboratory: normal 56.9%, VP 9.2%, VPH 2.2%, VPL 3.6%, PCT 4.6%, AIP 5.5%. The only other large category is that where samples are inadequate for diagnosis; this is usually where either the urine or stool alone is submitted, but not both. This accounts for 14.3%. Note too that these represent samples received and not patients. Patients with AIP in particular, and to a lesser extent PCT, are over-represented since they tend to be tested repeatedly to assess disease activity. This is rarely so for VP, most of whom are tested once for diagnostic purposes only.

7.3 EVALUATION OF DIAGNOSTIC TECHNIQUES FOR THE ROUTINE LABORATORY

Until the identification of the PPO gene, biochemical diagnosis by quantitative TLC coupled with careful evaluation of the clinical symptoms remained the final arbiter of diagnosis in our laboratory, and therefore in the country as a whole. The system has served us, and the country, well for many years. There are however two important problems associated with biochemical diagnosis of porphyria, and of VP in particular. These are discussed here.

Problems with the diagnosis of VP

Laborious nature of the technique

Preparation, measuring and weighing of urine and stool, drying and weighing of stool, extraction of porphyrins, application of sample to TLC plate, running, developing and scanning of the plate, and transferring of data into the database is labour-intensive. Exposure to stool and to the fumes of organic solvents are unpleasant for staff. Nor do patients enjoy the necessity to provide stool samples. Indeed, more than 20 years ago Day et al (1978b) wrote:

"Excreta are unpleasant to work with and may take several days to collect. Porphyrin concentrations are prone to large fluctuations from day to day in both urine and stool, whereas RBC and plasma porphyrin levels very only slightly according to measurements by ourselves and others (Allen et al 1975; Copeman et al 1966). Many subjects are reticent to donate excreta for analysis, most are prepared to give a few millilitres of blood. This is especially the case in surveys of families exhibiting porphyria where most members feel they are well even if they do prove biochemically porphyric."

Lack of a definitive test for VP

The single largest obstacle to the efficient diagnosis of VP in South Africa has been the absence of a practically useful and highly accurate test with which ultimately to confirm or refute a diagnosis of VP. In the absence of such a test, it was impossible to reach a final conclusion in the individual with an equivocal result other than by repeat testing, nor to assess the significance of equivocal findings as a group and hence to devise objective TLC criteria by which to remove this uncertainty.

This lack of precision sometimes encountered in assigning a diagnostic category has proved a problem in issuing diagnoses efficiently and in recommending appropriate management to patients and their doctors. It seemed that further refinement is of these techniques for the biochemical separation, identification and quantitation of porphyrin species in excreta would not overcome these problems. Therefore, from an early stage, the necessity for the identification of new methodology is which would result in a much higher accuracy for the diagnosis of both presence and the absence of VP in South Africa was recognised.

Determination of PPO activity and the diagnosis of VP

A diagnostic technique which has proved useful in some circumstances is the determination of PPO enzyme activity. Determination of erythrocyte PBG deaminase activity in particular is widely used for the diagnosis of AIP, though a number of problems with this technique have been recognised. Some families with classic AIP have been shown to have normal erythrocyte PBG deaminase activity. It has been shown that in these families, PBG deaminase activity in tissues other than erythrocytes is reduced by 50%, and the mutations in these families have been shown to reside in the initial part of the DNA sequence, encoding a part of the protein which is cleaved off in erythrocytes (Herrick et al 1989, Grandchamp et al 1989). Secondly, a

significant proportion of patients show results falling into an indeterminate range which complicates diagnosis.

The determination of enzyme activity is not suitable for the routine diagnostic laboratory. As a mitochondrial protein, PPO is absent from mature erythrocytes. Accordingly its activity can only be Measured in non-erythroid tissues. Hepatocytes might expected to show the greatest activity, but the risks in obtaining hepatic tissue by biopsy rule this out for the routine diagnosis of VP. Though skin fibroblasts and peripheral lymphocytes express PPO, they have little activity and therefore express extremely low levels of PPO activity. Furthermore, the PPO activity assay is poorly suited to routine use. Our extensive experience with the assay shows that in most cases the enzyme contribution to the total return of fluorescence is a fraction of the non-specific background rate of protoporphyrinogen auto-oxidation, and the difference between normal and reduced activity from normal activity is often less than the intra-assay variation. Furthermore, the assay is difficult, time-consuming and potentially hazardous since the operator is exposed to mercury vapour during the production of the sodium-mercury amalgam used for the reduction of the substrate. Our unit has shown that EBV-transformed lymphoblasts express sufficient PPO activity for a reliable estimation of that activity, and that the 50% reduction in activity typical of VP persists after transformation (Meissner et al 1986). Yet the techniques of transformation and subsequent culture of the lymphoblasts are themselves time-consuming and costly and are therefore not suited to routine use.

Biochemical criteria for the diagnosis of VP

Thus, the biochemical diagnosis of VP by urine, stool and plasma porphyrin analysis has remained the mainstay of porphyria diagnosis in our laboratory. A shortcoming of the biochemical descriptions of VP reported by previous authorities (Eales et al 1963, Dean 1971, Grosser and Eales 1973, Eales 1974, Day 1986, Day et al 1978b, Eales et al 1980, Day and Eales 1983, Moore and Disler 1985) is that they largely describe the classic and characteristic profiles of clearly expressed VP. Thus a diagnosis is easy in the patient with a high stool coproporphyrin, protoporphyrin, C5 and pseudopentacarboxylic porphyrin (pseudo-C5) in the stool who also has PU in the plasma. Problematic is the patient with minimal abnormalities, particularly a moderately raised stool protoporphyrin. This has led us to introduce indeterminate categories into our diagnostic categorisation (Table 7-2) as follows:

Probable VP. Though not entirely diagnostic, there is a substantial suspicion (>50% probability) that VP is present. This is represented by category 17 on the database, and will be referred to by the abbreviation VPH (VP: high probability) in the following chapters.

Possible VP. The results are not entirely consistent with normality, and there is a low order of suspicion (<50% probability) that VP is present. This is represented by category 18 on the database, and will be referred to by the abbreviation VPL (VP: low probability) in the following chapters.

Code	Category	Definition	Abbreviation
1	*No evidence of porphyria*	With normal limits: porphyria not suspected.	**Non-VP, NAD**
11	*Definite VP*	Unequivocally diagnostic of VP.	**VP**
17	*Probable VP*	VP (high probability). Not unequivocally diagnostic of VP but thought to have a high probability of being VP.	**VPH**
18	*Possible VP*	VP (low probability). Results do not fall clearly within normal limits but there is insufficient evidence to suspect VP strongly. (In practice, such patients often show an elevated stool protoporphyrin in the absence of any other porphyrin abnormality.)	**VPL**

Table 7-2. Possible diagnostic categories following interpretation of biochemical results for patients suspected of having VP.

Broadly speaking, the features of each classification category are as follows:

Diagnostic of VP

- Raised stool protoporphyrin
- Raised coproporphyrin
- Raised C5
- Raised pseudo-C5)
- Raised tricarboxylic porphyrin (C3).
- Presence of PU in plasma
- Presence of raised urine porphyrins, with or without raised ALA and PBG.

Apparently normal; VP not suspected

- Stool protoporphyrin normal or at most mildly elevated
- Normal coproporphyrin
- Absence of pseudo-C5 and C3
- Absence of "porphyrin activity" in urine
- Absence of PU in plasma

Indeterminate category

This we separated into two categories: "probable" VP and "possible VP", in which the probability of the actual presence of VP was thought to be higher and lower respectively.

- ***Probable VP (VPH):*** A markedly elevated stool protoporphyrin on its own, with or without slight elevations in other characteristic porphyrins.
- ***Possible VP (VPL):*** Presence of a moderately elevated stool protoporphyrin with little other evidence of porphyrin activity.

These diagnostic categories are elastic and there remains an important subjective and experiential element in the interpretation of biochemical porphyrin results.

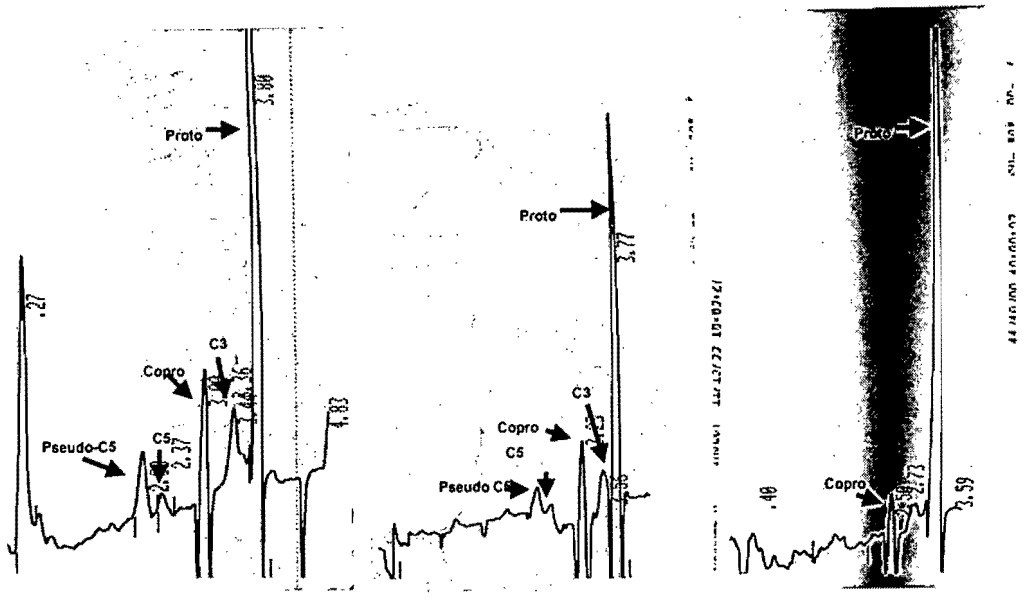


Figure 7-1. Some illustrative thin-layer chromatograms. On the left is a trace from a DNA-positive patient with the classic profile of VP, including elevations in pseudo-C5, C5, coproporphyrin, C3 and protoporphyrin. In the middle panel is a trace from a DNA-positive patient who shows a mild elevation of pseudo-C5, C5 and coproporphyrin but by quantitation, a low protoporphyrin. On the right is a trace from a patient with a markedly elevated protoporphyrin alone. This was initially diagnosed as "VPL". This patient is however DNA-negative.

7.4 DIAGNOSIS IN OTHER SOUTH AFRICAN LABORATORIES

No South African laboratory has had the benefit of the sustained interest and wide access to diagnostic specimens and to patients as the UCT laboratory. However, a number of State and private chemical pathology services in South Africa have offered a biochemical diagnostic service to patients. Typically this consisted of no more than the Watson-Schwartz test and the screening of urine and stool for porphyrins. One or two have offered solvent extraction or qualitative TLC, where porphyrins have been separated by TLC but have not been quantitated: the relative proportions of porphyrin present on the chromatogram are assessed visually (and therefore subjectively).

As the national referral centre for porphyria in South Africa, we have over the years had contact with a large number of patients who have been given diagnoses, both positive and negative, by the laboratories practising these techniques. It has been apparent that both the sensitivity and the specificity of these techniques is poor, particularly in the case of VP, and that this was distorting the spectrum of VP in South Africa. We became increasingly concerned about the numbers of patients diagnosed in other laboratories in whom symptoms quite atypical for VP were being ascribed to a porphyria. The problem was compounded by indeterminate laboratory results interpreted by both patient and doctor as giving support to the diagnosis, rather than as disproving it. In some instances patients were even admitted to hospitals as having acute attacks, yet subsequent testing in our laboratory failed to provide evidence of porphyria at all. It appeared that a tentative clinical diagnosis of VP, often based on spurious grounds, tended to become a self-fulfilling prophecy. Doctors send specimens to laboratories labelled "Suggestive VP symptoms". The technologist, making a subjective assessment of stool fluorescence, is influenced by the clinical information and issues a tentative report such as "Suggestive of VP"; thus establishing a vicious cycle in which both doctor and technologist confirm each other's confidence in a basically erroneous diagnosis.

7.5 OBJECTIVES OF THE STUDIES WHICH FOLLOW

- To determine the degree of agreement in the interpretation of quantitative porphyrin data between experienced interpreters.
- To determine by means of the mathematical techniques of discriminant analysis and classification tree analysis the most appropriate criteria for a diagnosis of VP by chromatography.
- To assess the accuracy of simple screening procedures.
- To assess the accuracy of plasma fluoroscanning for the recognition of VP.

7.6 STATISTICAL TECHNIQUES USED IN THE FOLLOWING CHAPTERS

Two statistical techniques which are useful for the recognition of the factors most predictive of a given outcome and which may be useful in establishing criteria for diagnosis of the techniques of discriminant analysis and classification tree analysis. Their use, and interpretation of the values they return, is briefly discussed below.

Discriminant analysis

Discriminant analysis is used to determine those variables which best discriminate between two or more groups. Computationally, discriminant functional analysis is related to analysis of variance (ANOVA). The underlying concept is that the test determines whether two or more groups are significantly different from each other with respect to the mean of a particular variable: if so, then it is fair to infer that this variable discriminates between the two groups. The final significance test of the ability of a variable to discriminate between groups is the F test, which is computed as the ratio of the between-groups variance in the data over the average within-groups variance. If the between-group variance is significantly larger, this suggests a significant difference between means.

In many instances, multiple variables are used (as will be the case in the analysis of our porphyrin biochemical data). All variables are then studied to determine those which contribute most to the discrimination between groups. This gives rise to a matrix of total variances and co-variances and a matrix of pooled within-groups variances and co-variances. These matrices are compared via multivariate F tests to determine whether any significant differences exist between groups with regard to all variables. This procedure is fundamentally identical to that of multivariate analysis of variance (MANOVA).

The discriminant function analyses in the following chapters are performed with the Statistica software package (StatSoft Inc, Tulsa, USA) which automatically determines some optimal combination of variables to produce independent functions for this distinction. The number of functions that are produced is equal to n-1 (where n is the number of diagnostic classes which must be distinguished), or the number of variables, whichever is the smaller. Whereas discriminant functions indicate which factors most reliably discriminate between diagnostic classes, the related classification functions may be used prospectively to determine to which group a particular case most likely belongs. There as many classification functions as there are diagnostic groups. Cases are classified as belonging to the group for which they have the highest classification score; these are additionally weighted in terms of the prior probability of a specific diagnosis.

Classification matrix

The classification matrix represents a cross-tabulation of predicted diagnostic class (predicted on the basis of the classification functions returned by the discriminant function analysis) versus the actual diagnostic class recorded on the database. This allows us to determine, for each diagnostic class, the probability of both the correct diagnosis being returned, and the probability of an incorrect diagnosis and the overall accuracy of the classification function in terms of diagnosis, from which such characteristics as sensitivity, specificity, positive and negative predictive values may be calculated.

Ranking of individual variables by relevance to diagnosis

In the process of *forward stepwise discriminant analysis* each variable is entered into the function sequentially, and its overall contribution to the discriminant power of the function is assessed. This allows us to determine which variables are most useful in directing the diagnosis toward one diagnostic category or another. These data are combined with the preceding data in some of the tables which follow. The statistical terms shown in the tables are defined as follows:

- Variable: the variable entered at each step
- Step: the step number
- Wilks' lamda: a measure of the power of discrimination between different categories. 0 represents perfect discrimination; 1 represents no discrimination at all. Where shown as part of a stepwise analysis, the overall lamda becomes progressively smaller as additional variables are entered, reflecting an increasing power of discrimination with successive steps.
- F value: the higher the F value, the greater the contribution of a particular variable to the diagnostic power. In the tables which follow, variables are classified from the highest F value to the lowest F value; this implies that the first-mentioned variables have the highest discriminating power whereas the last variables are the weakest in terms of discrimination.
- Partial lamda: this is Wilks' lambda associated with that particular variable's unique contribution to power of discrimination of the model. Again it ranges between 0 (perfect discrimination) and 1 (no discrimination).
- F value associated with the partial lamda
- r^2—defined as 1-tolerance; a measure of redundancy. A value of 0.1 for instance means that the variable is 90% redundant with a respect to other variables. The higher the tolerance, the more important the variable is to the discrimination.

Classification tree analysis

Classification trees are used to predict membership of a particular category based on the measurement of one or more predictor variables. Essentially a classification tree analysis produces an algorithm; the analysis determines decision points (or nodes) at which critical values of a single variable are used to steer the diagnosis in one or other direction. The analysis attempts to determine the combination of variables, and their critical values, which will best fit the eventual diagnosis.

Classification tree analysis is a valuable technique for the identification of underlying predictors in determining eventual assignment to a diagnostic category, and may also result in the production of clinically useful algorithms. However, trees must always be examined critically since it is possible to force 100% accuracy by subclassifying samples into ever

smaller classes until every single exception and aberrant value has been correctly assigned; this leads to the incorporation of misleading, counter-intuitive and even erroneous steps which will not be useful in *a priori* prediction.

7.7 EPIDEMIOLOGICAL TERMINOLOGY

The utility of both diagnostic techniques and of statistical manipulation of porphyrin data are discussed in the chapters which follow. Table 7-3 defines the epidemiological terms employed in those chapters.

	Disease present	**Disease absent**
Test positive	a (true positive)	b (false positive)
Test negative	c (false negative)	d (true negative)

Value	**Formula**	**Definition**	**Explanation**
Prevalence (prior probability)	(a+c)/ (a+b+c+d)	All patients with VP/all patients tested	
Sensitivity	a/(a+c)	True-positive test/all patients with VP	% of subjects with VP correctly identified by the test
Specificity	d/(b+d)	True-negative test/all patients without VP	% of subjects without VP correctly identified by the test
False-negative rate	c/(a+c)	False-negative test/all patients with VP	% of subjects with VP missed by the test
False-positive rate	b/(b+d)	False-positive test/all patients without VP	% of subjects without VP erroneously labelled positive
Positive predictive value (PPV)	a/(a+b)	True-positive tests/all positive tests	% of positive tests which actually denote VP
Negative predictive value (NPV)	d/(c+d)	True-negative test/all negative tests	% of negative tests which actually denote the absence of VP
Overall accuracy	(a+d)/ (a+b+c+d)	(true-positive test+true-negative test)/all tests	

Table 7-3. Terms used in interpreting the utility of a diagnostic test.

CHAPTER 8:

THE CORRELATION OF DIAGNOSTIC CATEGORIZATION BETWEEN EXPERT INTERPRETERS

In the following three chapters, the practical utility of biochemical porphyrin analysis in the diagnosis of VP is assessed. In this chapter, the correlation between the interpretation is placed on biochemical data by three expert observers is studied. In the following chapter, statistical techniques are employed to assess, retrospectively, the importance of specific abnormalities of porphyrin excretion in the diagnosis of VP and to determine which, if any, objective criteria account for the equivocal diagnostic categories. These two chapters lay the groundwork for the work of Chapter 10 in which the biochemical effects of VP are for the first time correlated with the presence or absence of a VP-associated mutation, on the basis of which the most appropriate predictors for a diagnosis of VP are selected.

8.1 INTRODUCTION

There is a subjective element in the interpretation of biochemical tests for porphyria. This subjective element enters the interpretation process at two levels:

- In assessing the *outcome* of a biochemical test *per se*. This is particularly an issue in assessing the degree of deviation from normal in tests such as the Watson-Schwartz reaction, porphyrin fluorescence under ultraviolet light, and thin-layer chromatograms assessed qualitatively without recourse to direct quantitation by fluoroscanning.
- In interpreting the *significance* of the results returned by testing. Even where chromatography is employed, ultimately the decision must be made as to whether a particular chromatographic pattern and the porphyrin concentrations associated with it represent normality or not. In particular, the diagnosis of VP may be complicated by a class of chromatographic porphyrin results which appear to exceed normal limits and yet are not unequivocally diagnostic of VP (Chapter 7): these are assigned to the diagnostic categories high-probability (VPH) and low-probability (VPL).

Throughout the period covered by this study, the issuing of final diagnostic reports in our laboratory has been in the hands of just three people, a biochemist, a professional nurse and a clinician (and author of this dissertation). None has fewer than 12 years' experience, and their opinions are, in South Africa, regarded as definitive. This study set out to assess how well the diagnoses made by these three experienced interpreters agree.

8.2 OBJECTIVES

- To assess the degree of inter-interpreter variation in interpreting urine, stool and plasma screening data and quantitative porphyrin data produced by TLC.

8.3 METHODS

A representative sample of laboratory results were extracted from the database and reproduced in the form of a table from which all identifying data and diagnostic information had been removed. The table is reproduced in Appendix 8.

Screening results

Urine and stool screening results alone were presented. The three interpreters were asked to assign a diagnosis, chosen from a list of possible answers (Table 8-1), to each set of results. Interpreters had no opportunity to modify their choice in the light of their subsequent exposure to the corresponding quantitative data, nor were results discussed.

TLC results

Urine, stool and, where available, plasma data were presented to each interpreter in the form of a table. The three interpreters were asked to assign a diagnosis, chosen from a list of possible answers (Table 8-2), to each set of results.

1	The screenings suggest a **normal result**.
2	The screenings are **equivocal** – could be either normal or VP
3	The screenings suggest **either VP or PCT**
4	The screenings **strongly suggest VP**.

Table 8-1. Diagnostic categorization for screening results.

There is no doubt about the biochemical diagnosis – either VP or normal.	
1	The trace is unequivocally normal
4	The trace is unequivocally diagnostic of VP
There is an element of doubt about the biochemical diagnosis as results are not unequivocally diagnostic.	
2	There is a suspicion that VP may be present, but the probability is low.
3	There is a suspicion that VP may be present, but the probability is high.

Table 8-2. Diagnostic categorization for TLC results. Categories 1,2,3 and 4 correspond to the classes NAD, VPL, VPH and VP as defined previously.

Following completion, the results were entered into a database and related to the original diagnostic information. The final number of samples available for comparison was 210.

Data handling

Data were entered into a Microsoft Access database and analysed with the Statistica software programme.

Statistical methods

The coefficient of concordance was calculated by Friedman analysis of variance. Partial correlations were compared by Kendall tau correlations; and gamma correlations were performed as a second test. The Kendall tau represents a probability and is the difference between the probability that two variables are in the same order in the observed data versus the probability that they are in different orders. The gamma statistic is preferable to the Kendall tau or Spearman R in the case of data which contain many tied observations as is the case here, and is computed as the difference between the probability that the rank ordering of the two variables agree minus the probability that they disagree divided by one minus the probability of ties.

8.4 RESULTS

These are summarised in Table 8-3. The three interpreters are identified by the letters *A, B* and *C*. The routine diagnostic category actually assigned to that sample in the database at the time of testing is labelled as *Original.*

Screening results

The spread of categorizations issued by the three interpreters is shown in Figure 8-1. It is seen that one interpreter (A) recognises a result as abnormal more frequently than the other two interpreters.

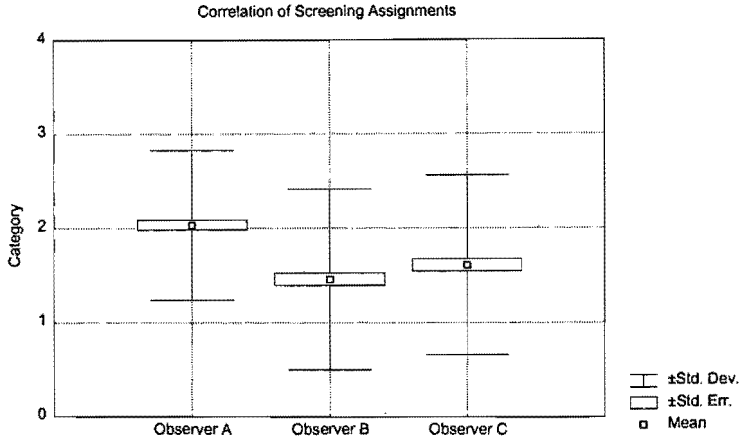


Figure 8-1. Spread of screening categorizations issued by the three interpreters.

A statistical difference was shown for the screening results ($p<0.0001$, Friedman ANOVA χ^2): the major difference lies between the scores of interpreter A and those of B and C. The Kendall coefficient of concordance of 0.36090 indicates a rather poor agreement between interpreters overall.

TLC results

The spread of categorizations issued by the three interpreters is shown in Figure 8-2. It is seen again that interpreter A recognises a result as suspicious of VP slightly more frequently than do interpreters B and C, though in this case no significant difference in mean scores was shown between interpreters ($p=0.97$, ANOVA).

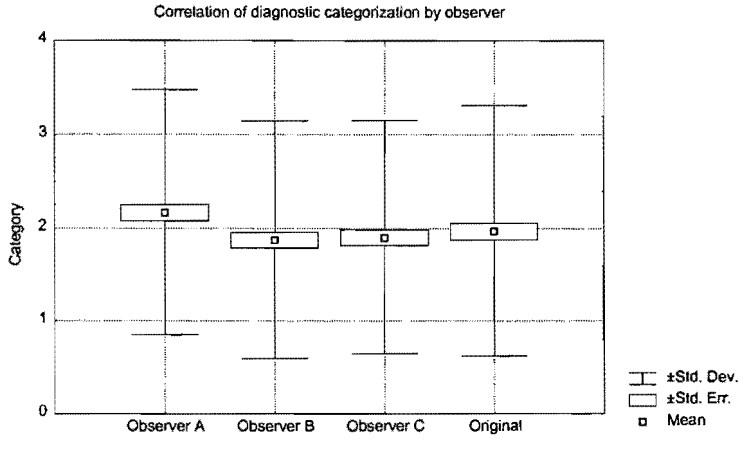


Figure 8-2. Spread of TLC categorizations issued by the three interpreters and as originally recorded in the database ("Original").

The coefficients of concordance are 0.13 between interpreters and 0.09 between interpreters and the database, suggesting week agreement. Indeed, it is considerably weaker than the paired correlation coefficients between interpreters, the lowest of which is 0.84, suggesting that, whereas two of the three interpreters agreed reasonably frequently, agreement by all three was less common.

Screening

	Kendall Tau Correlations				Gamma Correlations				
	A	**B**	**C**			**A**	**B**	**C**	
A	—	0.74	0.70		**A**	—	1.00	0.99	
B	0.74	—	0.77		**B**	1.00	—	0.99	
C	0.70	0.77	—		**C**	0.99	0.99	—	

Friedman ANOVA chi square p<0.0001

Kendall coefficient of concordance 0.36090

TLC

	Kendall Tau Correlations				Gamma Correlations				
	A	**B**	**C**	**Original**		**A**	**B**	**C**	**Original**
A		0.79	0.82	0.73	**A**		0.96	0.96	0.92
B	0.79		0.84	0.84	**B**	0.96		0.97	0.97
C	0.82	0.84		0.81	**C**	0.96	0.97		0.94
Original	0.73	0.84	0.81	1.00	**Original**	0.92	0.97	0.94	

Friedman ANOVA chi square P<0.0001

Kendall coefficient of concordance

between interpreters 0.13

between interpreters and original 0.09

Table 8-3. Partial correlations for three interpreters A, B and C independently assigning diagnostic categories to 210 sets of porphyrin results. For the TLC results, the correlations with the original assignment in the porphyrin laboratory database are additionally included. A correlation of 1 represents perfect agreement; 0 represents no agreement at all.

Extent of agreement on diagnosis between interpreters

In Table 8-4, the interpretation is are broken down by the degree of agreement between interpreters. "Agree" signifies that all three interpreters made the same diagnosis. "Close" means that the range of diagnoses spanned at most 2 adjacent categories; i.e. *VP and VPH*, *VPH and VPL* or *VPL and Normal*. "Remote" signifies that there were widely discrepant diagnoses spanning more than two categories, i.e. *VP and VPL* or *Normal, VPH* and *Normal*. It is seen that in only 69% of cases in which at least one interpreter diagnosed VP, was there complete agreement; in a further 13% the remaining interpreters allowed a high probability VP, whereas in 18% at least one interpreter put forward a diagnosis of low-probability or even of normality. For the categories of VPH and VPL, the agreement is poor. Inspection of those

records in which the greatest discrepancy occurred showed that these were largely those with high stool protoporphyrin values but little other evidence for VP. A smaller group showed elevations of C5 or pseudo-C5 alone.

	Highest level selected by any interpreter							
	4 (VP)		3 (High probability)		2 (Low probability)		1 (Normal)	
Agree	43	69%	4	21%	2	10%	107	100%
Close	8	13%	2	11%	19	90%	—	—
Remote	11	18%	13	68%	—	—	—	—

Table 8-4. Degrees of agreement on diagnostic category between three independent interpreters independently assigning diagnostic categories to 210 sets of porphyrin results. Close agreement implies a difference in agreement of no more than one class between highest and lowest assignments; remote implies a spread of more than one class between highest and lowest assignments.

8.5 CONCLUSIONS IN BRIEF

There is a subjective element in the diagnosis of VP among the three interpreters, resulting in a degree of variability, and there is frequently disagreement on diagnostic categorisation in borderline cases. This may result from inconsistencies in the weight placed on specific abnormalities of the porphyrin excretion profile. In the chapter which follows, an attempt is made to determine retrospectively which factors are rated most heavily in the diagnostic categorisations contend in the UCT porphyria laboratory database, following which, in Chapter 10, new biochemical criteria are sought and validated against the presence or absence of a VP-associated mutation. All results, including those in this chapter, are discussed in Chapter 14.

CHAPTER 9:

THE CHROMATOGRAPHIC DIAGNOSIS OF PORPHYRIA

The preceding chapter has shown that there is a degree of variability in the interpretation of biochemical excretion profiles, particularly in the equivocal group which overlaps both the unequivocal VP and the normal groups. In this chapter, the reasons for this are studied further.

9.1 INTRODUCTION

The standard diagnostic technique in the UCT porphyria laboratory is the chromatographic diagnosis of porphyria by TLC. Samples of urine, stool, plasma and erythrocytes are screened for the presence of porphyrins and are then subjected to chromatographic separation and quantitation.

The biochemical diagnosis of VP has traditionally been based largely on the finding of an elevated stool protoporphyrin. Our experience has shown a considerable overlap in protoporphyrin values between patients with clinically confirmed VP and patients believed on clinical and other grounds to be normal. This has led to the identification of a significant number of patients, occupying this overlap or grey area, whose diagnosis is in doubt. During the period reflected in this study, these have been further subdivided into the categories VPH and VPL. The criteria for the two intermediate categories VPH and VPL have never been formally defined, but arose from the need to make some sense of those results of an indeterminate nature at a stage when there was no easy recourse to enzyme activity assays or DNA studies to support or refute a diagnosis of VP. It would seem that VPH includes patients with either a markedly elevated protoporphyrin alone or with moderately elevated stool protoporphyrin coupled with at least some other non-diagnostic indication of abnormal porphyrin activity while patients with VPL typically have modest elevations in stool protoporphyrin alone. Thus the interpretation of porphyria biochemical profiles in our laboratory is largely intuitive and based on long experience. For the purposes of standardisation, it is always preferable to establish objective, numeric and mathematical criteria for individual diagnoses. There are two statistical techniques well-suited to the analysis predictive data which may serve this purpose: discriminant analysis and classification tree analysis. Both are applied retrospectively to the accumulated data of the porphyria laboratory database for the period 1993-1998.

9.2 OBJECTIVES

- To characterise VP biochemically.
- To identify, retrospectively, those variables most predictive of assignment of porphyrin results to a specific diagnostic class.

9.3 METHODS

Samples

All samples recorded on the UCT porphyria laboratory from January 1993 to December 1998 for which sufficient data for diagnosis were present were analysed.

Screening tests and quantitative TLC

Biochemical tests were performed as described in Appendix 1.

Diagnostic categories

Results were categorised as described in Chapter 7. In the case of suspected VP, results are classified as definite VP (VP), probable or high-probability VP (VPH) and possible or low-probability VP (VPL). All diagnoses are as recorded in the database and no attempt has been made to alter them *post hoc*.

Categorisation of continuous porphyrin data

Discriminant analysis suggested that in most instances the fact of elevation of a particular porphyrin was of greater importance than the actual measured concentration. To assess this, porphyrin values were reworked to introduce a categorical element (present/absent) for each variable, further refined by the introduction of a semi-quantitative scale for each variable, to give some indication of degree of elevation. Thus, for each variable, a ranking of 0 was applied if the variable fell within the laboratory normal range, and values of 1, 2 and 3 for progressive elevations. These were defined on an arbitrary scale, selected as to span the range of values encountered in laboratory practice, as reflected in Table 9-1. Discriminant analysis was then performed with the substitution of the categorical values for the original data.

	0	1	2	3	
Urine uroporphyrin	<=20	>20	>40	>160	>1280
Urine coproporphyrin	<=240	>240	>480	>960	>1920
Urine C7	<=1.5	>1.5	>3	>12	>96
Stool uroporphyrin	<=1.7	>1.7	>3.4	>10.2	>81.6
Stool coproporphyrin	<=50	>50	>75	>100	>200
Stool protoporphyrin	<=200	>200	>250	>300	>400
All other porphyrins which are normally undetectable by TLC	0	>0	>2	>8	>64
Urine ALA	<=45	>45	>60	>90	>120
Urine PBG	<=16	>16	>32	>64	>128

Table 9-1. Definitions for the categorisation of porphyrin variables for discriminant analysis.

Data handling

Data were entered into a Microsoft Access database and analysed with the Statistica software programme.

9.4 RESULTS

Figures 9-1 to 9-3 show the stool protoporphyrin, coproporphyrin and C5 categorised by the diagnostic classes VP, VPH, VPL and NAD. Values are not normally distributed but tend to cluster at the lower levels with a long tail extending upwards. Note that a logarithmic scale is used for protoporphyrin and coproporphyrin, and a linear scale for C5. The large box includes 90% of all values.

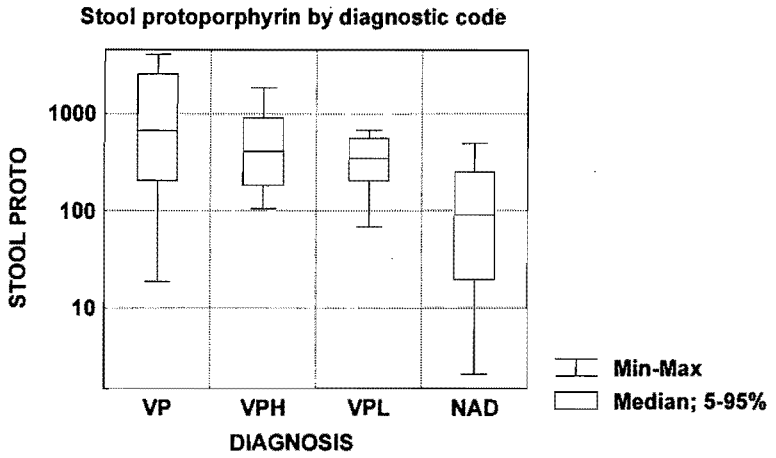


Figure 9-1. Stool protoporphyrin concentrations (nmol/g dry weight) categorised by diagnosis.

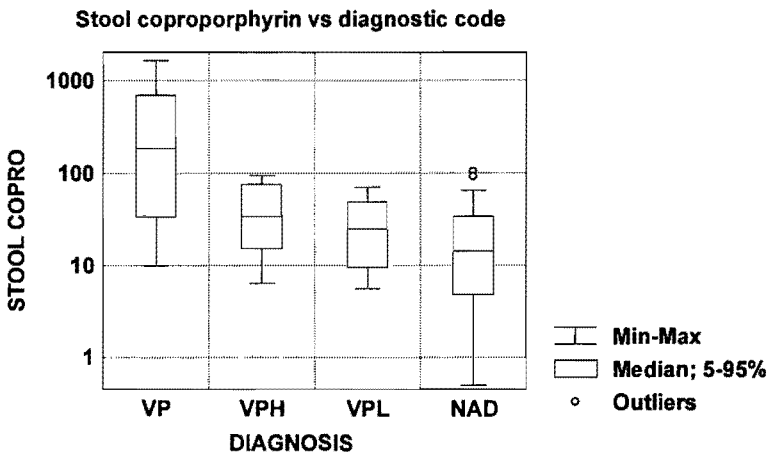


Figure 9-2. Stool coproporphyrin concentrations (nmol/g dry weight) categorised by diagnosis.

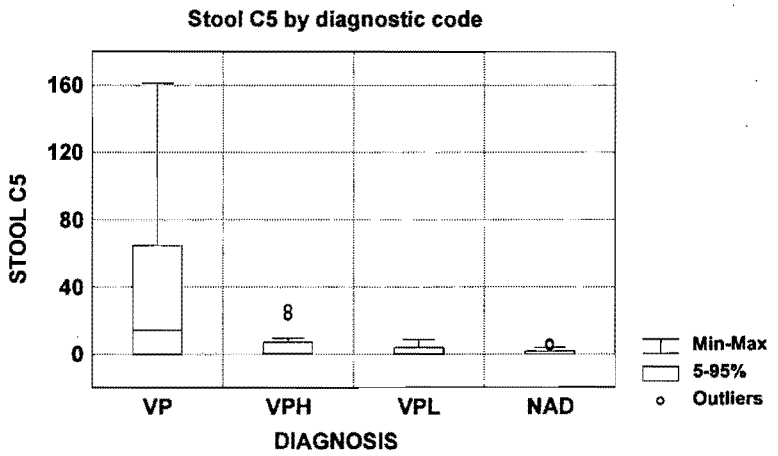


Figure 9-3. Stool C5 concentrations (nmol/g dry weight) categorised by diagnosis.

Discriminant analysis

The classification functions returned by discriminant analysis of the porphyrin data have a sensitivity for detection of VP of only 76.9% (Table 9-2). The sensitivity for PCT and AIP is better. The model fails to recognise the categories VPH and VPL most of the time, preferring to classify these as normal. However, discriminant analysis using the categorised data as defined in Table 9-1 is more discriminatory. Table 9-3 shows the relative importance of the variables, listed in order of their importance in the discrimination between diagnoses. It is seen that the categorical data are in general more discriminatory than the original numeric data, particularly in the case of the stool porphyrins, and substitution of these semi-quantitative values improves the ability of the discriminant analysis to discriminate between diagnostic classes (Table 9-4). The accuracy of a predicted diagnosis for both PCT and VP is now over 90%, and there is a major increase in the detection rate of the two indeterminate categories VPH and VPL. For the rest of this chapter, only these categorical variables are employed.

	Predicted diagnosis						
Actual diagnosis	**Sensitivity**	**NAD**	**VP**	**VPH**	**VPL**	**PCT**	**AIP**
NAD	100.0	88	0	0	0	0	0
VP	76.9	27	93	1	0	0	0
VPH	14.3	6	0	1	0	0	0
VPL	0.0	9	0	0	0	0	0
PCT	84.2	6	0	0	0	32	0
AIP	100.0	0	0	0	0	0	2
Total	81.5	136	93	2	0	32	2
PPV		64.7%	100%	25%	-	100%	50%

Table 9-2. Accuracy of classification functions applied to raw urine, stool and plasma porphyrins and urine ALA and PBG.

Variable	F
Stool isocopro (categorised)	384.8
Urine PBG (actual)	167.1
Stool copro (categorised)	115.0
Urine copro (actual)	74.9
Plasma C5 (categorised)	24.4
Stool proto(categorised)	23.6
Urine copro (categorised)	12.8
Stool pseudo C5 (categorised)	10.4
Urine uro (categorised)	8.0
Plasma proto (actual)	4.9
Plasma uro (categorised)	4.2
Urine ALA (actual)	2.6
Urine PBG (categorised)	2.6
Stool C5(categorised)	**2.6**
Stool C5 (actual)	*2.4*
Stool isocopro (actual)	1.7
Urine C7 (categorised)	1.4
Plasma PU (categorised)	1.2
Stool C6 (actual)	1.1

Table 9-3. Ranking of the variables in order of discriminatory value. (The higher the F value, the greater the contribution of the variable to discriminatory power of the model.) Those labeled "categorised" have been reworked on to an arbitrary scale reflecting elevation above normal or otherwise. It is seen that for most variables, the fact of elevation is more useful than the actual value in predicting the presence of porphyria correctly.

	Predicted diagnosis						
Actual diagnosis	**Sensitivity**	**NAD**	**VP**	**VPH**	**VPL**	**PCT**	**AIP**
NAD	**92.0**	81	0	3	4	0	0
VP	**91.7**	4	111	2	3	0	1
VPH	**28.6**	2	0	2	3	0	0
VPL	**55.6**	2	0	2	5	0	0
PCT	**97.4**	1	0	0	0	37	0
AIP	**100.0**	0	0	0	0	0	2
Total	**89.8**	90	111	9	15	37	3
PPV		**90.0%**	**100.0%**	**33.3%**	**22.2%**	**100.0%**	**66.7%**

Table 9-4. Classification accuracy of classification functions applied to categorical values of urine, stool and plasma porphyrins and urine ALA and PBG.

Ranking the variables in terms of discriminatory power

Table 9-5 shows the tabulation of porphyrin variables in the order of their contribution to the discrimination between the various forms of porphyria.

Step	Variable	Lambda	F-value
1	Stool isocopro	0.1186	384.79
2	Stool copro	0.0369	216.92
3	Urine PBG	0.0205	146.03
4	Stool proto	0.0142	110.94
5	Plasma C5	0.0098	93.69
6	Stool pseudo-C5	0.0081	79.10
7	Urine uro	0.0069	68.92
8	Plasma C6	0.0065	59.95
9	Urine copro	0.0062	53.07
10	Plasma copro	0.0058	47.94
11	Stool C3	0.0055	43.77
12	Plasma proto	0.0052	40.18
13	Plasma uro	0.0050	37.19
14	Urine C7	0.0049	34.49
15	Plasma C7	0.0048	32.18
16	Stool C5	0.0047	30.15

Table 9-5. Ranking of the categorical values in terms of their contribution to the power of the discriminatory model. It will be seen that as each variable is introduced, so the overall value of Wilk's lamda approaches 0 (perfect discrimination) more closely; the F value is a reflection of the discriminatory power of the variable.

Resolving the "grey area": discriminating between VP, AIP, PCT and normal alone.

Where the two categories of VPH and VPL are omitted from the calculations, the diagnostic accuracy of the of discriminant analysis improves further, with sensitivities of 97%, 94%, 97% and 100% for normality, VP, PCT and AIP respectively (data not shown). Clearly it is these two intermediate categories which the model has difficulty in resolving.

Resolving the "grey area": discriminating between VP, VPH, VPL VP and NAD

In an attempt to maximise discrimination between the various grades of VP, that is NAD versus VP, VPH and VPL, the analyses were repeated on those samples diagnosed as NAD, VP, VPH or VPL alone (Table 9-6).

Actual diagnosis	Predicted diagnosis				
	Sensitivity	NAD	VPL	VPH	VP
NAD	96.8%	2430	39	42	0
VPL	39.4%	43	74	71	0
VPH	29.4%	19	54	32	4
VP	86.5%	31	13	7	328
Total	89.9%	2523	180	152	332
PPV		96.3%	41.1%	21.1%	98.8%

Table 9-6. Results of discriminant analysis using urine, stool and plasma porphyrins (though not ALA or PBG) to distinguish between the grades of VP.

Whereas the predictive value for both normality and for unequivocal VP is good, the difficulty in resolving these grades of VP persists. VPH is largely identified as VPL, whereas VPL is frequently confused with VPH and NAD. Both however appear to be fairly well distinguished from unequivocal VP. The following ranking (Table 9-7) indicates which variables are most important in predicting the appropriate diagnostic class: NAD, VP, VPH or VPL. It appears that the major distinction is based on the height of the stool coproporphyrin and protoporphyrin; these make approximately equal contributions to the discrimination. By contrast, the other porphyrins including stool C5, pseudo-C5 as well as the precursors ALA and PBG are hardly weighted at all in the analysis.

	Step	Resultant Lambda	F-value	Partial Lambda	F-remove
Stool copro	1	0.2390	443.65	0.5899	95.94
Stool proto	2	0.1150	270.83	0.5411	117.02
Stool C5	3	0.1056	170.91	0.9603	5.71
Stool pseudo-C5	4	0.1040	123.81	0.9863	1.92

Table 9-7. Breakdown of discriminant analysis using urine, stool and plasma porphyrins to distinguish between the grades of VP.

Sequential investigation

Each adjacent pair of categories was then compared to assess which predictors best discriminate between the two (Table 9-8).

	Percent Correct	VP	VPH	Predictors (ranked in order of importance)
Distinguishing VP from VPH				
VP	89.7%	340	39	Stool copro
VPH	88.1%	13	96	
Total	89.3%	353	135	
Distinguishing VPH from VPL				
VPH	52.9%	46	41	Urine C7, stool copro, stool proto, stool pseudo-C5
VPL	85.8%	21	127	
Total	73.6%	67	168	
Distinguishing VPL from NAD				
VPL	79.7%	118	30	Stool proto
NAD	95.8%	62	1401	
Total	94.3%	180	1431	

Table 9-8. Distinction between the grades of VP (VP, VPH, VPL and NAD) by discriminant analysis using urine, stool and plasma porphyrins. Where a variable is not listed as a predictor, it may be assumed that it is of negligible importance.

Classification tree analysis

If one allows sufficient complexity on the tree, it can be guaranteed to produce 100% accuracy. This positive aspect is offset by two factors: firstly that the tree becomes excessively complicated, and secondly, that it will increasingly diverge from the true situation in that it will now exhaustively cover every possibility, including such perverse predictors as outliers, unusual cases or incorrectly classified samples. Thus, in analyzing the examples which follow, the intention is to identify those factors most predictive of a particular diagnosis, and to draw tentative conclusions as to how best to combine these factors to maximise diagnostic accuracy. Classification tree analysis is in the first instance an exploratory technique (though clinically useful algorithms may follow), and it is not our intention to imply that the following trees are of immediate utility in the diagnostic laboratory.

Discrimination between all hepatic porphyrias

The software programme returns the classification tree diagram depicted in Figure 9-4. The branching points represent decision points; the terminal boxes represent the terminal nodes where the eventual diagnosis is read.

With 15 decision splits and 16 terminal nodes, a high degree of accuracy is achieved as shown in Table 9-9. Indeed, the only inaccuracy is at a single node where, along with 79 normals, 1 VP and 1 VPL are trapped on the basis of the following sequence: low pseudo-5-carboxylic porphyrin, low urine uroporphyrin, low stool coproporphyrin and low stool protoporphyrin.

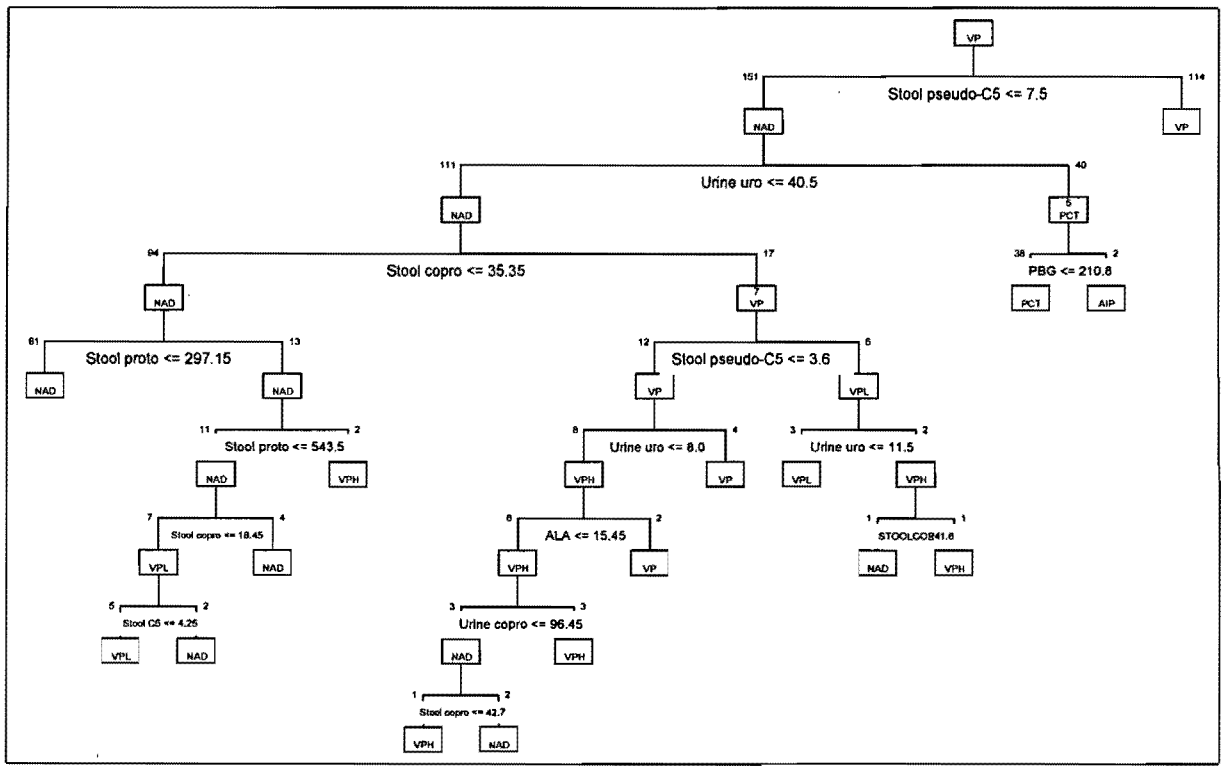


Figure 9-4. Classification tree for the diagnosis of porphyria based on porphyrin values returned by TLC.

Actual diagnosis	Predicted diagnosis						
	Sensitivity	NAD	VP	VPH	VPL	PCT	AIP
NAD	**100.0%**	88	0	0	0	0	0
VP	**99.2%**	1	120	0	0	0	0
VPH	**100.0%**	0	0	7	0	0	0
VPL	**88.9%**	1	0	0	8	0	0
PCT	**100.0%**	0	0	0	0	38	0
AIP	**100.0%**	0	0	0	0	0	2
PPV		**97.8%**	**100.0%**	**100.0%**	**100.0%**	**100.0%**	**100.0%**

Table 9-9. Accuracy of the classification tree depicted in Figure 9-4 (n=265)

How useful are plasma values?

The plasma values are relatively unimportant and are not used at all in the initial tree. Accordingly, the calculations were rerun to see the effect of omitting the plasma porphyrins (Table 9-10); this had the additional advantage of greatly increasing the number of cases available for learning.

Actual diagnosis	Predicted diagnosis						
	Sensitivity	NAD	VP	VPH	VPL	PCT	AIP
NAD	**100.0%**	226	0	0	0	0	0
VP	**97.8%**	3	225	0	0	2	0
VPH	**87.0%**	2	1	20	0	0	0
VPL	**71.0%**	7	1	0	22	1	0
PCT	**98.7%**	1	0	0	0	77	0
AIP	**77.8%**	0	2	0	0	0	7
PPV		**94.6%**	**98.3%**	**100.0%**	**100.0%**	**96.3%**	**100.0%**

Table 9-10. Accuracy of a classification tree including urine and stool porphyrins and urine precursors, but excluding plasma (n=597).

The model suffers a slight loss in discriminatory ability between VP, VPH, VPL and PCT, but overall accuracy remains high. Inspection of the variables found by the model to be most predictive of the eventual classes reveal the following ranking, ranked from 100 (most useful) to 0 (least useful): (Table 9-11).

	Ranking
Stool pseudo-C5	100
Stool proto	98
Stool C5	88
Stool copro	85
Stool isocopro	71
Stool C6	63
Urine C7	62
Stool C7	62
Urine uro	60
Urine C6	57
Urine C5	53
Stool uro	47
ALA	39
PBG	31
Urine copro	28
Stool C3	14

Table 9-11. Ranking of variables for their predictive value in the model.

How important are the urine precursors?

The following classification tree is directed at the diagnosis of VP, PCT and the grades VPH and VPL alone and performs well (Figure 9-5, Table 9-12). Addition of the Ehrlich's screen result or the direct measurement of PBG gives no additional benefit in terms of increased accuracy of diagnosis.

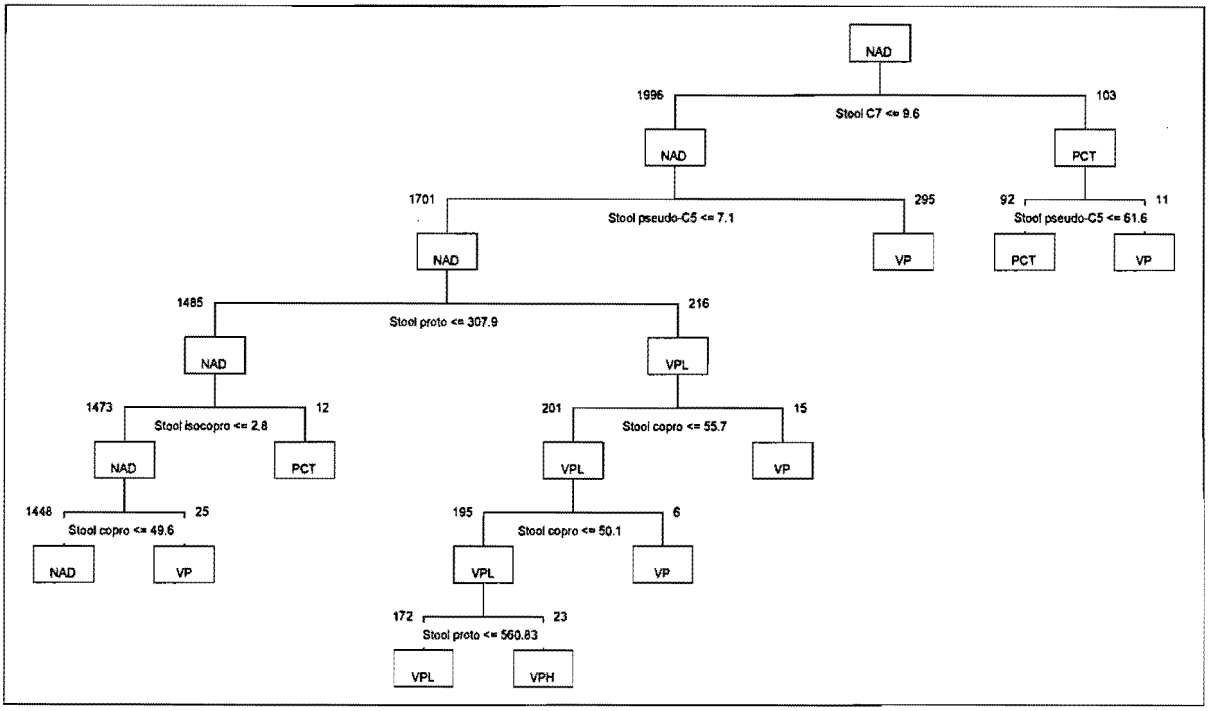


Figure 9-5. A classification tree for the diagnosis of VP, PCT and the grades VPH and VPL.

Actual diagnosis	Predicted diagnosis					
	Sensitivity	NAD	VP	VPH	VPL	PCT
NAD	**97.9%**	1414	6	2	23	0
VP	**95.0%**	5	305	4	3	4
VPH	**65.5%**	6	14	57	10	0
VPL	**54.5%**	41	8	17	79	0
PCT	**93.1%**	2	0	5	0	94
PPV		**96.3%**	**91.6%**	**67.1%**	**68.7%**	**95.9%**

Table 9-12. Accuracy of the classification tree shown in Fig 9-5, including urine and stool porphyrins, but excluding plasma and urine precursors (n=2099).

Discriminating between the grades of VP

The same approach may be used to further define the distinction between VP, VPH, VPL and NAD. In contrast to discriminant analysis, classification tree analysis produces a simple tree which functions well (Figure 9-5, Table 9-14). Experimentation shows that neither the urine precursors nor the plasma porphyrins are ranked highly in the analysis. Accordingly they are omitted from the model; this allows a larger and therefore more representative sample. The overlap between the grades of VP is again noted, as is the overlap between NAD and VPL.

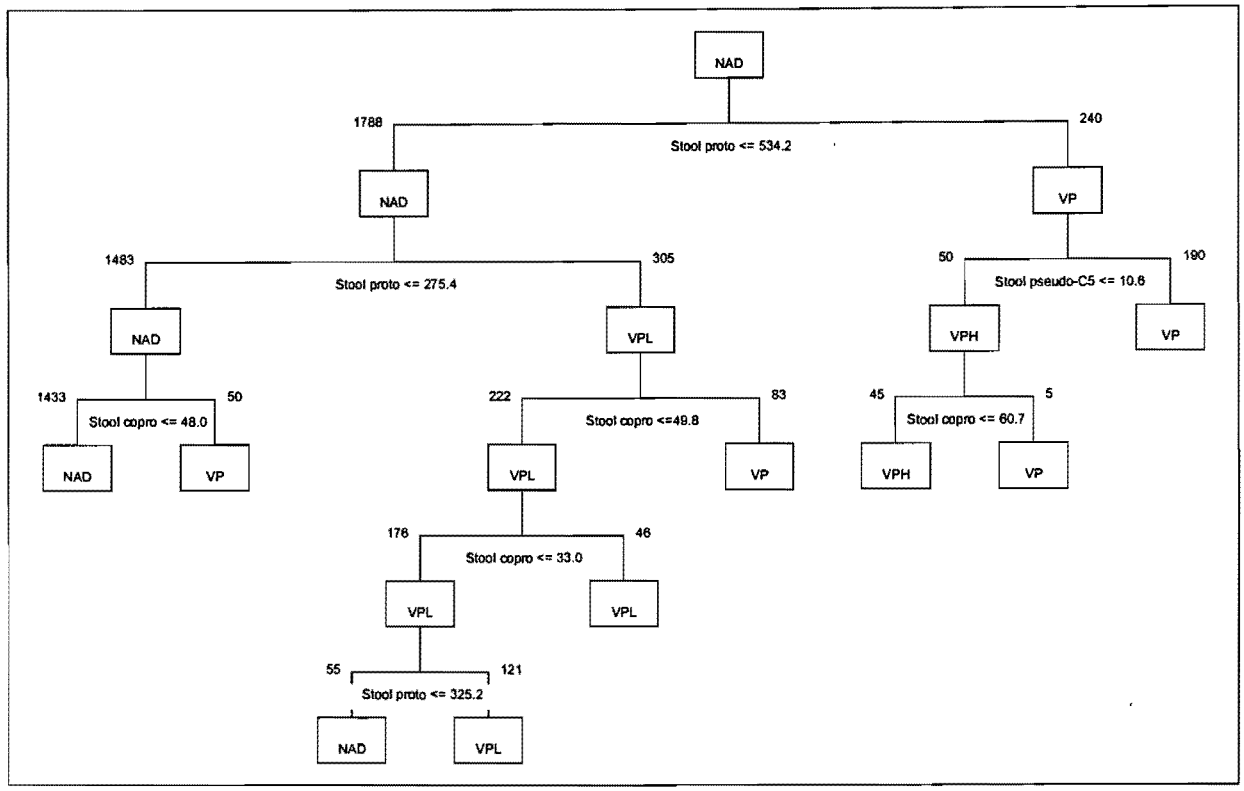


Figure 9-6. Classification tree for the diagnosis of VP.

Actual diagnosis	Predicted diagnosis				
	Sensitivity	**NAD**	**VP**	**VPH**	**VPL**
NAD	**97.4%**	1425	7	0	31
VP	**91.8%**	13	303	7	7
VPH	**29.9%**	5	12	26	44
VPL	**57.4%**	45	6	12	85
PPV		**95.8%**	**92.4%**	**57.8%**	**50.9%**

Table 9-13. Accuracy of an abbreviated classification tree including urine and stool porphyrins, (n=2028).

Predictive accuracy of the Classification Tree

Since incorrectly classified or atypical values may bias the classification tree, it is advisable to test the algorithm against a subset of samples for which the correct classification is known, but which were not used in the determination of the algorithm. In this way the true predictive accuracy of the tree can be assessed. Such a test of the classification tree follows.

The database was randomly divided into two groups; a *learning group* and a *test group* by assigning all those with an odd laboratory number to one group and with even laboratory number to the other. AIP was excluded because of the small numbers. All variables were incorporated. The classification tree was then developed based on the learning group only (n=137). This produces a tree with 100% accuracy for classes NAD, VP, VPH, VPL and PCT.

All variables, including ALA and PBG as well as plasma porphyrins, were used. The tree shown in Figure 9-7 was produced. One counter-intuitive decision point is noted: node 14 which traps 2 VP cases with low porphyrin values.

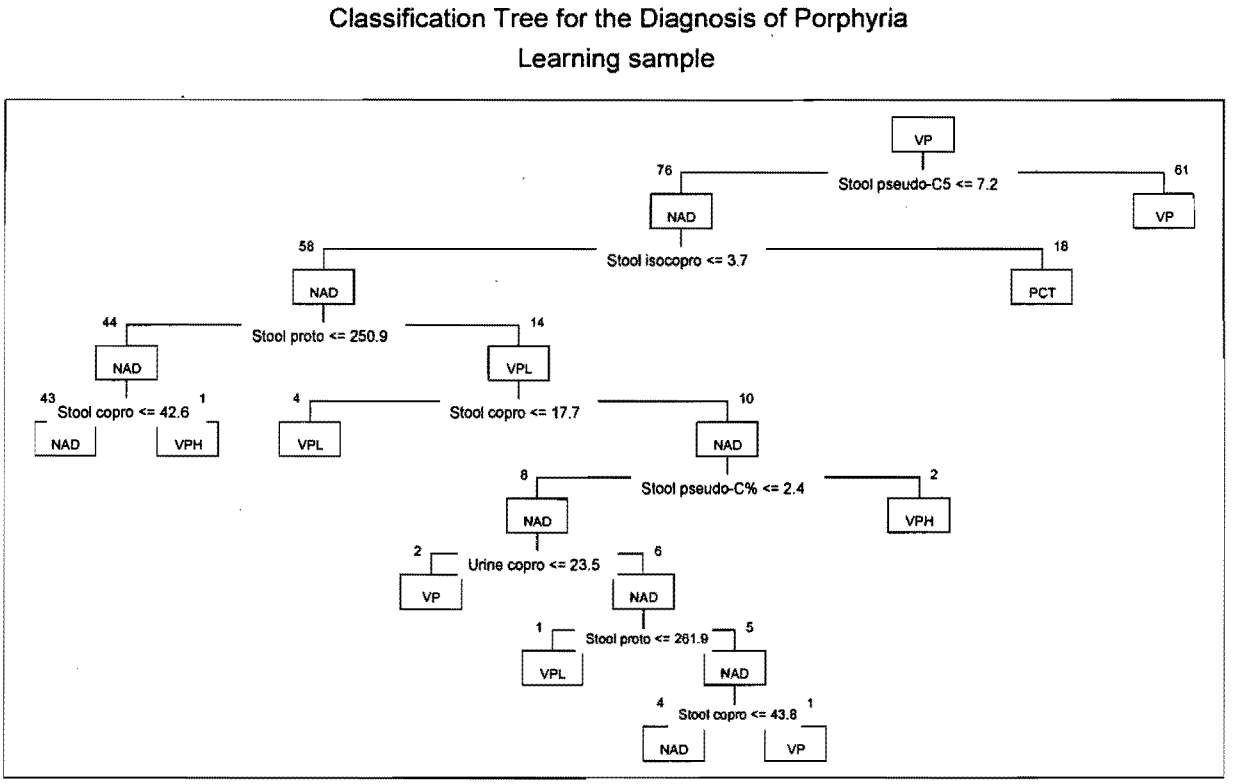


Figure 9-7. Classification tree for the diagnosis of porphyria as applied to the learning sample (n=137).

This tree was then applied to the test sample (n=139) with the results as shown in Table 9-14.

Actual diagnosis	Predicted diagnosis					
	Sensitivity	NAD	VP	VPH	VPL	PCT
NAD	**85.4%**	35	1	3	2	
VP	**98.2%**		56			1
VPH	**25.0%**	3		1		
VPL	**25.0%**			3	1	
PCT	**95.0%**	1				19
PPV		**89.7%**	**98.2%**	**14.3%**	**33.3%**	**95.0%**

Table 9-14. Performance of the classification tree shown in Figure 7 on a test population of samples (n=139).

A high degree of accuracy (considerably higher than that shown for discriminant analysis) is shown for NAD, VP and PCT, though the intermediate categories of VPH and VPL are again poorly categorised.

9.5 CONCLUSIONS IN BRIEF

This study has shown that patterns of porphyrin excretion, whether measured as categorical variables by discriminant analysis or by classification tree analysis, are more important than absolute values in the diagnosis of porphyria. Classification tree analysis yields better results than discriminant analysis, and produces algorithms with a high degree of diagnostic accuracy. The stool coproporphyrin emerges as an important predictor of VP. Even where stool pseudo-C5 and stool protoporphyrin are used to branch in the direction of VP, it is frequently the stool coproporphyrin on which the final distinction is based. It is also noted that the grades VP and VPH, and VPH, VPL and NAD are subject to a large degree of overlap, suggesting that the criteria used for the diagnosis are not absolute.

The traditional biochemical criteria for a diagnosis of VP are now, in the following chapter, tested against the presence or absence of VP as defined by the carriage of a VP-associated mutation. All results are discussed together in Chapter 14.

CHAPTER 10:

CORRELATING BIOCHEMICAL DATA WITH THE DNA-BASED DIAGNOSIS OF PORPHYRIA

10.1 INTRODUCTION

The great majority of patients with VP in South Africa carry a single mutation, the R59W mutation. This enables us, by reference to DNA analysis, to state with a high degree of accuracy whether an individual carries a VP gene or not: the only exceptions will be those subjects carrying other mutations, many of which we have in any event identified (Chapter 4). It is now possible to correlate biochemical porphyrin profiles produced by TLC with the absolute presence or absence of a gene for VP, and to apply this simply and quickly for large numbers of subjects in a way that would not be possible using the determination of PPO activity in EBV-transformed lymphocytes. We are therefore now in a position to determine accurate normal reference ranges for porphyrin excretion, and to determine which variables most reliably predict a diagnosis of VP.

10.2 OBJECTIVES

- To determine normal ranges for porphyrin and precursor concentrations in urine and stool
- To identify the most reliable biochemical predictors of VP.

10.3 METHODS

Subjects

All subjects with adequate urine and stool quantitative data whose DNA status for the R59W and 537delAT mutations were known were included in the study. These two mutations were included since data on a large number of subjects, both DNA-positive and DNA-negative, were available, and since both mutations are thought to be associated with complete loss of function of the protein coded by the mutant allele. Subjects carrying other mutations were excluded, as were an additional four subjects whose DNA status is in doubt. Two subjects showed high levels of stool coproporphyrin and protoporphyrin, but when retested one month later showed values well within the normal range. Two additional subjects showed repeated high levels of coproporphyrin and protoporphyrin and are R59W-negative. One has not been further investigated; in the second subject, SSCP/heteroduplex analysis has shown no abnormality of any exon, including the untranslated region of exon 1 and as yet no mutation has been identified.

DNA analysis

DNA samples were screened for the R59W mutation by *AvaI* restriction assay. Samples were screened for the 537delAT mutation by direct sequencing and/or *MvaI* restriction assay as described in Chapters 3 and 4 and Appendix 4.

Biochemical analysis

Urine, stool and plasma porphyrins were measured by quantitative TLC. Urine precursors were measured by ion-exchange chromatography. The methods are described in Appendix 1.

Data handling

Data were entered into a Microsoft Access database and analysed with the Statistica software programme.

10.4 RESULTS

Porphyrin values

The effect of puberty on porphyrin excretion patterns is clearly seen. In Figures 10-1 and 10-2, which use a logarithmic scale, no subject with VP aged less than 16 years shows a stool coproporphyrin or protoporphyrin higher than those shown by normal children. It is also apparent that the lowest quartile of adults carrying a VP mutation have porphyrin values which overlap those of the normal subjects, suggesting a rate of non-expression (or silent VP) of approximately 25%.

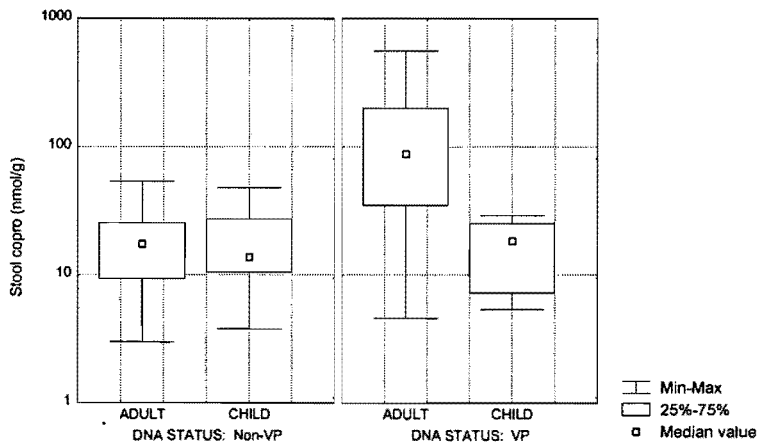


Figure 10-1. Biochemical expression of VP as reflected by stool coproporphyrin excretion. Adult is defined as age ≥16 years.

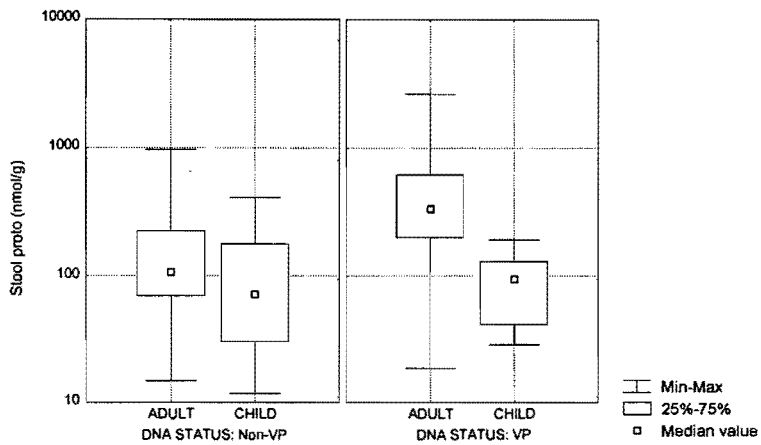


Figure 10-2. Biochemical expression of VP as reflected by stool protoporphyrin excretion. Adult is defined as age ≥16 years.

10.5 VALIDATION OF A NORMAL RANGE FOR THE LABORATORY

Using the absence of the R59W and 537delAT mutations to define normality, the following values are obtained for the mean and standard deviation (SD) (Table 10-1). Potential upper limits of the normal range (ULN) defined by the mean+2*SD, mean+3*SD levels, 95th and 98th percentiles, in addition to the ULN currently defined for the UCT porphyria laboratory, are shown. In the analyses which follow, the ULN is defined as mean+2*SD.

Statistical values					Prospective values for ULN				
					Current	Percentile		Upper limits	
	n	Median	Mean	SD		95th	98th	Mn+2SD	Mn+3SD
Urine ALA	41	9.5	12.0	7.0	45	27.4	29.6	25.9	32.9
Urine PBG	41	2.1	2.8	2.3	16	6.7	9.5	7.4	9.7
Urine uro	94	6.0	6.9	6.5	20	18.4	24.6	20.0	26.5
UrineC7	94	0.0	0.1	0.6	0	0.3	2.2	1.4	2.0
UrineC6	94	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
UrineC5	94	0.0	0.0	0.2	0	0.0	0.0	0.3	0.5
Urine copro	94	79.6	92.3	71.0	240	230.0	288.5	234.2	305.2
Stool uro	125	0.0	0.4	1.0	0	2.3	3.7	2.3	3.3
Stool C7	125	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
Stool C6	125	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
Stool ps5	125	0.0	0.7	3.0	0	3.1	10.3	6.6	9.6
Stool C5	125	0.0	0.2	0.9	0	1.9	2.4	2.0	2.8
Stool isocopro	125	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
Stool copro	125	16.7	19.0	11.1	50	39.3	44.2	41.2	52.4
Stool C3	125	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0
Stool proto	125	105.3	170.2	187.5	200	481.7	913.3	545.3	732.8

Table 10-1. Current upper limit of the normal range for porphyrin and precursor values in the Porphyria Laboratory, with potential values derived from subjects shown not to have VP by DNA screening. Units are nmol/10 mmol creatinine (urine) and nmol/g dry weight (stool).

The current reference range is comparable to the updated ULN indicated by mean+2SD with the following exceptions: the ULN for stool protoporphyrin must be substantially increased to 545.3 nmol/g dry weight; the ULN for stool coproporphyrin should be slightly reduced to 41.2 nmol/g dry weight and, the values for urine ALA and PBG should be reduced to 25.9 and 7.4 mmol/10 mmol creatinine respectively.

10.6 COMPARATIVE STATISTICS

Significant differences at the 5% level were shown between normal and VP subjects for the following porphyrins and precursors: stool protoporphyrin, stool coproporphyrin, urine uroporphyrin, urine C5, stool C5, stool pseudo-C5, urine ALA, urine coproporphyrin, urine C7, urine C6, urine PBG, plasma PU (Table 10-2).

		p-level	n	min	5th cent	median	95th cent	max
Statistically significant differences								
Stool proto	Non-VP	0.0000	125	11.8	21.4	105.3	482.4	966.7
	VP		73	18.7	42.0	332.0	2155.7	2620.1
Stool copro	Non-VP	0.0000	125	3.0	4.4	16.7	39.5	53.5
	VP		73	4.6	9.0	87.5	368.0	557.2
Urine uro	Non-VP	0.0000	60	0.0	0.0	6.0	21.0	38.0
	VP		63	0.0	0.0	14.0	260.0	1329.0
Urine C5	Non-VP	0.0000	94	0.0	0.0	0.0	0.0	1.6
	VP		63	0.0	0.0	0.0	56.4	431.5
Stool C5	Non-VP	0.0000	125	0.0	0.0	0.0	2.0	7.3
	VP		73	0.0	0.0	6.3	34.8	64.9
Stool ps5	Non-VP	0.0000	125	0.0	0.0	0.0	3.4	25.9
	VP		73	0.0	0.0	16.1	100.3	200.4
ALA	Non-VP	0.0000	41	3.2	4.2	9.5	27.4	32.4
	VP		37	6.2	7.8	19.8	72.1	331.2
Urine copro	Non-VP	0.0002	94	12.3	15.7	79.6	236.7	311.1
	VP		63	9.6	21.8	148.7	557.7	1367.6
Urine C7	Non-VP	0.0002	94	0.0	0.0	0.0	0.8	4.4
	VP		63	0.0	0.0	0.0	156.6	469.8
Urine C6	Non-VP	0.0004	94	0.0	0.0	0.0	0.0	0.0
	VP		63	0.0	0.0	0.0	17.7	381.8
PBG	Non-VP	0.0062	41	0.3	0.8	2.1	6.7	11.9
	VP		37	0.4	0.6	3.8	40.8	51.3
Plasma PU	Non-VP	0.0237	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	26.2	32.1
Differences not statistically significant								
Plasma uro	Non-VP	0.0518	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	8.3	8.3
Stool uro	Non-VP	0.0710	125	0.0	0.0	0.0	2.3	5.6
	VP		73	0.0	0.0	0.0	4.1	12.5
Plasma proto	Non-VP	0.1572	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	27.0	46.9
Stool C7	Non-VP	0.2141	125	0.0	0.0	0.0	0.0	0.0
	VP		73	0.0	0.0	0.0	0.0	2.4
Stool C6	Non-VP	0.2141	125	0.0	0.0	0.0	0.0	0.0
	VP		73	0.0	0.0	0.0	0.0	2.4
Stool isocopro	Non-VP	0.2141	125	0.0	0.0	0.0	0.0	0.0
	VP		73	0.0	0.0	0.0	0.0	11.6
Plasma C7	Non-VP	0.3291	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	8.2	8.2
Plasma C6	Non-VP	0.3291	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	26.7	26.7
Plasma C5	Non-VP	0.3291	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	12.7	12.7
Stool C3	Non-VP	1.000	125	0.0	0.0	0.0	0.0	0.0
	VP		73	0.0	0.0	0.0	0.0	0.0
Plasma copro	Non-VP	1.000	13	0.0	0.0	0.0	0.0	0.0
	VP		25	0.0	0.0	0.0	0.0	0.0

Table 10-2. Porphyrin and precursor concentrations in subjects separated by DNA analysis into normals and carriers of VP. Units are as defined earlier. VP subjects are aged 16 years and older: non-VP subjects are of any age. Min=minimum, max=maximum, cent=centile.

10.7 SIGNIFICANCE OF ELEVATED PORPHYRINS

The newly-derived ULN values for stool coproporphyrin and protoporphyrin, defined by *mean+2SD,* were then applied to those subjects with DNA-proven VP: these limits were therefore set at stool coproporphyrin ≤41.2 nmol/g dry weight and stool protoporphyrin ≤545.3 nmol/g dry weight respectively. With these limits, 22 of 73 adult subjects (30%) with DNA-proven VP fell within the normal range for stool coproporphyrin, and 54 of 73 subjects (74%) fell within the normal range for stool protoporphyrin. All porphyrin data for these 22 subjects in whom both stool coproporphyrin and protoporphyrin fell within normal levels were then individually reviewed. 19 subjects, by our subjective assessment, showed normal urine and stool porphyrin profiles, and all had been diagnosed as biochemically normal. One subject, R59W-positive, has a trace suggestive of low-level PCT, but does not meet the definition for dual porphyria, since there is no biochemical evidence for VP. The remaining two subjects had been labelled VPH on the basis of moderately raised stool protoporphyrin values alone.

10.8 CORRELATION WITH BIOCHEMICAL RESULTS: PREDICTIVE VALUES

The sensitivity of biochemical testing for the diagnosis of VP is now examined. All the following analyses are confined to VP subjects aged 16 or more: the control group includes non-VP subjects of any age, since no statistical difference can be demonstrated in the porphyrin concentrations shown in normal adults and children.

Correlation with expert interpretation

Table 10-3 illustrates the agreement between the diagnostic category assigned to a particular sample by the panel of expert interpreters (Chapter 8) and DNA status. Categories are grouped by majority opinion: if 2 of 3, or 3 of 3 interpreters agree, then their score is accepted; if none agree, then the average is taken. Seven children with DNA-proven VP aged less than 16 were encountered: all were unequivocally negative for VP biochemically and are excluded from the calculations which follow.

	Diagnosis by DNA status				
Biochemical consensus opinion	**Non-VP**	**VP**	**Totals**	**Probability of being normal**	**Probability of being VP**
Normal	97	15	112	0.87	0.13
VP: low probability	21	6	27	0.78	0.22
VP: high probability	6	3	9	0.67	0.33
VP: definite	1	47	48	0.02	0.98
Total	**125**	**71**	**196**		

Table 10-3. Probability of being DNA-positive or negative for VP classified by the consensus interpretation of biochemical prophyrin profiles.

Interpretation: Predictive values

The result is similar to that derived by reference to the new normal values. Of 71 adult subjects with DNA-positive VP, 15 (21%) will have unequivocally normal porphyrin profiles, a further 12% will have equivocal results, of which two-thirds are regarded as being of low probability. For an adult, an unambiguously normal TLC trace carries a probability of having VP of 0.13; this increases to 0.22, 0.33 and 0.98 for TLC diagnoses of VPL, VPH and definite VP respectively. Note however that these probabilities are calculated on diagnoses assessed by three independent interpreters; in view of the variation seen between interpreters, these probabilities may not hold true for individual interpreters. Furthermore, these probabilities hold for a special set of results: largely drawn from family screening studies in which the prior probability for VP will be high. The probabilities will be considerably lower in routine laboratory practice. The effects of progressively relaxing the threshold for making a diagnosis of VP on TLC results from VP to VP and VPH, and to VP, VPH and VPL are shown in the Table 10-4.

	Criterion for a positive biochemical test		
	VP	**VP/VPH**	**VP/VPH/VPL**
Sensitivity	66.2%	70.4%	78.9%
Specificity	99.2%	94.4%	77.6%
PPV	97.9%	87.7%	66.7%
NPV	83.8%	84.9%	86.6%
False Positive Rate	0.8%	5.6%	22.4%
False Negative Rate	33.8%	29.6%	21.1%
Prevalence	36.2%	36.2%	36.2%
Overall Accuracy	87.2%	85.7%	78.1%

Table 10-4. Predictive performance of biochemical profile, using successively more lenient diagnostic criteria (VP alone, VP or VPH, VP, VPH or VPL).

Is it possible to predict the mutation status of a subject with an equivocal biochemical diagnosis?

Individual values as well as the mean values for each porphyrin were then inspected for all subjects with a consensus diagnosis of VPH. Only one consistent difference was noted: the urine coproporphyrin is markedly and significantly higher in those positive for VP by DNA than for those negative (p=0.02, Student's t-test). The stool coproporphyrin also tended to be higher; by contrast, the pseudo-C5 and C5 porphyrins were indistinguishable; the stool protoporphyrin was actually lower in the VP group than in the non-VP group.

Repeating this procedure with consensus diagnosis of VPL, the following trends were noted: a higher urine ALA, stool pseudo-C5, stool C5 and stool coproporphyrin in the VP group (all significantly p=0.05 or less by Student's t-test). When the two uncertain categories are examined jointly, the informative porphyrins are stool C5 and stool coproporphyrin; perversely, the stool protoporphyrin is lower in the VP group than in the non-VP group. A slight trend to higher values is seen in the urine ALA, urine coproporphyrin and stool pseudo-C5.

Discriminant analysis

A discriminant analysis (not shown) correlating all porphyrins with sufficient variance with the results of R59W or 537delAT testing yields a sensitivity of 58.3% for DNA-positive VP, with a PPV for a function-returned diagnosis of VP of 100%. It incorporates only the stool coproporphyrin and uroporphyrin, all others, including stool protoporphyrin, are non-discriminatory.

Classification tree

A computer-generated classification tree (not shown) suggests that only the stool coproporphyrin contributes substantially to the distinction between those who are DNA-positive and those who are DNA-negative, with a cut-off value of 44.2 nmol/g dry weight.

DNA status		As predicted by the function	
	Sensitivity	Non-VP	VP
VP	67.2%	22	45
Non-VP	97.8%	87	2
PPV	97.8%	87.9%	95.7%

Table 10-5. Sensitivity and predictive value of using stool coproporphyrin alone to diagnose DNA-positive VP.

10.9 CORRECTION FOR VP EXPRESSION

Thus both discriminant analysis and classification tree analysis leave an irreducible minimum of approximately 30% of patients with DNA-positive VP who are not detectable biochemically, the same proportion who were shown earlier to have both normal coproporphyrin and protoporphyrin levels. These appear to represent those VP patients who are truly biochemically silent, and no further statistical manipulation is likely to provide useful predictors for their identification. It would appear to be more useful to divide all subjects with DNA-positive VP into two classes: *biochemically expressed* and *biochemically silent*. The problem is as always how best to define these classes. For the purpose of this analysis, we have chosen to define, and tentatively, biochemically expressed VP initially as *an elevation of any one of the following stool porphyrins above the new URL defined as mean+2SD: C5, pseudo-C5, coproporphyrin and protoporphyrin.*

Table 10-6 shows the results of applying these biochemical parameters to the subjects. All subjects with expressed VP have (by definition) elevated porphyrin markers; conversely, all subjects with silent VP have normal porphyrin markers, as do all DNA-positive children. However, and isolated elevation of any of the stool porphyrins is clearly not specific for VP since an elevated concentration of at least one of these markers was shown in 13 normal adult subjects as well, yielding a specificity of 92.2% and a positive predictive value of just 81.2% (Table 10-7). Using discriminant analysis and classification tree analysis, we therefore attempted to refine these diagnostic criteria so as to increase their specificity.

	Expressed VP	Non-VP	Child-VP	Silent VP
At least one elevated porphyrin marker	56	13	0	
Normal porphyrin markers	0	154	9	17

Table 10-6. Refining the definition of biochemically-expressed VP. Elevated porphyrin markers imply an increase above mean +2SD in at least one of the following porphyrins: pseudo-C5, C5, coproporphyrin or protoporphyrin.

Sensitivity	100.0%
Specificity	92.2%
PPV	81.2%
NPV	100.0%
False Positive Rate	7.8%
False Negative Rate	0.0%
Prevalence	25.1%
Overall Accuracy	94.2%

Table 10-7. Diagnostic utility of elevated porphyrin markers defined in Table 10-6 for the diagnosis of biochemically expressed VP.

Discriminant analysis

A discriminant analysis using all porphyrins for which sufficient variance is present results in the following classification matrix. The discriminant function is 100% sensitive in detecting normality, but still fails to recognise VP in nearly 40% of cases (Table 10-8).

DNA status		As predicted by the function	
	Sensitivity	Non-VP	Expressed VP
Non-VP	100.0%	89	0
Expressed VP	62.5%	18	30
PPV		83.2%	100%

Table 10-8. Performance of a discriminant function for the diagnosis of biochemically expressed VP using fully-quantitative porphyrin data.

If however, the raw porphyrin values are replaced by categorical values on a semi-quantitative scale has described in Chapter 9, the performance improves though the function is still somewhat insensitive in detecting biochemically expressed VP (Table 10-9). Table 10-10 indicates the relative utility of the various porphyrins in classification: it is seen that stool coproporphyrin and stool C5 between them account for almost all the discriminatory power of the model. Stool protoporphyrin is hardly predictive; stool pseudo-C5 (not shown) even less so.

DNA status		As predicted by the function	
	Sensitivity	Non-VP	Expressed VP
Non-VP	100%	89	0
Expressed VP	81.3%	9	39
PPV		91%	100%

Table 10-9. Performance of a discriminant function for the diagnosis of biochemically expressed VP using categorical (semi-quantitative) porphyrin data.

	Wilks' Lambda	Partial Lambda	F-remove	1-Tolerance (R^2)
Stool copro	0.304154	0.754241	41.05533	0.276647
Stool C5	0.251978	0.910420	12.39766	0.453518
Stool C7	0.240206	0.955038	5.93186	0.256805
Stool uro	0.234990	0.976235	3.06734	0.135127
Stool proto	0.230828	0.993836	0.78149	0.302422

Table 10-10. Relative contribution of the various porphyrins to the predictive power of the analysis.

Classification tree analysis

Classification tree analysis produces the algorithm shown in Figure 10-3. This simple algorithm uses just two variables: stool coproporphyrin and stool C5, and appears to constitute a sensitive and specific test, as summarised in Table 10-11.

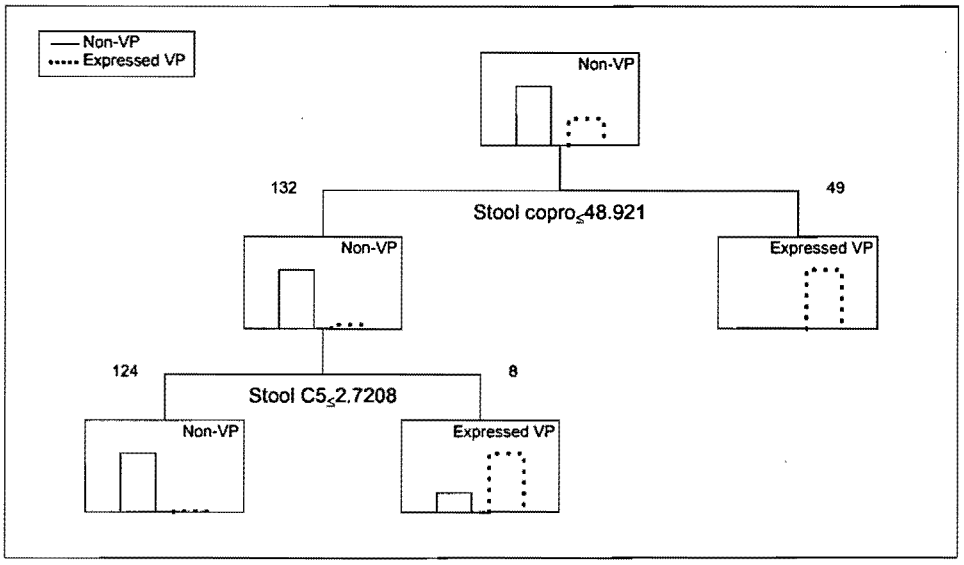


Figure 10-3. Classification tree for the recognition of biochemically expressed VP.

DNA status	Sensitivity	As predicted by the function	
		Non-VP	Expressed VP
Non-VP	98.4%	122	2
Expressed VP	94.7%	3	54
PPV		97.6%	96.4%

Table 10-11. Results returned by the classification tree shown in Figure 10-3.

The three individuals erroneously predicted by the function to have expressed VP include an 81 year-old man with a stool coproporphyrin of 53.5 nmol/gram dry weight though a low stool protoporphyrin of 173.7 nmol/g dry weight, a 51 year-old man with a very mild elevation in stool C5 and a 50 year-old man with marked elevations of stool C5 (25.9 nmol/g dry weight) and stool pseudo-C5 (7.3 nmol/g dry weight) though a normal stool coproporphyrin and protoporphyrin: Though he is R59W negative, these results are concerning and it is possible that he has one or other disturbance of porphyrin metabolism, whereas the first two subjects would appear to be real false positives.

Two subjects classified as expressed VP were misclassified as normal by the algorithm. Their stool coproporphyrin values are 46.2 and 47.3 nmol/g dry weight respectively which closely approach the cut-off value of 48.9 nmol/g dry weight. In neither were stool C5 or stool pseudo-C5 detectable; the first had a stool protoporphyrin of 482 nmol/g dry weight whereas stool protoporphyrin was low in the second. Both were only classified as expressed VP since their stool coproporphyrins exceeded the level of 41.2 nmol/g dry weight set at *Mean+2SD*: it would not be inappropriate to regard these two individuals, particularly the second, as non-expressed VP: this would improve the negative predictive value of the test to 100%.

Sensitivity	96.4%
Specificity	97.6%
PPV	94.7%
NPV	98.4%
False Positive Rate	2.4%
False Negative Rate	3.6%
Prevalence	30.9%
Overall Accuracy	97.2%

Table 10-12. Utility of the diagnostic algorithm shown in Figure 10-3.

Table 10-13 shows the relative importance of the various porphyrins as predictors of the presence of biochemically-expressed VP.

Stool C5	100
Stool copro	82
Stool pseudo-5	67
Stool proto	33
Stool C7	14
Stool uro	3

Table 10-13. Relative importance of individual porphyrins in the prediction of biochemically expressed VP.

Application of the classification tree to the porphyria database.

This algorithm was then retrospectively applied to the entire porphyria database. The following correspondence with the diagnoses recorded on the database were obtained (Table 10-14). This algorithm is highly predictive, agreeing with the diagnosis recorded in 95.0% of cases of VP and in 97.3 % of cases judged NAD. It would classify 17.6% of those recorded as VPL and 43.1% of those recorded as VPH as having VP, which accords well with the probabilities for the presence of VP of 0.22 and 0.33 calculated earlier.

Recorded diagnosis	As predicted by the algorithm	
	Non-VP	Expressed VP
NAD	2443	68
VP	19	360
VPH	62	47
VPL	155	33

Table 10-14. Comparison of the algorithm applied retrospectively with the diagnoses originally made.

10.10 CONCLUSIONS IN BRIEF

All children and at least 30% of all adults carrying a PPO mutation associated with VP exhibit normal TLC porphyrin profiles, and cannot be detected by urine and stool chromatography. The distinction between biochemically expressed and non-expressed VP is somewhat arbitrary. Analysis suggests values of 48.92 nmol/g dry weight for coproporphyrin and 2.72 nmol/g dry weight for stool C5 as critical values which are highly discriminatory between normal subjects and DNA-positive subjects in whom VP is biochemically expressed as indicated by an elevation in at least one of the terminal porphyrins excreted in stool. Stool protoporphyrin is a poor predictor of the presence or absence of VP. These results, and problems inherent in the diagnosis of VP in general, are discussed further in Chapter 14.

CHAPTER 11:

PORPHYRIN PROFILES WITHIN BILE, SMALL BOWEL AND COLON

The preceding chapters have shown that it is impossible to diagnose VP with 100% sensitivity and specificity on the basis of porphyrin excretion profiles alone. Furthermore, despite the belief that the protoporphyrin is the porphyrin most relevant to a diagnosis of VP on the grounds that protoporphyrinogen is the natural substrate for PPO, this work has shown that protoporphyrin is less predictive of VP than the stool coproporphyrin and stool C5. A small project we had undertaken to assess the fidelity of stool porphyrin profiles as a reflection of hepatic porphyrin metabolism is therefore relevant, and is discussed briefly in this chapter.

11.1 INTRODUCTION

The biliary porphyrin profile has been shown to be dissimilar to that of stool, with a preponderance of coproporphyrin (Aziz and Watson 1969, Logan et al 1991). The discrepancy between faecal and biliary porphyrins is thought to be due largely to the effects of bacterial metabolism within the bowel: other possible sources of variation include the effects of intestinal bleeding and dietary sources of haem. The contribution of these factors to the stool porphyrin profile were discussed in Chapter 6. We had previously undertaken a study to delineate the differences between biliary and faecal porphyrins and to determine at which level of the bowel the transition from the biliary pattern to the faecal pattern occurs. This is relevant to our finding that stool protoporphyrin appears to be a poor predictive of VP and this study is accordingly described here.

11.2 SUBJECTS AND METHODS

Subjects

Permission was obtained from the relatives of 7 subjects to take samples of bile and bowel content when the organs were removed for transplantation. All subjects met the medical and legal criteria for brain-death. Vital functions were being supported on a ventilator and their legally-competent relatives had given their consent for the subjects' organs to be removed for transplantation. The stool values are those of 125 subjects shown to be negative for the R59W and 537delAT mutations (Chapter 10).

Methods

During laparotomy, samples for porphyrin analysis were taken from four sites. Using a syringe, 5 ml of bile were aspirated from the gallbladder, and liquid bowel contents were aspirated from the mid-jejunum. Solid or semi-solid samples of the bowel contents were removed from the caecum and from the sigmoid colon through a small incision. Samples were esterified with 5% sulphuric acid in methanol and porphyrins were extracted into chloroform. Porphyrins were separated by thin layer chromatography and quantified by fluoroscanning as described in Appendix 1. Results for liquid samples (bile and jejunum) were expressed in nmol/l; solid and semi-solid samples (caecum and colon) as nmol/g dry weight.

Data handling

Data were entered into a Microsoft Access database and analysed with the Statistica software programme.The data were analysed for significant differences by ANOVA.

11.3 RESULTS

Absolute values are not reported since it is the comparison of the proportions of each porphyrin present in each type of sample which are meaningful. The mean porphyrin profiles for samples derived from each source are listed in Table 11-1 and are shown graphically in Figure 11-1. Sigmoid porphyrin profiles were very similar to those encountered in normal stool samples, and both consisted largely of protoporphyrin with a small contribution from coproporphyrin and little or no C3. In contrast, the bile contains a large proportion of coproporphyrin and a significant proportion of C3 with very little protoporphyrin. The change is highly significant ($p<0.00001$, ANOVA). Post-hoc comparison of means by Tukey's honest significant difference test for unequal n demonstrates a highly significant difference between the porphyrin profiles of both bile and jejunum and those of caecum, sigmoid or stool; bowel and jejunal profiles do not vary significantly, nor do caecum, sigmoid and stool profiles.

Source	N	Copro %	C3 %	Proto %
Bile	7	55.0±4.8	23.3± 3.7	20.9±5.7
Jejunum	7	52.0±5.6	20.1±3.0	27.9±6.3
Caecum*	7	22.2±6.1	7.2±2.0	70.6±7.1
Colon*	7	11.5±2.8	7.9±4.0	80.6±2.9
Normal stool*	125	0.15±0.11	0.00±0.00	0.85±0.11

Table 11-1. Porphyrin profiles (proportional) at varying sites. *Significant with respect to bile) and jejunum.

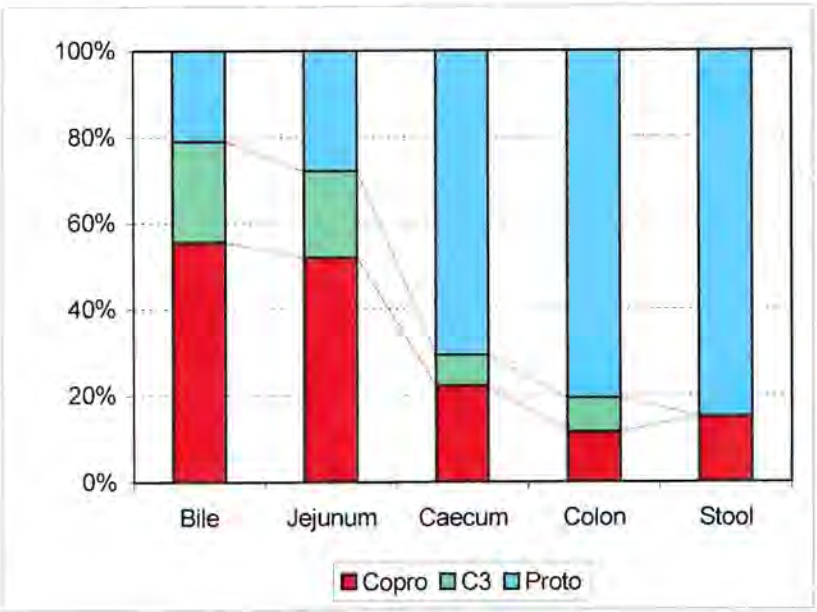


Figure 11-1. Varying porphyrin profiles with site of sampling.

11.4 CONCLUSIONS IN BRIEF

The porphyrin profile of bile and stool are highly dissimilar. The porphyrin content of bile and jejunum are essentially similar, as are the profiles of caecal and sigmoid colonic content and stool. Thus the stool porphyrin profile is at best a most indirect reflection of metabolic processes occurring within the liver. This may account in part for the poor predictive value of stool protoporphyrin in the diagnosis of VP. These results are discussed further in chapter 14.

CHAPTER 12:

SIMPLE SCREENING TESTS FOR THE DIAGNOSIS OF PORPHYRIA

In the following two chapters, two further biochemical methods for the diagnosis of VP are examined. The first is the method of simple qualitative screening for porphyrins in urine and stool; though an old (indeed the original) method of porphyrin analysis, this is still used in some South African laboratories for diagnosis. The second, to be discussed in chapter 13, is the method of plasma fluorescence scanning, which has been suggested to represent a more sensitive test for the diagnosis of VP than chromatography.

12.1 INTRODUCTION

Simple screening tests for the presence of urinary PBG, urinary porphyrins and stool porphyrins have been widely used in South Africa for the diagnosis of porphyria. These tests are largely qualitative, but are usually performed in a semi-quantitative fashion using such intervals as *negative, trace, one plus, two plus and three plus positive.* In practice, urine which screens strongly positive for PBG is considered likely to represent AIP (either in remission or in an acute attack) or VP in the acute phase; a markedly positive stool screen is regarded as strongly suggestive of VP and an isolated strongly positive urine porphyrin screen as suggesting PCT. Difficulty however arises when fluorescence is more equally spread between the urine and stool; in South Africa, such a situation is not unusual and the diagnosis may lie between PCT and VP .

Though the use of screening tests as an initial step in the diagnostic evaluation of patients with suspected porphyria is still recommended (Bonkovsky and Barnard 1998), we believe that it should always be followed up with quantitative methods. Even today, qualitative screening tests as the only test for porphyria are still performed by some private and hospital laboratories in South Africa.

In the UCT laboratory, porphyrin quantitation by TLC is always preceded by qualitative screening for two reasons. Firstly, screening results give a prompt indication of the likelihood of an abnormality, and secondly, they suggest the amount of sample to be extracted and applied to the TLC plate so as to maximise the yield while reducing the possibility of quenching. Thus we have accumulated a large body of data comprising both qualitative screening results and quantitative results on the same specimen. In the work described here, the data resulting from six years' qualitative porphyrin screening in the UCT laboratory are compared with the quantitative and diagnostic results subsequently obtained by quantitative TLC on the same specimens.

12.2 OBJECTIVES

- To determine the correlation between results of screening tests and actual porphyrin values as measured by quantitative methods
- To determine the predictive value of screening tests in the diagnosis of porphyria.

Correlation of stool screening results with stool porphyrin concentrations

There is a strong correlation between the stool screening results and total porphyrin concentration as measured by quantitative techniques. The relationship is again not linear (r^2 =0.39), but logarithmic (r^2=0.51) (Figure 12-3). Figure 12-4 shows a large degree of overlap of porphyrin concentrations at all values of the stool screening result. The median values, interquartile distance and range attached to the various classes of stool screening are shown in Table 12-2.

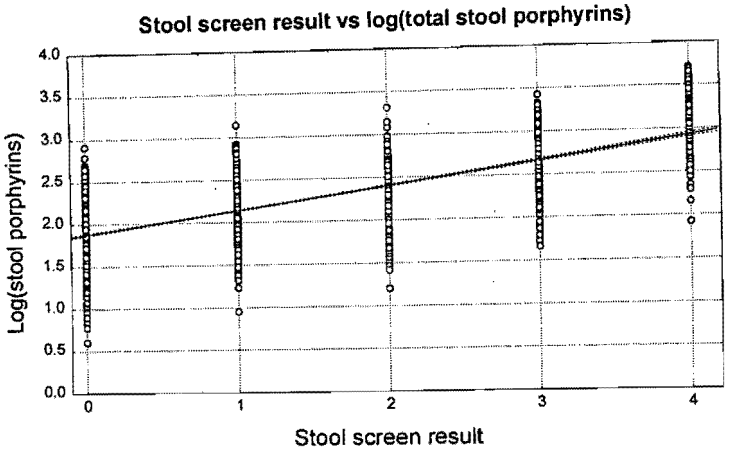


Figure 12-3. Correlation of observed stool screening results with subsequent estimation of total stool porphyrin (nmol/g dry weight) by quantitative TLC.

Screen Result	n	Median	Inter-quartile range	Range
Negative	1535	80	50-132	4-820
1 (Trace)	671	143	95-218	9-1392
2 (+)	688	217	139-341	16-2050
3 (++)	327	411	276-629	46-2662
4 (+++)	268	1221	802-1980	83-5078

Table 12-2. Stool screening. Quantitative porphyrin concentration compared with screening category.

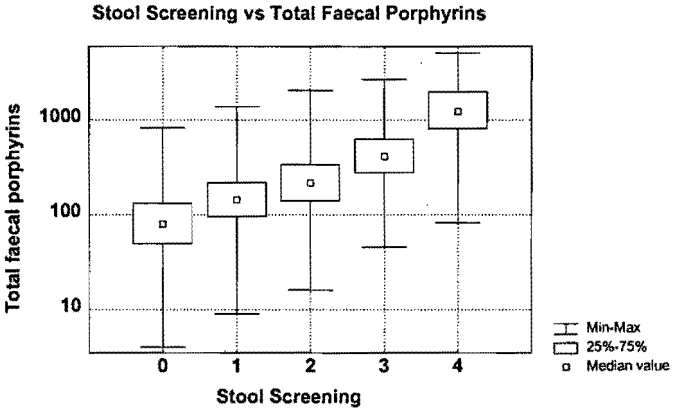


Figure 12-4. Total stool porphyrin concentrations (nmol/g dry weight) associated with each interval of the stool screen.

Correlation of urine PBG screening results with urine PBG concentrations

This is shown in Figure 12-5. In this case, the relation is linear rather than logarithmic. Again there is a fair correlation (r^2 =0.55), however a large degree of overlap exists for each screening interval.

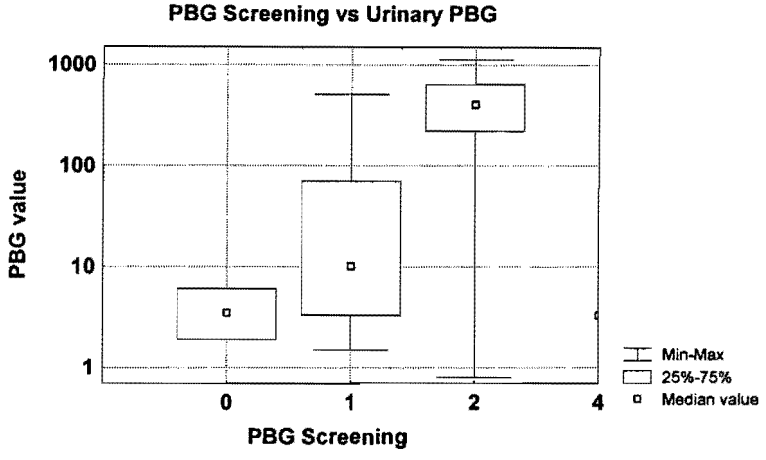


Figure 12-5. PBG concentrations (μmol/10 mmol creatinine) associated with each interval of the PBG screen.

Predictive accuracy of screening results

Table 12-3 shows the performance of stool screening as a diagnostic test for the presence of VP. The effect of changes in performance as a result of altering two variables are shown: (1) establishing the diagnostic threshold for VP at decreasing degrees of positivity of the stool screening test result, and (2) broadening the TLC criteria for a diagnosis of VP to include not only definite VP, but also probable VP (VPH), as defined in Chapter 7.

	Diagnostic class					
	VP only			VP and VPH		
Screening result defined as positive:	2-4	3-4	4 only	2-4	3-4	4 only
Sensitivity	95.6%	80.9%	54.6%	92.5%	73.3%	45.3%
Specificity	73.3%	91.6%	98.2%	75.1%	92.9%	98.6%
PPV	28.5%	51.8%	77.4%	35.7%	60.7%	83.0%
NPV	99.3%	97.7%	95.1%	98.5%	95.9%	92.3%
False Positive Rate	26.7%	8.4%	1.8%	24.9%	7.1%	1.4%
False Negative Rate	4.4%	19.1%	45.4%	7.5%	26.7%	54.7%
Prevalence	10.1%	10.1%	10.1%	13.0%	13.0%	13.0%
Overall Accuracy	75.5%	90.5%	93.8%	77.4%	90.4%	91.7%

Table 12-3. Performance of stool screening as a diagnostic test for VP.

Use of urine screen alone for the diagnosis of VP

Urine screening tests alone are entirely worthless in the recognition of VP. A strongly positive urine screening result (grade 4) has a sensitivity of 16.4% and a positive predictive value of only 30.9% for definite VP, interestingly however, it has a specificity of 96.2%; an indication of the high prevalence of VP in this sample.

Discriminant analysis

Discriminant analysis returns the following classification when applied to these data (Table 12-4).

As recorded on database	As predicted by the function				
	Sensitivity	Normal	VP	AIP	PCT
Normal	94%	3133	173	0	30
VP	74%	71	224	0	7
AIP	26%	12	9	8	2
PCT	44%	48	49	3	78
PPV		96.0%	49.2%	72.7%	66.7%

Table 12-4. Predictive accuracy of a discriminant function using observed screening values for the diagnosis of porphyria.

Classification tree analysis

Analysis returns the classification tree shown in Figure 12-6. Diagnoses of AIP are excluded from the analysis; since relatively few samples are included in the database, they result in a more complicated tree without materially altering the diagnostic accuracy of the tree for VP and PCT. The diagnostic matrix returned by the tree is shown in Table 12-5.

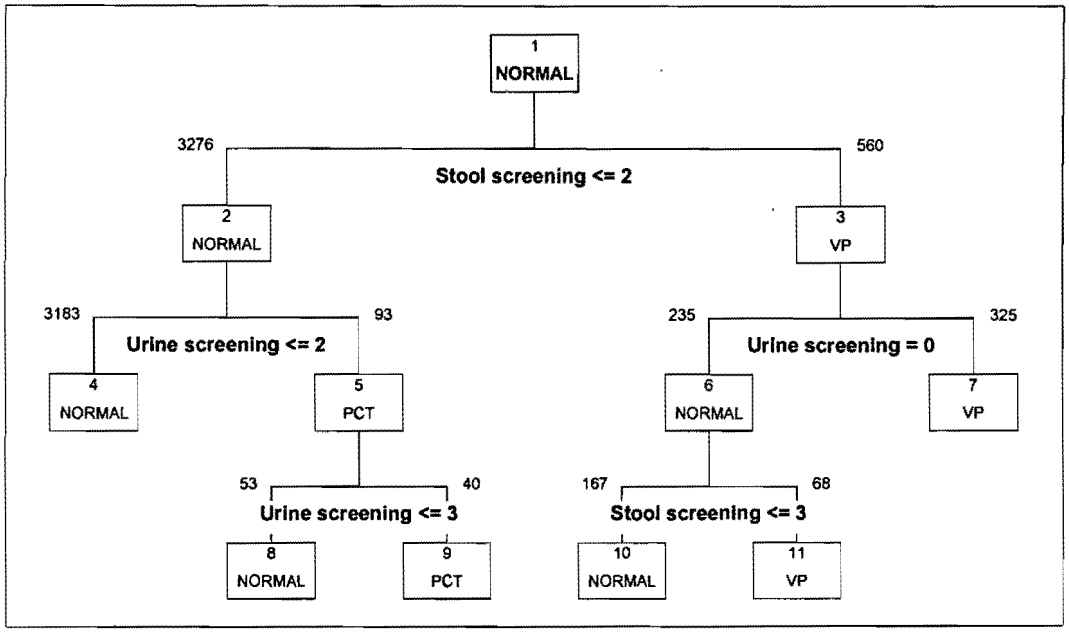


Figure 12-6. Classification tree for the diagnosis of VP and PCT based on screening results.

As recorded on database	As predicted by the function			
	Sensitivity	**Normal**	**VP**	**PCT**
Normal	97.30%	3175	81	8
VP	60%	180	273	2
PCT	41%	48	39	30
PPV		93.3%	69.5%	75.0%

Table 12-5. Predictive accuracy of the classification tree using observed screening values for the diagnosis of porphyria.

Correlation of screening with TLC result

How good is the correlation between a screening result and the eventual diagnostic classification? For the following statistical tests, the TLC and screening scores were adjusted so as to fall into three categories: *unequivocally normal*, *equivocal* and *unequivocally VP*. Cross-correlations for each of the three interpreters tested in Chapter 8 are shown in Table 12-6. The correlations for each interpreter were then assessed; the Kendall tau values were 0.46, 0.74 and 0.74 respectively, the gamma coefficients were 0.86, 0.96 and 0.93. This suggests that, in experienced hands, screenings are quite predictive of the final outcome, though the extent will vary between individuals.

As diagnosed on screening results alone	As diagnosed on TLC results		
	NAD	**Equivocal**	**VP**
Interpreter A			
NAD	**89.4%**	7.5%	3.0%
Equivocal	16.1%	**39.2%**	44.6%
VP	4.8%	9.5%	**85.7%**
Interpreter B			
NAD	**86.8%**	11.1%	1.9%
Equivocal	40.3%	**26.1%**	33.6%
VP	0.0%	0.0%	**100.0%**
Interpreter C			
NAD	**85.1%**	9.3%	5.6%
Equivocal	3.8%	**30.7%**	65.4%
VP	0.0%	0.0%	**91.3%**

Table 12-6. Correlation of an interpreter's diagnosis based on screening tests only with their eventual diagnosis based on quantitative porphyrin data. The figures indicating concordance are highlighted.

12.5 CONCLUSIONS IN BRIEF

Though screening results correlate well with final TLC results, there is a wide spread within each class which makes prediction of actual porphyrin content inaccurate. In experienced hands however, screening results are fairly predictive of the eventual diagnosis returned by chromatography. These results are discussed further in Chapter 14.

CHAPTER 13:

PLASMA SCANNING AS A DIAGNOSTIC TOOL IN VP

13.1 INTRODUCTION

Long et al (1993) have suggested that plasma scanning is a more sensitive test for VP than faecal porphyrin analysis. A study was therefore undertaken to assess the sensitivity and specificity of this test in comparison with the technique of chromatographic detection of porphyrins in urine, stool and plasma; both were compared with DNA status as the ultimate arbiter of VP carriage.

13.2 OBJECTIVES

- To determine the utility of plasma fluoroscanning as a method for the diagnosis of VP in the South African context.

13.3 METHODS

Subjects

Samples were largely drawn from family studies in two extended families shown to carry the R59W and 537delAT mutations. Some additional subjects in whom both biochemical analysis and DNA analysis had been performed were included.

Methods

The R59W and 537 delAT mutations were recognised by restriction assay and/or direct sequencing as described in Appendix 4. Biochemical porphyrin quantitation was by quantitative TLC. Plasma was diluted 1:10 with phosphate-buffered saline and the emission spectrum was scanned from 580 nm to 650 nm with the excitation monochromator set at 405 nm. Peak heights quoted in this chapter are those measured at a standard sensitivity and are therefore comparable. All methods are fully described in Appendix 1.

Data handling

Data were entered into a Microsoft Access database and analysed with the Statistica software programme.

13.4 RESULTS

Correlation with DNA status

In 86 subjects both a DNA result and a plasma scanning result were available. Of these 65 were aged 16 or more; 21 were children. A typical plasma scan is shown in Figure 13-1, and the results are summarised in Table 13-1.

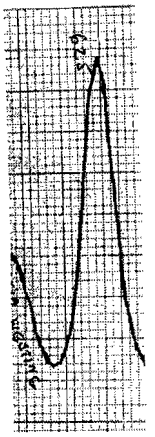


Figure 13-1. Typical plasma fluorescence scan in VP. The emission spectrum has been scanned from 580 nm to 650 nm with a constant excitation wavelength of 405 nm. A fluorescence peak is shown with a maximum at 625 nm.

DNA status	Peak at 625 nm absent	Peak at 625 nm present	Other peak present	Total
Mutation negative	38	2	4	44
Mutation positive	5	37	0	42

Table 13-1. Summary of results: correlation of presence of peak at 625 nm with presence of a VP-associated mutation.

Four subjects showed plasma fluorescent peaks at other wavelengths: three at 619 nm suggesting PCT, 1 at 630 nm suggesting EPP. These diagnoses were confirmed by TLC in these subjects.

False negatives

Of 42 subjects with mutation-proven VP, 37 were positive by plasma scanning. Of the 5 subjects negative by plasma scanning, 3 were children aged 6, 11 and 12; the remaining two subjects were aged 23 and 55. Thus, only two false-negative results were encountered in adults. The first patient, aged 55, was positive for the R59W mutation. His urine precursors, urine porphyrins and stool porphyrins were low, with a stool coproporphyrin of 20.9 and a stool protoporphyrin of 267 nmol/g dry weight. The second, aged 23, was positive for the 537 delAT mutation. No stool data were available.

False positives

Two DNA-negative subjects showed peaks at 625 nm. One patient, aged 70, demonstrated a peak of 4 mm but is clearly free of VP since she belongs to the family carrying the 537delAT mutation and was shown to be negative. Her stool protoporphyrin was somewhat high at 482 nmol/g; however coproporphyrin and all other porphyrins were low. In all subjects with DNA-proven VP who demonstrated a peak, the height of the peak was at least 5 mm. Long et al (1993) set their threshold for positivity at a 3 mm deflection: it would appear appropriate for us to set our limit at 5 mm. The second subject, aged 19, had been tested biochemically at age 16, apparently in view of a family history of porphyria though further information is lacking: she was found to be negative. She was not tested further on this occasion in view of

the negative R59W result. Though VP is unlikely, the possibility of a non-R59W mutation is however not excluded.

Applying the criteria that the test is only indicated in adults aged more than 16, and that a peak must exceed 5 mm or more in height to be significant, the results of the test may be summarised as follows (Table 13-2).

	Plasma scan	
DNA status	**Positive**	**Negative**
DNA-negative	1	28
DNA-positive	30	2

Table 13-2. Results returned by fluoroscanning in a sample of patients in whom mutation status is known: restricted to adults and a positive scan defined as a peak height exceeding 4 mm.

The diagnostic utility of this test in the identification of VP in adults may be calculated as follows (Table 13-3), and is contrasted with the results of TLC assessment in the same group of patients: strict criteria for the diagnosis of VP (unequivocal VP) had been applied. Similar results are obtained where the biochemical diagnosis is based on an elevated stool coproporphyrin and C5 as described in Chapter 10.

	Scan	TLC
Sensitivity	93.8%	66.7%
Specificity	96.6%	100.0%
PPV	96.8%	100.0%
NPV	93.3%	70.4%
False Positive Rate	3.4%	0.0%
False Negative Rate	6.3%	33.3%
Prevalence	52.5%	55.8%
Overall Accuracy	95.1%	81.4%

Table 13-3. Utility of plasma fluorescence scanning in comparison with chromatography as a diagnostic test for VP.

The test is therefore highly sensitive and specific, with excellent predictive value in this population. It is considerably more sensitive than TLC diagnosis which, though specific, is of poor sensitivity.

Correlation with biochemistry

A quantitative measure was applied to the height of the peak by measuring its displacement in mm at the standard settings of the fluorescence detector. Though these values are arbitrary, they will correlate with intensity of the plasma fluorescence. Some features of the peak at 625 nm are shown in Table 13-4.

	DNA status	
	Mutation positive	Mutation negative
N	32	33
Mean	95.0	1.2
Minimum	0	0
Maximum	467	23
SD	121.0	4.5

Table 13-4. Height (mm) of the peak at 625 nm.

Correlation with individual porphyrin concentrations in urine, stool and plasma.

The height of the peak at 625 nm correlates positively with stool pseudo-C5 (r=0.68), stool C5 (r=0.76), stool coproporphyrin (r=0.87), stool protoporphyrin (r=0.82), plasma PU (r=0.96) and plasma protoporphyrin (r=0.6); these correlations are significant at the 5% level. Multiple regression analysis indicates that the height of the peak correlates most strongly with the stool coproporphyrin. The relationship between the height of the fluorescence peak and the stool C5, coproporphyrin and protoporphyrin concentration is shown graphically in Figures 13-2, 13-3 and 13-4 .

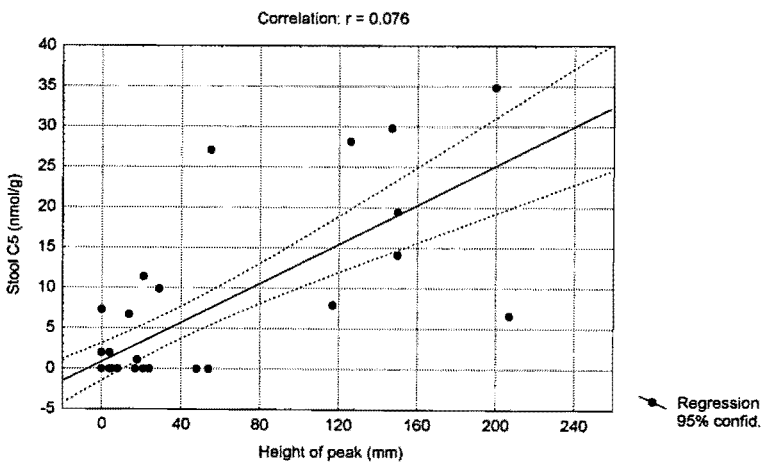


Figure 13-2. Correlation between height of peak at 625 nm and stool C5.

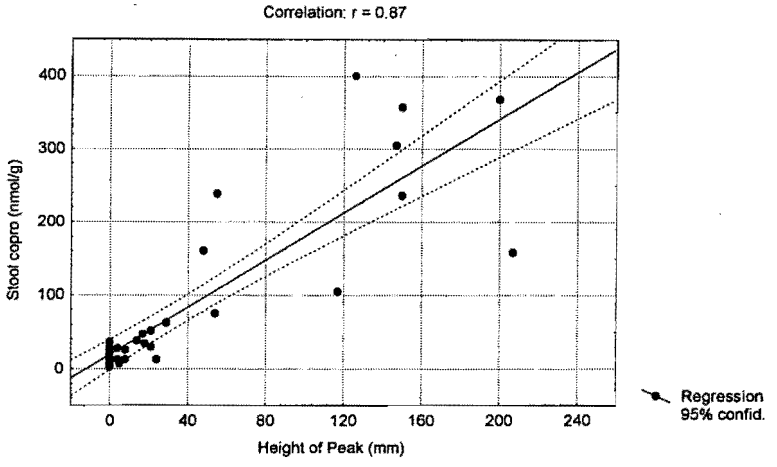


Figure 13-3. Correlation between height of peak at 625 nm and stool coproporphyrin.

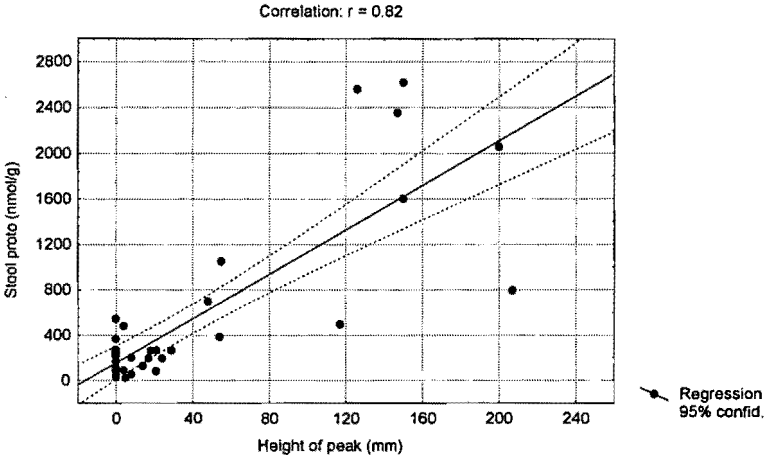


Figure 13-4. Correlation between height of peak at 625 nm and stool protoporphyrin.

Sensitivity of plasma scanning in comparison with TLC

The results of plasma fluoroscanning were then compared with the chromatographic diagnosis originally assigned to the specimens; these had been assigned without reference to plasma fluoroscanning results. Not all of these have had their DNA status checked. Of those with a negative peak (Table 13-5), 13 could not be assigned owing to the inadequacy of the specimens received (e.g. insufficient stool received), and 12 were assigned to categories of uncertain diagnostic significance: probably normal or VPL: only one of these 8 samples was positive by fluoroscanning. Since we have determined a false negative rate of approximately 6% (Table 13-3), no more than 1 of these 12 specimens is likely to represent VP. Using plasma fluoroscanning therefore, these samples could with a high degree of confidence be reported as non-VP. Conversely, 9 of 116 samples which were normal biochemically had a positive scan result. In view of the false-positive rate of 3.4% shown for plasma fluoroscanning (Table 13-3), it is likely that at least 8 of these samples are indeed from

patients with VP. Thus plasma fluoroscanning would detect a further 8-9 subjects with VP over and above the 32 with biochemical evidence of VP.

Chromatographic diagnosis	Peak at 625 nm absent	Peak 625 nm present
Normal	107	9
Probably normal	1	0
Inadequate specimen	13	0
VP	1	32
VPL	11	1
Unclassified abnormal	0	1

Table 13-5. Assigned biochemical diagnoses in adult subjects tested by plasma fluoroscanning.

13.5 CONCLUSIONS IN BRIEF

Plasma fluoroscanning is a sensitive test for the detection of VP. In this study, it was 100% sensitive for patients with biochemically-expressed VP and over 90% sensitive for all subjects carrying the R59W and 537delAT mutations: the sensitivity of TLC by contrast is approximately 67%. (We have however subsequently identified 2 patients, not included in this study, in whom biochemically-expressed VP was associated with a negative plasma scan: one subject carrying the Y348C mutation, the other the R59W mutation.) Plasma scanning appears to be a most useful test as a first-line investigation in the recognition of VP and will be associated with fewer erroneously classified or equivocal cases than stool porphyrin quantitation. It is also suited to screening studies in the family of affected patients, though occasional cases will be missed; it is of no value in children. The height of the fluorescence peak correlates well with the stool porphyrin concentration, and most strongly with the stool coproporphyrin concentration. These results are discussed further in Chapter 14, and additional experience with plasma fluoroscanning in a single kindred with the R59W mutation is described in Chapter 17.

CHAPTER 14:

DISCUSSION: THE BIOCHEMICAL FEATURES OF VARIEGATE PORPHYRIA

This sequence of studies illustrates the relationship between the presence of an inherited mutation in PPO and biochemical expression of VP. We have shown that the biochemical criteria for a diagnosis of VP are imprecise: though many subjects have profiles which appear unequivocally normal or unequivocally diagnostic of VP, there remains a group with minor changes in porphyrin concentrations—particularly elevated protoporphyrin—whose significance was unknown. Furthermore we have shown that three experienced interpreters may place different weights on abnormalities of porphyrin excretion. We have attempted to define more predictive biochemical criteria for diagnosis of VP, by analysing urine and stool porphyrin data in the light of a knowledge of the DNA status of the subject. Finally we have assessed two further diagnostic methods: screening analyses, which are shown to be at best moderately predictive, and plasma fluorescence scanning, which appears to be an important addition to the diagnostic armamentarium. These individual studies are now discussed.

14.1 VARIABILITY IN DIAGNOSIS

The initial study (Chapter 8), in which the predictions of three experienced interpreters were compared, highlights the diagnostic problems inherent in the biochemical diagnosis of VP. The variability shown in diagnosis does not in any way invalidate the diagnostic work of our laboratory over many years. Firstly, the samples we studied constitute a small subset of all the profiles produced by our laboratory and were deliberately selected to include a range of diagnoses, amongst which the equivocal group are over-represented: between them, the two equivocal categories constitute just 5% of the positive diagnoses on our database. Secondly, the data were presented to the interpreters without any accompanying clinical data whereas a knowledge of the clinical circumstances is often helpful in assessing the prior probability of a positive result. Thus an equivocal result in a young person with a strong family history of VP is very much more likely to represent a true positive result than a similar result in an older person with no family history and atypical symptoms. Thirdly, when an equivocal diagnosis is made, the patient is always advised to submit further specimens which may then help to confirm or reject a diagnosis of VP.

Our analysis of the diagnoses assigned to a specimen set of porphyrin results by three experienced interpreters suggested a significant degree of individual variation. One interpreter predicted the presence of VP more readily than the other two on screening tests, and a Kendall's coefficient of concordance of 0.36 suggests that agreement on individual results may be poor. Similarly, the Kendall's coefficient of concordance of 0.13 for the results of TLC diagnosis Indicates poor overall agreement among interpreters. Interestingly, as shown in Table 8-3, the agreement between pairs of interpreters is close while the overall agreement is poor; suggesting that whereas two interpreters may agree, agreement between all three is less frequent. Thus complete agreement on a diagnosis of VP was only seen in 69% of cases. In 18%, at least one interpreter suggested a diagnosis of low-probability VP or even interpreted the profile as normal. When the original records were reviewed, it appeared that the greatest discrepancy occurred as a result of one interpreter having placed more emphasis on a raised stool protoporphyrin as a marker of VP than the others: a further source of

disagreement was the weight placed on elevations of C5 or pseudo-C5 in the absence of other markers of VP (Appendix 8).

These results suggest that the three interpreters are not using uniform criteria for the diagnosis of VP. The two intermediate categories of VPH and VPL in particular are vague in their definitions. No explicit biochemical criteria for the diagnosis of VP, nor for assignment to the equivocal categories outlined in Chapter 7 exist, largely because there could be no certainty about diagnosis in the absence of absolute proof of the presence or absence of VP. Until recently, such proof could only be determined on the basis of lymphoblast PPO activities. The number of samples in which PPO activity would have to be assayed to allow for correlations between biochemical findings and PPO status is too large to be feasible. The literature offers no help in this regard. The biochemical criteria put forward by Day (1978), Eales et al (1980) and others serve usefully to define that group of patients with unequivocal biochemically-expressed VP but offer little assistance with the interpretation of the more difficult borderline TLC profile. They may be regarded as caricatures: when dealing with a sample set of several thousand cases, exceptions are to be expected. Hence the biochemical criteria for a diagnosis of VP have always been somewhat subjective, relying largely on abnormalities of stool protoporphyrin in particular, as well as on abnormalities in stool coproporphyrin, stool pseudo-C5, urinary porphyrins and plasma PU.

14.2 DIAGNOSTIC CATEGORISATION ON THE BASIS OF TLC PORPHYRIN PROFILES

The subsequent study (Chapter 9) defined the porphyrin excretion profiles observed in our subjects and attempted retrospectively to determine which patterns most reliably predicted the diagnoses which had been assigned to those subjects in the course of our routine laboratory work. Figures 9-1 to 9-3 show that there is a continuous range of stool coproporphyrin and protoporphyrin values, with no obvious distinction between patients with VP and those who are normal. This led us to introduce the categories of low-probability and high-probability VP (VPL and VPH) to accommodate specimens yielding intermediate values. Though stool porphyrins are on average higher in those with a diagnosis of VPH, there is significant overlap with both the VPL and the unequivocal VP categories, as well as some overlap with the normal category. These results suggest that it will be difficult to distinguish these categories on absolute values of porphyrin alone; if they are to be resolved, it may be on the basis of the pattern of accumulation of porphyrins as suggested by Moore and Disler (1985).

Statistical analysis

To determine which porphyrins contribute most to our diagnostic categorisation, we undertook a discriminant analysis, a form of multifactorial analysis which, in addition to determining which variables contribute most strongly to an observed outcome, has the ability to predict membership of a diagnostic class on the basis of those variables. Discriminant analysis suggests that normality is relatively easy to diagnose, but that mathematical models have difficulty in predicting VP as a diagnosis and largely fail with VPH and VPL. Interestingly, discriminant analysis became significantly more predictive when variables were categorised into present/absent with an element of semi-quantitative scoring. This supports the contention that it is not so much the absolute values of porphyrins, but rather the pattern of their accumulation, which is of greatest diagnostic significance. In differentiating all forms of porphyria, the stool isocoproporphyrin and urine PBG (Table 9-5) appear most discriminatory. This will be due to their utility in recognising PCT and AIP respectively. (Had more patients with VP been identified during the acute attack in this series, the urine PBG

would presumably have been less useful.) The most important finding however, one which recurs repeatedly is the diagnostic utility of the stool coproporphyrin as a reliable predictor of VP.

The equivocal groups, VPH and VPL, greatly complicate the biochemical diagnosis of porphyria. If these categories are omitted, discriminant analysis produces a sensitivity exceeding 90% for all forms of porphyria. We then investigated the range of "VP like profiles" obtained in our patients, by contrasting adjacent categories: Table 9-6 reaffirms the difficulty in reliably separating these categories when the categories VP and VPH, VPH and VPL, and VPL and NAD are contrasted. This clearly has implications for the biochemical diagnosis of porphyria both at the upper end (likely to be VP) and at the lower end (likely to be normal) of the spectrum. Breakdown of the analysis (Table 9-7) indicates that the major discriminators are the stool coproporphyrin and stool protoporphyrin. Stool C5 and pseudo-C5 are of poor discriminatory ability, at least in discriminant analysis. In comparing contrasting pairs of diagnoses however (Table 9-8) an interesting pattern emerges. It would appear that the distinction of VP from the VPH is largely on the basis of stool coproporphyrin, and this distinction is fairly sensitive. The distinction of VPH from VPL is however less sensitive: it appears to pick out the presence of urinary porphyrins and stool pseudo-C5 in addition to the stool coproporphyrin and protoporphyrin. The major distinction between normality and VPL is on the basis of the stool protoporphyrin, suggesting that the VPL category largely contains those results in which the only abnormality is an elevation of stool protoporphyrin.

Classification tree analysis has advantages over discriminant analysis. Firstly, it is intrinsically more suited to categorical predictors than discriminant analysis; and the importance of categorization has been shown above. Secondly, it lends itself to the production of algorithms which, unlike discriminant analysis, may be tested against common sense, and may also be used for the development of diagnostically useful clinical algorithms. Classification trees function well with the original quantitative porphyrin data since in effect they categorise the data themselves by splitting sets of samples into groups which fall above or below critical values. The classification tree shown in Figure 9-4 is highly predictive, with an overall accuracy of 99.2%, easy to apply and lends itself to an automated diagnostic process. The logical path may be traced as follows:

Step 1

Stool pseudo-C5, (cut-off 7.5 nmol/g dry weight). Most cases of VP (95%) are trapped at this point.

Step 2

Urine uroporphyrin (cut-off 40.5 nmol/10 mmol creatinine). This traps all cases of PCT (100%) along with cases of AIP, which are easily distinguished by a raised urine PBG.

Step 3

Stool coproporphyrin (cut-off 35.4 nmol/g dry weight). This branch gives rise to two hierarchies, leading to the identification of the two major classes: NAD and VPH on the one hand; the remaining VP and VPH on the other.

Subtree 1

Step i.

Stool protoporphyrin (cut-off 297.15 nmol/g dry weight)

This traps 90% of normal samples.

Step ii onwards.

Stool protoporphyrin, stool coproporphyrin and stool C5 serve to split off 2 cases of VPH from VPL and a further 2 cases of NAD as well as 5 cases of VPL; however the split between VPL and NAD appears counter-intuitive, with VPL having lower values of stool coproporphyrin and stool C5 than the normals; these are likely to represent inconsistencies in classification. Indeed, by tracing this sequence of key values through the database, it was possible to identify the anomalous results; they turn out to be that group with moderately raised stool protoporphyrin but no other abnormalities, which appear to have been classified as NAD or VPL arbitrarily.

Subtree 2.

Numbers are small on this branch, which uses rather subtle distinctions to trap the remaining 2 cases of VP and 5 cases of VPH, as well as 3 cases of NAD and 3 of VPL. The algorithm employs two parallel systems; the use of an elevated stool pseudo-C5, and evidence of a modest increase in urine porphyrin activity, with some use of the stool coproporphyrin.

The rather complicated classification tree for all porphyrias shown in Figure 9-4 is diagnostically highly accurate (Table 9-9). The important information is encapsulated in the first few nodes. Stool pseudo-C5 detects the majority of patients with VP (114 of 120), suggesting an importance for this porphyrin which was not revealed by discriminant analysis. PCT is detected along with AIP on the basis of a high urine uroporphyrin; PCT and AIP are then distinguished on the basis of the PBG.

Figure 9-5 shows an interesting variation on this model. A high stool C7 is used to detect some cases of VP and most cases of PCT; the stool pseudo-C5 being used to make this distinction. Thereafter, most cases of VP are trapped by a high stool pseudo-C5. In the next step, stool protoporphyrin divides the remainder into two sub-trees. An important observation is that the VP cases are evenly divided by the stool protoporphyrin value of 370.9 nmol/g dry weight: stool protoporphyrin is by itself a poor predictor of VP. In both sub-trees, stool coproporphyrin is used to distinguish VP from the other categories of NAD, VPL and of VPH. This is supported by the classification tree shown in Figure 9-6 which is restricted to samples from the VP spectrum: NAD, VPL, VPH and VP. The stool protoporphyrin discriminates poorly between VP and the other grades, and as many cases of VP have stool protoporphyrin values below the cut-off as above it. It is the stool coproporphyrin which separates normality from VP. The stool protoporphyrin alone effectively serves only to distinguish normality from VPL.

Incomplete data

Whereas discriminant analysis suggested slight reductions in accuracy when plasma porphyrin and urine precursor concentrations were omitted, classification tree analysis identifies them as unimportant, and classification proceeds on the basis of urine and stool values alone. In the case of the diagnosis of VP in particular, even urine values are rejected and classification is on the basis of stool results only. This suggests that in practice our diagnosis need be based almost entirely on stool data, and urine and plasma data, contrary to our teaching, are for practical purposes unnecessary *for the diagnosis of VP*. Clearly however, they retain importance for the diagnosis of AIP and PCT, and in assessing the biochemical activity of VP. In effect, classification tree analysis tells us that, while diagnostic urine and plasma profiles may be seen in overt cases of VP (as described in the classic descriptions by Day and Eales), diagnostically they add little or no value to a careful interpretation of the stool data alone.

14.3 THE CORRELATION OF BIOCHEMICAL DATA WITH DNA-PROVEN VP

The purposes of porphyria diagnosis are essentially two-fold. Firstly, one needs to diagnose porphyria in people presenting with suggestive symptoms and signs. Secondly, it is important to identify the asymptomatic relatives of people with a positive diagnosis of porphyria so that they may be adequately counselled and protected. The final arbiter of a diagnosis of VP is the presence or absence of a causal mutation in the PPO gene, and this provides the "gold standard" with which other diagnostic tests should be compared. Following our identification of the R59W mutation and the other mutations which have followed, we are for the first time in a position to validate our biochemical tests against a real and not an assumed standard, and thus potentially to improve their accuracy.

Our results confirm the observation that the biochemical expression of VP is a post-pubertal phenomenon. No subject aged less than 16 years in this study showed evidence of VP biochemically. Biochemical screening for VP in prepubertal children is therefore useless, and alternative methods must be employed. Hitherto the only reasonably reliable test was the measurement of PPO activity, held by most to be an unsuitable test for the routine laboratory (Long et al 1993, Hift et al 1997) though it has been used to detect the inheritance of VP in experimental studies (Meissner et al 1986, Da Silva et al 1995).

The porphyrin reference values in use in the UCT laboratory were established at least ten years ago, at a time when, for practical purposes, VP or its absence could only be diagnosed on the basis of biochemical testing. By DNA screening, we are now able to determine the presence or absence of VP with near-100% accuracy: the only reason for less than perfect sensitivity is the ever-present possibility of novel mutations in the population referred for testing. The ability to prove normality allowed us to determine a new normal normal range of porphyrin concentrations. The results suggest that the upper limit of the normal range in use in our laboratory is valid with two exceptions with an important bearing on the diagnosis of VP. It is clear that the stool protoporphyrin concentration of 200 nmol/g dry weight is inaccurate. This ULN was originally set approximately 15 years ago by Dr RS Day at a somewhat low level in order to increase the sensitivity of TLC for the diagnosis of VP (PN Meissner, personal communication). A new upper limit of normal (ULN) set at *Mean+2SD* would suggest a limit of 545.3 nmol/g dry weight. Secondly, the stool coproporphyrin, currently set at 50 nmol/g dry weight, should be reduced to 41.2 nmol/g dry weight to encompass *Mean+2SD*. Raising the cut-off value for stool protoporphyrin will immediately reduce the number of suspected cases of VP, particularly that group labelled VPL, a diagnosis which appears largely to be influenced by elevations in the stool protoporphyrin in the absence of other diagnostic markers of VP. Conversely, lowering the stool coproporphyrin will to a lesser extent increase the number of abnormal results.

When the upper limit of normal for the most significant stool porphyrins was set at *mean+2SD*, 33% of DNA-positive adults fell within the normal range. We may therefore conclude that at least 33% of all adults subjects carrying a PPO mutation known to be associated with severe loss of functional activity will demonstrate normal stool porphyrin excretion patterns. Indeed the figure may be higher as a result of some referral bias since those patients who are biochemically or clinically expressed are more likely to present themselves for DNA testing than their asymptomatic relatives. Since most of the subjects entered into these calculations are however members of two large families in whom all members, irrespective of symptoms, were screened, this effect is likely to be small and the figure of 30% is probably valid. Furthermore, this is in keeping with our findings in the large R59W kindred to be described in Chapter 17 where only 52% of adult gene carriers showed unequivocally positive stool biochemistry and 36% appeared unequivocally negative.

Therefore, in any family screening project, all children and approximately 30% of affected adults will escape detection if screening is confined to biochemical analysis alone. Interestingly, these figures are similar to those predicted by Disler et al (1982) who suggested that up to 40% of gene carriers might have silent VP.

Highly significant statistical differences between normal and VP subjects were found for stool coproporphyrin, protoporphyrin, C5 and pseudo-C5, for urine uroporphyrin, C5, C7, C6, ALA and PBG, and for plasma PU. Unfortunately, as shown in Table 10-2, there are large areas of overlap between normal and VP. We determined that 33% of our adult subjects carrying a PPO mutation demonstrated faecal concentrations of pseudo-C5, C5, coproporphyrin and protoporphyrin which fell within the new normal range. Indeed, 20 of 22 subjects had originally been diagnosed as normal biochemically, and the remaining two had been diagnosed as equivocal VP on the basis of a raised stool protoporphyrin only.

Using DNA testing as the diagnostic standard for VP, we were now able to assess the significance of the low probability and high probability (VPL and VPH) diagnoses assigned in our database. In this study, the probabilities of carrying a VP mutation were as follows: for a normal VP result, 0.13; for VPL, 0.22; for VPH, 0.33; for definite VP, 0.98. Examined retrospectively, several trends became apparent. A diagnosis of definite VP was favoured in subjects with higher stool coproporphyrin and stool C5 values whereas the stool pseudo-C5, urine coproporphyrin and urine ALA were of lesser value and the stool protoporphyrin non-predictive. Classification tree analysis (Figure 10-3) suggests, in retrospect, that the stool coproporphyrin served as the most predictive porphyrin.

Since 30% of all DNA-positive subjects appeared to be truly biochemically silent, there is little purpose in attempting to define biochemical values to detect 100% of these subjects. We therefore divided all the subjects with DNA-positive VP into two classes: biochemically-expressed and biochemically silent. The distinction itself raises the problem as to how to distinguish the two groups since, as shown in Figures 10-1, 10-2 and 10-3, all the important stool porphyrins appear to be widely distributed, rather than falling into distinct normal and abnormal classes. There is therefore a danger of establishing a circular argument: first establish the criteria which define a diagnosis of biochemically-expressed VP, then prove retrospectively that those criteria meet the diagnosis. It would appear logical that biochemically expressed VP will at the very least be associated with elevation of at least one of the terminal porphyrins excreted in stool: elevations of these porphyrins are readily recognised in unequivocal VP and have emerged repeatedly as the most relevant porphyrins in the discriminant and classification tree analyses. As an initial step in definition, we therefore selected all DNA-positive subjects showing an elevation of any one of the following stool porphyrins above the new ULN defined as *mean+2SD*: C5, pseudo-C5, coproporphyrin and protoporphyrin. We made the assumption that biochemically-expressed VP would constitute a subset of this group of subjects, and then undertook discriminant and classification tree analyses to determine the best criteria for separating those with DNA-proven VP from those who are DNA-negative. Table 10-7 shows that the larger group, those with an elevation in any one of these porphyrins, overlaps the normal group to an unacceptable extent, thus these loose criteria are not themselves sufficiently predictive of VP for an elevation in any of these porphyrins to define biochemically-expressed VP.

Tables 10-8 and 10-9 shown that discriminant analysis is still rather poor at detecting this group of expressed VP; though the use of categorical rather than ordered data improves the sensitivity. Stool coproporphyrin and stool C5 emerge as the most important predictors (Table 10-10) whereas stool protoporphyrin has very little discriminatory power. Classification tree analysis returns a diagnostically useful result (Figure 10-3, Table 10-11). The stool coproporphyrin and C5 again emerge as the most diagnostically useful predictors of DNA-

positive VP within this group. The stool pseudo-C5 and stool protoporphyrin are shown to be less discriminatory.

This would therefore suggest that the most appropriate biochemical predictors of VP are:

- a stool coproporphyrin exceeding 48.92 nmol/g dry weight *or*
- a stool pentacarboxylic porphyrin exceeding 2.72 nmol/g dry weight.

Note however that these criteria are, standing alone, suitable only for the differentiation of normal results from VP. Many samples from patients with PCT will meet these criteria, but these will in most cases be easily distinguished by including critical values of urinary porphyrins and stool isocoproporphyrin. As with all diagnostic algorithms, exceptional cases will be encountered which give false results: it may be expected that that small group of subjects with active phases of VP, who show the increased excretion of urinary porphyrins, along with patients with dual VP, will have to be separated from those with PCT.

These values are based on a relatively small learning sample of 181 cases. As experience grows, the learning sample will increase and classification tree analysis will further refine the algorithm. It is unlikely however that the final values will diverge much from those given here. An important conclusion is that the true "equivocal VP" result is not that with an isolated high protoporphyrin, but rather that whose stool coproporphyrin or C5 values straddle these critical values.

This algorithm was then retrospectively applied to the entire porphyria database, excluding those samples with diagnoses other than NAD and the grades of VP, with the following results shown in Table 14-1. There is a slight increase in the number of cases diagnosed as VP, and 5% of cases originally diagnosed as VP would now be regarded as normal. An important advance is that the VPL and VPH groups disappear completely: 17.6% of the former and 43.1% of the latter would satisfy the criteria for VP: these values agree well with the values obtained in the small sample for whom DNA values are available.

Original diagnosis		As predicted by the new values	
		VP	Normal
NAD	2511	68 (2.7%)	2443 (97.3%)
VPL	188	33 (17.6%)	155 (82.4%)
VPH	109	47 (43.1%)	62 (56.9%)
VP	379	360 (95.0%)	19 (5%)

Table 14-1. Effect of applying the new criteria retrospectively to the porphyrin values on the porphyria laboratory database.

14.4 PORPHYRIN PROFILES WITHIN BILE, SMALL BOWEL AND COLON

An important finding emerging from this study is the dethroning of the stool protoporphyrin as the most important predictor of VP: it appears that coproporphyrin is most suited to this, with pentacarboxylic porphyrin assisting this role. In large part this would appear due to the significant and variable switch in porphyrin profiles from coproporphyrin predominance to protoporphyrin predominance between bile and stool, which introduces an element of uncertainty into the assessment of the significance of an elevated stool protoporphyrin. The results of the study described in Chapter 11 clearly show that the belief that faecal porphyrins largely reflect the porphyrins delivered to the bowel in bile is untenable. A mechanism must

be sought to account for the shift in porphyrin predominance from coproporphyrin in bile to protoporphyrin in caecum and stool. Two mechanisms seem possible. It is possible that the earlier, more hydrophilic porphyrins are preferentially reabsorbed from the small bowel, or secondly, that there is an interconversion of porphyrins from coproporphyrin to dicarboxylic porphyrins within the bowel itself. That intestinal cells are capable of reabsorbing porphyrins has been well shown by the convincing evidence for an enterohepatic cycling of porphyrins put forward by several investigators (Ibrahim and Watson 1968, Lemberg and Legge 1949, Stathers 1966, Pimstone et al 1982, Pimstone et al 1987). Pimstone et al (1987) showed a significant reabsorption of porphyrins over the first few centimetres of the duodenum downstream of the hepatopancreatic ampulla, but did not suggest that any particular porphyrin was absorbed in preference to another. Furthermore, since jejunal and biliary profiles are essentially similar, such selective reabsorption would have to take place in the ileum. This must remain conjectural: no study has ever examined the ileal absorption of porphyrins.

There is however good evidence that the intestinal microflora actively alter porphyrin profiles as discussed in Chapter 6 (England et al 1962, Elder et al 1980, Beukeveld et al 1987). The abrupt change in porphyrin profile between small bowel and large bowel would be entirely consistent with a major role by intestinal bacteria, since the small bowel is sterile whereas caecum and and sigmoid are not. It would seem unlikely that exogenous sources of dicarboxylic porphyrin, whether from diet or from intestinal bleeding, could contribute in any meaningful fashion to this major difference in profiles. Indeed, the essential similarity of biliary and jejunal porphyrin profiles argues against the significant addition of exogenous dicarboxylic porphyrin to bile as it enters the duodenum.

Were accurate quantitation of the total amounts of porphyrin present over the length of the bowel possible, it would be easier to delineate the precise nature of this change in bowel porphyrins more definitively. Unfortunately, as the total volume of intestinal content passing through the bowel changes so dramatically as a result of water reabsorption, it is impossible to extrapolate from the porphyrin concentrations determined here to total amounts of porphyrin present, and therefore impossible to determine whether the net aggregate porphyrin present increases or decreases with transit through the bowel.

Logan et al (1991) have shown, as we have, that the predominant porphyrin in bile is coproporphyrin and in stool is protoporphyrin. They have also demonstrated that the difference in porphyrin content noted between normal subjects and those with VP is far higher in bile than in stool. To believe, as many do, that the raised stool protoporphyrin of VP is a direct consequence of the reduction in conversion of protoporphyrinogen to protoporphyrin in the liver as a result of diminished PPO activity may therefore be false. Clearly factors operating within the bowel, most probably bacterial-mediated breakdown of haem and interconversion of porphyrins, is substantially modifying the porphyrin profile present in bile such that it is almost unrecognisable in stool. The limited literature available on this process has all suggested that it is predominantly the dicarboxylic porphyrins which result from this process. It is probably the extreme variability in stool dicarboxylic porphyrins, coupled with the TLC reporting system which labels all dicarboxylic porphyrins generically as protoporphyrin, which leads to the inaccuracy of protoporphyrin in predicting the presence of VP.

14.5 THE DIAGNOSTIC UTILITY OF SIMPLE SCREENING TESTS

Though the median values for urine PBG, urine porphyrin and stool porphyrin concentrations correlate well with the results of simple screening tests, several difficulties are evident. Firstly, the relationship is logarithmic rather than directly proportional; thus strongly positive screening values for urine and stool porphyrins should carry more weight than low or

intermediate values. Secondly, the range of porphyrin concentrations associated with any given degree of positivity on screening tests is wide. This results in a poor compromise between sensitivity and specificity. A strongly positive stool screen (+++) is highly specific for the diagnosis of VP (Table 12-3) but this is at the expense of sensitivity, and were the threshold for a positive result set that this level, nearly 50% of patients with unequivocal VP would be missed. Clearly shown is the reciprocal decline in specificity which results if lesser grades of positivity are set as the threshold for diagnosis in order to improve sensitivity: thus setting the threshold at + would have a positive predictive value of only 28.5%.

Even the higher grades of stool porphyrin positivity are not entirely specific for VP, and a significant proportion have other diagnoses (Table 14-2). Approximately half of the non-VP cases at the higher levels in fact have PCT; most of these will however be recognisable by a strongly positive urine screen, the utility of which is shown in the discriminant and classification tree analyses. In practical terms, a high grade stool porphyrin screening result is highly suggestive of VP; however 3% with a grade 4 screening result will in fact be normal and 11% will have other diagnoses, of which about half will be PCT. For patients whose stools are scored as grade 3, 28% will be normal, 15% will have a diagnosis other than VP and a further 13% will have indeterminate values: thus 58% of such patients will have neither definite nor even probable VP. These data show the limitations of simple screening tests for diagnosis even in a reference laboratory with extensive experience and expertise in their use. Stool screening alone is clearly inadequate for the detection of VP; sensitivity is low and even a strongly positive (+++) result has a positive predictive value of only 83%.

These calculated values are dependent on the population from which the samples are drawn. Therefore, in extrapolating the results to other laboratories, caution must be exercised. The results are heavily influenced by the high prevalence of VP in our database (10% for definite VP; 13% for probable VP). This will not hold true for laboratories attempting porphyria diagnosis for any population other than a South African population. However, the results should be generalisable to other South African laboratories, with the proviso that a small element of bias may have crept into our database sample owing to a proportion of "difficult" diagnostic problems having been submitted to the UCT porphyria laboratory as the referral centre offering definitive diagnoses.

Screening result	0	1	2	3	4
Definite VP	10	7	56	101	209
Probable VP	7	13	39	38	15
Possible VP	20	38	82	42	7
Normal	1767	573	428	89	7
PCT	19	24	44	29	13
Other	17	9	23	18	15
Total	1840	664	672	317	266

Table 14-2. Predictive value of stool screening results for VP as assessed against eventual TLC-based diagnosis.

Discriminant function analysis

Several of our patients with AIP are sampled repeatedly as a result of frequent presentations with acute symptoms. This will have the effect of a weighting the AIP results in favour of the more severely expressed cases. In practice therefore, the diagnostic accuracy of this discriminant analysis is likely to be considerably reduced for AIP, skewed as it is in favour of

the diagnosis of the more severe form. With PCT, some of these patients have been sampled serially during treatment during which time they have shown a trend towards biochemical improvement. The effect of this redundancy will be to overestimate the predictive value of the discriminant analysis for AIP and to reduce the predictive value for PCT. However, the problem of redundancy is not a significant problem with VP, since few patients in our database are sampled repeatedly once the diagnosis has been established.

The only factor which is strongly weighted is the PBG score, because of its utility for diagnosis of AIP. Interestingly, the stool screening result is only moderately weighted in favour of VP indicating the lack of precision of this variable in diagnosing VP. Use of these classification functions, in which all subjectivity is removed (other than in the determination of the original screening grades) provides an upper limit for the diagnostic accuracy of these screening tests. In particular it yields the highest accuracy in that it is derived *a posteriori* - which must yield more accurate results than *a priori* arguments. This suggests that the use of simple screening tests for the diagnosis is of porphyria in South Africa is no more accurate than a maximum value of 49.2% for VP, 72.8% 7% for AIP and 67% for PCT. Even then, these figures will represent an over-estimate, since they are compared only with patients with biochemically expressed porphyria and not with all patients, both silent and expressed.

Classification tree analysis

The diagnostic algorithm returned by classification tree analysis in Figure 12-6 appears sensible, and approximates the process of diagnosis an experienced interpreter would intuitively make. The initial step is to separate out those samples screening ++ or +++; these are then successively assigned to *VP* and *Normal* depending on the magnitude of the urine and stool screenings. Those with low stool screenings, on the other hand, are successively subclassified into *PCT* and *Normal*. Note that this tree rejects the PBG screening result as being unnecessary to the classification. The predictive accuracy is shown in Table 12-5. It is seen that use of the classification tree approach is less sensitive for the detection of VP than decision analysis; approximately 60% versus 74%, but has a higher PPV. Negative screening tests are shown to have imperfect negative predictive value; 7% of all samples returned as negative will in fact be diagnosed as VP or PCT by TLC.

Use of stool screening results alone results in a fall in the detection rate of VP from 60% to 45% and a drop in PCT detection from 41% to 0%. Use of urine screening results alone results in a drop in detection of VP to just 15%; a further 13% will be recognised as abnormal but misclassified as PCT. As shown in Table 12-4, the best positive predictive value reached by a stool screening test is 77.4%: this is if only a very strongly (+++) result is regarded as positive. If lesser grades of reaction are regarded as positive, the PPV drops to 28.5%. A negative screening results however has a negative predictive value of 99.3%, though a false-negative rate of 4.4% remains.

Despite the poor predictive accuracy of screening tests as contained in our database, screening tests in the hands of our three experienced interpreters proved surprisingly predictive of the eventual diagnosis (Table 12-6). For a screening diagnosis of VP, the agreement was 85-100%; for normality, it was 85-89%. It is possible to improve the reproducibility of screening tests by modifying the methods more precisely. Thus the proportions of urine or stool and Dean's solution employed can be standardised and results corrected for urine concentration. Colour detection can be improved by automation, by replacement with spectroscopy or even by the provision of colour-comparison charts. Modifications of the Watson Schwartz reaction, including the use of anion exchange resin to separate PBG from interfering substances in urine, with PBG being measured by absorbance at 555 nm, will improve sensitivity: it was suggested that this may be useful for the detection of latent AIP (Schreiber et al 1989). Though a survey conducted by the Australian association

of clinical biochemists porphyrin working party showed good sensitivity (75-97.5%) for urinary porphobilinogen screening, this appears to be over-optimistic. Buttery et al (1990) have evaluated the Watson-Schwartz method for PBG detection and found a lower sensitivity (42-69%); this declined further to 28-53% in the presence of coloured or concentrated urine. It has been shown that in practice, screening tests alone for porphyria perform poorly. In an assessment of 201 cases referred to a British hospital, routine screening in the referring laboratories was shown to be unreliable, with both false negative and false-positive results. Only 43% of porphyrics were correctly identified. It was suggested that alternative semi-quantitative tests for porphyrins employing spectrophotometric scanning of acidified urine or faecal extracts would be preferable (Deacon 1988).

This study has shown that screening of samples by the Watson-Schwartz and Dean's methods in the South African population will correctly classify less than 70% of patients with biochemically proven VP, and will fail to identify approximately 6% of patients with normal screening values as being porphyric. Even where a porphyria is recognised, screening tests are poorly discriminatory in separating VP from PCT with a substantial degree of overlap between the two diagnoses. Furthermore, neither urine nor stool screenings in isolation have worthwhile predictive value. We conclude that there can be no justification for the continued practice of some laboratories issuing screening results as the final laboratory result. Without exception, every subject in whom the diagnosis of porphyria is entertained requires diagnostic testing with higher predictive values than are achievable with screening.

14.6 PLASMA FLUOROSCANNING

Several authors have suggested that plasma fluoroscanning is a more sensitive test for the detection of VP than porphyrin analysis. In the study by Da Silva et al (1995), 50% of biochemically negative VP gene carriers, as identified by lymphocyte PPO activity, were positive by plasma fluoroscanning. Long et al (1993) report that that the sensitivity of an increased faecal porphyrin concentration as a test for asymptomatic VP is 36 to 38%, whereas the sensitivity of plasma fluorescence scanning is 93% (95% confidence interval 78-99%). Our findings are in accordance with these results. We determined a sensitivity of 93.8% for plasma fluoroscanning as compared with a sensitivity for stool porphyrin analysis of 66.7%.

Accepting the false-positive and false negative rates shown in Table 13-3, plasma fluoroscanning would prove very useful in refining our diagnostic criteria for VP. As shown in Table 13-5, in a sample of 176 subjects, plasma fluoroscanning would detect a further 9 subjects with VP over and above the 32 positive by TLC, and would exclude 11 of 12 subjects with equivocal TLC results as normal.

The height of the porphyrin peak correlated closely with the concentrations of stool porphyrins, suggesting that the intensity of the fluorescence peak is proportional to the activity of the porphyria as measured by porphyrin excretion. Interestingly, coproporphyrin correlated most strongly with the plasma fluorescence peak. The preliminary work done on the nature of the compound responsible for the fluorescence in the plasma (Poh-Fitzpatrick 1980, Longas and Poh-Fitzpatrick 1981) suggested that the plasma contained a mixture of unbound porphyrins as well as a tightly-bound complex, probably of a dicarboxylic porphyrin, with albumin. Coproporphyrin was not shown, though Day had suggested that coproporphyrin was the major porphyrin present in the plasma of patients with VP, and that the PU porphyrin present in plasma and characteristic of VP was most likely a coproporphyrin-peptide conjugate (Day et al 1978). We have not yet studied a sufficient number of plasma samples chromatographically as well as by plasma fluoroscanning to make such correlations. The association between faecal coproporphyrin and plasma fluorescence may suggest that the fluorescent complex in plasma is indeed coproporphyrin, though it may

just be that the correlation reflects a non-causal relationship between two powerful markers of VP.

Da Silva et al (1995) view their failure to identify the VP carriers status in 50% of biochemically-silent carriers pessimistically. However, if the purpose of the test is viewed differently, plasma fluoroscanning is a most useful addition to our range of available diagnostic techniques. It is possible to establish a DNA-based diagnostic test for more than 90% of all families with VP without much difficulty: currently, in our experience reported in Chapter 4, it rarely requires more than 4-6 weeks to determine diagnostically useful information at the DNA level for a family with a novel mutation. Even before the exact mutation is determined, it is often possible to use the abnormal behaviour of the relevant exon on SSCP or heteroduplex analysis to identify gene carriers. The primary purpose of plasma scanning therefore is not for family screening studies, but rather for the immediate identification of VP in patients presenting to the laboratory for the first time. If positive by plasma scanning, it is appropriate to proceed to urine and stool porphyrin analysis and thereafter to DNA studies. We have shown that plasma fluoroscanning is almost 100% sensitive in detecting those patients with VP who will prove positive by chromatographic examination of urine and stool: just 2 subjects—one being R59W-positive, the other Y348C positive—have shown negative plasma fluoroscanning results in the face of positive stool biochemistry. The reason for this perverse result is unknown. Thus, other than in exceptional circumstances, faecal porphyrin analysis need not be undertaken in subjects who test negative by plasma fluoroscanning.

14.7 THE PREVALENCE OF VP IN SOUTH AFRICA

Dean (1963) suggested an overall prevalence of 3 per thousand in the white population of South Africa, and a figure of 8000 ± 2000 for the country as a whole. Allowing for the natural increase in the population—the white population currently stands at 5 million—the current figure would be approximately 15 000. It must be noted however that testing was performed by relatively insensitive stool screening, Our own results, reported in Chapter 12, indicate a sensitivity for stool screening for chromatographically-proven VP of 50-90% (a mean of 70%), depending on the intensity of the screening reaction regarded as positive. A chromatographic diagnosis of VP in turn has a sensitivity of approximately 70% for DNA-positive VP (Chapter 10). This suggests that Dean's prevalence should be increased by a factor of $1/(0.7^2)$, that is approximately doubled. The projected prevalence, assuming that Dean's figure was correct, would then be 6 per thousand population and a current total of approximately 30 000 gene carriers, of whom less than half will be symptomatic (Chapter 16).

SECTION 3

THE CLINICAL EXPRESSION OF VARIEGATE PORPHYRIA

Sections 1 and 2 of this dissertation described the molecular biology and biochemical features of variegate porphyria respectively. In Section 3, the clinical expression of VP is examined. The relevant literature is reviewed in Chapter 15. In the following chapter, we report our experience with four patients with homozygous VP, and the underlying mutations and clinical features are described and correlated. In Chapter 17 we describe a study of a single, large family carrying the R59W mutation, and show that approximately 60% of adults are clinically silent whereas skin disease is present in 40% and the probability of an acute attack now low. In Chapter 18 a personal experience with the management of more than 100 episodes of the acute porphyric crisis in Groote Schuur Hospital is reviewed. Unusual presentations and complications are discussed, and the outcome of treatment reported. The results presented in these chapters are discussed as a whole in Chapter 19.

CHAPTER 15:

THE CLINICAL FEATURES OF VARIEGATE PORPHYRIA: AN INTRODUCTION AND REVIEW OF THE LITERATURE

The principal clinical features of variegate porphyria are two-fold: the acute attack and skin disease. The following areas of interest are relevant to the original work of this dissertation: the proportion of patients carrying the R59W mutation who express VP clinically, a description of the acute attack observed in a large cohort of patients in the Groote Schuur Hospital, Cape Town, and the clinical and molecular features of the homozygous VP syndrome. For this reason the published clinical experience with VP including the prevalence of skin disease, and the pathogenesis, clinical features and management of the acute attack are described in detail, but the pathogenesis of the skin disease is not discussed.

This chapter begins with a review of homozygous or compound heterozygous VP (both of which are collectively abbreviated here as HVP), which has been mentioned on several occasions in Section 1. Both the molecular biology and the clinical features of HVP are reviewed, reported and discussed in this section, and our personal experience is reported in Chapter 16.

15.1 HOMOZYGOUS PORPHYRIA

Introduction

Two of the porphyrias are described as autosomal recessive conditions. These are Doss porphyria (ALA dehydratase deficiency, plumboporphyria) and congenital erythropoietic porphyria (CEP). The other porphyrias are inherited as autosomal dominant traits and may therefore present clinically in the heterozygous state. All have now additionally been reported in the homozygous state. The clinical features of these homozygous subjects tend to fall into two groups: firstly, those symptoms characteristic of the heterozygous state, but usually presenting earlier in life—usually in early childhood—and often in exaggerated fashion, and secondly, unusual features peculiar to the homozygous state and possibly relating to haem deficiency during embryonic development, as has been postulated for the skeletal and neurological features of homozygous AIP, HCP and VP (Roberts et al 1998).

Most of these homozygous variants were described before the introduction of molecular investigation. The presence of severe symptoms in an infant or child with a biochemical profile suggestive of porphyria raised the possibility of the homozygous state, and in some cases, corroboration by the demonstration of severely reduced activity of the appropriate haem-synthetic enzyme in the subject, perhaps accompanied by evidence of a reduction in activity in keeping with the heterozygous state in the parents, was offered. Conclusive proof followed years later when heteroallelic inheritance of two mutations—the compound heterozygous state—or homoallelic inheritance of a single mutation were shown.

Occupying an interesting and as yet not fully defined position is EPP. The mode of inheritance is known to be complex, and to have features suggestive of both dominant and recessive inheritance, usually described as "autosomal dominant with low penetrance". It has been suggested that biochemical evidence of disturbed porphyrin synthesis alone is a feature of the heterozygous state, whereas clinical expression requires the inheritance of two abnormal alleles.

The homozygous porphyrias are summarised in Table 15-1 and are described more fully below.

Enzyme	Inheritance	Heterozygosity	Homozygosity
ALA dehydratase	AR	Nil	Doss porphyria
PBG deaminase	AD	AIP	Homozygous AIP
Uroporphyrinogen cosynthase	AR	Nil	CEP
Uroporphyrinogen decarboxylase	AD	Familial PCT	Hepatoerythropoietic porphyria (HEP)
Coproporphyrinogen oxidase	AD	HCP	Homozygous HCP Harderoporphyria
Protoporphyrinogen oxidase	AD	VP	Homozygous VP (HVP)
Ferrochelatase	AD/AR?	EPP (?)	EPP (?) EPP with hepatopathy (?)

Table 15-1. Summary of the mode of inheritance of the porphyrias and of the clinical syndromes associated with the heterozygous and the homozygous or compound heterozygous state. AD: autosomal dominant, AR: autosomal recessive.

Homozygous porphyrias other than VP

ALA dehydratase (ALAD) deficiency

Also known as plumboporphyria or Doss porphyria, this is a rare autosomal recessive disorder of haem synthesis. Five cases have been reported since the first description by Doss et al (1979). In each case, both parents have been shown to have substantially reduced ALAD deficiency, in keeping with the heterozygous state, but are not clinically affected. The patients however exhibit a predominantly neurological syndrome similar to the acute attack, with abdominal pain and vomiting progressing to a motor neuropathy and paralysis. Two of the reported patients first developed their symptoms at the age of 15 (Doss et al 1979); a third patient was noted to have severe symptoms at birth (Thunnell et al 1987). This child underwent a liver transplant at the age of 6 for recurrent crises with paralysis and his clinical condition has subsequently improved (Plewinska et al 1991). A fourth patient presented with neuropathy for the first time at the age of 63 (Hassoun et al 1989). The fifth patient is an elderly Japanese woman who presented for the first time with a severe acute attack at the age of 69 (Muraoka et al 1995). A 20 year follow-up has now been reported on the first two patients. The initial acute attacks had been successfully treated by infusion of glucose and haem arginate and both patients have remained free of symptoms since (Gross et al 1998).

The diagnostic features on biochemical analysis include marked elevations of ALA and normal or near-normal PBG excretion—the reverse of the pattern seen with AIP. Erythrocyte ALAD activity is barely detectable, in keeping with the homozygous state. The molecular defects have been reported in three of these patients; all are shown to be compound heterozygotes bearing single base substitutions, unique to each patient, on each allele (Sassa 1998).

AIP

Homozygous AIP was first described in 1990 (Beukeveld et al 1990). This was a retrospective diagnosis in a Dutch female child who had died in 1964. Clinical features included severe neurological abnormalities, including mental retardation, associated with porencephaly. Her teeth fluoresced under UV light. She remained in a persistent vegetative state and required tube-feeding for her entire life and died at the age of 8. Her mother had typical AIP; her father was initially thought to be normal, but sensitive testing later suggested that he too had a subtle deficiency in PBG deaminase activity. Re-examination of the proband's urine indicated features of AIP, though with some departures from the typical pattern, including an abnormal uroporphyrin isomer I:III ratio (Beukeveld et al 1990). Her parents were ultimately shown to carry two different mutations in exon 10 of the PBG deaminase gene; each parent was heterozygous for one mutation and presumably the child had been a compound heterozygote (Picat et al 1990). Both these mutations have been shown to be associated with AIP in heterozygotes (Delfau et al 1990). An unusual patient described by Gregor et al (1977), a child who experienced convulsions and bilateral cataracts and demonstrated the biochemical features of AIP as well as an increased erythrocyte protoporphyrin concentration, is likely to have been an unrecognized case of homozygous AIP (Llewellyn et al 1992).

Subsequently Llewellyn et al (1992) described a three-year-old girl admitted to hospital following febrile convulsions. She was described at the age of 18 months to have ataxia, intention tremor and dysarthria, partial agenesis of the cerebellar vermis, bilateral cataracts and right optic nerve hypoplasia. She was found to have an elevated PBG excretion and severely depressed erythrocyte PBG deaminase activity. Her younger brother had a markedly reduced erythrocyte PBG deaminase activity and elevated urinary PBG excretion. Though he initially showed no obvious clinical deficits, he subsequently developed ataxia. Their parents, who were unrelated and asymptomatic, were shown to have elevated PBG levels and moderately reduced erythrocyte PBG deaminase activities, in keeping with latent AIP. They were shown to be heterozygous respectively for a C to T and a G to A transition in the same codon in exon 10; the children were compound heterozygotes. A further patient, reported from Spain, presented clinically with psychomotor retardation, hepatosplenomegaly and bilateral cataracts (Xu et al 1995).

All the patients described with homozygous AIP have shown markedly elevated urinary ALA and PBG excretion and severely reduced erythrocyte PBG deaminase activities. They have been shown to be either homozygous or compound heterozygotes for mutations which are known to be associated with AIP in the heterozygous state, but which appear in *in vitro* studies to retain some residual catalytic ability (Elder 1997). An interesting point is that none of these children have developed acute attacks (Elder 1997).

CEP

The clinical features relate directly to the accumulation of large quantities of uroporphyrin, C7, C6, C5 and coproporphyrin, all of isomer series I, in bone marrow, erythrocytes, plasma, the skeleton, skin and urine. Characteristic clinical features include haemolysis, mild splenomegaly, anaemia, and photocutaneous sensitization which frequently gives rise to a severely destructive process. In addition to the development of erosions and blisters in sun-exposed areas, there may be photomutilation with contraction of digits, loss of nose, lips and ears, and hypertrichosis. These changes usually begin in early life, rather than post-pubertally as is the case with the more typical autosomal dominant disorders.

In keeping with the recessive nature of the condition, uroporphyrinogen co-synthase (UROS) activity is reduced to less than 50% of normal values. The molecular basis of CEP has recently been reviewed by Desnick et al (1998). Eighteen mutations have been identified

in the UROS gene, comprising deletions, insertions, splice mutations and single-base substitutions. All but one of the known CEP missense mutations occurred in apparently highly conserved peptide sequences of the UROS gene. Some patients are homoallelic for a specific mutation whereas other patients are heteroallelic for two mutations. CEP is encountered in populations around the world, and several shared mutations have been identified in patients from apparently unrelated families. Particularly striking is the C73R mutation which was homoallelic in five of the subjects and was present in 22 unrelated families in total.

There is some evidence for genotype-phenotype correlation in CEP. The C73R mutation results in the loss of more than 99% of normal activity when expressed in *E coli*; human homozygotes for this mutation demonstrate an extremely severe phenotype which may include profound anaemia, hydrops fetalis and transfusion-dependency at birth. Patients who carry both C73R and a second mutation expressing somewhat more residual activity may demonstrate a moderately severe phenotype; whereas patients who carry two mutations associated with a higher degree of residual activity have milder forms of CEP (Desnick et al 1998).

PCT

34 uroporphyrinogen decarboxylase (UROD) gene mutations have been identified in patients with familial PCT (Elder 1998, Mendez et al 1998, Christiansen et al 1999, McManus et al 1999). Most are restricted to single families. Where patients are homoallelic for a UROD mutation, or are heteroallelic for two such mutations, a severe phenotype known as hepatoerythropoietic porphyria (HEP) may result. This is associated with a severe deficiency of UROD activity in all tissues and is expressed clinically as severe photosensitivity and photomutilation, with onset in childhood (Hift et al 1993a, Elder and Roberts 1994, Meguro et al 1994, Roberts et al 1995b, Moran-Jimenez et al 1996). The clinical expression is variable; severely affected subjects may resemble patients with CEP, whereas others show a milder disease more in keeping with PCT (Elder 1997). Rarely, HEP may be associated with hepatosplenomegaly (Smith 1986) or other features, including hemiparesis (Parsons et al 1994). Interestingly, the mutations associated with HEP are rarely identified in patients with heterozygous familial PCT (Elder 1997). Presumably, in order to be compatible with life, these mutations must code for proteins with significant residual activity, and in the heterozygous state, this residual activity is sufficiently high not to result in clinical expression (Roberts et al 1995b, Meguro et al 1994).

HCP

In addition to 5 polymorphisms, at least 19 mutations have been identified in patients with HCP (Rosipal et al 1999). These include missense mutations, nonsense mutations, deletions, frameshift mutations and splicing mutations. HCP was first described in the homozygous state in a patient who showed evidence of growth retardation, skin pigmentation and hypertrichosis (Grandchamp et al 1980, Grandchamp et al 1977). Acute attacks were not encountered in early childhood, though she later had typical acute attacks at the age of 10 years and again at the age of 20 years. She was shown to be homozygous for an R231W mutation in the gene for coproporphyrinogen oxidase; this mutation decreases but does not entirely abolish the activity and stability of the enzyme, and a residual activity of approximately 22% has been measured (Martasek et al 1994). Compound heterozygous HCP has subsequently been described (Doss et al 1999).

Recently a variant coproporphyrinogen oxidase deficiency syndrome has been described and labeled harderoporphyria (Lamoril et al 1995, Lamoril et al 1998). The oxidation of coproporphyrinogen to protoporphyrinogen proceeds in two steps, with the first step leading to a tricarboxylic porphyrinogen intermediate, harderoporphyrinogen. Where, as a result of a

mutation, the second step alone is impaired, this intermediate accumulates giving rise to the characteristic syndrome (Elder 1997). In this condition, haematological manifestations predominate and include jaundice, severe haemolytic anaemia and hepatosplenomegaly, as well as skin photosensitivity present from birth (Nordmann et al 1983, Doss et al 1984). In one family, three affected siblings were homoallelic for a K404E mutation; in the second family, affected subjects were heteroallelic for the K404E mutation and for a T to G transversion in the third position of the donor splice site in intron 6 which resulted in exon skipping (Lamoril et al 1998). Thus the K404E mutation appears to be specifically associated with a deficiency in the conversion of harderoporphyrinogen to protoporphyrinogen.

EPP

The inheritance of EPP is complex. Though typically described as an autosomal dominant trait with incomplete penetrance, there is evidence to suggest that, in some families at least, particularly those in whom hepatopathy occurs, an autosomal recessive mechanism appears to explain the observed pattern of inheritance more accurately. Studies of enzyme activity in affected families further suggest complex patterns of inheritance. Most patients with manifest disease have severely reduced ferrochelatase activity, which is in keeping with a disorder carried on both alleles. This has led to the belief that co-inheritance of two defects; one, presumably more severe, from the biochemically abnormal parent and the second, less severe, from an apparently normal parent, is necessary before clinical EPP becomes apparent. Since only one parent can be shown biochemically to carry EPP, the disease appears to be dominant, yet its actual inheritance will be recessive (Sarkany et al 1994). A family has been reported in which a clearly mutated allele was complemented by a poorly expressed ferrochelatase allele associated with markedly reduced mRNA expression (Gouya et al 1996). It is not known whether a similar mechanism is common to all families with EPP.

Homozygous VP

The first report of HVP appeared in 1984 (Kordac et al 1984) and further reports have followed (Murphy et al 1986, Coakley et al 1990, Gandolfo et al 1991, Norris et al 1990, Mustajoki et al 1987). The eight reported cases were, with one exception, remarkably similar. Both sexes were affected. Typically patients presented with photosensitivity within days to months of birth. Many were mentally retarded. Delayed neurological development was common and most experienced seizures. Nystagmus was common. Structural abnormalities of the hands were frequently reported, ranging from clinodactyly to severe deformity. Growth retardation was frequent. Reported activities of PPO in these cases ranged from 0 to 20%. With one exception, none were reported to have developed elevated porphyrin precursor concentrations nor the acute attack. These clinical features are discussed more fully in Chapters 16 and 19, and are summarised in Table 19-1.

Molecular biology of HVP

The mutations present in several patients with HVP have now been reported. Kauppinen et al (1997) identified heteroallelic I12T and P256R mutations in a Finnish child believed to have HVP. The first mutation occurs in an evolutionarily conserved region of exon 2 and may thus have a significant functional effect, whereas the second mutation was shown to affect enzyme activity in bacterial cells but not in mammalian cells: this may indicate that the second mutation is less deleterious to the activity of PPO than the first. Frank et al (1998b) identified the patient with homozygous VP first reported in 1990 (Norris et al 1990) as a compound heterozygote. Heteroduplex analysis of exon 6 revealed a heteroduplex in the proband and her father and a G to A transition at nucleotide 505 of the PPO cDNA was shown. This leads to a

substitution of glycine by glutamic acid at position 169 (G169E) in the paternal allele. A second heteroduplex was noted in exon 10 in proband and mother, and was shown to be associated with a G to A transition at nucleotide 1071 of the PPO cDNA resulting in a glycine to arginine substitution at position 358 (G358R).

Roberts et al (1998) investigated the genetic basis of apparently homozygous variegate porphyria in 5 of 11 unrelated patients. Two of these had not previously been reported; three had been reported previously (Kordac et al 1984, Norris et al 1990) One subject is the same subject characterised by Frank et al (1998b). Mutations were confirmed by sequencing or restriction digests of genomic DNA. Two of the five patients were shown to be homoallelic for missense mutations (D349A and A433P). Both were the product of consanguineous unions. The remaining three were heteroallelic (G169E/G358R; G358R/Intron 7 deletion; A219KANA/Intron 11 transversion). Both intronic mutations are functionally significant. The intron 11 transversion results in a decrease in normal mRNA content in excess of 90% whereas the intron 7 deletion results in the compete deletion of exon 7. Six of these mutations produce an abnormal pattern on DGGE or heteroduplex analysis. A further 104 unrelated patients with heterozygous VP were screened using these techniques; none of these mutations were identified nor have they been previously reported in heterozygous VP. The seventh mutation (the Intron 11 transversion) was not present in any of 28 VP patients in which this region had been sequenced or in 55 normal subjects screened by allele-specific oligonucleotide hybridisation. This paper draws some important conclusions on the nature of the mutations which, in combination, lead to the development of HVP. These are discussed in more detail in Chapter 19.

15.2 THE CLINICAL PROFILE OF PATIENTS WITH VARIEGATE PORPHYRIA

There have been relatively few descriptions of series large enough to give insight into the typical patterns of clinical expression of patients with VP. Summarised here are those reports which attempt to delineate those patterns, described by the country of origin, as there is some evidence that the relative frequency of skin disease is determined by the country of residence, presumably as a result of varying exposure to sunlight (Mustajoki 1978).

South Africa

Eales et al (1980) delineated the clinical spectrum of VP as seen at Groote Schuur Hospital over a 30 year period preceding 1980. More than 300 index cases had been seen, and this abundance of clinical material allowed the authors to give a definitive clinical and biochemical description of VP. The sex and clinical presentation of these 300 index patients are presented in Table 15-1.

	Cases	%
Sex		
Male	129	43
Female	171	57
Clinical presentation		
Pure acute	51	17
Acute/cutaneous	62	21
Pure cutaneous	156	52
Biochemical only	31	10

Table 15-1. Details of the presentation of 300 patients in Cape Town drawn from Eales et al (1980).

There was a slight female preponderance. 260 patients were white and 40 of mixed race; a ratio of 0.87/0.13. Most cases presented for the first time in the third and fourth decades of life; smaller numbers were encountered in the 5th, 6th, 7th and even 8th decades. A small proportion presented between the ages 10 and 20, but there were no clinical or biochemical presentations before puberty.

A striking observation is the large proportion of patients who had developed an acute attack—34% of the total group. Also striking is the small proportion (just 10%) of patients known to have porphyria who were clinically silent. Thus 90% of all patients with VP manifested symptoms; 52% with skin disease alone, 73% in total with skin disease and 17% with acute symptoms alone. What is however not clear from this study is how many of these patients with acute symptoms alone develop skin disease subsequently; our own experience suggests that it is rare for a patient with VP who has suffered acute symptoms to remain free of skin disease.

The Netherlands

Several Dutch families with VP have been described. Westerhof and Smit (1981) examined a three-generation kindred in Amsterdam. The biochemical diagnosis of porphyria was based on the quantitation of ALA, PBG, uroporphyrin and coproporphyrin in the urine and coproporphyrin and protoporphyrin in the stool. This family's genealogy could be traced back to 1630, though it was unfortunately not possible to demonstrate kinship with the South African immigrant families. 27 adults drawn from 3 generations were physically examined. VP was diagnosed biochemically in 10 subjects. Thus, 37% of the adult members of this family were shown to have biochemical evidence of VP. Since 50% of family members would be expected to carry VP, this may indicate that a substantial proportion (approximately a further 10%) are both biochemically and clinically silent. Only 4 subjects were reported to be symptomatic: all had skin disease and three had had acute attacks. The remaining six biochemically-positive subjects were asymptomatic and were described as latent cases.

Subsequently two Dutch families were described by Te Velde and Noordhoek (1970). In each family, the first male progenitor had been born in Deventer. Since it is known that Gerrit Jansz, one of the ancestral couple thought to have imported VP to South Africa, was born in Deventer, it was believed possible that one or other of these Dutch families might be related to the South African kindred, though this could not then be proved. VP was diagnosed biochemically in 6 of 18 members in the first family and in 6 of 16 members in the second family. However, 2 members of each family were younger than 14 and biochemically

expressed VP would not be expected in these youngsters, thus the incidence of expressed VP in these families is 37% and 43%, as compared with an expected 50%, respectively. This study therefore suggested that approximately 40% of a patient family carrying VP will be proven biochemically to carry the disease, suggesting that approximately 10% will be biochemically silent and and undiagnosable by biochemical means alone. Since half the family members are expected to have inherited VP, the implication is that approximately 20% of gene carriers are not detectable biochemically.

Te Velde et al (1989) revisited four families who had been described previously, in the years between 1920 and 1933, to have "cutaneous porphyria" and "acute porphyria". Two of these families were shown to have HCP and AIP respectively. A third family appeared to have VP. This investigation provided no useful evidence about the penetrance of VP in this family since insensitive qualitative tests alone were used: 4 subjects were detected by the screening of urine for porphyrins whereas a further 22 subjects tested negative. The fourth family, shown to carry VP, was however informative. 62 members of the second family were tested (of whom 12 fall in the last generation; ages are not given but some may be prepubertal). 19 members had biochemical evidence of the disease; 17 had porphyric skin lesions and 3 had had acute attacks. There are therefore substantially fewer patients with biochemically proven VP than would be expected from a condition with autosomal dominant inheritance, suggesting a significant rate of "silent" carrier status, which, by inference, is about 38%. Furthermore, one may induce that a high proportion of patients with biochemically expressed VP will demonstrate skin disease (17 of 19 patients, 90%), which is of interest as it does not support the contention that patients with VP in Europe are less likely to express skin disease than those in South Africa.

Finland

Though the number of patients with VP in Finland is substantially less than in South Africa, and also less than the number of patients with AIP, many useful reports have emanated from that country, largely because of the excellence of clinical record keeping, the maintenance of a central registry of porphyria, as well as the careful interest shown in the disease by Finnish workers. An unexpectedly large numbers of patients with VP were identified (Mustajoki and Koskelo 1976). Tellingly, all were diagnosed only during the 10 year period following the introduction of faecal porphyrin analysis. This suggested that VP might be considerably more prevalent than had hitherto been suspected. The authors suggested that VP might escape recognition because of a lower incidence of skin disease in temperate climates or because of misdiagnosis as some other form of porphyria. Thus VP with skin disease might be misdiagnosed as PCT and acute symptoms as evidence of AIP in the absence of careful faecal porphyrin analysis.

Subsequently Mustajoki (1980) described a 12 year experience with VP in Finland. During this period, 57 persons with VP were diagnosed in the Helsinki laboratory. Since this is the only laboratory in Finland where faecal porphyrins were tested, it was thought likely that this included all patients with diagnosed VP in that country. Of these 57 patients, 37% were male and 63% female. The patients belonged to 9 families. Using church records, the investigators were able to trace family trees back to the first half of the 19th century. Of all first-degree adult relatives studied, 45% were found to have VP, which might suggest a silent-carrier rate of approximately 5% in a family, or about 10% of all gene carriers.

An acute attack had been diagnosed in 18 patients (32%), of whom only two were male and 16 female. Using the population older than 14 years as a basis for calculation, Mustajoki computed the prevalence of VP in Finland to be 1.3 per 100 000. His studies led him to believe that VP was endemic within the Finnish population and had not been imported from

elsewhere. He commented that the prevalence of VP is by European standards high in Finland. He surmised that this might be due to a founder effect, as in South Africa, or that more careful screening with faecal porphyrin analysis in Finland was detecting patients with VP whereas such cases might be missed elsewhere as a result of a failure to employ faecal testing, as had been suggested by several authors (Doss et al 1978, Fromke et al 1978, Hamnström et al 1967, Perrot et al 1978).

Penetrance of the gene had been reported to be almost complete by Dean (1971) and Fromke et al (1978). The occurrence of porphyria in 45% of the first degree relatives of the Finnish families in this study was in keeping with this. However, Mustajoki identified several obligate carriers without detectable biochemical or clinical abnormalities which demonstrated that the gene is not always expressed. He also pointed out factors which would make accurate estimation of penetrance impossible. Thus the selection of clinically manifest cases would exaggerate the penetrance while the inclusion of cases with new mutations or "spurious" offspring would lower it.

New England

Muhlbauer et al (1982) identified 5 families with VP in the space of 2 years in New England, a restricted geographical area, which led them to believe that VP may be more prevalent than previously appreciated. This study confirmed that cutaneous disease appeared to be milder in non-South African patients, an observation thought to be due to the less intense sunlight of the higher latitudes. They sounded a warning therefore that patients with VP and acute symptoms but no skin disease might erroneously be diagnosed as having AIP.

Forty individuals belonging to five unrelated families with VP were evaluated. A female preponderance of symptomatic patients was shown. Nine patients had manifest porphyria diagnosed on the basis of skin disease (6 females, 2 males) with or without acute symptoms (5 females, 1 male). No patient died during an acute attack. A striking finding was the propensity of female patients to present with clinical symptoms.

All nine symptomatic patients had characteristic VP excretion patterns with elevated stool coproporphyrin and protoporphyrin. A further six family members were regarded as having latent VP since faecal porphyrin excretion was increased but they had no symptoms. The faecal porphyrin content was lower in this group than in the manifest group. A further 6 subjects were thought to have questionable porphyria because of slight elevations of faecal or urine porphyrin excretion. 19 patients were classified as normal on the basis of lack of symptoms and normal stool examinations. This points to a significant rate of silent carriage, with only 15 of an expected 20 patients having clear biochemical evidence of VP, of whom nine were symptomatic. Muhlbauer et al (1982) quote references suggesting that patients with VP may complain of neuropsychiatric or abdominal symptoms in the absence of an acute attack, including headaches, depression, abdominal pain, paraesthesia, constipation and personality disturbances. In this series, two subjects had had unexplained periods of depression and three had had migraines. The authors do however advise caution in view of the widespread nature of such conditions amongst the general population.

Denmark

With (1983) described all cases of VP and HCP listed in the Denmark Porphyria Register at that time. The prevalence of VP was found to be slightly higher than that of HCP (20 and 11 cases respectively) but considerably lower than that of AIP and PCT (153 and 131 respectively). The male:female ratio was 6:14 in VP; this again suggests a greater propensity for females to present clinically than males. Of the 20 cases of VP, six had both skin disease and acute attacks whereas six had skin disease alone and seven had had acute attacks alone.

Only 1 biochemically-positive asymptomatic subject had been registered. The prevalence of VP in Denmark appeared to be about 0.4 per 100 000. With suggested that a much higher percentage of these families must contain biochemically latent carriers since very few latent cases were proven amongst relatives.

15.3 SKIN MANIFESTATIONS OF VARIEGATE PORPHYRIA

Eales (1960) reported that more than 80% of patients with VP had skin disease, and that this was the presenting symptom in half (Eales 1974). The skin disease encountered in Eales' series (1980) may be regarded as typical of VP, and his description remains pertinent. A cardinal feature was the increased fragility of the skin of the sun-exposed surfaces of the face and the dorsal surfaces of the hands. Typically, even minor trauma led to detachment of the epidermis from the dermis with a resulting blister or raw area oozing serum. These lesions were described as healing rapidly with minimal scarring in the absence of infection, whereas secondarily affected lesions led to disfiguring pigmented scars or to areas of depigmentation. Occasional patients who suffered repeated trauma and constant exposure to the sun developed progressive pseudosclerodermatous changes on the hands and fingers. Facial features included hypertrichosis.

Acute photosensitivity was not usually a feature of uncomplicated VP, but was described as occurring when VP was accompanied by intercurrent diffuse liver disease or biliary obstruction (Eales 1963). Under these circumstances, it manifested with massive formation of bullae thought to result from a high circulating porphyrin concentration following the deviation of porphyrin excretion from stool to urine. The presence of an immediate photosensitivity in patients with VP, more typical of EPP, has been reported in other studies and the responses to experimental irradiation have included erythema, weal formation, oedema and vesiculation (Mustajoki and Koskelo 1976, Levene 1968, Rimington et al 1967, Runge and Watson 1962). Mustajoki (1980) has suggested that the worsening of skin manifestations sometimes noted during pregnancy or during therapy with the oral contraceptive might also be explained on a basis of hepatic dysfunction.

The high proportion of patients with VP (80%) reported by Eales to have skin symptoms does not appear to be typical of the experience in more temperate climates. In Finland, skin fragility was reported in half of 45 patients and was mostly mild, with a history only being elicited on direct questioning (Mustajoki and Koskelo 1976). Interestingly, all patients had been diagnosed during an acute attack or during investigation of family members; none had had skin manifestations as their presenting symptom. In a subsequent report on 53 patients, Mustajoki (1980) reported that 29 (55%) had had no skin symptoms, whereas 45% (10 males and 14 females) had noted excessive fragility of the skin of the sun-exposed areas. This was particularly marked on the backs of the hands, though 6 subjects also had facial involvement and one reported skin problems in the legs. In all patients, skin fragility was first noted after puberty. Eight subjects reported that the skin was more fragile in summer whereas the other patients had not noticed a seasonal variation. Of the 15 patients who had had acute attacks, 7 had had skin symptoms as well and 8 had not. Mustajoki also compared porphyrin values in patients with skin disease and those without. In general, urinary and faecal porphyrin excretion was higher in those with symptoms but there was a wide variation and statistical significance was only found for the urinary coproporphyrin.

Other commentators have also reported relatively low levels of expression for skin disease in VP in North America and Europe (Holti et al 1958, Hamnström et al 1967, Taddeini and Watson 1968, Cochrane and Goldberg 1968, With 1969, Doss et al 1977, Fromke et al 1978, Doss et al 1978). This has led some commentators, including Mustajoki (1978), to postulate that the skin manifestations in VP may be milder in temperate and cold climates than they are

in South Africa as a result of the lower degrees of sun exposure. As discussed by Mustajoki and Koskelo (1976), the maximal irradiation in South Africa is similar to that in northern Europe in the height of summer, but has a considerably longer duration. Mustajoki therefore postulated that the higher incidence of skin problems in South Africa was due to chronic exposure to strong sunlight.

There is also experimental evidence that epidermal melanin protects against porphyrin photosensitisation. The mean erythematous dose for light of several wavelengths was assessed in a patient with both VP and vitiligo (Westerhof et al 1981). The vitiliginous skin, melanin-free, had only one quarter the resistance to photosensitisation of normal skin at 405 nm and half the resistance at 310 nm and 500 nm.

15.4 THE ACUTE ATTACK

Pathogenesis

The acute porphyrias include ALA dehydratase deficiency, AIP, HCP and VP. Both ALA dehydratase deficiency and AIP are marked by acute symptoms only, whereas HCP and VP may, in addition to a propensity to the acute attack, be associated with skin disease as well. The characteristic clinical picture of the acute attack is that it is episodic, may or may not be associated with an obvious precipitating event (such as the administration of porphyrinogenic medication or menstruation), and is marked by a typical constellation of symptoms; notably severe abdominal pain accompanied by few clinical signs and by an absence of peritonism, features of autonomic neuropathy (Yeung-Laiwah et al 1987, Blom and Atsmon 1996)—particularly hypertension and tachycardia, vomiting, ileus and constipation (Kirsch et al 1998). This may proceed to a typical motor neuropathy resembling the Guillain-Barre syndrome (McEneany et al 1993, Bont et al 1996). In severe cases, this leads to a severe flaccid quadriparesis and respiratory failure requiring ventilation. Pathologically, the neuronal injury is characterised by severe axonal necrosis though at times there may be a lesser element of demyelination as well (Windebank and Bonkovsky 1992, Cavanagh and Mellick 1965, Hierons 1957, Gibson and Goldberg 1956, Mustajoki and Seppäläinen 1975, Albers et al 1978, Flügel and Druschky 1977). The histological changes associated with acute neuropathy in VP appear to be no different from those with other forms of porphyria. The cardinal features are those of severe qualitative and quantitative changes in myelinated and unmyelinated fibres suggesting an axonopathy in keeping with the dying-back phenomenon (Thorner et al 1981). Once established, the neuropathy is slowly reversible and typically months to years are required before full function is regained.

The pathogenetic mechanisms whereby the acute attack is established are poorly understood. The most likely hypotheses include ALA neurotoxicity and haem deficiency, acting either directly within the neuron or via a deficiency of one or more essential haemoproteins. The subject has recently been concisely and elegantly reviewed by Meyer et al (1998).

ALA-induced neurotoxicity

The observation that the acute attack is always accompanied by an elevation in ALA concentrations has led some authorities to suggest that ALA is itself neurotoxic. Typically ALA has been suspected in preference to PBG since acute lead poisoning, ALA dehydratase deficiency and hereditary tyrosinaemia type 1 deficiency, all of which are accompanied by elevated ALA levels but not by elevated PBG levels, result in a syndrome similar to the acute attack. There is little evidence that either PBG or the porphyrins are themselves neurotoxic. Furthermore, treatments such as carbohydrate loading or haematin infusions appear to lower

ALA excretion and are accompanied by clinical resolution. However, it has never been possible to prove a causal link between high ALA excretion and the acute attack. In particular, many asymptomatic patients (particularly with AIP) excrete levels of ALA in excess of those seen in other patients (typically those with HCP and VP) who are actively experiencing symptoms. Secondly, experimental administration of ALA to healthy volunteers, to subjects with AIP (Dowdle et al 1968, Meyer et al 1972, Shimizu et al 1978, Mustajoki et al 1992) and to experimental animals (Edwards et al 1984a, 1984b) does not precipitate the syndrome. Additionally, there is experimental evidence in rodents to suggest that the blood-brain barrier is poorly permeable to ALA (Meyer et al 1998). In patients with the acute attack, ALA concentrations in the CSF have been reported to be much lower than those measured simultaneously in blood, representing only 2-3% of blood levels (Percy and Shanley 1977, Bonkovsky et al 1971, Gorchein and Webber 1987). Though this may protect the central nervous system against the toxic effects of ALA, the peripheral nervous system would however remain exposed to the full blood ALA concentration, and levels at which *in vitro* effects on nerve function have been demonstrated (Bonkovsky and Schady 1982, Jordan et al 1990). There are other lines of evidence to suggest that under certain circumstances ALA *in vivo* is detrimental. Administration into the cerebral ventricle has excitatory effects (Shanley et al 1976, Pierach and Edwards 1978) and reduces seizure latency in rodents (Yeung-Laiwah et al 1987). There is also evidence that ALA at low concentrations can alter neurophysiological function, depress spinal reflexes and induce depolarization of muscle in animals (Bonkovsky and Shady 1982, Jordan et al 1990, Loots et al 1975, Nicoll 1976, Becker et al 1975), and is neurotoxic in chick embryos (Percy et al 1981). Yet ALA at considerably higher concentrations has not proved toxic to human spinal cord neurons in culture (Gorchein 1989).

Researchers have also been struck by the similarity in chemical structure between ALA, the inhibitory neurotransmitter GABA and the excitatory neurotransmitter L-glutamic acid. Evidence of interaction between ALA and these neurotransmitters has been sought (Muller and Snyder 1977, Brennan and Cantrill 1979, Brennan and Cantrill 1981). There is some suggestion that ALA can act as a partial GABA-agonist *in vitro*, but there is as yet little evidence of an effect of ALA on the GABA-ergic system *in vivo*, though ALA has been shown to mimic the inhibitory effect of GABA on the rat pineal gland (Puy et al 1996b). This may have a clinical counterpart in that a marked decrease of both day and night-time melatonin concentrations has been shown in patients with AIP (Puy et al 1993).

Haem deficiency

The acute porphyrias appear to be characterised by intracellular haem deficiency, leading to derepression of ALA synthase activity and the overproduction of porphyrins and their precursors. Since haem plays a vital role in oxidative metabolism and is found in a wide variety of biological compounds, including haemoglobin, myoglobin, enzymes of the respiratory chain such as cytochromes b and c, and the cytochrome P450 system, it has been postulated that the acute attack may represent the clinical effects of intracellular (and more specifically intraneuronal) haem deficiency (Meyer et al 1998). There is experimental evidence in both normal humans and in porphyric patients of a functional deficiency in some haem-containing enzymes. Thus impaired cytochrome P450-mediated drug metabolism has been demonstrated in patients with porphyria (Mustajoki et al 1994, Anderson et al 1976, Ostrowski et al 1983, Birnie et al 1987, Tokola et al 1988, Herrick et al 1987, Mustajoki et al 1992, Bonkovsky et al 1991), and a return to normal activity can be shown following the administration of exogenous haem. There is however no direct evidence for intraneuronal haem deficiency, and there is evidence that brain ALAS in rats is not directly induced by drugs such as phenobarbital which are known to induce hepatic ALAS strongly. This suggests

that the brain is passively affected by changes in haem synthesis occurring in extraneuronal sites, rather than being the prime seat of a disturbance in haem concentrations itself (De Matteis and Ray 1982). Secondly, when haem is administered exogenously both to humans and to rats, it has not been possible to detect a corresponding rise in the CNS haem content (Lamon et al 1979, De Matteis et al 1981). A reading of the available evidence therefore suggests that direct intraneuronal haem deficiency is unlikely to be the prime cause of the neuropathy associated with the acute attack.

There is however a reasonable possibility that intracellular haem deficiency may indirectly play a role in nerve damage via a secondary deficiency in haemoproteins. Of particular interest is the enzyme tryptophan dioxygenase. This is the rate-limiting enzyme in the metabolism of tryptophan to kyurenine. Thus, when tryptophan dioxygenase activity decreases, the levels of tryptophan increase. Since tryptophan is the substrate for tryptophan hydroxylase, the effect is an increase in serotonin formation. This sequence of events has been shown indirectly as has its corollary in that administration of exogenous haem can prevent and reverse the increase in serotonin. Several investigators have demonstrated elevated blood levels of tryptophan and serotonin and increased urinary excretion of the serotonin metabolite 5-HIAA in patients with AIP (Puy et al 1993, Bonkovsky et al 1991, Price et al 1959) and have shown that these changes in tryptophan metabolism could be reversed by the administration of haem. Thus in summary, though no causal relationship has been proven, there is experimental evidence to support a derangement in the concentrations of the neurotransmitter serotonin in patients with AIP, and to link this experimentally to haem deficiency. Since serotonin has a wide range of physiological actions in many organs of the body, including those which are the seat of the characteristic symptoms of the acute attack, there would appear to be a reasonable possibility that a haem-deficiency-induced elevation in serotonin levels may account at least in part for some of the features of the acute attack.

Haemoproteins are also involved in mitochondrial electron transport. It has thus been postulated that haem deficiency might lead to deficiencies in energy production, particularly in neuronal tissue leading to axonal degeneration. There is some evidence to support the postulate that energy production is impaired in porphyria. Muscle biopsy studies in patients with AIP have shown decreased cytochrome oxidase activity (Goldberg et al 1985), and blood lactate levels may be higher after glucose-loading in patients than in controls (Herrick et al 1990). The evidence remains tenuous. In particular, known disorders of mitochondrial function such as the mitochondrial myopathies differ in symptomatology from the acute attack of porphyria. Other haemoproteins, including nitric oxide synthase, have also been implicated, but as yet there is little experimental evidence to support these hypotheses.

Though various forms of porphyria are encountered in non-human mammals, there is, perhaps surprisingly no natural animal model of an acute porphyria in which these hypotheses can be tested. Meyer et al (1998) have produced PBG-deficient mice by a process of double gene-knockout; such mice are in effect compound heterozygotes for AIP. These mice showed evidence of neuropathy with slow movement, ataxia, impaired motor coordination and proprioceptive insensitivity. Electroneurophysiological and histological studies reveal axonal abnormalities. However, these features are seen in all mice including those who have not been treated with porphyrinogenic drugs (and have thus not been precipitated into an overt acute attack) and who presumably do not show the suddenly raised ALA levels characteristic of the acute attack. It is possible that the these mice are more a model of homozygous porphyria (which is known to be associated with vegetative dysfunction even in the absence of acute attacks as described above), rather than representing a true model for the acute attack *per se*. A strain of mice bearing the R59W mutation in the gene for PPO has recently been established by gene-knockout techniques and is now under study in our laboratory in collaboration with Dr HA Dailey of the University of Georgia.

Therapy of the acute attack

The glucose effect

Carbohydrate loading was the first specific therapy advocated for the acute porphyric attack. Early clinical studies suggested that approximately 75% of patients would respond favourably to intravenous or oral glucose administration (Rose et al 1961, Doss and Verspohl 1981, Doss et al 1985). There is evidence that ALAS activity and porphyrin synthesis are repressed when liver cells grown in culture are exposed to porphyrinogenic medication in the presence of high carbohydrate concentrations. Carbohydrate intake also inhibits the induction of hepatic ALAS in experimental porphyria and in human AIP. Conversely, there is evidence that deficient carbohydrate intake and fasting may precipitated the acute attack of porphyria, and may even increase porphyrin excretion both in healthy subjects and in experimental rats (Lahav et al 1984). This, the so-called glucose effect, is demonstrable in AIP, VP and even in non-acute porphyrias such as EPP and PCT (Doss and Verspohl 1981). In addition to glucose, fructose and glycerol appear to share this ability to reduce porphyrin synthesis. No dose-response curve has however been shown for the effect of glucose in suppressing the induction of hepatic ALAS.

Haem administration

It has been known for over 30 years that administration of exogenous haem reduces ALAS activity in both experimental systems and in patients with porphyria, and there is now an extensive literature on the effects of haem administration in patients with porphyria. This effect is mediated via a repression of ALAS by a process of negative feedback. When first introduced into clinical practice (Bonkovsky et al 1971), haem was typically given in the form of haematin. Many reports followed attesting to the efficacy of haematin in aborting the acute attack (Lamon et al 1979, Pierach et al 1980, Bissell 1988, Watson 1975, McColl et al 1981). Concerns about the safety of haematin, which appeared to be associated with transient coagulopathies, renal dysfunction, thrombophlebitis and AST elevation (McColl et al 1981, Pierach 1982, Pierach 1986, Goetsch and Bissell 1986, Dhar et al 1978) led to the introduction of haem arginate in which haem is coupled to arginine (Tenhunen et al 1987). This compound exhibits a greater stability then haematin and has a longer shelf-life; widespread experience and numerous clinical reports attest to a more favourable safety profile (Mustajoki et al 1986, Tenhunen et al 1987, Tokola et al 1987, Volin et al 1988) and greater efficacy in rapidly reducing ALA and PBG levels in the acute attack (Kirsch et al 1998, Hift et al 1997, Elder et al 1997, Mustajoki et al 1986, Volin et al 1988). Haem arginate administration is typically followed within 48 hours by evidence of clinical resolution. It is however known that neuropathy is not reversed by the administration of haem (Pierach 1982, Mustajoki 1985), and haem must therefore be administered early in the course of the acute attack before the neuropathy has become established.

Though haem arginate is effective in aborting the acute attack, the effect is short-lived. Typically ALA, PBG and porphyrin levels begin rising within 48 hours of the last of a four-day course of injections (Timonen et al 1990a, Herrick et al 1989b). Thus the role of haem arginate is to abort a crisis rather than as a prophylactic measure in patients with porphyria. It has been suggested that weekly or monthly prophylactic injections of haem arginate may serve in some cases to suppress the clinical effects of porphyria in patients subject to repeated acute attacks (Lamon et al 1978). Yet porphyrin excretion, initially suppressed in four patients with stable VP given weekly infusions of haem arginate for four weeks, and returned to pretreatment levels, and there was no improvement in skin disease (Timonen et al 1990b). Since haem is known to induce the enzyme haem oxygenase and thus to mediate its own catabolism, tolerance would appear a reasonable assumption. In our experience, prolonged,

repeated use of haem arginate has led to an apparent decrease in efficacy, suggestive of tolerance, which has been improved by the inhibition of haem oxygenase. This is discussed below, and our experience is described in Chapters 18 and 19.

Haem oxygenase inhibitors

Since haem is catabolised by the enzyme haem oxygenase, itself induced by haem, the use of haem oxygenase inhibitors to maintain haem levels has been mooted. A number of substituted metalloporphyrins will inhibit haem oxygenase; these include tin protoporphyrin, tin mesoporphyrin and zinc mesoporphyrin. Tin protoporphyrin markedly inhibits the induction of hepatic ALAS by allylisopropamide in adults rats; a dose of 50 μmol/kg body weight resulting in a 60% reduction in ALAS activity. This is accompanied by a decrease in the urinary excretion of ALA and PBG. At the highest dose, excretion was totally abolished (Galbraith et al 1985). In normal volunteers, administration of tin protoporphyrin results in a mean 38% decrease in serum bilirubin and a mean 47% decrease in biliary bilirubin (Berglund et al 1988). This is accompanied by an increased excretion of endogenous haem in bile for 48 hours. Tin protoporphyrin is rapidly cleared from plasma with a half-life of 3.4 hours though the inhibition of haem oxygenase lasts for at least four days. The activity of microsomal haem oxygenase has been measured directly in liver samples and has shown to be diminished by tin protoporphyrin (Berglund et al 1988). This work has been extended to experimental studies in patients with acute porphyrias. Administration of both tin protoporphyrin and tin mesoporphyrin, whose potency *in vivo* is approximately 5 to 10 times greater (Drummond 1987), led to significant decreases in urinary ALA and porphyrin excretion in patients with AIP and VP (Galbraith and Kappas 1989). Experience has shown that tin protoporphyrin alone is of no value in reducing ALA and PBG excretion in patients with the acute attack (Dover et al 1993). Furthermore, the combination of haem arginate and tin protoporphyrin was no more effective than haem arginate alone in reducing ALA and PBG excretion in an attack but there was evidence that tin protoporphyrin prolonged the biochemical remission induced by haem arginate.

Suppression of menstrually-induced exacerbations of porphyria

Recurrent acute attacks of porphyria related to the menstrual cycle are a problem in some female patients. The proportion of patients in whom this is a relevant factor is not known, though in the series of Kauppinen and Mustajoki (1992), approximately 30% of women with both AIP and VP reported an onset of abdominal pain prior to menstruation which might reflect an exacerbation of porphyria, though only two had actually required hospitalisation. Despite the belief that these premenstrual attacks are the results of rising oestrogen or progesterone, some patients have apparently improved while taking the oral contraceptive, including the two described above. Other patients have however suffered exacerbations on exogenous steroid therapy, leading to a search for safer and more effective therapy. Danazole, a synthetic steroid with weak androgenic activity and which decreases gonadotropin secretion, was administered to two female patients with AIP who were experiencing cyclical attacks of porphyria, but appeared to exacerbate the porphyria (Lamon et al 1979).

In view of their efficacy in suppressing the normal hormonal fluctuations of the menstrual cycle, gonadotrophin-releasing hormone analogues appeared promising candidates for the treatment of menstrually-related acute attacks (Anderson et al 1984, Bargetzi et al 1989, Anderson 1989). Anderson et al (1990) assessed the use of daily gonadotrophin releasing hormone analogue administration in six female patients with well-documented AIP and frequent cyclical exacerbations. Premenstrual attacks of AIP were reduced or eliminated and the authors concluded that this provides a safe alternative to the use of oestrogen and progesterone therapy. Herrick et al (1990) assessed the efficacy of the luteinising hormone

releasing hormone (LHRH) analogue buserelin in 7 patients with apparent menstrually-related attacks of AIP. The median number of attacks fell from seven during the baseline observation period to three during treatment. The response was variable, and the greatest improvement was seen in those patients in whom the association between attacks and the menstrual cycle was strongest.

Savage et al (1992) reported the use of a subcutaneous testosterone implant in a patient with life-threatening premenstrual attacks which were not adequately suppressed with gonadotrophin releasing hormone agonist therapy. Menstruation returned three months after discontinuation of buserelin and no serious attacks occurred thereafter. Repeated implantations were performed at 6 monthly intervals. The authors suggested that testosterone had had a beneficial clinical effect.

Case reports continue to appear, reporting the beneficial effects of LHRH analogues in single female patients with cyclical porphyria. As expected, osteopaenia is noted (Yamamori et al 1999), though supplemental oestrogen in the form of low-dose oestrogen patches appears to be tolerated (De Block et al 1999).

Other forms of therapy

Propranalol, which is a useful agent in controlling the adrenergic overactivity of the porphyric crisis, has also been suggested in several case reports to have independent activity in suppressing porphyria (Flacks 1970, Atsmon and Blum 1970, Atsmon et al 1972, Blum and Atsmon 1976, Douer et al 1978a, Douer et al 1978b). This has not found widespread use, nor has the effect ever been convincingly shown in a series of patients. It is difficult to separate any effect of propranolol from the natural tendency of the acute attack to remit once patients are admitted to hospital, precipitating factors are removed and other potentially active therapy such as carbohydrates or haematin is in progress.

15.5 CLINICAL FEATURES AND PROGNOSIS OF THE ACUTE ATTACK

Initially the prognosis of patients suffering from acute attacks was believed to be very poor and mortality rates of 18–58% have been described (Waldenström 1937, Waldenström 1957, Goldberg 1959, Eales 1963, Darocha 1971, Sorensen and With 1971, Beattie and Goldberg 1976, Moore et al 1987). However, no long-term follow-up studies had then been published and therefore the lifetime risks of manifest symptoms of porphyria amongst patients with both VP and AIP were not initially known. Later reports however suggest both a lower rate of occurrence of the acute attack as well as a more favourable outcome.

Early South African experience

Eales (1963) reviewed his experience with porphyria and reported a mortality of 22% for patients treated between 1939 and 1963. Seventeen years later, the mortality was significantly lower (Eales et al 1980). From 1963 to 1971, there had been only 3 deaths, and none from 1971 to 1980. Eales attributed this improved survival to improved treatment—particularly the introduction of mechanically assisted respiration—and also to a falling incidence of the acute attack. The number of acute attacks, which had varied between 5 and 9 cases per year from 1952 to 1968, fell dramatically to average about 2-4 per year. This striking decline was thought to be due to the initiation of a family screening program in 1963.

The review by Eales et al (1980) described the main clinical features of the acute attack in their preceding 60 patients. Though the male:female ratio of patients with skin involvement

alone was 1:1, the ratio for patients with acute attacks was 1:3. The clinical features of the acute attack are listed in Table 15-2 and are depicted graphically in Figure 19-1 where they are contrasted with the figures observed during the course of the study reported in Chapter 18. The most common features were abdominal pain, which was present in 100% of patients, tachycardia and hypertension, present in 83 and 63% of patients respectively, and vomiting and constipation, present in 78 and 64% respectively. Eales provides a memorable description of the abdominal pain which has stood us in good stead in distinguishing the pain of the porphyric crisis from that associated with other conditions:

> *The pain was often described as being continuous, deep seated, gnawing, with fluctuations in intensity which often became intolerable, leading to incessant pleas for medicinal relief. It could be relieved temporarily only with pethidine and morphine injections. Little relief was afforded by the conventional analgesics. It could be located in any quadrant of the abdomen but was most frequently situated in the upper abdomen, especially in the epigastrium but could also affect the central area or the right or left ileum fossae. Palpation often evoked complaints of severe tenderness which however was unassociated with true involuntary rigidity or rebound tenderness. In 23% of patients it was associated with severe backache and in a few patients it extended to the lower sternal area.* (Eales et al 1980).

Eales et al described neuropsychiatric features which might accompany the acute attack as follows: excessive over-reaction by the patient with hyperventilation; hysterical behaviour; and infrequent frankly psychotic behaviour. In many patients a severe confusional state associated with seizures was present, and the authors believed that this was due to water intoxication (thus presumably accompanying hyponatraemia) which they ascribed to iatrogenic inappropriate fluid administration.

	Eales et al (1980)* (%)	**Mustajoki (1980) (%)**
Abdominal pain	100	95
Abdominal tenderness	—	80
Non-abdominal pain	—	70
Backache	23	—
Hypertension	63	60
Tachycardia	83	90
Vomiting	78	65
Constipation	64	80
Epileptic seizures	27*	25
Psychiatric symptoms	36*	40
Motor disturbances	62*	80
Cranial nerve abnormalities	32*	20
Respiratory muscle paralysis	27	80
Objective sensory disturbance	20*	40
Red urine	—	80

Table 15-2. Symptoms and signs of the acute attack reported from South Africa and Finland. *Approximate figures read from a graph since exact figures are not quoted.

Neurological involvement was common in their series. In approximately 65% of cases, neuropathy was noted. Typically, this was preceded by aching pains in the limbs which were regarded as a precursor to motor paralysis. Though arreflexia was sometimes evanescent and reversible, it was usually followed by the development of profound flaccid quadriplegia affecting the proximal musculature of the pelvic and shoulder girdles. 27% of the patients in this series demonstrated involvement of the bulbar centres controlling respiration and heart action; though presumably it was profound paralysis of the diaphragm and chest wall resulting in respiratory failure rather than any problem with the central control of respiration that the authors intended.

Electrolyte disorders in the acute attack

Most patients described by Eales et al showed evidence of an elevated serum urea during the acute attack. Additionally the authors described a strikingly high incidence of hyponatraemia and hypochloraemia, with a substantial though less striking incidence of alkalosis and hypokalaemia. This confirmed their findings in several previous reports (Eales 1963, Eales and Dowdle 1971). The incidence of hyponatraemia in particular was dramatic, with 95% of patients showing this feature and 51% of patients having severe hyponatraemia, as defined by a serum sodium <120 mmol/l. In one group of patients, there was evidence of gross sodium depletion in the absence of marked urinary losses. The cause here was thought to be gastric sodium loss coupled with poor sodium intake. A second group had a high urinary sodium excretion. One possibility was a primary renal tubular sodium-losing state which the authors ascribed to a direct effect of ALA on the renal tubule. The second possibility was the syndrome of inappropriate ADH secretion (SIADH); the basis for which was poorly understood in 1980. The authors expressed doubts as to whether this syndrome would play any role in porphyria.

Precipitants of the acute attack

Many of the acute attacks described in this series were thought to have been precipitated by drugs. Table 15-3 indicates those drugs which had been implicated.

Drugs	Exposures (n)	Only precipitant (n)
Barbiturates	81	31
Analgesics	16	5
Sulphas	16	5
Non-barbiturate hypnotics	15	4
Unidentified sedatives	14	10
Miscellaneous	12	3
Anticonvulsants	10	5
Alcohol (acute abuse)	9	4
Hormonal	6	4

Table 15-3. Drugs thought to have been instrumental in inducing the acute attack, as reported by Eales et al (1980).

The Finnish experience

Mustajoki (1980) described his experience with the acute attack of VP. The mean age at the onset of the first attack in the 18 patients who had had such an episode was 33; the youngest patient was 20 and 4 patients were aged more than 40. Twelve patients had had acute symptoms once, 5 patients twice and one patient three times during their lives. barbiturates were implicated in 4 patients and infection in two. In the remaining 12 patients no precipitating factors could be traced or the history was inadequate. Fourteen patients had experienced neurological symptoms of whom ten had received barbiturates during an established acute attack, before the appearance of paresis. Two had also been given sulfonamides and one had been given meprobamate.

The symptoms and signs of the acute attack are listed in Table 15-2. Abdominal pain occurred in all but one patient and was usually associated with constipation and vomiting. Motor weakness was present in 80% of the cases and respiratory muscle paralysis in 40%. Seizures were noted in 25%. 80 patients were reported as having psychiatric symptoms, often mild, but a quarter of the patients were frankly delirious and psychotic. Seven patients (39%) died during an acute attack. Of the 48 patients still alive at the end of 1978, nine had had an acute attack previously whereas the others had always been asymptomatic.

Since Dean (1971) had suggested that the ancestors of porphyric families did not exhibit any excess mortality until after modern drugs became available, Mustajoki (1980) attempted to determine whether life expectancy for patients with VP had been higher before the modern era of readily-available drug therapy, and consequent increased likelihood of exposure to potentially porphyrinogenic medication. He assessed the life-expectancy of 13 obligate gene carriers; eleven of whom had died before 1920 and a further two of whom had died before 1940. This he compared to the life expectancies of 66 matched controls, subjects from the same family who appeared not to carry the VP gene, and whose birthdates were within 10 years of those of the carriers.There was a slight but non-significant difference in life expectancy; the mean age at death of the carriers being 62.1 and of the controls 65.1. None of the gene carriers had died before the age of 40, and more than ten of them had lived longer than 50 years.

The frequency of the acute attack in these Finnish patients was similar to that reported in South Africa by Eales (1963). Mustajoki (1980) showed that half of these attacks had occurred before 1960, when there was a poor understanding of porphyria in Finland, and that the prognosis had improved considerably since that time. He had also observed that subjects with latent VP appeared to be at low risk of developing symptoms since only two patients had experienced symptoms once the diagnosis of porphyria had been established. He advanced two theories to explain this: firstly, that education and information about the precipitating factors had allowed them to avoid acute attacks; secondly, that many patients with VP would remain symptom-free throughout their lives in any event, their identification as biochemically-positive for VP being incidental. His series included 12 asymptomatic subjects aged more than 50.

In a subsequent paper, Kaupinnen and Mustajoki (1992) conducted a prospective study amongst patients with AIP and VP, and coupled this with a retrospective review of the risk factors for the acute attack. The authors introduced an important principle when they divided their patients in the follow-up cohort into two groups. The first comprised those patients in whom porphyria was only diagnosed following a first acute attack; the second comprised those in whom the diagnosis had been established as a result of family screening before the first attack developed. The Finnish Porphyria Registry, which had been founded in 1966, contained details of 195 patients with AIP from 39 families and 73 patients with VP from 14 families who had reached the age of 18 years by the end of 1989. 206 patients with AIP or

VP, who were still alive in 1970, were followed to assess their natural history. A strength of this study is that it includes both patients with symptoms and those without and thus gives information about the prognosis amongst unselected patients with acute porphyria in general; most previous studies had included a selected group—those who expressed symptoms.

Prognosis of the acute attack

31 patients died during an acute attack, of whom 29 died before 1974. Only 2 subjects died thereafter, both were women, not included in this follow-up, who were believed to have been treated inadequately in their local hospitals. Before 1967, 44% of attacks were associated with paralysis whereas only 18% were associated with paralysis after 1967. The difference is statistically significant. Of the 16 patients who developed paralysis after 1967, porphyria had not been diagnosed in twelve at the time of the attack. Of the 4 patients who are known to have porphyria before they develop the attack associated with paralysis, two were heavy drinkers and the other two were believed to have received inadequate treatment. The difference in frequency of deaths during the acute attack prior to 1966 and after 1966 is also highly significant. During the period of follow-up, 13.6% of patients had a subsequent acute attack. An interesting finding of this study was that the likelihood of an acute attack was significantly higher in patients who had had previous attacks than in those who had not. This difference was statistically significant. No difference in the risk of an attack could be shown between those with AIP and those with VP.

Thus acute attacks had become less frequent and mortality significantly lower during the two decades prior to publication. Severe attacks accompanied by paralysis had also become less frequent. This supports the opinions expressed by other authors (Bonkovsky and Schady 1982, Kappas et al 1989, Tschudy and Lamon 1980) that the prognosis for patients with AIP and VP has improved with time. The authors suggest three reasons why the prognosis had improved. Firstly, disease was now detected earlier because of better diagnostic testing and an increased awareness amongst doctors. Secondly, treatment of the acute attack had improved. Thirdly, asymptomatic patients diagnosed early were able to take precautions and, in the event of acute symptoms, the acute attack was recognised and treated more quickly.

Prevalence of acute symptoms by self-reporting

Questionnaires were sent out to all patients requesting information on symptoms of their porphyria. 75 patients (47% of the total group, 56 with AIP, 19 with VP) reported some symptoms which they had interpreted as due to their porphyria. Those who had had acute attacks previously reported more symptoms and there was a male predominance (56% vs 32%); the difference was statistically significant. Fewer than 14% of patients with AIP or VP in this study experienced acute attacks requiring hospitalistation. The authors correctly caution that this figure of 47% may be excessive as a result of symptoms due to other causes being ascribed to the porphyria. However, they do conclude that milder symptoms of porphyria may occur much more frequently than severe acute attacks, and are more common amongst patients who have had a previous attack than amongst those who have not.

Precipitating factors for acute symptom by self-reporting

Kaupinnen and Mustajoki's subjects reported the following factors as having contributed to their symptoms: infection (29%), alcohol (25%), fasting (12%), drugs (10%), menstruation (30%). An interesting finding was that acute symptoms were often associated with several simultaneous precipitating factors. An important corollary was that many of the patients regularly use drugs, many of which were believed to be unsafe in porphyria, yet remained well. Aggravation of porphyria was also an uncommon complication of surgery and

anaesthesia. It further appeared that moderate use of alcohol seldom causes problems, though heavy drinking may be associated with an increased risk of the acute attack.

Thus the authors drew the important conclusion that, though patients were frequently exposed to precipitating factors, these were seldom associated with serious problems. They therefore suggested that strict avoidance of all known precipitating factors was unnecessary amongst patients with AIP or VP unless they were prone to the acute symptoms. This latitude however extends only to patients in the quiescent phase, and not to those who have had frequent attacks or who are experiencing an acute attack currently.

Menstrual effects, pregnancy and sex hormone therapy

30% of 95 women had cyclical symptoms suggestive of porphyria, though the authors suggest that some of these symptoms were probably not related to porphyria. The symptoms were described as abdominal pain accompanied by pain in the extremities a few days before menstruation and extending through the first few days of the menstrual period. It was often associated with constipation. The frequency varied from 3-12 times per year. No difference could be found in the appearance of this symptom in those who had previous attacks and those who had not. These findings were not confined to patients with AIP, and 31% of women with VP also reported these symptoms. Two patients required hospitalisation repeatedly in the premenstrual phase because of acute attacks, and these were prevented by the administration of the oral contraceptive. Pregnancy was uneventful in 92% of cases. Acute symptoms however developed in 14 pregnancies (8%) in 12 women (9 with AIP, 3 with VP). The acute attack followed delivery in seven of these pregnancies. Of 12 women who had had symptoms of porphyria previously, four subsequently became pregnant without aggravation of the porphyria. A single patient reported symptoms of porphyria during all three of her pregnancies, but porphyria had not been diagnosed until after this series of events.

44 of 95 women (46%) had used sex hormone preparations either for contraception or for menopausal symptoms. Six of these women (14%) had had suggestive symptoms during the taking of these preparations though only 2 (4.5%) had had an actual acute attack requiring hospitalisation. Thus the authors concluded that cyclical symptoms were relatively common but that menstrually induced acute attacks requiring hospitalisation were rare. Pregnancy was rarely associated with the aggravation of porphyria, and most pregnancy-associated attacks had occurred in patients not yet known to have porphyria, which may indicate that other precipitating factors were also present. Oral contraceptives appeared generally safe for patients with porphyria and fewer than 5% had experienced an acute attack during their use.

Operations and anaesthesia

In total, 142 of 158 patients (90%) had had local anaesthesia for dental or other minor procedures without any complications. Ninety (57%) had undergone 163 operations while in an asymptomatic phase, but there were only3 post-operative acute attacks (1.8%). All of these were in patients with a history of previous acute attacks.

Other associations with porphyria

There was evidence that hypertension and renal failure were more common amongst patients with porphyria than amongst the general population, and a clear association was shown between hepatocellular carcinoma and AIP. Cardiovascular disease and diabetes were not over-represented. 43 of the 158 patients (27%) reported neuropsychiatric symptoms during the asymptomatic phase though only three had actually received psychiatric care. The age- and sex-specific prevalence rates of these symptoms were no higher amongst patients with porphyria than in the general Finnish population with the exception of females with porphyria aged 30-44 years, who appeared to have a slight excess of mild psychiatric symptoms.

The influence of anaesthesia and pregnancy on porphyria, and the associations of hypertension, renal disease, psychiatric symptoms and carcinoma are described in more detail later in this chapter.

Causes of death

96 subjects were deceased by the time this study was terminated. Porphyria had been the cause in 31 cases (32%), with a mean age of death of 36 years (range 17-67). There were 8 cases with carcinoma and 5 cases of other malignancies, whereas the other deaths were due to a variety of causes.

The United States

More recently a study has been published from America (Jeans et al 1996) assessing the cumulative survival of 136 patients with AIP who were hospitalised for porphyric attacks between 1940 and 1988. At the time of diagnosis, patients had an average age of 32 years (range 9-75); the male:female ratio was 43:93. Recurrent attacks were the rule, and were experienced by 109 patients. At the time of follow-up, 19 males (44%) and 31 females (33%) were deceased. Compared to age-matched controls, the standardised mortality ratio was 3.2. Most deaths had occurred during the initial porphyric attack (20%) or a subsequent attack (38%). Interestingly, suicide had occurred in five subjects. There was evidence for improved survival in the group who had had acute attacks after 1971, the year in which haematin became available, though this did not reach statistical significance.

Current opinion on the incidence of the acute attack

Currently acute attacks are relatively uncommon; and most patients with any of the acute porphyrias will never experience an attack at all. Indeed, the incidence of acute symptoms has been estimated to be 10-20% for AIP (Kappas et al 1995), 30% for HCP (Martasek 1998) and 9%, our own initial estimate, for VP (Kirsch et al 1998). No large series of patients with the acute attack have appeared recently, though single case reports highlighting aspects of management, such as anaesthesia, pregnancy and the puerperium (Wenger et al 1998), complications such as paralysis and metabolic disturbances, and alternative forms of therapy, such as somatostatin combined with plasmapharesis (Medenica et al 1997), antioxidant administration (Thunell et al 1997) and cimetidine (Rogers 1997) continue to appear.

Anaesthesia and porphyria

That porphyria is unpredictable in its response to agents known to be capable of inducing the acute attack is shown by the work of Mustajoki and Heinonen (1980), who studied the effects of anaesthesia in Finnish patients with AIP or VP. They identified 37 patients (23 with AIP, 14 with VP) who had previously suffered acute symptoms, but were free of symptoms at the time of anaesthesia. These 37 patients received a total of 62 general anaesthetics. None developed an acute attack, despite the use of potentially dangerous medication. Specifically, 18 patients (11 with AIP, 7 with VP) received barbiturates during 29 anaesthetic exposures; 4 of these patients had received barbiturates on 2 occasions, and 2 patients had received barbiturates 4 and 5 times respectively without ill effect. By contrast, 14 patients underwent general anaesthesia in the presence of acute symptoms of porphyria. Ten of these patients underwent laparotomy or appendicectomy and no cause for abdominal symptoms, which presumably were due to porphyria, was found. Ten of these patients receive thiopental. In seven, the severity of the porphyric symptoms worsened postoperatively and three patients developed paralysis.

The conclusion drawn from this study is that patients in a latent stage of porphyria incurred only a slight risk of progressing to a porphyric crisis during anaesthesia, even where powerful inducing agents such as barbiturates are administered. In contrast, administration of such agents to patients who are already in an established acute attack is extremely hazardous. Thus the authors suggest that drugs known to induce acute porphyria may in many instances be safely used in patients with quiescent forms of these diseases; which in turn implies that case reports indicating safe use of a particular agent in an individual porphyric are of relatively little value. Precipitation of the acute attack is a complex event, and probably involves the synergistic results of more than one factor.

Pregnancy and porphyria

Initial reports on the outcome of pregnancy in patients with the acute porphyrias were disquieting (Nielsen and Nielsen 1958). Thus Hunter (1971) reviewed 72 reported cases with a total maternal mortality of 27%; amongst primigravidae, maternal mortality was 52%, though Bloch (1965) had reported a better outcome for VP. Brodie et al (1977) analysed the obstetric histories of 50 women with acute porphyria: 39 with AIP, 8 with HCP and 3 with VP. 54% of patients with AIP had an acute porphyric attack in pregnancy and the puerperium though only 24% of pregnancies were affected. The figure for VP was 1 of 3 patients in a total of 8 pregnancies. Two-thirds of the acute attacks occurred antenatally and one-third after delivery. The total fetal wastage was 13% and babies born following an acute attack were smaller than those with an uneventful pregnancy. Pregnancy was thought to be safer in patients with HCP than with AIP since 25% of the patients had had an attack but only 4 of 26 pregnancies were affected. Fetal wastage was 16%.

Of the 50 women, 25% had an initial diagnosis of porphyria made only during pregnancy or in the puerperium. However, the attack rate was stated to be no different in those diagnosed during pregnancy as compared with those in whom the porphyria was known before pregnancy. Thus, the authors suggested that inadvertent administration of medication alone was not the most important factor. In only 8 attacks were porphyrinogenic drugs implicated and these were usually barbiturates. There was one maternal death in this series. The total fetal wastage of 9% compares well with the 12% abortion rate quoted from a survey of normal women at the time. Thus the fetus did not appear to be deleteriously affected. Brodie et al postulated that the high incidence of acute attacks in pregnancy was most likely hormonal in origin. The article concludes that despite the high rate of attack during pregnancy in this study, the mortality was low and this therefore suggested that the porphyric pregnancy has a better prognosis for both mother and fetus than a reading of the earlier literature would suggest. Case reports of acute attacks occurring during pregnancy and the puerperium continue to appear (Keung et al 2000).

15.6 OTHER ASSOCIATIONS WITH PORPHYRIA

Psychiatric manifestations

Crimlisk (1997) has recently reviewed the associations between porphyria and psychiatric illness. The first indication that there might be an association was the description by Karlbling et al (1961) which reported that 35 of 2500 psychiatric patients admitted to a short term psychiatric unit screened positively for PBG with the Watson Schwartz reaction. Twelve of these were reported to have manifest AIP on clinical grounds. This gave a point prevalence of 0.48%. A follow-up study 11 years later in Australia (McEwin 1972) used a quantitative PBG analysis alone and found a prevalence of 0.16%. Subsequently, Tishler et al (1985) screened

nearly 4000 psychiatric patients by the Watson Schwartz reaction, confirming positive results by the measurement of 24-hour excretion of ALA and PBG. 70 patients screened positive, of whom 8 were thought to have manifest AIP on the basis of further tests which included PBG deaminase assay. A further 10 positive patients were thought to have AIP though no association could be made between their clinical picture and the condition since the absence of raised urinary porphyrin precursors suggested quiescence. They concluded that the point prevalence for AIP was 0.21%. The psychiatric diagnoses included schizophrenia, schizo-affective disorder and atypical psychosis. It is currently believed that the studies may well have overestimated the presence of porphyria in view of the deficiencies of Watson-Schwarz screening for PBG alone as a diagnostic test for porphyria. In no study was any control group employed and the prevalence of porphyria in the community from which the patients admitted to these institutions were drawn is unknown. Nor has the eventual outcome of these patients been reported. It is not known whether withdrawal of psychotropic medication in any way modified their clinical histories. The link between porphyria and overt psychosis in the absence of the acute attack remains unproven.

The association between porphyria and psychiatric illness, supposedly identified by the demonstration of an excess of porphyria among psychiatric poulations, has become a commonplace in both the medical and the lay literature, yet careful studies in unselected populations of porphyric patients have failed to lend significant support. Wetterburg's study in Swedish patients (Wetterburg 1967) had suggested that neuropsychiatric symptoms are not associated with AIP or VP in remission. The study by Kaupinnen and Mustajoki (1992) confirmed these findings, though there was a suggestion of a slightly increased risk of generalized anxiety in females during early middle age. Since this was purely based on a self-reported questionnaire, the case was thought to remain unproven.

Patience et al (1994) undertook a study to determine whether major mental illness co-segregated with acute intermittent porphyria in families where the two conditions are found. They identified 16 individuals who had had psychiatric contact from the case records of 344 consecutive patients held by the porphyrias research group in Glasgow. Twelve of these were available for study. Of these twelve probands, 9 were asymptomatic carriers of AIP. Forty relatives were interviewed for a history of mental illness and for current symptoms. No association was found between AIP and schizophrenia or manic-depressive illness. Only one patient with schizophrenia was identified amongst these 344 subjects. Bipolar illness was identified in two families with AIP but failed to co-segregate with the disease. The commonest psychiatric diagnosis assigned in these patients was generalised anxiety.

In contrast, confusion and psychosis occurring as part of the acute attack are reported in reliable series (Elder et al 1980, Mustajoki 1980), but this does not appear to extend to patients with quiescent porphyria.

Hypertension and renal failure

An association between hypertension, renal disease and AIP has been suggested, though the data on which these claims are made have always been difficult to interpret. In a large family with acute porphyria (Church et al 1992), a prospective study showed a significant difference between mean systolic and diastolic blood pressure and plasma creatinine between porphyric and non-porphyric subjects. Five of 19 porphyric subjects in the third generation died of the complications of chronic hypertension, with renal failure in three. Overall, 62% of those subjects tested had hypertension and 50% showed renal impairment; neither hypertension or renal failure were noted in the apparently non porphyric subjects. Yet this extraordinarily strong association does not appear to hold in general; one suspects that the presence of a

second inherited condition which, interestingly, must co-segregate with porphyria in this family.

A recent population-based case-control study based on mortality data (Andersson and Lithner 1994) suggested that hypertension is more common in patients with severe AIP (56%) in comparison with controls and patients with latent AIP (23% and 16% respectively). Renal disease did not appear more common in patients with AIP than in controls, though renal function was impaired in 3 of 8 patients with severe recurrent AIP; one of these however had another cause for this in the form of nephritis in association with systemic lupus erythematosus. Hypertension was commonly registered on the death certificates of patients with manifest AIP (68%), compared with 21% of those with latent AIP; however neither myocardial infarction nor stroke were more common. The authors suggest that hypertension is more common in patients with manifest AIP than in those with latent AIP or control subjects. By contrast, they found no evidence of an association between hypertension or renal disease and VP.

Subclinical neuropathy

Severe motor neuropathy is a well recognised complication of the severe or untreated acute attack of porphyria. Indeed, most of the symptoms of the acute attack are ascribed to an autonomic neuropathy. Arising from the observation that a subclinical neuropathy may be present despite an absence of symptoms in lead poisoning (Seppalainen and Hernberg 1972), Mustajoki and Seppalainen (1975) investigated peripheral nerve conduction velocities in 20 patients with AIP, 5 with VP and 25 age- and sex-matched controls. Only nine of these porphyric patients had never had symptoms of porphyria. A further thirteen had had symptoms of acute porphyria 1-12 years earlier (acute neurological symptoms or acute abdominal symptoms) and two of these showed slight neurological deficits. The other three subjects complained of recurrent non-specific pains or paraesthesiae. The results suggested a subclinical neuropathy. The conduction velocity of slow motor fibres of the ulnar nerve, and the sensory conduction velocity of the median and ulnar nerves were significantly slower in patients when compared with controls. The conduction velocity of slower fibres was the same in subjects who had had previous acute symptoms and those who had not. Motor and sensory conduction velocity showed a trend towards lower values in those who had had acute symptoms as compared to those who had not, but the difference was not significant. Nine patients were studied with electromyography. One of these had no prior symptoms and was normal. The other eight had all had prior symptoms and showed changes compatible with denervation. No correlation could be shown with age, sex, type of porphyria or the excretion levels of porphyrins.

In their discussion, Mustajoki and Seppalainen (1975) state that abnormalities in the conduction velocity of slower fibres were also evident in porphyric patients who had never had symptoms. These results are however not given directly, and the frequency or importance of this finding cannot be assessed from this report. The authors make the point that the tests of neuronal function they employed are more sensitive than the usual routine tests used in neurophysiological laboratories which may explain why they found abnormalities even in asymptomatic patients; others have reported normal motor conduction velocities or rapidly reversible abnormalities even in patients with neurological manifestations (Simpson 1962, Maytham and Eales 1971). That patient who have previously experienced an acute attack retain objective neurological deficits years later is perhaps not surprising. A more interesting conclusion from this study is that patients who have never experienced symptoms may demonstrate a subclinical neuropathy. Insufficient data are given in this paper to assess the importance of this, and to determine whether it applies to VP as well as AIP. Relevant,

however, are the histological findings in sural nerve biopsies of 2 patients with HCP reported by Di Trapani et al (1984). They found evidence of axonal degeneration in 2 patients with HCP, one of whom had had an acute attack six months before, the second of whom had never experienced an acute attack though he had clinical and electrophysiological evidence of chronic progressive neuropathy.

Similar results have subsequently been presented for other patients with AIP (Kochar et al 2000). In a study of 25 patients, all of whom had had previous acute attacks and were now in the latent phase, 19 demonstrated one or other abnormal neurophysiological parameter, which included reduced motor and sensory nerve conduction velocities, late responses and median nerve somatosensory evoked potentials. Reduced nerve conduction velocity appeared to have no relationship with age, sex, or duration of illness or the number of attacks which had been experienced. Others have failed to demonstrate reliable abnormalities in patients with latent AIP and in those with no symptoms whereas obvious changes were present in patients with clinical neuropathy (Flugel and Druschky 1977).

Tests of autonomic functions have also been reported in acute intermittent porphyria. Yeung-Laiwah et al (1985) reported evidence of both parasympathetic and sympathetic dysfunction during the acute attack in subjects with AIP, which improved during remission. However, mild parasympathetic dysfunction was also detectable doing remission and in latent asymptomatic AIP.

Hepatocellular carcinoma

An association between hepatocellular carcinoma and AIP in Sweden has been suggested by Lithner and Wetterberg (1984). Kauppinen and Mustajoki (1988) searched the Finnish register of porphyrias and recovered details of 82 patients, of whom 63 had AIP and 19 VP, who had died since 1929. The cause of death was reported as hepatocellular carcinoma in 7 AIP patients and 1 VP patient. This gives a relative risk of dying of hepatocellular carcinoma of 61. There is a strong association with AIP, and at first sight the relative risk must be greatly increased in VP (1 patient of 8). The authors caution however that no conclusions can be drawn on the basis of one patient. Insufficient details were available for the importance of other risk factors for hepatocellular carcinoma to be adequately assessed. Thus hepatitis B surface antigen was tested in only one patient, who was negative. One patient had a history of hepatitis and a period of alcohol consumption. There were however no grounds to believe that factors other than AIP had been present in these patients. More recent studies have confirmed the increased risk of cancer in AIP and PCT, largely as a result of an excess of hepatocellular carcinoma, though a small excess of lung cancer (and of mortality from cirrhosis and emphysema) was found. Any association with VP was not specifically assessed (Linet et al 1999).

A case report from Tidman et al (1989) reported the onset of porphyric skin disease in a 70 year old female in whom a hepatocellular carcinoma was diagnosed. Investigation of porphyrin excretion patterns was thought to demonstrate a pattern typical of VP. One notes however that the plasma emission scan was maximal at 620 nm, which would suggest PCT rather than VP. The authors suggest that the most likely explanation for the association in this patient was that the tumour was coincidental, but resulted in some intrahepatic cholestasis with reduced biliary excretion of porphyrins and precipitation of the cutaneous syndrome as has previously been described for VP (Fowler and Ward 1975). A second possibility was that the hepatocellular carcinoma resulted in a tumour-induced secretion of porphyrins as a true paroneoplastic phenomenon. This association has previously been reported for PCT (Solis et al 1982, Grossman and Bickers 1978) but is rare. No definitive diagnosis was however possible in this patient and the discussion cannot be taken further.

Cholelithiasis

An association between EPP and cholelithiasis has been recognised since the first report by Magnus et al (1961). Gallstones associated with EPP contain protoporphyrin (Cripps and Scheuer 1965), which is to be expected since EPP is associated with the increased biliary excretion of protoporphyrin. Herrick et al (1991) reported four patients with VP who were diagnosed as having cholecystitis. Gallstones were recovered at surgery in two patients; the first contained an excess of protoporphyrin, the second was not assayed but fluoresced under ultraviolet light. VP is associated with the increased excretion of protoporphyrinogen in bile (Bloomer et al 1990), and the authors postulated that protoporphyrinogen undergoes auto-oxidation to protoporphyrin in bile with consequent stone formation.

15.7 THE CLINICAL STUDIES

The clinical features of VP, the syndrome of homozygous VP and the pathogenesis, presentation and management of the acute attack have been reviewed in this chapter. Our personal experience with the clinical presentation of VP is described in the following chapters. In Chapter 16, we describe the South African experience with HVP. In Chapter 17, the clinical features of VP in a large family, fully characterised by the presence or absence of the R59W mutation, are described. In Chapter 18, a personal experience with the acute attack of both AIP and VP is described. Brief conclusions are drawn at the end of each chapter and a full discussion follows in Chapter 19.

CHAPTER 16:

HOMOZYGOUS VARIEGATE PORPHYRIA: THE SOUTH AFRICAN EXPERIENCE

16.1 INTRODUCTION

The syndrome resulting from homo-allelic inheritance of a single mutation or hetero-allelic inheritance of two mutations in the PPO gene (HVP) appears to be distinct from the clinical syndrome accompanying heterozygous VP (Chapter 15). It is noteworthy that HVP does not appear to be common in South Africa despite the very high prevalence of the R59W mutation. Our experience with HVP in South Africa is described.

16.2 OBJECTIVES

- To describe the clinical features associated with the syndrome of homozygous VP.
- To identify and describe those mutations associated with homozygous VP in South African patients.

16.3 METHODS

Subjects

Four patients with biochemical evidence of VP in whom we have diagnosed HVP on the basis of clinical appearance and DNA studies are described.

Biochemical diagnosis

Urine, stool, plasma and erythrocyte porphyrins were analysed by quantitative thin-layer chromatography and quantitative fluoroscanning as described in Appendix 1.

PPO activity

In the first subject, PPO activity was determined on Epstein-Barr virus transformed lymphoblasts, using the methods described in Appendices 2 and 3.

Identification of mutations

The mutations discussed in this chapter have been described in Chapters 3 and 4. The R59W and R168C mutations were first identified in cloned sequences derived from lymphoblasts of the first subject. The R59W mutation was identified in the remaining three subjects by *AvaI* restriction assay. The Y348C and R138P mutations were first identified by SSCP/heteroduplex analysis and confirmed by direct automated sequencing. For family studies, the R59W mutation is identified by *AvaI* restriction assay, the R168C mutation by *BsaJI* restriction assay, the Y348C mutation by *MaeIII* restriction assay and the R138P by SSCP/HD analysis. All methods are described in Chapters 3 and 4 and Appendix 4.

16.4 RESULTS

The porphyria diagnostic service at UCT has diagnosed HVP in 4 subjects. The DNA findings are summarised in Table 16-1, and the patients are described below.

Family	Subject	Allele I	Allele II
I	LO	R59W	R168C
II	CvdM	R59W	Y348C
III	DdP	R59W	R138P
	AN	R59W	R138P

Table 16-1. Summary of the molecular findings in three families in whom HVP has been recognised.

Proband 1: LO

Clinical description

This female child was born in 1986. Severe photosensitivity was noted from birth, when she developed severe blistering of the face and hands during phototherapy for neonatal jaundice. Porphyria was not diagnosed until the age of 9 months, when porphyrins were sent to the UCT laboratory for screening. As she grew older, she showed evidence of blisters, erosions and scars on the hands and face (Figure 16-1). In contrast with heterozygous VP, skin disease was also apparent on the scalp, with consequent loss of hair. Skin changes were also noted in areas not exposed to the sun, and the skin was thickened and infiltrated on the proximal arms and legs, and on the trunk and buttocks. Skin biopsies from sun-exposed areas were abnormal with striking perivascular PAS-positive non-fluorescent hyaline deposits in the papillary and upper-reticular dermis. Features of acute and chronic photo-damage with basket weave hyperkeratosis, epidermal atrophy, necrotic keratinocytes and marked dermal solar elastosis were also present. Histological changes were also noted in the non-exposed skin but were less marked, consisting largely of hyaline deposits around papillary dermal vessels. During the first seven years of life, she experienced a recurring problem of staphylococcal skin infection, particularly of the fingers, which was complicated by septicaemia on several occasions. Permanent cloxacillin prophylaxis proved highly effective in preventing these infections and was continued until the age of 9.

Other obvious features were severe myopia and a pendular nystagmus. Particular striking is the severe brachydactyly (Figure 16-2). The fingers and toes are markedly foreshortened. This has resulted in severe functional impairment such that she cannot grasp a pencil nor use a pair of scissors. Radiographs (Figure 16-3) showed brachydactyly with broad short metacarpals and phalanges and a delayed bone age. Of particular concern to the parents was a tendency to self-mutation; particularly compulsive gnawing and biting of the damaged fingers. This did not appear to be accompanied by pain. Furthermore, her parents had observed that the child had on several occasions burnt her fingers on a stove without any apparent discomfort. A paediatric neurologist confirmed the presence of a gross sensory neuropathy of the hands and feet. Electrophysiological studies of nerve conduction demonstrated a reduced amplitude of conduction in both median sensory nerves in keeping

with a sensory neuropathy. No motor abnormalities were demonstrable. This is in marked contrast to the typical neuropathy accompanying the acute attack of heterozygous VP, which is predominantly motor. Histology was performed on a sural nerve biopsy. A paucity of small unmyelinated and myelinated fibres was shown as well as some Wallerian degeneration, but the most noticeable feature was a marked widening of the nodal gaps with a loss of Schwann cell cytoplasm over the gap in keeping with early segmental demyelination. Neurological development was delayed and at 6 years her perceptual and motor development were about 1 year behind the expected stage of development. She demonstrated appropriate verbal skills and, in all other respects, normal intelligence. Though she was reported to have had infrequent complex partial seizures; electroencephalography is normal and the evidence for this is not convincing.

She is currently 13 years old (Figure 16-4). She remains in a good health. The skin and finger changes are more severe and she is of small stature. She is performing adequately at a special school for the physically handicapped. She is reported to have behavioural abnormalities including a reduced attention span, hyperactivity and a low threshold for frustration. She has been receiving methylphenidate (Ritalin®) with good effect and with no apparent deterioration of porphyria. In the past five months she has begun menstruating. This is not been associated with any aggravation of porphyria. At no stage has she suffered symptoms suggestive of an acute attack.

Biochemical results

The results are compatible with VP (Table 16-2). An elevated erythrocyte protoporphyrin concentration is additionally noted.

PPO activity

A severe reduction in activity to less than 10% of the control value was demonstrated in the proband. Her mother showed a 50% reduction in activity in keeping with heterozygous VP. PPO activity in her father was reduced by approximately 25%, which is significantly less than the 50% reduction shown in a large group of heterozygous South African patients with VP (Meissner et al 1986).

DNA studies

These are fully described in Chapters 3 and 4. The proband is an R59W/R168C compound heterozygote (Meissner 1996). The mother carries the R59W mutation and expresses VP biochemically though she is asymptomatic. The father, who is originally of Dutch and German descent, carries the R168C mutation and is both biochemically and clinically silent.

Family studies

There was no family history of VP in either the maternal or the paternal lineages. The proband has 2 younger siblings. Neither has shown clinical or biochemical evidence of VP. The elder sibling carries neither the R59W or the R168C mutation: the younger child has yet to be tested.

Proband 2: CvdM

The second subject is currently aged 7 years. She is of Afrikaner descent, the daughter of unrelated parents. She first presented at the age of 10 months with severe skin disease following which VP was confirmed by biochemical testing.

Subjects	ULN	LO	CvdM	DdP	AN
Urine precursors (umol/10 mmol creatinine)					
ALA	<45	16	16	106	84
PBG	<16	9	8	106	24
Urine (nmol/10 mmol creatinine)					
Uroporphyrin	<20	31	12	1464	384
C7	<1.5	0	18	570	222
C6		0	0	178	55
C5		0	6	1296	203
Coproporphyrin	<240	101	177	4538	478
Stool (nmol/g dry weight)					
Uroporphyrin	<1.7	5	2	33	0
C7		0	0	73	0
C6		0	0	120	0
Pseudo C5		0	23	376	130
C5		13	2	161	0
Isocoproporphyrin		0	0	114	0
Coproporphyrin	<50	67	69	1212	620
C3		0	0	634	0
Protoporphyrin	<200	698	432	1804	407
Plasma (nmol/l)					
Uroporphyrin	<2.5	2	2	13	10
C7		0	0	4	0
C6		0	0	10	0
C5		0	0	0	0
PU		0	20	23	26
Coproporphyrin	<1.0	26	0	0	0
Protoporphyrin	<4.5	46	44	16	51
Red cells (nmol/l)					
Uroporphyrin		0	0	0	—
C7		0	0	0	—
C6		0	0	0	—
C5		0	0	0	—
Coproporphyrin	<80	0	0	0	—
Protoporphyrin	<800	4406	5500	2580	—

Table 16-2. Biochemical values in 4 subjects with HVP. ULN: Upper limit of normal

Clinical description

The subject shows a clinical picture markedly similar to that of LO. She has obvious skin changes in sun-exposed areas compatible with a diagnosis of VP: blisters, scars, pigmentary changes and milia. Unexposed skin also appears to have an abnormal consistency. She has marked brachydactyly (Figure 16-5), photo-onycholysis, myopia, nystagmus, a sensory neuropathy and problems with concentration but is otherwise of normal intelligence. She has never experienced symptoms suggestive of an acute attack.

Biochemical results

These are shown in Table 16-2. The results are compatible with VP. The erythrocyte protoporphyrin is additionally raised.

DNA studies

These are described in Chapter 4 and in a subsequent publication (Corrigall et al 2000). The proband was found to be a R59W/Y348C compound heterozygote. The R59W mutation is carried on the maternal allele and the previously unreported Y348C mutation on the paternal allele. Several maternal and paternal family members carry the R59W and Y348C mutations respectively. The Y348C mutation was shown to be a *de novo* mutation in the father.

Family studies

Neither parent reports a family history of VP, and neither was aware of porphyria prior to diagnosis in the child. The proband's mother demonstrated a positive biochemical porphyrin profile for VP whereas the father's was normal. He did however demonstrate a small peak on plasma porphyrin scanning. The results of genetic and biochemical testing are summarised in the pedigree shown in Figure 4-20. No relative has ever experienced clinical symptoms of porphyria. Her mother (R59W-positive) and a paternal half-brother (Y348C-positive) show a biochemical profile compatible with VP, whereas no other first-degree relatives have biochemical evidence for VP either on TLC or on plasma fluoroscanning.

Patients 3 and 4: DdP and AN

Clinical description

Proband 3 is a young mixed-race female patient who first presented to our unit at the age of 19 with an acute porphyric crisis. No diagnosis of porphyria had been made either in the proband or the family before this. She is currently 26 years old. She has subsequently experienced two mild acute attacks. She has otherwise remained in good health, and has two healthy children. Her skin disease is unusually severe in comparison with that of typical patients with VP. Her hands and face show evidence of chronic blistering, thinning and atrophy of the skin and hypertrichosis (Fig 16-6). She shows deformities of the hands with a degree of shortening of the fingers and deformities of the finger joints (Figure 16-7), though reasonable function is maintained. She shows no ocular, developmental or neurological abnormalities. The clinical features are considerably less marked than those of probands 1 and 2, and the possibility of HVP was not initially entertained.

Patient 4 is her 23 year-old sister. She developed typical skin changes of VP at the age of 19. Since VP had ready been described in her sister, the diagnosis was made promptly. The skin disease is typical of VP and would be classed as moderately severe. There are no finger deformities and no evidence of skeletal, ocular or neurological disorder. She has never experienced acute symptoms.

Biochemical studies

Both patients show a porphyrin profile compatible with VP (Table 16-2); the sample shown for DduP was taken during the her first acute attack and therefore shows elevated ALA and PBG concentrations. Her erythrocyte protoporphyrin is additionally elevated.

DNA studies

Initially these two subjects were diagnosed as VP, unqualified, though the skin disease was felt to be unusually severe in Proband 3. When DNA testing became possible, both were confirmed to carry the R59W mutation. However, we subsequently considered the possibility of a second mutation in DduP in view of the unusually severe phenotype. SSCP/HD analysis followed by direct automated sequencing revealed an additional R138P mutation in DdP and subsequently in AN (Chapter 4). Both are therefore R59W/R138P compound heterozygotes.

Family studies

Both Proband 3's prepubertal sons have been shown to carry the R59W mutation. Patient 4's 13-year-old-daughter is negative for the R59W mutation and carries the R138P mutation. Biochemical testing has confirmed VP in the mother, and was negative in two brothers. The father has refused testing. Family studies are incomplete.

16.5 CONCLUSIONS IN BRIEF

The clinical, biochemical and molecular features of four subjects with HVP have been described. No subject is an R59W homozygote: all are compound heterozygotes heteroallelic for the R59W and a second mutation. There is evidence of considerable phenotypic variation, ranging from severe abnormalities in the R59W/R168C and R59W/Y348C compound heterozygotes to milder abnormalities in the R59W/R138P compound heterozygotes. Furthermore, clinical severity was itself variable in two sisters carrying the R59W/R138P genotype. These results are discussed further in Chapter 19.

Figure 16-1. Proband 1: The face of LO as a young child. Marked skin disease is evident.

Figure **16-2.** Proband 1. The hands of LO. Severe skin disease and marked brachydactyly are present.

CHAPTER 17:

THE CLINICAL AND BIOCHEMICAL EFFECTS OF THE R59W MUTATION IN A SINGLE FAMILY

17.1 INTRODUCTION

The proportion of subjects carrying a VP-associated mutation who express the disease, either biochemically or clinically, is not known with certainty. Some subjects who express VP biochemically appear to remain asymptomatic thoroughout their lives and it is known that other "silent" subjects have normal stool porphyrin excretion profiles. Such cases were initially identified as obligate carriers who had transmitted the defect to the next generation despite themselves or who were shown to have reduced PPO activity in lymphoblast PPO assays (Meissner et al 1986) yet appeared normal on biochemical testing. More recently, silent carriers have been directly identified by the demonstration of a VP-associated mutation associated with normal porphyrin excretion patterns (Chapter 5).

Two previous studies have attempted to assess the extent to which VP is clinically expressed in South Africa. Eales et al (1980) suggested that 90% of patients with VP experienced acute attacks, skin disease or both. A later survey (Hift et al 1997) indicated that there had been a change over the period between the two studies, with a decreasing proportion experiencing acute attacks, correspondingly more experiencing skin disease only and a large group being clinically silent but detected as a result of the investigation of affected families (Table 13-1).

	Skin	Acute	Both	Latent
Eales et al (1980)	52%	17%	21%	10%
Hift et al (1997)	45%	10%	9%	38%

Table 17-1. Frequency of clinical manifestations of VP in Cape Town as reported in 1980 and 1997.

In both studies, the sample population consisted of patients proven biochemically to have VP, and are therefore weighted in favour of those with biochemically manifest disease. Silent carriers would not be included since they would have been undetectable before the advent of DNA testing. Furthermore, there is a strong element of ascertainment bias in that a substantial proportion of patients are tested as a result of their symptoms. To determine the true prevalence of symptoms among an unselected VP population, it is appropriate to test all members of a sufficiently large family or group of families, such that both symptomatic and asymptomatic gene carriers stand equal chances of been identified. This information is then correlated with the presence of biochemical and clinical abnormalities.

Accordingly, with simple and accurate methods for the identification of the R59W mutation at our disposal, we initiated a study to determine the degree to which carriage of the R59W mutation is expressed both biochemically and clinically in an extended South African family.

17.2 OBJECTIVES

- To characterise a single family in terms of R59W carriage.
- To determine the prevalence of biochemical expression associated with the R59W mutation
- To determine the prevalence and nature of clinical symptoms arising from VP.
- To determine the effect of age, menarche, menstruation and menopause on clinical symptoms.
- To identify associations between VP and other medical illnesses.

17.3 METHODS

Subjects

We chose a family which had been studied by Professor Eales 20 years previously. Key people within this family were contacted and, with their help, the family tree was revised and updated (Figure 17-1). All family members were contacted by letter and their participation invited. As far as possible, parents were tested for the R59W mutation first; where they tested negative, subsequent generations in that line were not examined further.

DNA analysis

Genomic DNA was extracted from peripheral whole blood by the method of Parzer and Mannholter (1991). Exon 3 was amplified using the primers listed in Table 4-1. Approximately 1 μg of PCR product was digested with *AvaI* using a sufficient quantity to allow for complete digestion, at the temperature and for the time recommended by the manufacturers of the enzyme. The products were then electrophoresed on a 6% polyacrylamide gel and visualised by ethidium bromide staining. Pre-and post-digestion products for both patient and a control were always included in the gel. The technique is fully described in Appendix 4-13.

Biochemical analysis

Urine, stool and plasma porphyrin concentrations were measured by the methods described in Appendix 1.

Plasma fluoroscanning

Plasma was diluted 1:10 with phosphate-buffered saline and the emission spectrum was scanned from 580 nm to 650 nm with the excitation monochromator set at 405 nm. The method is fully described in Appendix 1.

Clinical details

A questionnaire in both English and Afrikaans, reproduced in Appendix 10, was sent to all subjects. It was designed with the following intentions:

- To collect essential demographic data.
- To obtain information about the diagnosis; in particular, whether previous biochemical testing for porphyria had been undertaken.
- To determine whether the subject had ever experienced acute symptoms.
- To determine whether the subject had ever experienced cutaneous symptoms.
- To identify any effect of ageing, menarche, puberty, menstruation and the menopause on clinical expression.
- To identify any association between VP and a history of hypertension, heart disease and psychiatric or emotional illness.

Those who did not return the questionnaire initially were sent a reminder and further questionnaire. At the conclusion of the survey, those subjects who were R59W-positive but had not yet returned their questionnaires were contacted telephonically and the questionnaire was completed over the telephone.

Data handling

All data were entered into a Microsoft Access database. Statistical analyses were performed with the Statistica software package.

17.4 RESULTS

Inheritance of the R59W mutation

75 members of this extended family were tested. Following the exclusion of those found to belong to non-informative branches of the family, 62 subjects were included. These are shown, classified by DNA status, sex and age, in Table 17-2. Five obligate carriers in generation I and two subjects in generation II are deceased. All living subjects have been tested with the exception of three subjects in generation III who declined to participate. Of the subjects enrolled, 55 were aged 16 or more and are classified as "adult" in the following analysis. The autosomal dominant mode of transmission is confirmed. Excluding the two positive females in generation I (since they, unlike the succeeding generations are not randomly tested), there are 29 DNA-negative subjects and 31 DNA-positive subjects. 39.4% of the males and 67% of the females tested positive for the R59W mutation. The difference is not significant ($p=0.07$, Yates-corrected χ^2).

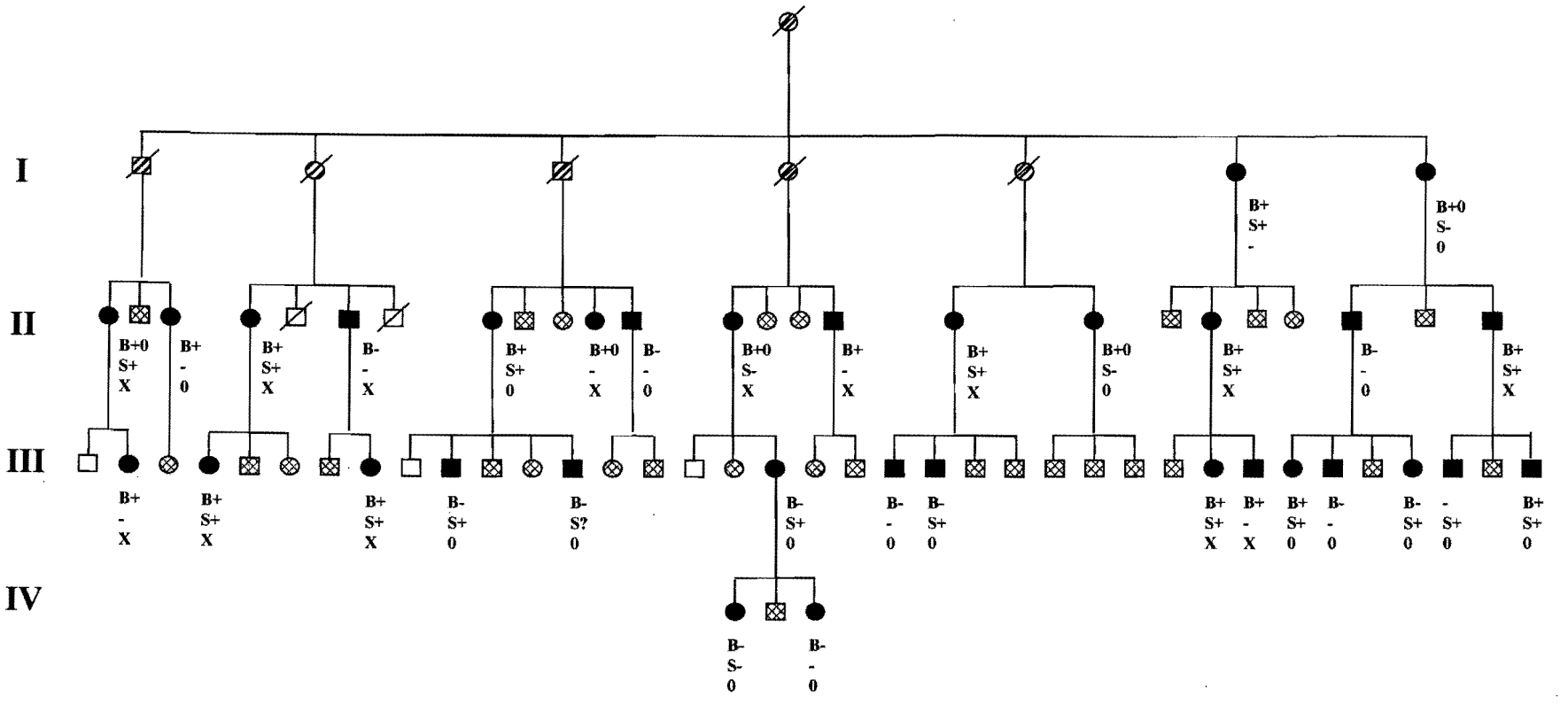


Figure 16-1. Abridged pedigree illustrating 4 generations of the R59W-positive family. R59W-positive subjects are indicated by a solid symbol, R59W-negative subjects by a shaded symbol and subjects who decline testing by an open symbol. A diagonal slash indicates that the subject is deceased. R59W-positive subjects are further characterised as follows:

B+ Positive VP profile on TLC. **B-** Negative VP profile on TLC. **B+0** Positive VP profile previously documented but currently negative.

S+ Positive plasma fluorescence scan. **S-** Negative plasma fluorescence scan. **S?** Equivocal plasma fluorescence scan.

X VP clinically expressed. **0** VP clinically silent.

- No information.

	Any age		Adults > 16 years	
	Female	Male	Female	Male
Untested	4	7	1	
R59W-negative	9	20	8	17
R59W-positive	20	13	17	12

Table 17-2. Results of DNA testing in the extended family. The 12 subjects not tested include 9 subjects in generations I and II who are deceased and 3 subjects in generation III who declined to participate.

Correlation with stool biochemistry

44 adult subjects provided adequate specimens for biochemical analysis (Table 17-3). Of those R59W-negative, 16 (89%) had negative stool biochemistry, while a further 2 (11%) showed equivocal results. Of the R59W-positive subjects, 14 (54%) had positive stool biochemistry, a further 3 (12%) had equivocal results and 9 (35%) were unequivocally negative, giving a significant false-negative-rate. Negative stool results were not confined to younger subjects; the mean age of the R59W-positive subjects with normal stool porphyrin profiles was 43 (range 21-64). Furthermore, three female subjects, aged 50, 56 and 64, who currently are biochemically negative, are known to have tested positive biochemically previously.

Correlation with plasma fluoroscanning

87.5% of the R59W-positive subjects were positive for a peak at 625 nm by plasma fluoroscanning, while all the R59W-negative subjects had normal scans. Plasma fluoroscanning is thus more sensitive than stool porphyrin analysis in the detection of carriers. Two subjects with plasma scan results have not been tested biochemically. In those subjects in whom both tests were performed, plasma fluoroscanning was shown to be more sensitive. 10 R59W-positive subjects with a stool profile diagnostic of VP, 8 subjects with a normal profile and 2 subjects with an equivocal profile had a positive plasma fluoroscanning result. Three subjects, aged 57, 64 and 73 had negative plasma scans. One of these had a normal stool profile, the second was equivocal and the third had not been tested. However, three other subjects, though counted here as positive, demonstrated very small peaks, of the order of a 2 mm deflection only. All had normal biochemistry.

Experience with previous testing

Only 7 of 11 (64%) of R59W-negative respondents and 14 of 28 (50%) of R59W-positive respondents reported having been tested previously by stool porphyrin analysis. Thus, even within a large well-characterised family known to carry VP, a high proportion of potential carriers had not previously offered themselves for testing. Of the R59W-negative group, five (71%) were aware of a previous negative result on porphyrin analysis, one had been given an equivocal result and one had been tested but was unaware of his result. Of the R59W-positive group, 10 (71%) were aware of a previous positive result on biochemical testing, three knew of a negative result (21%) and one knew that he had been tested, but was unaware of his result.

	Porphyrin analysis (n=43)			Plasma fluoroscanning (n=21)	
	NAD	VPL	VP	Negative scan	Positive scan
R59W-negative	16 (89%)	2 (11%)		9 (100%)	0 (0%)
R59W-positive	9 (35%)	3 (12%)	14 (54%)	3 (12.5%)	21 (87.5%)

Table 17-3. Biochemical results and plasma fluoroscanning correlated with DNA status in adults. (Note that the number of patients tested by porphyrin analysis and by fluoroscanning differ, and percentages rather than absolute numbers should be compared.)

Clinical symptoms of VP

No subject aged less than 16 had experienced either skin disease or acute symptoms. The discussion which follows is therefore limited to those aged 16 or more.

Acute attacks

Clinical details were available on 28 of the 29 R59W-positive adults (97%). The results of the study are summarised in Figure 17-2. Only one patient had had an unequivocal acute attack. This was a single attack in a female patient, then aged 35, in 1965 for which she was treated in hospital for 3 weeks. At that time she had already been aware of typical skin lesions for 5 years. The attack was not complicated by paralysis and there has been no recurrence. A further 5 subjects reported that they suspected that they had previous acute attacks. Two of these could quite clearly be ruled out as having had acute attacks on the basis of an atypical history. The evidence in the remaining two subjects is not compelling and is regarded at best as equivocal; were these acute attacks at all, we would classify them as mild, with no recurrence and no complications such as neuropathy. Both had skin disease. Assuming that perhaps one of these attacks was genuine, a reasonable estimate would be that acute attacks were experienced in approximately 2 (7.1%) of these subjects, with a minimum of 1 (3.6%) and a maximum of 3 (10.7%).

When specifically asked about experiences with abdominal pain, 6 of 28 R59W-positive subjects (21.4%) and 2 of 11 R59W-negative subjects (18%) reported experiencing significant abdominal pain in the past. The difference is not statistically significant ($p=0.74$, κ^2), indicating that a mere history of abdominal pain is an extremely unreliable diagnostic indicator of acute symptoms in VP.

Skin disease

Of the 28 R59W-positive subjects, 11 had a history of unequivocal photocutaneous sensitivity compatible with VP: A further 3 described skin symptoms which seemed unlikely to represent porphyric skin disease. From this study therefore, it appears that the proportion of patients with R59W-positive VP experiencing skin disease is 39.3%. The skin disease was most marked on the dorsal surfaces of the hands In 10 subjects and on the face in one. In addition to involvement of the hands, skin disease was reported on the face, forearms and feet in 3, 2 and 4 subjects respectively.

Sex and skin disease

Skin disease was experienced in 8 of 14 females as opposed to 3 of 11 males (57% vs 27%). It is unclear whether this reflects a true sexual bias or is an artefact produced by small numbers. The difference is not statistically significant ($p=0.135$, κ^2).

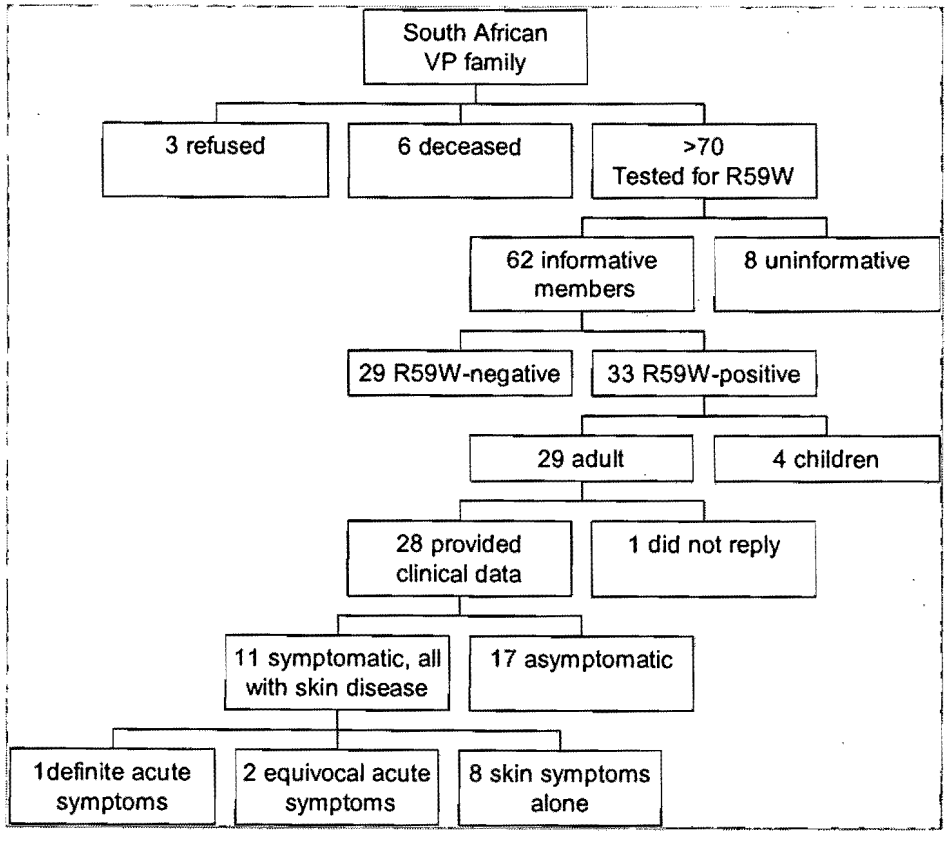


Figure 17-2. Results of the clinical study.

Age and skin disease

There is also no significant difference in age between those subjects showing skin disease and those who do not (Figure 17-3). The average ages and standard deviations are 44.1±17.3 and 40.4±18.0 respectively. The difference is not statistically significant (p=0.6, t test). There appeared to be a strong association between advancing age and a subjective perception of improvement in skin disease. The ages of the 5 oldest subjects (3 female, 2 male) range from 48 to 73; four of these (3 female, 1 male) reported an improvement in skin disease as they had grown older. One of these are females was postmenopausal; the other two were not. None of the subjects younger than 45 had noticed any improvement in their skins. It therefore appears that the skin disease of VP has a tendency to improve as the patient ages, but that that this effect is not evident until after the age of 45.

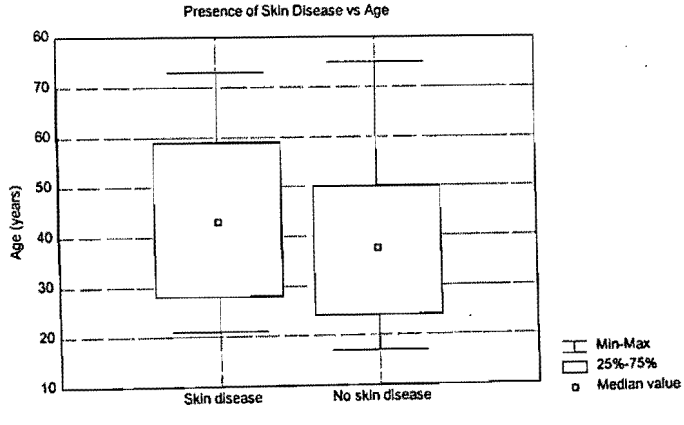


Figure 17-3. Presence or absence of skin disease correlated with age.

Psychiatric problems

Of 28 R59W-positive respondents, 7 (25%) reported problems of anxiety or depression and most had consulted a doctor for this. By contrast, none of 11 R59W-negative respondents reported these problems. The difference is however not significant (p=0.26, Yates-corrected χ^2).

Other medical problems

There were no associations between R59W status and hypertension or heart disease.

17.5 CONCLUSIONS IN BRIEF

We have confirmed that neither biochemical nor clinical evidence of VP will be found in subjects aged less than 16 years. We have shown that approximately 35% of DNA-positive adults will show no biochemical evidence for VP on stool porphyrin testing. Plasma fluoroscanning is more sensitive with a false-negative rate of 12.5% and thus, as with stool porphyrin chemistry, becomes more likely in older subjects. Approximately 60% of all adults with VP are asymptomatic, experiencing neither skin nor acute symptoms. Furthermore, in contrast with hospital-based studies, the acute attack of VP is now a rare in event in the lives of most of families carrying VP, with a single confirmed attack in a single subject in this family. These results are discussed further in Chapter 19.

CHAPTER 18:

THE ACUTE ATTACK: A PERSONAL SERIES

18.1 INTRODUCTION

The last large series of acute attacks are those those reported by Eales (1980), Mustajoki (1978, 1985) and Kaupinnen and Mustajoki (1992). These reports documented a high rate of serious complications of the acute attack, but suggested that the prognosis has improved with time. Our experience with all acute porphyric crises treated at Groote Schuur Hospital over a 12 year period is reviewed here.

18.2 OBJECTIVES

- To describe the acute attack of porphyria as seen at Groote Schuur Hospital.
- To describe the clinical features and compare these with those reported previously.
- To identify the precipitating factors, clinical consequences, therapy and outcome.
- To compare the clinical features of AIP and VP.

18.3 METHODS

Subjects

All patients admitted to Groote Schuur Hospital with a diagnosis of the acute porphyric attack during the period 1 July 1986 to 30 April 1999 were admitted and treated under our direct supervision. A diagnosis of the acute attack was based on the following grounds:

- Severe abdominal pain
- Minimal or absent abdominal tenderness
- Frequently accompanied by nausea, vomiting and anorexia
- A clinical presentation essentially identical to a previously documented attack
- Absence of any other cause for the symptoms.

All first attacks, as well as any attack with atypical features or in which there was doubt about the diagnosis, were further confirmed by a positive Watson-Schwartz test and confirmed by the direct measurement of urine PBG concentrations.

Methods

Laboratory diagnosis

Urine was screened for PBG with the Watson-Schwartz test and ALA and PBG concentrations were determined by iron-exchange chromatography. The diagnosis of porphyria was proven in every patient by quantitative thin-layer chromatography. All methods are described in Appendix 1.

Clinical data

The following details were recorded for every admission.

Antecedents

- Possible precipitating factors, with particular reference to drug exposure, menstruation, infection and pregnancy.
- Duration of the symptoms before presentation.

On admission

- Symptoms: pain, nausea, vomiting and constipation.
- Physical examination, with particular reference to abdominal tenderness, pulse rate and blood pressure.
- Measurement of serum sodium, potassium, urea and creatinine.

While in hospital

- Frequent clinical examination, never less than once daily and, in the early stages of an attack, more frequently, with daily recording of clinical condition in the hospital notes.
- Recording of all complications, with particular reference to neuropathy, paralysis, seizures or confusion and of phlebitis at the site of infusion of haem arginate.
- Frequent recording of pulse and blood pressure.
- Repeat estimations of sodium, potassium, urea and creatinine in patients who failed to improve rapidly or in whom initial values had been significantly abnormal.
- Recording of all drugs administered with times and doses.

Summarisation

At the conclusion of the study, all folders were reviewed and the following data extracted, summarised and recorded on data sheets.

- Length of hospital stay.
- Highest blood pressure and pulse rate observed during the admission and the day on which the highest value occurred.
- Lowest sodium or potassium, and highest urea or creatinine observed during the admission and the day on which the lowest sodium was measured.
- A retrospective assessment of the severity of symptoms for each day of the admission, based on a reading of the clinical notes for that particular day and made without reference to the pethidine requirements for that day. This was recorded on the following scale:
    - 2 = severe pain
    - 1 = moderately severe pain or pain which had clearly lessened in intensity
    - 0 = substantially pain-free or pain which had markedly lessened in intensity.
- The number of days till disappearance of pain.
- Total number of doses of antiemetics and beta-blockers received.
- Total dose of pethidine given per day. In the few instances where other opiates such as morphine or fentanyl had been administered, the dose was converted to the equivalent dose of pethidine thus: 100 μg fentanyl=10 mg morphine=100 mg pethidine.
- Daily dose of haem arginate and the number of days on which haem arginate had been given.
- Number of doses of tin protoporphyrin given.

Information storage, retrieval and statistics

At the conclusion of the study, a master list was made of all patients admitted with acute porphyria during the study period correlated with diagnosis, sex, date of birth, diagnosis and total number of admissions for the acute attack. All data were entered into a Microsoft Access database. Data were analysed using Microsoft Access and statistical comparisons were performed with the Statistica software package. Where data were essentially normal in

distribution, comparisons were made with the Student's t-test, otherwise the Mann-Whitney U test was employed. 2x2 tables were analysed by χ^2. Sequential data were analysed by one-way ANOVA. The specific tests employed are stated in the text.

18.4 RESULTS

All subjects admitted to Groote Schuur Hospital with an acute attack during the period of the study have been included with two exceptions. One patient (HK) remained almost continuously in hospital with severe, essentially unrelenting porphyric crises. She experienced more than 100 sequential attacks prior to her death. She is not included in the following analyses as her over-representation would distort the data. A second patient (MJ) gradually developed a similar profile during the course of the study. Having suffered her first attack in 1992, she had by late 1996 suffered 33 acute attacks. Data pertaining to these attacks, which were more typical of AIP in that she experienced periods free of pain and was able to return home between attacks, have been retained, but details of later attacks, during which she was hospital-bound, are not included. The course of both these patients is described in detail later in this chapter.

Twenty-five individual patients were treated for the acute attack during the study period; 15 with AIP (13 female, 2 male) and 10 with VP (5 female, 5 male). These data are summarised in Table 18-1.

	Patients		Attacks	
Diagnosis	**Male**	**Female**	**Male**	**Female**
AIP	2	13*	14	73
VP	5	5	18	7
Total	7	18	32	80

Table 18-1. Incidence of the acute attack. *One subject (HK) is counted as a patient with AIP, but her attacks have been censored as explained above.

Patients and demography

Following exclusion of data specific to HK, details of 112 attacks experienced by 24 individuals were analysed. The female:male ratio is significantly higher in AIP than VP ($p<0.0001$, χ^2). The mean age of the patients, averaged across all attacks, is shown in Figure 18-1. The distribution is skewed towards a younger age, and patients with AIP are younger than those with VP (Figure 18-2). The difference in age is significant ($p<0.0001$, Mann-Whitney U test). The median age at the first attack for AIP is 23.5 years and for VP 30 years.

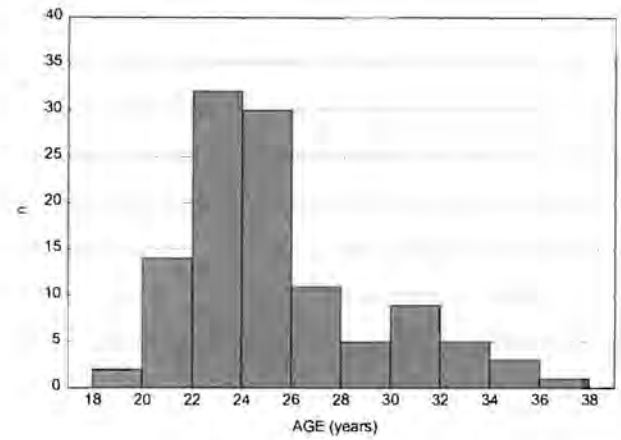


Figure 18-1. Age incidence for all attacks.

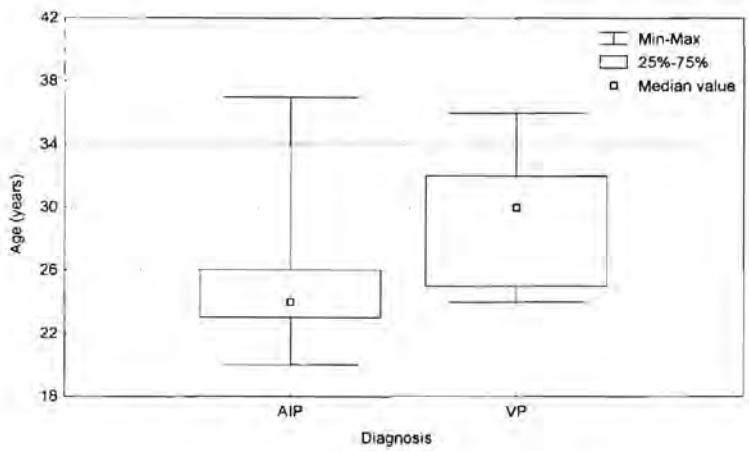


Figure 18-2. Age distribution, comparing AIP with VP.

Number of attacks experienced per patient

The average number, median number and range of attacks are shown classified by sex and diagnosis in Table 18-2.

	VP			AIP			All
	Male	**Female**	**Total**	**Male**	**Female**	**Total**	**Total**
n	5	5	10	2	12	14	24
Mean	3.6	1.4	2.5	7	6	6.1	4.6
Median	2	1	1	7	2.5	2.5	1
Minimum	1	1	1	1	1	1	1
Maximum	8	3	8	13	33	33	33

Table 18-2: Average number, median number and range of attacks classified by sex and diagnosis.

Several patients had repeated attacks: these are commoner in AIP (Figure 18-3). The difference is not statistically significant (p=0.5, Mann-Whitney U test). Males are more prone

to recurrent attacks then females. This assumption is probably valid for VP, though the difference is not statistically significant (p=0.2, Mann-Whitney U test), but the apparent high frequency of acute attacks in males with AIP is not valid since the mean and median are derived from just two male patients, one of whom had 13 attacks (Figure 18-4).

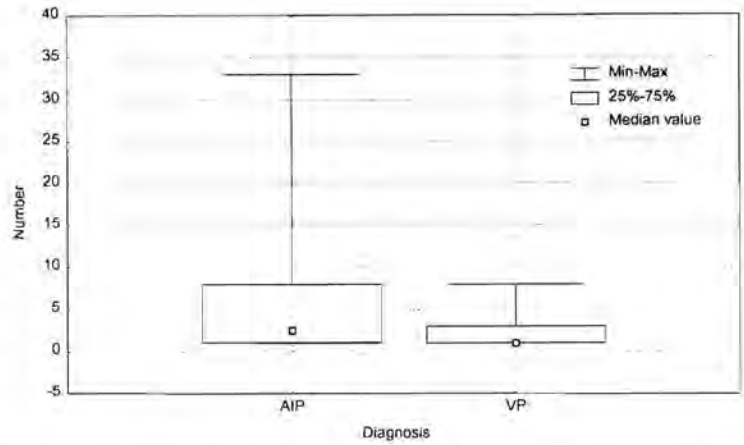


Figure 18-3. Number of admissions in the acute attack per individual patient.

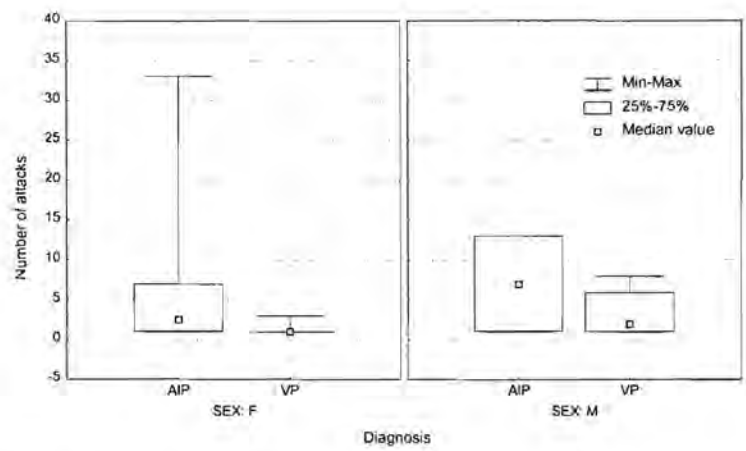


Figure 18-4. Number of admissions in the acute attack per individual patient, categorised by sex and diagnosis.

Presenting symptoms and signs

The median duration of symptoms before admission was 2 days (range 0-18 days) and was unaffected by diagnosis. The clinical features are shown in Figure 18-5. The most common presenting symptom is severe abdominal pain. Though very prevalent, it is not invariable, and 3 of 112 attacks were accompanied either by no pain or by mild pain that was not particularly troublesome. Nausea and vomiting are common. Constipation by comparison was rarely a prominent symptom. Tachycardia and hypertension, are frequent, but may not be noticeable at the time of the first presentation, since they tend to become more obvious during the ensuing days, on average reaching a maximum 48 hours after admission. Systolic blood pressure tended to be the most sensitive parameter: it was elevated above 120 (a generous upper limit of normal in view of the predominately young and female population) in 67% at the time of admission and in 91.5% at some stage of the admission.

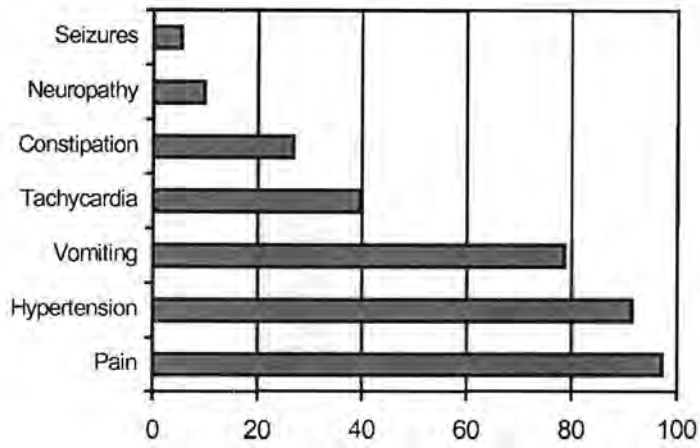


Figure 18-5: Clinical features of the acute attack (%). The figures for hypertension and tachycardia reflect the incidence at any stage of the attack; for neuropathy and seizures, the development of new or further manifestations during the attack.

	Admission	**Highest value at any stage**
Pulse: mean (SD) (/min)	85 (15.4)	92 (14.2)
Percentage of admissions with pulse >90	23.6%	39.6%
Systolic blood pressure: mean (SD) (mmHg)	137 (21.6)	154 (22.1)
Percentage of admissions with systolic >120	67.9%	91.5%
Diastolic blood pressure: mean (SD) (mmHg)	85 (15.6)	92 (14.2)
Percentage of admissions with diastolic >80	46.2%	72.6%

Table 18-3. Tachycardia and hypertension: mean values at admission and mean of the highest value recorded at any stage.; percentage of attacks in which the normal limit was exceeded.

Precipitating factors

The most likely precipitants of the acute attack are shown in Table 18-4, and are analysed graphically by sex and diagnosis in Figure 18-6.

	VP			**AIP**			
	Male	**Female**	**Total**	**Male**	**Female**	**Total**	**All**
Hormonal	—	0	0	—	12	12	12
Drugs	13	3	16	3	8	11	27
Infection	0	0	0	1	1	2	2
Pregnancy	—	1	1	—	0	0	1
Unknown	5	3	8	10	52*	62	70
Totals	18	7	25	14	73	87	112

Table 18-4: Suspected precipitants of the acute attack. *Many of these may be hormonal, though the relationship to the menstrual cycle was not clear-cut.

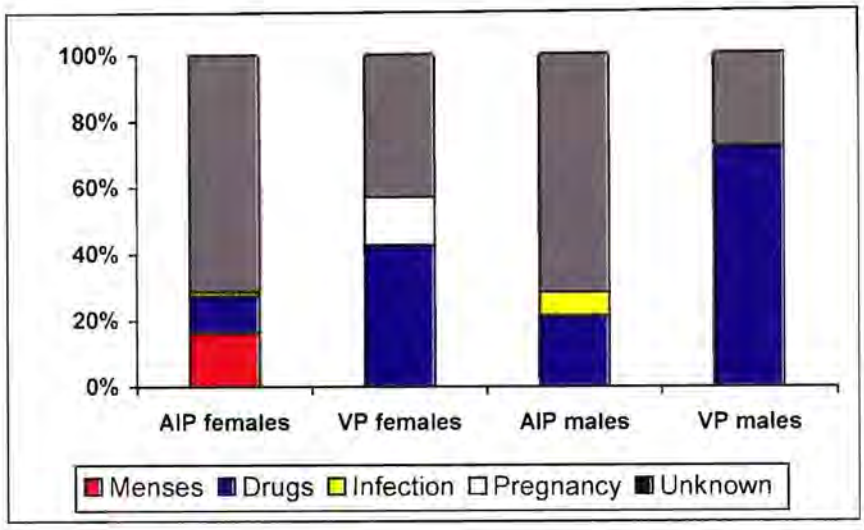


Figure 18-6. Relative importance of various precipitating factors by diagnosis and sex.

The most striking finding is the significant proportion of hormonally-related attacks in AIP and the high proportion of attacks in female AIP patients for which no precipitating factor could be proven. It is likely that some or even most of these may be related to the hormonal cycle. Drugs are frequently the precipitating factor in VP, and probably in males with AIP as well. These points are discussed in Chapter 19.

Drugs were implicated in 27 instances and comprised the following: alcohol (9 instances), cannabis (9 instances), combined oral contraceptive pill or progesterone (3 instances), erythromycin (1 instance), rifampicin or rifampicin/INH/pyrazinamide combination (2 instances), non-steroidal anti-inflammatory (type unknown, 1 instance) and phenytoin (1 instance). Particularly striking are the drugs of abuse, alcohol and cannabis, which may frequently be taken in combination. Indeed, these were responsible for most of the recurrent attacks in males (Table 18-5); reflected here are only those attacks in which there was clear evidence of exposure, but it is likely that they were implicated in other attacks as well.

Subject	Sex	Diagnosis	Drugs	Implicated in n attacks	Total number of attacks experienced
CK	M	AIP	Cannabis, alcohol	3	13
CH	M	VP	Cannabis, alcohol	5	8
DA	M	VP	Cannabis, alcohol	6	6

Table 18-5: Effect of drugs of abuse in inducing repetitive attacks in male patients with porphyria.

Biochemical and electrolyte disturbances

Hyponatraemia was common during the attack (Table 18-6), and 39.3% recorded a sodium value below 135 mmol/l at some stage of the admission. Severe hyponatraemia (Na<125 mmol/l) was noted in 5 admissions: three of these admissions were accompanied by seizures and four were accompanied by neuropathy. The lowest sodium values noted were 100 and 104 mmol/l. Elevated urea values were very common, and were present at the time of admission in 64%. This is likely to reflect dehydration, and values typically improved rapidly following admission and intravenous fluid administration.

	At time of admission	Lowest/highest value at any stage
Sodium: mean (SD) (mmol/l)	136 (4.6)	134 (6.1)
Percentage with a value <135 mmol/l	26.6%	39.3%
Potassium: mean (SD) (mmol/l)	4.2 (0.5)	4.1 (0.5)
Percentage with a value <3.5 mmol/l	6.5%	9.8%
Urea: mean (SD) (mmHg)	7.9 (2.9)	8.0 (2.9)
Percentage with a value >6.7 mmol/l	63.0%	64.1 %

Table 18-6: Biochemical abnormalities. Mean (SD) values at admission and mean of the lowest (sodium, potassium) or highest (urea) value recorded during admission; percentage of attacks in which the normal limit was transgressed.

There were no significant differences between patients with AIP and VP in these values with the exception of the serum potassium, where the values of 4.2 and 3.7 mmol/l for AIP and VP respectively were significantly different (p=0.001, Mann-Whitney U test). There is no obvious explanation for this. Since creatinine concentrations were similar in the two groups, it is unlikely to relate to differences in renal function. The biochemical values in males and females were similar.

The mean ALA and PBG values noted at the time of admission were significantly higher in AIP than in VP. Mean (SD) values were, for AIP and VP respectively, for ALA, 386.1 (226.5) and 189.1 (115.7) (p=0.02, t test); for PBG, 544.9 (242.0) and 168.9 (122.3) (p=0.02, t test).

Complications

Seizures

Seizures were encountered in 6 attacks (5 AIP, 1 VP). Three of these were associated with hyponatraemia. Two episodes, in a single patient with severe AIP (MJ), appeared to relate to the use of large doses of pethidine; 1975 mg total dose in one attack, and 10650 mg total dose in the second. In each case, the seizure was preceded for several hours by myoclonic jerks, which are typical of pethidine-induced seizures. Similar seizures were encountered in patient HK.

Neuropathy

Neuropathy developed at some stage in 11 of the 24 patients: 8 with AIP (57%) and 3 with VP (30%). However, in nearly all cases, neuropathy developed before admission to Groote Schuur Hospital, usually under the care of another hospital, and in most cases before porphyria had been diagnosed. In only 2 of 112 admissions did neuropathy supervene during the course of admission to Groote Schuur Hospital: in neither case had haem arginate been administered. In most cases neuropathy was limited to mild weakness of the extremities manifesting as wrist-drop and foot-drop. Only one patient developed a flaccid quadriparesis requiring ventilation. Neuropathy is discussed in more detail In Chapter 19.

Therapy

Pharmacological treatment received during admission is depicted in Table 18-7.

	Admissions	Median number of doses or dosage*	Range
Antiemetics	61 (54.5%)	3 doses	1-17
Beta-blockers	39 (34.8%)	6 doses	1-32
Haem arginate	75 (67.0%)	4 doses	1-8
Total dose		500 mg	125-1252mg
Tin protoporphyrin	4 (3.6%)	3 doses	2-4
Pethidine or other opiate	103/103** (100%)	1200mg	50-10650mg

Table 18-7: Drugs used in the treatment of the acute attack. *Antiemetics, beta-blockers and tin protoporphyrin) are reported in standard doses (prochlorperazine 6.25 mg by injection, metoclopramide 10 mg by injection, propranalol 40 mg orally or atenolol 50 mg orally; pethidine and haem arginate in mg. ** Records were incomplete in 9 cases.

Antiemetics and beta-blockers were administered in 54% and 35% of admissions respectively. The pain of the acute attack is severe. Opiates were universally required; the total dose required varied widely from 50 mg to over 10 000 mg, with a median dose of 1200 mg. Doses of 75-100 mg, hourly, 2-hourly or 4-hourly were frequently required.

Figure 18-7 shows the median daily requirement for pethidine, which is significantly associated with hospital day ($p<0.0001$, Kruskal-Wallis ANOVA). (The lower requirement on day 1 is artefactual since many patients were admitted in the afternoon or night, and hence were not present for a full 24 hours.) It is seen that the pain is worst on the second day, tends to persist for days 3 and 4, and thereafter improves rapidly. This is mirrored by a highly significant decrease in symptoms as shown in Figure 18-8 ($p<0.0001$, Kruskal-Wallis ANOVA).

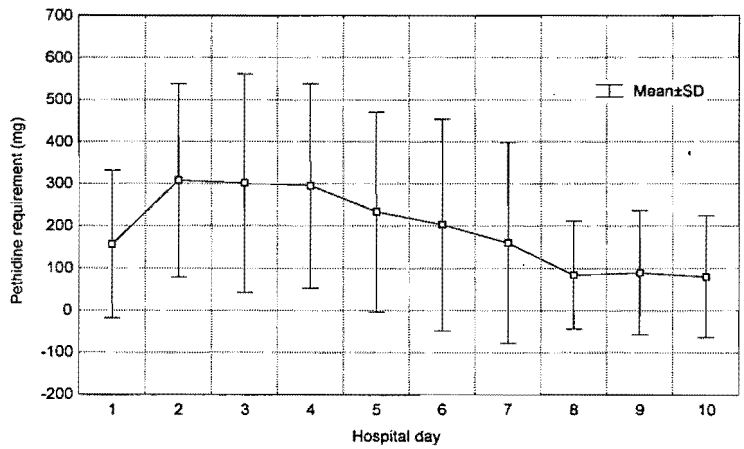


Figure 18-7. Mean daily requirement of pethidine by hospital day.

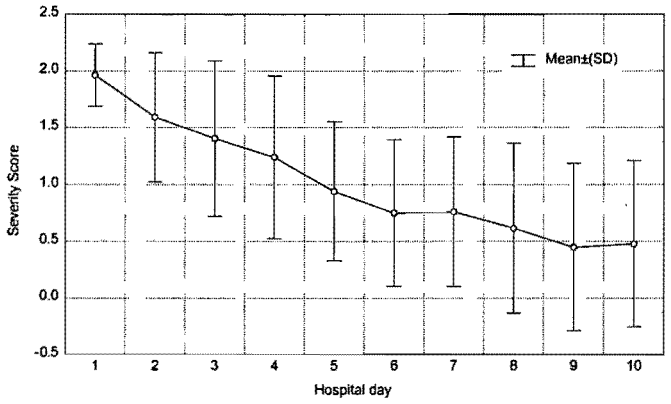


Figure 18-8. Mean daily severity score by hospital day.

Haem arginate

Haem arginate was used in 75 admissions (67%). The first few patients were treated with a dose of 3 mg/kg. Thereafter a standard dose of 125 mg, irrespective of body mass, was used. In 40 instances (53%) a full 4-day course was given; in 23 (31%), treatment was stopped after 3 doses following rapid improvement. In 8 cases only 1 or 2 doses were used. In 5 cases, 5 doses were given. In 4 cases, a second course of haem arginate was given, following an inadequate response to the first course, with a total of 6-8 doses. Patient MJ, with severe AIP, was responsible for 6 of the instances where more than 4 doses were used, and tin protoporphyrin was additionally used in three of these. The other three patients who required additional haem arginate all had AIP, and received 5 doses.

The median time for starting haem arginate was 2 days, that is on the day following admission, with a range of 1-12. Figure 18-9 shows the symptom score by treatment day, ranging from day 8 pre-treatment (-8) to day 8 post-initiation of haem therapy (+8). Clearly seen is the effect of haem arginate on severity score; values remain high until the initiation of haem therapy, whereafter they fall. Figure 18-10 shows the pethidine requirement before and after initiation of haem therapy. In this case pethidine requirement rises steadily until the decision is taken to commence haem therapy, whereafter the requirement falls. This interesting pattern is likely to represent the influence of those patients whose attacks were initially judged relatively mild, as shown by relatively low pethidine requirement. However, symptoms failed to improve and in many cases deteriorated as the attack progressed, until haem therapy was instituted. The decline in both severity score and pethidine requirement are highly significant ($p<0.0001$, one-way ANOVA).

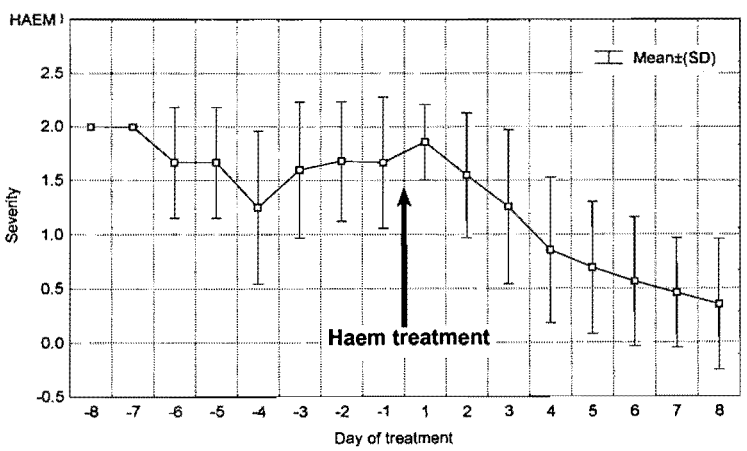


Figure 18-9. Effect of initiation of haem therapy (day +1) on symptom severity score.

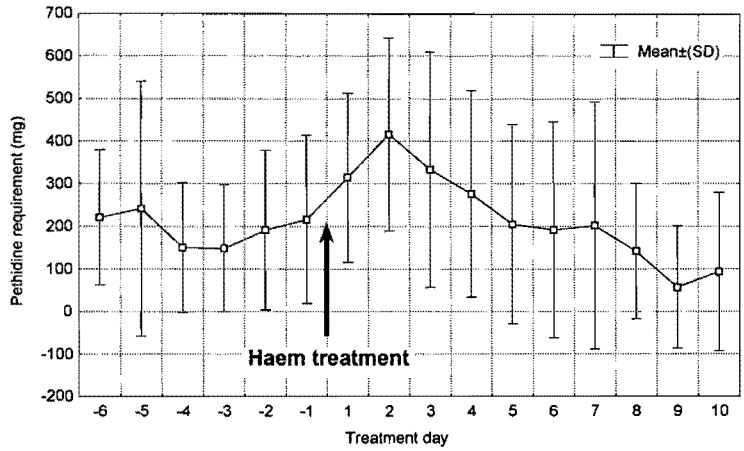


Figure 18-10. Effect of initiation of haem therapy (day +1) on pethidine requirement.

Duration of hospitalization

The median time till resolution of symptoms was 6 days (range 1-19, interquartile range [IQR] 4-7). The median length of hospital stay was 7 days (range 1-56, IQR 5-9). Three patients who remained longer than 30 days all had severe, complicated or unresolved attacks: 1 developed profound quadriparesis requiring ventilation; a second moderate paralysis complicated by staphylococcal septicaemia requiring prolonged intravenous therapy; a third was HIV positive and her course was complicated by intracranial infection.

Relative severity of AIP and VP

Some markers of severity of the acute attack are summarised in Table 18-8. Blood pressure, pulse and haem arginate requirement were significantly higher in patients with AIP than in patients with VP. No significant difference could however be shown in the length of admission or in the time taken for the patient to improve after admission.

	AIP			VP			
	Mean	**SD.**	**n**	**Mean**	**SD**	**n**	**p**
Maximal systolic BP	156.5	22.00	83	145.7	20.63	23	0.036
Maximal diastolic BP	98.2	16.09	83	86.7	14.74	23	0.002
Maximal pulse rate	93.3	14.18	83	85.2	12.75	23	0.015
Doses of haem arginate	3.9	1.14	69	2.7	0.90	11	0.002
Pethidine requirement	1746	1620	83	1278	1053	20	0.22
Length of admission (days)	8.3	6.8	87	8.0	9.4	25	0.89
Interval before improvement	6.3	3.3	85	5.4	3.7	25	0.21

Table 18-8. Relative severity of the acute attack.

Mortality

Three patients in this series have died. Their histories are summarised here.

Patient CK

This 22 year old man was proven to have AIP following an acute attack in a provincial town. He is the cousin of patient HK, whose history follows. He was admitted to Groote Schuur Hospital 13 times over a period of 25 months with acute attacks. In three of these, the use of recent cannabis was proven by the demonstration of positive urinary cannabinoids, and collateral history suggested that this was a major contributory factor to his recurrent attacks. His course was considerably complicated in that he returned to his home town, 800 km from Cape Town, between attacks, and his attacks would begin while there. Despite efforts to arrange for continuity of care and a reliable supply of haem arginate for him, his treatment was inconsistent. His local hospital was reluctant to provide haem arginate in view of its expense and the belief that his problem was largely self-induced by his continuing use of cannabis and alcohol. He would therefore return voluntarily to Groote Schuur Hospital with established acute attacks. After 29 months, he developed a further attack at home during which a neuropathy developed. Haem arginate was not given and he died. Whether the death was primarily due to paralysis or to a metabolic disturbance is unknown.

Patient HK

This 25 year-old woman was admitted to the intensive care unit of a hospital in a provincial town with severe flaccid quadriparesis and respiratory failure. She reported having experienced bouts of unexplained abdominal pain over the preceding months, and her mother had died of a similar and similarly undiagnosed complaint many years previously. Urine and stool samples were sent to our laboratory and a diagnosis of AIP was established. After several months, during which time she recovered sufficient muscle power to breathe spontaneously, she was transferred to Groote Schuur hospital for rehabilitation. With intensive physiotherapy, she recovered sufficiently to walk with crutches. Her course was one of increasing severity and frequency of the acute attack. Initially, attacks were experienced at approximately three-weekly intervals. Over the course of several years, the interval between attacks progressively shortened until she was essentially hospital-bound.

Several of these attacks were extremely severe. On two occasions she required admission to the intensive care unit. During one episode, she developed severe metabolic disturbance with low serum levels of sodium, porassium, magnesium and calcium, accompanied by massive losses of these cations in the urine. She was rescued with haem arginate, ventilation and supportive care. However, her attacks became progressively more frequent and her pethidine requirement high. This led to frequent clashes between patient and nursing staff who were not always convinced that her incessant demands for pethidine were warranted by any actual pain. However, on every occasion when haem arginate was withheld, objective evidence of the acute attack would followed shortly, usually by a deterioration in neuropathy and increasing weakness of the extensors of the hands and feet, confirming that her complaints were genuine.

Her course was complicated by a number of problems. She developed tuberculosis in hospital; choice of antituberculous therapy was difficult. Since she was in any event more or less continually in a state of the acute attack, it was difficult to show any association between specific drugs and an aggravation of her porphyria. Eventually she received only streptomycin and ethambutol, which is a very poor combination for the treatment of tuberculosis. The radiological lesions cleared, only to relapse within 6 months. She then received nine months treatment with streptomycin, ethambutol and amoxycillin-clavulinate. Despite this unusual therapy, she recovered. A lack of venous access became a problem. On many occasions she required central venous cannulation for the administration of fluids and haem arginate, and

she received a number of surgically implanted central venous catheters and synthetic arteriovenous fistulae. On several occasions she developed staphylococcal graft sepsis. This required treatment repeatedly with vancomycin or flucloxacillin without any obvious aggravation of her porphyria.

Experience showed that she could not cope without haem arginate and any delay in administering it resulted in an aggravation of neuropathy. With time, the response to haem arginate became incomplete, with little reduction in symptoms. Tin protoporphyrin, (a method for preparation of which was kindly shared with us by Professor Michael Moore) was produced in our hospital pharmacy and was added to her therapy. This was beneficial; her symptoms would improve more rapidly and more completely after the combination. A pattern of treatment was established whereby every alternate course of haem therapy would require the co-administration of tin protoporphyrin for an improvement in her symptoms. Thus it appeared that the effects of tin protoporphyrin persisted for 3-4 weeks, while haem arginate was required every 10-14 days.

Three other modalities of therapy were attempted. Prophylactic administration of haem arginate in the interval between attacks proved useless. Parenteral GnRH agonist therapy for a period of six months was not accompanied by any reduction in the frequency or severity of attacks despite documented amenorrhoea. On one occasion, a subcutaneous implant of testosterone was performed as suggested by Savage et al (1992) but was ineffective.

This course continued for 5 years. Finally her condition deteriorated to the point that she was never pain-free with an ongoing requirement for massive doses of pethidine. Haem arginate, even with tin protoporphyrin, failed to clear her symptoms, though it would appear to prevent a deterioration to the point of severe neuropathy and paralysis. Finally, following a series of discussions between patient, medical and nursing staff, chaplain and ethicist, a decision was taken to scale down the intensity of therapy. No further haem arginate was given, and she died, well-sedated, of respiratory failure.

Patient MJ

This young woman with AIP first presented with an acute attack aged 22. Over the following four years, she experienced 33 acute attacks; these attacks are incorporated in the series described above. Thereafter, her course converged with that of HK. The intervals between admissions shrank until she too was permanently hospital-bound. She required alternating cycles of haem arginate and haem arginate plus tin protoporphyrin to reduce the intensity of her attacks. Though she developed a foot-drop and wrist-drop, she never became paralysed or required intensive care unit admission. She required a series of surgically implanted venous cannulae for venous access; a subcutaneous injection port proved the most effective route of access. Ultimately she too was never pain-free and her quality of life became intolerable. A decision was taken to scale down the intensity of therapy and she died of respiratory failure.

Autopsy findings

Autopsies were performed in both HK and MJ. Unexpected findings were noted in the livers, which had a slate-grey or green colour. Bridging fibrosis without cirrhosis was noted histologically. There was heavy iron staining in acinar zone 3, in Kupffer cells and in splenic macrophages. An additional pigment was seen in the liver parenchyma which was PAS-negative and did not fluoresce. Interestingly, the bone marrow contained no excess of iron. In MJ, the kidneys showed glomerular sclerosis and iron pigment was noted within the cells of the proximal convoluted tubule. Intimal proliferation of small vessels was noted.

Comment on the autopsy findings

Neither patient had received blood transfusions but showed large amounts of iron in the reticulo-endothelial cells of liver and spleen. This appeared to cause no problem during life, and is thought to reflect iron-loading from intravenous haem administration. An unusual feature in both was the presence of an abnormal pigment. This may represent tin derived from tin protoporphyrin. It is our intention to determine tissue iron and tin levels in these patients spectrophotometrically.

18.5 CONCLUSIONS IN BRIEF

Significant differences between patients with VP and AIP have been shown, particularly in terms of the factors precipitating the acute attack. The major complications of severe neuropathy: seizures and respiratory paralysis are now rare. The mortality is currently low. In some patients, particularly with AIP, recurrent attacks are a problem. The treatment of the acute attack has been described and some evidence provided to suggest that haem arginate is indeed efficacious. These results, with further description of some of the more interesting features of the acute attacks we have accounted, are discussed fully in the chapter which follows.

CHAPTER 19:

DISCUSSION: THE CLINICAL FEATURES OF VARIEGATE PORPHYRIA

In the preceding three chapters, three aspects of the clinical presentation of variegate porphyria were reported: the syndrome of homozygous variegate porphyria, the clinical penetrance and presentation in a single large R59W-positive family, and a personal experience with the recognition and management of the acute porphyric crisis. The observations described in these chapters are now discussed.

19.1 THE SYNDROME OF HOMOZYGOUS VARIEGATE PORPHYRIA (HVP)

We had initially diagnosed HVP in our first patient, the proband LO, on the basis of her clinical features and the age of presentation, supported by the demonstration of extremely low PPO activity in EBV-transformed lymphoblasts. This was before it became possible to test for mutations in the PPO gene directly. A description of this patient, together with details of a second patient from the United Kingdom, was published in a paper in which we summarised the clinical features of HVP as a syndrome clearly distinct from that of typical heterozygous VP (Hift et al 1993b). The clinical and biochemical details of eight previously reported cases of HVP and of the four described in Chapter 16 are summarised in Table 19-1. Once DNA testing for mutations within the PPO gene became feasible, she was shown to be a compound heterozygote, as have our three subsequent patients.

Probands 1 and 2 conform to the pattern of clinical expression of homozygous and compound heterozygous VP (HVP) arising early in life ("early-onset HVP") established by several reports (Kordac et al 1984, Murphy et al 1986, Coakley et al 1990, Norris et al 1990, Gandolfo et al 1991, Mustajoki et al 1987, Hift et al 1993b). The clinical features of early-onset HVP may therefore be summarised as follows (Table 19-2). Additionally we have now provided evidence that, as exemplified by Probands 3 and 4, HVP may present later in life ("late-onset HVP"), with the features noted in Table 19-3. One case, described by Coakley (1990), was so exceptional that we suggested in our earlier review (Hift et al 1993b) that the diagnosis might have been mistaken. This child first showed signs of disease at the age of 12 years (10 years later than any of the other cases reported at that time), had no photosensitivity, experienced severe acute attacks and is the only patient to have died. The biochemical findings were also exceptional; a normal stool protoporphyrin was accompanied by a markedly raised coproporphyrin. With the benefit of a further 7 years' experience, we have to qualify our initial reluctance to accept the diagnosis. As described in Chapter 10, a raised faecal coproporphyrin in the absence of a raised protoporphyrin appears to be an acceptable diagnostic marker for VP. Secondly, as Probands 3 and 4 demonstrate, we now have evidence that HVP may be recognised for the first time after childhood, and may indeed be complicated by the acute attack. Without precedent however is the lack of photosensitivity. It is possible that Coakley's patient represents a form of HVP intermediate between the severe, early-onset form and the more benign, late-onset form described here, though the lack of photosensitivity would be most surprising. DNA studies on surviving family members would confirm the diagnosis, a method used to diagnose homozygous AIP retrospectively in a child who had died many years earlier (Picat et al 1990), but have not been reported.

Author	**1**	**1**	**2**	**2**	**3**	**4**	**5**	**6**	**7**	**7***	**8***	**8***	**8***
Sex	male	female	male	female	male	female	male	female	male	female	female	female	female
Age at onset	<1 mo	<1 mo	2 yr	9 mo	<1 mo	18 mo	<12 mo	12 yr	5 mo	<1 mo	10 mo	19 yr	19 yr
Mental retardation	Y	Y	N	N	N	Y	Y	Y	Y	N	N	N	N
Fits	Y	Y	nd	Y	N	Y	Y	Y	Y	?	N	N	N
Developmental delay	nd	nd	nd	nd	Y	nd	Y	Y	Y	Y	Y	N	N
Nystagmus	Y	Y	N	N	N	nd	nd	nd	Y	Y	Y	N	N
Growth retardation	nd	nd	Y	Y	Y	N	nd	nd	Y	Y	Y	N	N
Photosensitivity	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y
Hand deformities	nd	nd	Y	Y	Y	Y	Y	nd	Y	Y	Y	Y	N
Retarded bone age	nd	nd	nd	Y	nd	nd	nd	nd	N	Y	Y	N	N
Acute attacks	N	N	N	N	N	N	N	Y	N	N	N	Y	N
Death	N	N	N	N	N	N	N	Y	N	N	N	N	N
PPO activity*													
Proband	0.16	0.17	0.05	nd	0.08	0.14	0.20	0.17	0.46	0.1	nd	nd	nd
Mother	0.49	0.49	0.47	nd	0.56	0.68	0.60	0.50	nd	0.5	nd	nd	nd
Father	0.53	0.53	0.28	nd	0.48	0.66	0.50	0.50	nd	0.75	nd	nd	nd
Biochemistry													
Urine ALA	1.4	2.5	<1	nd	0.1	nd	0.6	19.4	nd	0.4	0.4	2.4	1.9
Urine PBG	1.0	1.7	<1	nd	0.1	nd	0.9	105.6	nd	0.6	0.5	6.6	1.5
Urine uroporphyrin	8.0	6.3	4.9	nd	0.7	8.1	2.9	131.0	6.7	0.9	0.6	73.2	19.2
Urine coproporphyrin	1.2	1.3	1.0	1.0	0.4	1.2	2.3	12.9	0.4	0.3	0.7	18.9	2.0
Faecal coproporphyrin	6.7	4.5	2.1	4.4	4.5	4.4	1.2	3.8	5.9	4.4	1.4	24.2	12.4
Faecal protoporphyrin	12.6	7.3	2.9	1.8	5.8	1.4	2.0	0.02	3.0	1.8	2.2	9.0.2	2.0
RBC protoporphyrin	nd	nd	1.8	21.61	8.1	6.8	5.3	nd	7.3	21.6	6.9	3.2	nd

Table 19-1. Summary of the clinical and biochemical features in all patients with the homozygous VP syndrome described thus far. The references are as follows (1) Kordac et al 1984; (2) Murphy et al 1986; (3) Mustajoki et al 1987; (4) Norris et al 1990; (5) Gandolfo et al 1991; (6) Coakley et al 1990; (7) Hift et al 1993; (8) Corrigall et al 2000. The four subjects described in Chapter 16 are marked with an asterisk.

Aspect	Clinical features	Comment
Sex	Male or female	
Age at onset	Days-months	
Neurological features	Mental retardation	Variable
	Seizures	Variable
	Developmental delay	
	Nystagmus	
	Sensory neuropathy	
	Behavioural abnormalities	Hyperactivity
Dermatological features	Photosensitivity	Severe
	Abnormalities of non-sun-exposed skin	
Skeletal abnormalities	Growth retardation	
	Brachydactyly	
	Finger joint deformities	
	Retarded bone age	
Acute attacks	Not a feature	
Life expectancy	No evidence yet for reduced life expectancy	

Table 19-2. Characteristic features of early-onset HVP.

Aspect	Clinical features	Comment
Age at onset	Mid to late adolescence	
Neurological features	Nil	
Dermatological features	Photosensitivity	Moderate-Severe
	Abnormalities of non-sun-exposed skin	Absent
Skeletal abnormalities	Brachydactyly	Variable
	Finger joint deformities	Variable
Acute attacks	Possible	

Table 19-3. Characteristic features of late-onset HVP.

Molecular biology of HVP

The prevalence of R59W-positive VP is believed to be sufficiently high in the white Afrikaner population of South Africa for homozygotes to have emerged. Yet no such patients have been identified, and we postulated that the homozygous state of the founder South African mutation would prove to be lethal. Indeed, in our paper reporting the first South African case of HVP, we wrote: "No case has yet been described in South Africa. We had previously believed that the homozygous state might be lethal. It is probable that HVP, like the heterozygous condition, is subject to variable expressivity, and that only the less-severely affected fetus will survive till birth. It is also possible that some cases of HVP will turn out to

be compound heterozygotes rather than true homozygotes when testing at the gene level is possible. It is an interesting observation that all the parents of the cases described thus far appear to have been latent carriers as none are reported to have expressed VP clinically." (Hift et al 1993b).

Since the advent of DNA analysis for VP, these predictions have been proved correct. With respect to our original case (LO), we suggested that the parents would be found to carry different mutations, and that the paternal mutation would be associated with a less severe impairment of activity. This we predicted on the basis of the father's lack of biochemical expression of VP, on the grounds that his PPO activity was reduced to lesser extent than was that of his wife, and on the premise that the combination of a less severe mutation and the South African founder mutation might allow some residual activity in the proband, thus bypassing our belief that the homozygous state would appear to be lethal. Indeed, she was proven to be an R59W/R168C compound heterozygote. All three subsequent patients with HVP have also proved to be compound heterozygotes.

There is evidence to suggest that some residual PPO activity is always present in the surviving homozygote or compound heterozygote. The R59W mutation exhibits essentially zero catalytic activity (Meissner et al 1996, Dailey and Dailey 1997), whereas the R168C mutation does appeared to be associated with some residual activity. This is consonant with experience in homozygous AIP, HCP and PCT (Elder 1997). Thus, as suggested by Whatley et al (1999), mutations that abolish activity are lethal in homozygotes and are found only in heterozygous VP, while the milder mutations are not clinically apparent in heterozygotes but may present with homozygous VP. Those mutations that abolish activity include frameshift mutations, those that induce premature stop codons and some missense mutations at particularly crucial sites. It is possible that some missense and splice-defective mutations may fall into an intermediate category causing VP with a low clinical penetrance. The Y348C mutation we demonstrated in the family of Proband 2 may represent such an intermediate mutation, since at least one family member expressed VP biochemically. The R168C and R138P mutations however would appear to fit Whatley et al's definition of "mild".

This point has further been stressed by Roberts et al (1998). In none of 5 HVP patients did parents or other relatives manifest VP clinically. Clinically silent VP is more common than overt disease (Chapter 17, Kirsch et al 1998) and PPO mutations are sufficiently frequent in European populations for rare cases of homozygosity and severe PPO deficiency to occur outside consanguineous unions. Indeed, one of the mild mutations in Roberts's study (G358R) was present in two HVP patients, one from the Czech republic and one from the UK. Two patients had both a chain termination or splice mutation and a missense mutation that were shown by family studies to be carried on the same allele. One of the two is mutant alleles in a Finnish compound heterozygote (Kauppinen etal 1997), P256R, was also observed in the United Kingdom in association with a splice mutation carried on the same allele; this mutation, which affects a poorly conserved residue, is a polymorphism present in about 10% of the French population (Whatley et al 1999).

This suggests that this and perhaps other relatively mild mutations may not be uncommon in the general population. Yet none of the mutations shown in their HVP subjects were present in a large group of western European patients with VP. The inference from their experience is that HVP results from the conjunction of two mutations, neither of which is associated with complete inactivation of the enzyme, and neither of which is thus sufficient in its own right to result in clinically expressed VP in the heterozygous state. This is in contrast to our experience in South Africa and to that in Finland—two high prevalence areas subject to VP founder effects—where the more severe mutations in subjects with compound heterozygous VP may indeed be associated with VP in heterozygotes.

Furthermore, Roberts et al (1998) provide evidence to support the hypothesis that at least one mutant allele must code for a protein with residual catalytic activity in HVP. Seven mutations were identified in this study: Four were missense mutations and the fifth a 12 bp insertion coded for the inserted sequence KANA. In this fifth case, sequence from the other allele was barely detectable. Differential quantitation of the mRNA produced from each allele in expression studies showed that about 90% of the mRNA produced by this patient was derived from the mutant allele. The complementary mutation was identified as a T to G transversion in intron 11, which segregated independently of the KANA allele and is situated in the polypyrimidine track of the exon 12 spice acceptor site. It was suggested that this mutation is likely to impair splicing efficiency with formation of an unstable, abnormal mRNA species. The remaining mutation, in intron seven, was associated with the complete deletion of exon 7. Both these intronic mutations were believed to result in complete loss of activity of the protein coded by that allele.

The four missense mutations and the KANA mutant were screened for residual PPO activity by testing their ability to rescue the growth of a *hemG* (PPO gene)-deficient SAS38X strain of *E. coli*. This strain requires very low levels of PPO to grow normally; therefore even low-activity mutants will complement it successfully. Four mutant PPO clones were able to rescue growth, and their activities relative to that of normal human PPO expressed in the same strain ranged from 9.5-25% of normal. Only the G169E mutation failed to sustain growth, suggesting that it is non-functional. It was also the only mutation of those identified in this paper that alters a highly conserved residue unchanged in human, mouse and a number of prokaryotic species. These results are summarised in Table 19-4, where those mutations able to rescue growth are indicated by a tick and those unable to do so, by a cross.

Subject	Allele I		Allele II	
I	D349A	√	D349A	√
II	A433P	√	A433P	√
III	G358R	√	G169E	*x*
IV	G358R	√	Intron 7	*x*
V	A219insKANA	√	Intron 11	*x*

Table 19-4. Mutations identified in 5 western European cases of HVP (Roberts et al 1998). A tick (√) indicates that the mutation appears to encode a protein with measurable residual activity. A cross (*x*) indicates that the protein appears to be non-functional or absent.

Two of the subjects are homozygotes: in each case the mutation is associated with residual activity. Three subjects are compound heterozygotes and in each case one of the alleles encodes a protein with residual activity. Thus, in summary, all five of these patients had a mutation which preserved some PPO activity on at least one allele. Severe mutations that abolished PPO activity or markedly decreased the concentration of normal mRNA were always allelic to milder mutations.

Mutational analysis in our subjects is consonant with these conclusions. We have shown that the R59W mutation is associated with a near-absence of catalytic activity. Though this has not been directly measured in the case of the R168C, Y348C and R138P mutations, the observation that no clinical disease has been shown to be associated with the heterozygous state and that biochemical expression is unusual or absent would suggest that they are not associated with a complete loss of catalytic activity. Work in our laboratory on the activity of

the R168C and Y348C mutants is in progress. Clearly some activity must be preserved to maintain the vital function of haem biosynthesis. But, as stated by Roberts et al (1998), what has been surprising in all these disorders has been the substantial loss of activity that can be tolerated albeit at the cost of disease of variable severity. Their study also showed no correlation between the presence of severe manifestations of VP and the extent of residual PPO activity. Indeed, the highest PPO activities, as determined by prokaryotic expression, were present in both the most and the least severely affected patients. Nor is the mechanism whereby point mutations in the PPO gene produce the variable non-porphyric features of homozygous VP known. Short stature and neurological defects are often present in homozygous variants of AIP and HCP, which may suggest that certain early stages of development are critically dependent on haem biosynthesis. Furthermore, as exemplified by the two sisters described in Chapter 16, DdP and AN, genotypically identical but phenotypically dissimilar, factors beyond the PPO gene must also influence clinical expression.

Our demonstration of late-onset HVP accompanied by the acute attack and also by urine and stool porphyrin profiles more typical of heterozygous VP (Table 19-1) also suggests that the lack of acute symptoms in early-onset HVP is more a feature of the prepubertal state than of HVP *per se*. Our first proband, LO, has recently begun menstruating. It is possible that she may now be prone to acute symptoms, and we now take all possible precautions to avoid this.

19.2 THE BIOCHEMICAL AND CLINICAL EXPRESSION OF VP

The study described in Chapter 17 is the first large kindred to be fully investigated by DNA testing as well as by biochemical and clinical analysis. Altogether 62 informative members of the family were tested and 33 were R59W-positive, which is, as expected, in keeping with an autosomal dominant mode of transmission. The sex imbalance in favour of females is probably fortuitous. The difference is not statistically significant. Nor has there ever been evidence to suggest that males are less likely than females to carry VP or to survive postnatally. Indeed, most studies have suggested that females are more likely to be clinically affected than males, at least in terms of acute symptoms.

The correlation of R59W status with stool biochemistry is in keeping with the findings described in Chapter 10. Diagnosis by urine and stool biochemistry was insensitive: only 52% of adult subjects showed unequivocal positive stool biochemistry and 36% were in fact unequivocally negative. Nor was the performance of stool biochemistry related to age; false-negative results could be found at any age, and several older patients who had been unequivocally positive previously now demonstrated normal stool excretion patterns.

In this family, the diagnostic utility of plasma fluoroscanning is confirmed. 87.5% of adult subjects who are R59W-positive were correctly diagnosed by plasma fluoroscanning, and 100% of those who are R59W-negative were correctly diagnosed as such. Interestingly, all three false-negative results were obtained in older patients (aged 57-73) who had previously shown positive biochemistry that, with age, had reverted to a normal excretion pattern. Plasma fluoroscanning gave no positive results in children aged less than 16. Taken together, these results suggest that stool porphyrin testing alone will only detect approximately 50% of patients carrying the R59W mutation, but that the detection rate rises to nearly 90% if plasma fluoroscanning is substituted for porphyrin testing. Neither is however 100% sensitive, and it appears that children and older subjects who have had VP for many years in particular are prone to false-negative results.

It is disturbing that only 50% of R59W-positive and 64% of R59W-negative respondents were aware of having been tested for porphyria previously. Two members recalled having been tested previously yet had not thought it important to apprise themselves of the result. Yet

this is a large family which has been known to carry porphyria for decades, and many of its older members have participated in several studies with the UCT Porphyria Centre. Indeed, every member of this family, when contacted, was aware of the presence of porphyria within the larger family. This suggests a high degree of complacency about porphyria amongst the South African VP population. Yet at the same time, this study may provide the explanation for this complacency. Since only one acute attack (35 years previously) and no deaths from porphyria had occurred in four generations, it would appear that many members do not regard VP as of any personal relevance.

62 informative members of the family were tested genetically. All were asked to provide clinical details in response to a questionnaire. The return rate was very low for those who had tested negative; in most cases these subjects, being aware of their status and asymptomatic, attached little importance to their answers. 26 of 29 R59W positive subjects returned the questionnaire, and a further 2 subsequently provided the information telephonically. Thus reliable information was forthcoming in 97% of those positive for the mutation. Patients were aware of their DNA status when they replied which has the potential to bias the answers, but this does not appear in practice to have been a problem. The questionnaire was designed to allow the patient to describe possible acute symptoms or skin symptoms in a manner which would allow us to distinguish symptoms typical of porphyria from those less likely to be significant. In the case of equivocal answers, the respondent was contacted telephonically and further questioned. Thus the final conclusions in terms of the prevalence of symptoms are reliable.

17 of these 28 adult patients were and always had been completely asymptomatic (60.7%). This is considerably greater than the 38.3% reported by us in 1996 and the 10% reported by Eales et al in 1980 (Figure 19-1). In large measure the difference between the present study and the 1996 study is the incorporation of many more true silent cases, i.e. subjects who would not have been recognised as DNA-positive by stool porphyrin testing in 1996 but who are now readily identifiable by genetic testing. This lack of ascertainment bias also allows the surprising, though perhaps not entirely unexpected, finding that the acute attack is a rare event amongst unselected patients with VP. It would appear to have affected only one, or perhaps two, individuals on a single occasion during their lifetimes. The low incidence of acute symptoms cannot be attributed to better education about porphyria alone, since this study has shown that approximately 50 to 60% of subjects even within a well-studied family are unaware of their status and presumably have not taken any special precautions for porphyria.

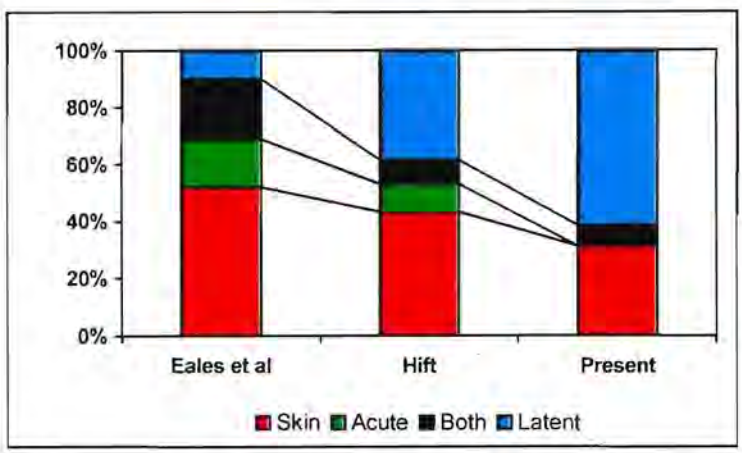


Figure 19-1. Proportions of patients expressing clinical symptoms in Cape Town: before 1980 (Eales et al 1980), 1997 (Hift et al 1997) and as revealed by genetic testing (present study).

Though Mustajoki (1980) reports an approximately equal sex-incidence of skin disease in his Finnish patients with VP (10 males and 14 females), females appeared more likely to experience skin disease in our family. Most reports do however suggest a higher incidence of acute symptoms in females. Since the prevalence of acute attacks was extremely low in this study, this point cannot be taken further here.

Since the presence or absence of skin disease does not, after puberty, correlate with age, other factors must be responsible for this phenotypic difference. Clearly the elevated levels of endogenous sex steroids present after puberty cannot be responsible for skin disease alone since not all post-pubertal subjects, male or female, develop it. Similarly, 4 of 5 subjects aged 48 and older had noted an improvement in skin disease, of whom two were females as yet premenopausal and one was male. This parallels the tendency we had noted for stool porphyrin excretion to decrease with advancing age; three subjects in the study who currently have normal stool porphyrin excretion patterns are known to have been positive previously. This improvement, both clinical and biochemical, with age is unexplained.

Currently active skin disease was nearly always accompanied by unambiguously diagnostic stool porphyrin profiles; in one woman only was an equivocal profile present. The converse did not hold true: 50% of patients without skin disease showed normal biochemistry, whereas 33% had a typical VP profile and 17% an equivocal result. The skin disease experienced by these subjects was typical of previous descriptions of VP. It was most marked on the dorsal surfaces of the hands and in two-thirds was limited to the hands. The face, forearms or feet were also affected in the remainder.

In this study, no apparent association with hypertension, renal disease or heart disease was apparent. However a small study such as this would be unlikely to detect any such association unless the magnitude were extraordinarily large, as in the family described by Church et al (1992). Rather, large epidemiological studies such as those by Andersson and Lithner (1994) are required to prove such an association, and these have not yet been attempted in South Africa. An interesting finding is the large number of our R59W-positive subjects who report minor psychiatric symptoms such as anxiety and depression. The difference between the prevalence of this in our DNA-positive and DNA-negative subjects is however not statistically significant. Though claims for significant psychiatric symptoms in association of porphyria have been made (McEwin 1972, Tishler et al 1985), studies in cohorts of patients with both AIP and VP have not confirmed this (Wetterburg 1967); Kaupinnen and Mustajoki (1992) found a slight excess of generalised anxiety during early middle age.

19.3 THE ACUTE ATTACK

Our experience with all patients admitted with acute symptoms of porphyria to a single hospital over a period of 12 years is reported in Chapter 18. The UCT Porphyria Service enjoys a reputation for excellence in South Africa, and patients with particularly severe problems are likely to be referred to us for diagnosis and management. Thus our experience is to a degree biased toward the more severe patient. A further potential source of bias includes socio-economic circumstances. In South Africa, the wealthier and the medically-insured community usually make use of private health services, whereas the remainder are served by state hospitals, including the academic hospitals such as our own. Patients requiring special expertise are however referred to an academic hospital such as Groote Schuur Hospital. VP in particular is largely a disease of white people in South Africa, who tend to use the private sector. Yet there are no institutions or doctors other than our own with any particular expertise or interest in porphyria. Since our advice has been sought in the few instances where haem arginate has been used elsewhere in South Africa, we are aware of most patients experiencing acute attacks—and certainly those experiencing acute attacks of any degree of severity—in

the country. This has been an infrequent occurrence, and we believe that our experience is generalisable to the experience of the country as a whole.

Clinical features

The cardinal features of the acute attack, pain, vomiting and autonomic symptoms, have not changed since those described in previous series (Figure 19-2). Thus pain remains the most common presenting feature and is almost invariable. Particular striking however are the exceptions. Three patients developed unusual manifestations following attacks in which pain was not prominent, and are described below. Hypertension was even more common in this series than has previously been described, with a systolic blood pressure in excess of 120 mm Hg being recorded at some time during more than 90% of admissions, while only 68% will have it at the time of presentation, suggesting that it is a manifestation which becomes progressively more frequent as the acute attack progresses. Tachycardia is less common; and a pulse rate exceeding 90/minute was present in less than half of all admissions. Nausea and vomiting are common. Constipation was not a prominent feature in our patients at the time of admission, which may reflect the short period between onset of symptoms and presentation. The most striking changes however are in the rate of major complications of the acute attack: seizures, neuropathy and respiratory failure. These are discussed later in this chapter.

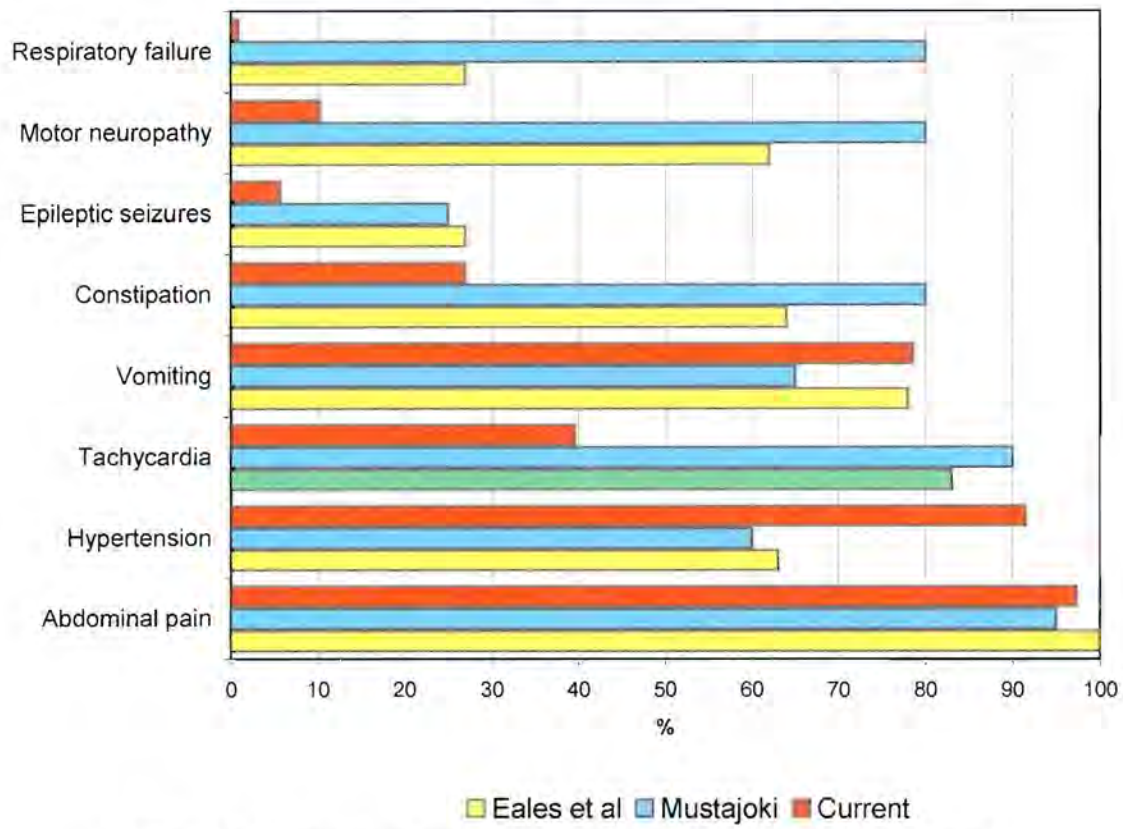


Figure 19-2. The symptoms and signs of the acute attack as reported by Eales et al (1980), Mustajoki (1980) and contrasted with the experience described here.

Painless attacks: unusual and misleading presentations of porphyria

Patient FW

This 27 year-old woman was not known to have porphyria. She was admitted to hospital in our absence with abdominal pain; and as part of her investigations, a Watson-Schwartz reaction was performed and was positive. VP was subsequently confirmed biochemically, and an elevated ALA and PBG were shown in keeping with an acute attack. When assessed by us 48 hours after admission, her abdominal pain had abruptly ceased, though her serum sodium was 114 mmol/l. There were no signs of neuropathy. The disappearance of the pain was interpreted as an improvement in her condition, and haem arginate was withheld. Within hours , she developed severe flaccid quadriparesis. She required prolonged ventilation in the intensive care unit and had a prolonged convalescence, though recovery was eventually complete.

Patient MH

This 32 year-old man was not previously known to have porphyria, though he was eventually shown to belong to a family known to have VP. He was admitted to another hospital with abdominal pain, and was found to have a positive Watson-Schwartz reaction. Our opinion was requested. At this stage his abdominal pain had abruptly ceased though he complained of discomfort in the legs. Neurological examination was normal. Haem arginate was withheld. He developed a moderately severe quadriparesis within hours. Ventilation was not required and recovery, though slow, was complete.

Comment

As suggested by Figures 18-7 and 18-8, recovery from the acute attack is usually a smooth process with a gradual lessening of pain over 72 or more hours. These two cases were exceptional in that the abdominal pain stopped abruptly. In both, quadriparesis followed very rapidly. In the first, the low serum sodium should have alerted us to the severity of the attack. We have therefore learnt that a sudden cessation of abdominal pain may be an ominous sign portending the onset of motor neuropathy. The reason for this is unknown. Secondly, we have learnt to recognise discomfort in the legs and buttocks as a warning of impending neuropathy. An abrupt cessation of abdominal pain, complaints of discomfort in the legs and hyponatraemia mandate the immediate administration of haem arginate.

Patient NG

This 25 year-old woman is a member of a family known to carry VP. She had been biochemically tested at the age of 16 and was found to be negative. She had not been tested subsequently. She now presented to a private physician with a sudden onset of confusion and abnormal behaviour. At no stage was pain of any description present. No cause for the confusion could be found, and there were no other objective neurological abnormalities. A brain CT scan was normal. Testing for the R59W mutation was positive. Unfortunately the urine was not tested for the presence of PBG. In a telephonic consultation, we were asked whether this was compatible with the acute attack of porphyria. We suggested that it was not, since no other patient in this series had presented with confusion in the absence of a demonstrable cause, and a presentation without pain was highly exceptional. We suspected a primary psychiatric disorder. Within 7 days she had recovered completely. We examined her two weeks later in the porphyria clinic at Groote Schuur Hospital. She is a bright, appropriate young person with no evidence or history of mental illness. She now complained of severe discomfort over her entire body. This was accompanied by a mild glove-and-stocking sensory loss and hyperaesthesia, in keeping with a diagnosis of causalgia or complex regional pain syndrome. She described typical features such as being unable to wear underclothes because

of the intense discomfort caused by the material against the skin. We have seen this syndrome previously in two patients with AIP, following a documented acute attack, though in these cases the attack had been typical, with severe abdominal pain and no confusion. Accordingly, it would appear in retrospect that NG did indeed suffer an acute attack of VP. Porphyria is an unusual cause of an acute confusional state without the classic features of the acute attack such as abdominal pain, constipation and vomiting, though isolated cases of AIP presenting with confusion have been reported (Tan and Yeow 1988). Perhaps porphyria indeed deserves the title *the little imitator* which Waldenström bestowed upon it.

Electrolyte abnormalities

A high proportion of patients (64%) demonstrated an elevated urea on admission. There was no further increase following admission, and in most cases resolution was prompt. This is most likely due to dehydration since there was a rapid improvement following admission to hospital, and all our patients receive intravenous normal saline-dextrose following admission. AIP has however been associated with chronic renal failure, and both patients with severe, recurrent AIP (HK and MJ) demonstrated chronically raised creatinine levels. Hyponatraemia is common; 26.6% of our patients demonstrated hyponatraemia on admission and nearly 40% had low sodium concentrations at some stage of the admission. This may in part be due to dehydration, though the slower response to intravenous normal saline as compared to urea suggests that other factors may be operative, as has been suggested by Eales (1980). Severe hyponatraemia is dangerous and is a marker of a particularly severe attack. Of five admissions complicated by severe hyponatraemia, four were complicated by the onset of neuropathy; the fifth patient (AS) experienced seizures and a very severe course which is described below. In these patients, urine sodium concentrations were high, suggesting either renal salt-wasting or the syndrome of inappropriate ADH secretion. In each case the serum sodium failed to improve in response to fluid restriction alone; and rose only after the infusion of hypertonic saline. In two instances, this large urinary sodium loss was accompanied by marked kaliuresis and calciuresis, suggesting a primary tubular defect. It is therefore our impression that the hyponatraemia of the acute porphyric attack is due to renal salt-wasting rather than to SIADH despite suggestions in the literature linking SIADH to the hyponatraemia of the acute attack (Lipschutz and Reiter 1974, Farese et al 1979, Chogle et al 1980). It is therefore our practice to correct severe hyponatraemia *ab initio* with hypertonic saline. Rapid correction of hyponatraemia in AIP has resulted in central pontine myelinolysis and cortical laminar necrosis (Susa et al 1999), and correction must therefore be slow.

An unusually severe attack accompanied by an adrenergic crisis

Patient AS is described here as the autonomic features of her presentation were so extreme as to suggest a phaeochromocytoma. She is a member of a family known to have AIP. She was admitted to a country hospital following a spontaneous abortion at the age of 22. There she developed abdominal pain and hypertension. She was transferred to Groote Schuur Hospital. On admission, the serum sodium was 130 mmol/l, systolic blood pressure 230 mmHg and diastolic blood pressure 130 mmHg; pulse rate was 144/minute. She appeared to have little or no abdominal pain, but was drowsy and confused. A brain CT scan showed multiple areas of attenuation in keeping with ischaemia or infarction. Her serum sodium declined to 120 mmol/l and she developed seizures. The blood pressure and pulse remained extremely elevated and failed to respond to high doses of beta-adrenergic blockers. The degree of hypertension and tachycardia, as well as the encephalopathy and neurological deficits, were unprecedented in our experience of the acute porphyric crisis. We considered the possibility of a co-incidental phaeochromocytoma. She was admitted to the intensive care unit, intubated and ventilated, and treated with intravenous magnesium sulphate, pethidine, beta-blockade

and haem arginate. Magnesium sulphate was highly effective in controlling both the adrenergic features and the seizures. Recovery was rapid and she was discharged from hospital, neurologically intact and completely asymptomatic, after a total admission of just 9 days.

Clearly this severe adrenergic crisis was due to porphyria. Acute hypertension mimicking phaeochromocytoma as the main presenting feature of AIP has been reported (Bravenboer and Erkelens 1989). The neurological deficits and defects noted on CT scanning in our patient appear to reflect reversible cerebral ischaemia, which has occasionally been described in AIP. Sudden, permanent occipital blindness was reported in a 28 year-old woman who developed a severe attack of AIP accompanied by convulsions and peripheral neuropathy (Lai et al 1977). Examination of the brain showed extensive infarction in both occipital lobes consistent with anoxia. Cerebral vasospasm as well as transient brain lesions presumably due to ischaemia, have also been reported in AIP (Black et al 1995, Kupferschmidt et al 1995). Multiple cerebral lesions have been seen on MRI in both cerebral hemispheres during an attack of AIP accompanied by seizures and hallucinations. Both the clinical and MRI lesions regressed following treatment, which would suggest that vascular mechanisms may underlie the pathogenesis of cerebral dysfunction in AIP (King et al 1991).

Though magnesium sulphate has been suggested for the control of seizures in AIP (Taylor 1981, Sadeh et al 1991), it was primarily used in our patient since it is the standard agent employed at Groote Schuur Hospital for the peri-operative management of of phaeochromocytoma where it controls adrenergic overactivity smoothly and reliably. Magnesium sulphate worked extremely well in patient AS, and we now recommend its use in any patient in whom the adrenergic or cerebral features dominate the presentation.

Complications

The rate of complications of the attack is markedly lower in this series than in those described by Eales et al (1980) and Mustajoki (1980). In most cases neuropathy was mild and was limited to wrist-and foot-drop; only one patient required mechanical ventilation.

This remarkable improvement in outcome requires explanation. Other authorities have commented upon an increasingly favourable outcome for the acute attack (Bonkovsky and Schady 1982, Kappas et al 1989, Tschudy and Lamon 1980, Kaupinnen and Mustajoki 1992). The admissions described here are all recorded over a recent period, largely in the 1990s. Earlier series by contrast included patients from the 1960s to the 1980s. During this time, several factors have changed which may have led to a better outcome. There is a greater awareness and understanding of porphyria among both doctors and patients. Diagnostic tests for porphyria and for the acute attack have become more reliable and accessible. Patients understand their illness better and are perhaps more likely to present to hospital early. Management protocols for the acute crisis have been refined and specific therapy, haem arginate, is now available.

A striking finding in this study is that most episodes of neuropathy, seizures and paralysis occurred during the first admission, or during an admission before the diagnosis had been established. Similar observations have been made elsewhere. Thus, of a series of 9 patients with AIP admitted to a Taiwanese hospital, six developed neuropathy, six had autonomic dysfunction, five had mental changes, four had seizures, two had hyponatraemia and one died of intractable seizures (Chen et al 1994). Such experiences suggest that it is not the disease which has become milder, but our ability to manage it which has improved.

Haem arginate

A large body of evidence supports the contention that haem arginate is highly effective in aborting the acute attack (Bonkovsky et al 1971, Watson 1975, Lamon et al 1979, Pierach et

al 1980, McColl et al 1981, Mustajoki et al 1986, Tenhunen et al 1987, Tokola et al 1987, Bissell 1988, Volin et al 1988, Hift et al 1997). The only controlled trial (Herrick et al 1989b) was unable to show a significant difference in outcome. Yet Figures 18-9 and 18-10 strongly support the hypothesis that haem arginate is responsible for a rapid and reliable improvement in symptoms. These figures include only those patients who, on the basis of their symptoms, were given haem arginate; those admissions in which symptoms settled spontaneously (33%) are not included. Mean severity scores had not improved for up to 8 days, and improvement began promptly once haem arginate was given. Similarly, in these patients, pethidine requirements rise progressively until haem arginate therapy is given, following which a statistically significant and marked reduction in pethidine requirements is noted.

Despite the suggestion that haem arginate infusion is associated with a lower rate of thrombophlebitis than haematin (Mustajoki et al 1986, Tenhunen et al 1987, Tokola et al 1987), haem arginate infusion was frequently followed by severe thrombophlebitis at the drip site. Phlebitis complicated 14 of 79 infusions, particularly early in this series when haem arginate was administered in saline. A change from plastic to glass infusion bottles did not help. Administration in human serum albumin however appeared to be associated with a far lower rate of phlebitis, and we accordingly established this as our standard practice. Renal failure and coagulopathy, as reported for haematin were not encountered.

No serious complications such as hyponatraemia, neuropathy or seizures developed in any patient once haem arginate had been started; in each case, complications had developed prior to administration of haem arginate, or were present at the time of admission. The only exceptions were the two most severe patients, MJ and HK, described in Chapter 18.

Tin protoporphyrin

We have accumulated some experience in the use of tin protoporphyrin. We believe it to be effective in maintaining the efficacy of haem arginate in the face of frequent dosing which, in our experience, may lead to the development of tolerance and consequent therapeutic failure. In contrast to the experience of Dover et al (1993), we have found no evidence that the attack-free interval is significantly prolonged by its use. A further observation is with regard to its safety. Despite the administration of as many as 120 doses of 25 μg, there has been no clinical evidence of toxicity. When patients HK and MJ were on two occasions inadvertently exposed to sunshine within 48 hours of administration, marked skin erythema was noted in sun-exposed areas. Marked darkening of the skin was also noted over time. We have however noted the appearance of apparent iron overload and the accumulation of an unusual pigment, which may represent tin, in the livers of both patients who received large amounts of haem arginate and tin protoporphyrin. Definitive identification of this pigment is awaited.

Neuropathy

Though neuropathy was an unusual development during those acute attacks managed by us in Groote Schuur Hospital, it was already present in several patients who had had their first attack elsewhere. In all cases, the neuropathy was largely reversible. Three patients who had been severely quadriparetic (of whom two were transferred to us during the recovery phase, and one, FW, had developed paresis under our care, improved with physiotherapy and orthopaedic supports; all regained the ability to walk. A noticeable phenonomen amongst those patients with recurrent AIP and pre-existing neuropathy was what we have termed the "barometer" phenomenon: within 2-3 days of the start of a recurrent attack, wrist dorsiflexion tended to become both subjectively and objectively weaker; following haem arginate administration and resolution of the attack there was a prompt and complete return to the *status quo ante*. The clinical impression was that these patients operate just below a threshold of clinically-evident nerve damage: with a slight increase in "haem-synthetic dysfunction",

nerve function deteriorates, but this initial deterioration is immediately reversible provided the metabolic defect is rapidly repaired. Only if the crisis was neglected, did a more long-lasting deterioration in function become apparent. In the light of this observation, it is our standard practice to ensure that every patient with a history of previous neuropathy receives haem arginate without delay, whereas other patients are allowed 24 hours to determine whether spontaneous remission will occur.

Unusual forms of neuropathy

In addition to patient NG described above, two patients demonstrated unusual patterns of autonomic and sensory neuropathy.

Patient GW

This young woman with AIP was first diagnosed after experiencing an acute attack in another centre. This was unaccompanied by motor neuropathy. She thereafter began complaining repeatedly of chronic, unbearable pain. Her physician labelled these as acute attacks and admitted her on several occasions, treating her pain with pethidine. Yet samples sent to our laboratory failed to reveal any further elevation in PBG during these admissions, and the possibility of opiate-addiction was raised. She was then referred to us for an opinion. Questioning quickly revealed that her problem, like that of NG, was one of a causalgia, or complex pain syndrome. She gave a graphical description of tight clothing or a crease in the bed sheets causing an unbearable sensation of pain. Though careful electrophysiological testing failed to reveal evidence of motor or sensory nerve dysfunction, our neurologists believed that her symptoms were in keeping with a residual small-fibre sensory neuropathy, which is difficult to detect in the laboratory. With adequate explanation, reassurance and simple analgesics, she required no further opiates and her condition improved over several months.

Patient TR

This 29 year-old woman with recurrent menstrually-associated attacks of AIP had a mild wrist-drop, which improved. Striking however were peripheral features of autonomic nerve dysfunction: light stroking of the dorsal surface of the forearm would bring about dramatic pilo-erection ("goose bumps") and profuse sweating in that arm.

Seizures

Seizures were rare in this series. In most cases, a precipitating factor was evident. In three cases, seizures accompanied severe hyponatraemia; the association between encephalopathy and seizures in porphyria and hyponatraemia has been noted previously (Dixon 1997, Usalan et al 1996). In two further instances, the seizures were typical of those associated with high doses of pethidine, and one patient had received no less than 10650 mg of pethidine during a single admission. Pethidine is metabolized *in vivo* to norpethidine, a metabolite which is epileptogenic (Schwark et al 1986, Czuczwar and Frey 1986, Armstrong and Bersten 1986, Hagmeyer et al 1993, Adair and Gilmore 1994, Kussman and Sethna 1998, McHugh 1999), and pethidine-related seizures hve been reported in a patient with HCP (Deeg and Rajamani 1990). Similar seizures were noticed in patient HK, and were typically preceded by myoclonic jerks. Both the seizures and the myoclonus were easily abolished by clonazepam, and we now prescribe clonazepam routinely in any patient whose pethidine requirements are unusually high.

Recent evidence indeed suggests that seizures are not as common in porphyria as was previously thought. Swedish investigators contacted a large group of patients with AIP and sought evidence for epilepsy (Bylesjo et al 1996). Just 10 of 268 patients (3.7%) reported seizures. Eight of these were women and two were men. Six had had tonic-clonic seizures and

four had had secondarily generalised partial seizures. In six patients, seizures were associated with an acute attack of AIP, and three patients had been severely hyponatraemic. This supports our contention that seizures in porphyria are largely a feature of the acute attack itself rather than the result of any latent tendency; furthermore, that in a substantial proportion, an immediate initiating event, typically hyponatraemia or the use of large amounts of pethidine, is present.

Variations in the profile of the acute attack: AIP versus VP

VP is much more common in South Africa than AIP. Over the period of this study, we have diagnosed 687 patients with VP and 48 with AIP. Yet the ratio of patients with acute attacks of VP to patients with AIP is 10:14 and the difference is highly significant ($p<0.00001$, $\aleph^2$). This suggests that the acute attack is more prevalent amongst patients with AIP than with VP. The symptoms of the acute attack of both AIP and VP are essentially the same, yet the patient profile is dissimilar, and they appear to differ in average degree of severity. Young females predominated in AIP, whereas the sex-ratio was equal in VP. Patients with VP were older than those with AIP. Recurrent attacks were also more likely in AIP than in VP. Recurrent attacks were particularly a feature of females with AIP, whereas a similar predisposition was not shown in females with VP. The reason for these interesting findings may lie largely with the precipitating factors. As shown in Figure 18-6, two important patterns are evident. Firstly, acute attacks in males with VP are largely related to drug exposure, and the number of recurrent attacks is dominated by two subjects who abused both cannabis and alcohol (Table 18-5). Once this pattern had been recognised, the patients were counseled and the frequency of attacks diminished. Indeed, with each further attack, a history of additional alcohol and cannabis abuse was obtained. Patient DA experienced 6 attacks over a period of 15 months, and CH experienced 5 attacks over a period of just 6 months, yet since the end of this short period, neither has required further admission. This suggests that the acute attacks arose in response to some exogenous factor, of which the exposure to alcohol and cannabis appear most likely. Similarly, one male subject with AIP, CK, described in detail in Chapter 18, experienced 13 attacks over a period of 25 months. He was unforthcoming about his habits, but was reported by his relatives to abuse cannabis and alcohol (even within the hospital); urine cannabinoids were positive, proving exposure to cannabis, on the three occasions late in his course on which they were sought. It would appear probable that more than just those proven three of his attacks were induced by drug exposure. The effect of cannabis on haem synthesis has not been reported, whereas several authors have discussed the effects of alcohol on porphyrin metabolism. Alcohol-induced changes have been shown in the enzymes of haem synthesis (Gajdos 1968, McColl et al 1980, Moore et al 1984). The acute effects of alcohol in AIP have been studied in an Indian population where 34 teetotal patients with AIP in remission were given 60 ml of 30% ethanol. The Watson-Schwartz test for porphobilinogen became positive in half of these patients, and in approximately one quarter a clinical attack was precipitated (Saksena et al 1991). Though alcohol in moderation is probably safe, there is evidence that alcoholic binges may be associated with acute attacks (Thunell et al 1992, Kauppinen and Mustajoki 1992).

Despite the high prevalence of porphyria, and VP in particular, precipitation of the acute attack by drugs appears to be rare in Cape Town. With the exception of these three substance-abusers, only 13 episodes in 12 years could be ascribed to drugs. This observation is in keeping with the conclusion that, in the stable patient with porphyria, administration of drugs known to be porphyrinogenic rarely results in mischief (Mustajoki and Heinonen 1980). Precipitation of the acute attack by drugs was proportionately more common in VP than in

AIP, probably by default since a high proportion of patients with AIP had either menstrually-related attacks or attacks of unknown cause.

The second striking feature is the proportion of females of AIP in whom attacks appeared to be menstrually-related. Coupled with this is the high proportion of cases in females in which the cause was unknown. It is possible that many of these may have reflected menstrually-related attacks, since our criteria were stringent and we have not recorded attacks as menstually-related unless there was a clear and reproducible relationship between the onset of menstruation and the onset of the attack, which typically predated the onset of menstruation by 2-3 days. If the remainder of these attacks in females with AIP were indeed hormonally induced, the relationship is not clear-cut and is difficult to prove. In many cases, a clear temporal relationship could not be established. Furthermore, the results of treatment with GnRH agonists in three patients have been contradictory. One subject with AIP, patient TR, experienced severe monthly attacks, requiring hospitalisation and associated with a neuropathy, with a clear association between the onset of menstruation and the onset of pain. She received GnRH agonist therapy with a complete absence of attacks for two years. Following cessation of therapy, she rapidly fell pregnant and has remained well ever since. Despite occasional episodes of mild pain, she has not required further hospitalisation. Two other subjects, HK and MJ, described in detail in Chapter 18, failed to respond to GnRH agonist therapy. Both became amenorrhoeic and suppression of oestrogen and progesterone was documented. Despite this, acute attacks continued. In the case of MJ, acute attacks initially continued at intervals of approximately four weeks, more or less coinciding with the expected onset of menstruation despite amenorrhoea. This is unexplained. The published experience with GnRH agonist therapy bears out this incomplete and not entirely explicable response to menstrual suppression; Herrick et al (1990) report a poorer response in patients in whom the relation between symptoms and menstruation is less typical.

A further point of interest arising from this study is the very low prevalence of acute attacks in pregnancy; with just a single attack, with a good outcome, arising in pregnancy in this series. Nor are we aware of any other attack in pregnancy having occurred elsewhere in South Africa during the past 12 years. Even patient TR, who required 24 months' GnRH agonist therapy to control typical menstrually-related attacks, had an uneventful pregnancy shortly after discontinuing therapy.

Severity of the attacks

The severe attack of VP could be just as severe as the AIP, as exemplified by FW, who developed hyponatraemia, seizures and profound quadriparesis requiring ventilation. However, it is our impression that the average acute attack of AIP is more severe than that of VP. Thus the mean systolic and diastolic blood pressures and the pulse rate and the proportion of patients requiring haem arginate were all significantly higher in AIP than in VP (Table 18-8. Though a lower requirement for pethidine, average hospital stay and duration of symptoms were shown in VP, the spread is wide and the differences not statistically significant. The rate of significant complications is equivalent in VP and AIP: 5.7% and 4% for seizures, 9.2% and 12% for neuropathy.

SECTION 4

CONCLUSIONS AND FUTURE WORK
REFERENCES
APPENDICES

CHAPTER 20:

CONCLUSIONS AND FUTURE WORK

20.1 THE DIAGNOSIS OF VARIEGATE PORPHYRIA

The work described in Section 1 of this dissertation has important consequences for the diagnosis of variegate porphyria in South Africa. The high prevalence of this mutation amongst the VP population of South Africa suggests a more important role for DNA testing in the first-line diagnosis of patients suspected of carrying VP than it has in countries where the disease is more heterogeneous.

We have shown in Section 2 that the sensitivity and specificity of traditional chromatographic examination of urine, stool and plasma for a diagnosis of VP are less than is desirable even in a specialised laboratory such as ours. We have also shown the deficiency of screening tests for first-line diagnosis, a technique which unfortunately persists in some laboratories in South Africa. An important consequence of this work has been the confirmation that as many as 30% of all adults carrying the R59W mutation are not detectable by urine and stool porphyrin analysis. The increased sensitivity shown by fluoroscanning indicates that this is a more reliable (and indeed easier) test for the routine porphyrin diagnostic laboratory. We have now altered our diagnostic approach in that all samples submitted to the UCT laboratory are first tested by plasma fluoroscanning, and stool chromatography is not performed unless the fluoroscanning is positive except under special circumstances.

It is clear that the non-specialised laboratory offering screening, non-quantitative and even quantitative chromatography is placed at a disadvantage in interpreting faecal porphyrin analysis. Experience has suggested that no South African laboratory other than the UCT laboratory is prepared or able to undertake the rigorous biochemical analyses required for anything approaching an accurate diagnosis. It is therefore our duty to provide guidance in the most appropriate and correct use of the diagnostic techniques available for the diagnosis of VP. This we have already begun, and the work continues. The standard approach we are recommending makes use of two of the newer diagnostic techniques discussed in this dissertation: demonstration of the R59W mutation by *AvaI* restriction analysis and the use of diagnostic plasma fluoroscanning. These techniques have the added advantage of requiring a blood specimen only. Thus, with the promotion and acceptance of our recommendations, we believe that simpler and more accurate diagnosis of VP is possible in other South African laboratories without the necessity to set up TLC or HPLC porphyrin separation and quantitation protocols. Our laboratory will continue to retain a central role in the maintenance of standards, the refinement of diagnostic techniques and the identification of non-R59W VP families and the mutations they carry. An important function of our laboratory is the comprehensive diagnostic, counselling and treatment service we offer. Since this comprehensive service is unlikely to be provided elsewhere, a possible deleterious aspects of the decentralisation of accurate testing for VP may be the uncoupling of diagnosis from education and advice on management.

An important task which remains is the education of clinicians and clinical pathologists in the correct use of the DNA test, as outlined in the algorithms in Chapter 6 and Appendix 6. In particular, it must be understood that a negative R59W test will not exclude patients with forms of porphyria other than VP, nor the unusual patient with an R59W-negative form of

VP. Additionally is important that the limitations of DNA testing in assessing clinical severity are understood. This is an educational task with which we continue.

20.2 FURTHER RESEARCH REGARDING THE MOLECULAR BASIS FOR VARIEGATE PORPHYRIA

Possible mechanisms for exploiting the genetic homogeneity for VP we have shown include the pursuing of structure-function relationships. As a first step, it is appropriate to recreate to these mutations by site-directed mutagenesis and then to express the mutant protein. Work is currently underway in our laboratory on the expression of the R168C, R59W and Y348C mutations. Such work may also throw light on genotype-phenotype relationships. An important avenue of research is the creation of an animal model for the elucidation of the clinical features of the porphyrias. An R59W-positive gene-knockout mouse model has been developed by Professor Harry Dailey and a colony is now established in our laboratory. These mice have been shown to have urine and stool porphyrin profiles in keeping with VP, and investigative work will commence shortly.

We are now in a position to establish more accurate prevalence data for VP in South Africa. Whereas Dean's figures were based on the results of thousands of stool porphyrin screenings, it is possible to reproduce this work more accurately and more easily by the application of the *AvaI* restriction assay to a representative population. Simultaneous use of SSCP/heteroduplex analysis would indicate the prevalence of non-R59W PPO mutations and polymorphisms.

An important goal of molecular biology research in all the porphyrias, as with genetic disorders generally, is the identification of safe and effective forms of gene therapy. For most of our patients with VP, this is not a priority at present, since, as described in this thesis, most are little troubled by their illness. It would however be of particular value in patients with HVP, in those with AIP with frequent and debilitating crises, and perhaps in those few of our patients with heterozygous VP in whom the skin disease is more than usually disfiguring.

20.3 FURTHER STUDIES OF THE BIOCHEMICAL FEATURES OF VARIEGATE PORPHYRIA

An interesting conclusion from this work is that the classic diagnostic stool porphyrin profiles described by the foremost authorities in the past may have led to an overly-simplistic belief that the diagnosis of VP by stool porphyrin testing is simple and clear-cut. This did not take account of the marked variability in stool porphyrin excretion patterns, particularly of the variation in stool protoporphyrin. It is now necessary to validate our new diagnostic criteria prospectively in larger numbers of subjects, normal and with VP. An increased reliance on a raised stool coproporphyrin may result in the misdiagnosis of HCP, a condition which is extremely rare in South Africa and was not encountered in the series of patients in which these criteria were established.

It is clear that very little is known of porphyrin handling within the bowel. Kinetic work on the excretion, reabsorption and enterohepatic cycling of porphyrins may provide interesting results: this may additionally have clinical implications for the use of sorbent therapy such as oral charcoal and cholestyramine in the therapy of porphyria.

20.4 THE CLINICAL FEATURES OF VARIEGATE PORPHYRIA

The work of Chapter 17 has confirmed that the majority of patients with the R59W mutation are asymptomatic. Particularly encouraging is the extremely low prevalence of acute attacks. Thus we now have objective evidence to suggest that variegate porphyria is clinically not as severe a problem as the early literature may suggest. We can only speculate as to the cause of the apparent improvement in the well-being of our VP patients. We believe that in part the difference is one of ascertainment: symptomatic patients draw attention to themselves whereas those who are biochemically or clinically silent do not. However, it is probable that at least some of the favourable clinical outcome for VP is due to the educational and support efforts of our Centre, particularly with improved diagnostic efforts, and active education programme and our important work of the extension and maintenance of the drug-safety database (Appendix 9). Such work must continue, and research into the mechanisms of drug-induction of porphyria, and of more accurate models for the prediction of porphyrinogenicity is required.

Our study is unable to demonstrate which factor or factors are responsible for expression, or conversely non-expression, of VP in subjects carrying the R59W mutation. This is an important and potentially fertile field for further research: it would appear reasonable to direct initial studies into the impact of factors such as endogenous hormones, low-grade exposure to potentially porphyrinogenic compounds and genotypic variation in haem-containing enzymes such as the cytochrome P450 group on haem requirement. This may ultimately lead to the discovery of new forms of therapy based on the modulation of the activity of these enzymes.

Our description of a large series of patients with the acute attack in Chapter 18 provides an overview of the presentation and management of this potentially fatal complication in the modern era. An important task is for us to educate clinicians both within South Africa and elsewhere who may be called upon to manage the patient with the acute attack in the light of our experience. By providing a comprehensive description of the clinical features of the acute attack as currently seen, and by recommending those forms of therapy which have contributed to a successful outcome in a high proportion of our patients, we may contribute to an improved prognosis for patients with porphyria generally. This educational task has already begun.

20.5 RESEARCH INTO THE ACUTE ATTACK

The central question as to how porphyria brings about in the classic neurovisceral features of the acute attack has not yet been satisfactorily answered. Further research into this question is required; intracellular haem deficiency, and possibly haem-deficiency of major haem-containing enzymes systems such as tryptophan dioxygenase, require further study. Our R59W-positive mouse model provides one experimental model which we intend to exploit.

20.6 HOMOZYGOUS VARIEGATE PORPHYRIA

We have extended the clinical description of HVP to include two subjects with a milder form of the syndrome, which we have termed the late-onset form of HVP. We have supported the proposition that a "lethal" mutation in HVP is always balanced by a less deleterious mutation on the second allele, since we have not encountered an R59W-positive homozygote, and we have provided evidence that the three additional mutations in our subjects appear to be less deleterious to the host than the our R59W mutation. The natural history and life expectancy of HVP are not yet known, and we shall be following our patients carefully in the years to come.

REFERENCES

Adair JC, Gilmore RL (1994). Meperidine neurotoxicity after organ transplantation. J Toxicol Clin Toxicol 32: 325-8.

Aizawa T, Hiramatsu K, Ohtsuka H, Niwa A, Yukimura Y, Yamada T, Kiyosawa K, Furuta S, Ogata H, Miyamoto H (1987). Defective hepatic anion transport in variegate porphyria. Am J Gastroenterol 82(11): 1180-5.

Albers JW, Robertson WC, Daube JR (1978). Electrodiagnostic findings in acute porphyric neuropathy. *Muscle Nerve* 1: 292-6.

Allen BR, Parker S, Thompson GG, Moore MR, Darby FJ, Hunter JAA (1975). The effect of treatment on plasma uroporphyrin levels in cutaneous hepatic porphyria. Br J Dermatol 93:37-42.

Allison BS, Schatz G (1986). Artificial mitochondrial presequences. Proc Natl Acad Sci USA 83: 9011-5.

Anderson KE (1989). LHRH analogues for hormonal manipulation in acute intermittent porphyria. Semin hematol 26:10-15.

Anderson KE, Alvares AP, Sassa S, Kappas A (1976). Studies in porphyria: V: drug oxidation rates in hereditary hepatic porphyria. Clin Pharm Therap 19:47-54.

Anderson KE, Spitz IM, Bardin CW, Kappas A (1990). A gonadotropin releasing hormone analogue prevents cyclical attacks of porphyria. Arch Intern Med 150:1469-74.

Anderson KE, Spitz IM, Sassa S, Bardin CW, Kappas A (1984). Prevention of cyclical attacks of acute intermittent porphyria with a long-acting agonist of luteinizing hormone-releasing hormone. N Engl J Med 311:643-5.

Andersson C, Lithner F (1994). Hypertension and renal disease in patients with acute intermittent porphyria. J Intern Med 236:169-75.

Armstrong PJ, Bersten A (1986). Normeperidine toxicity. Anesth-Analg 65:536-8.

Atsmon A, Blum I (1970). Treatment of acute porphyria variegata with propranolol. Lancet 1:196-7.

Atsmon A, Blum I, Fischl J (1972). Treatment of an acute attack of porphyria variegata with propranolol. S Afr Med J 46:311-4.

Ayres S (1987). Homozygous variegate porphyria: vitamin E as a possible therapeutic approach. J Royal Soc Med 80:129.

Aziz MA, Watson CJ (1969). An analysis of the porphyrins of normal and cirrhotic human liver and normal bile. Clin Chim Acta 26:525-31.

Bachman RC, Burnham BF (1969). Purification of porphyrin esters by gel filtration. J Chromatogr 41:394-9.

Bargetzi MJ, Meyer UA, Birkhaeuser MH (1989). Premenstrual exacerbations in hepatic porphyria: prevention by intermittent administration of an LH-RH agonist in combination with a gestagen. JAMA 261:864.

Barnes HD (1945). A note on porphyrinuria with a resume of eleven South African cases. Clin Proc 4:269-5.

Barnes HD (1958). Porphyria in South Africa: the faecal excretion of porphyria. S Afr Med J 32:680-3.

Barnes HD (1963). Counter-current analysis of ether-soluble stool porphyrins. S Afr J Lab Clin Med 9:177-82.

Beattie AD and Goldberg A (1976). Acute intermittent porphyria. Natural history and prognosis. In: Porphyrins and human disease. Doss M (ed). Karger: Basel, Munich, Paris, London, pp. 245-250.

Becker DM, Goldstuck N, Kramer S (1975). Effect of delta-aminolaevulinic acid on the resting membrane potential of frog sartorius muscle. S Afr Med J 49:1790-2.

Becker DM, Viljoen JD, Katz J, Kramer S (1977). Reduced ferrochelatase activity: a defect common to porphyria variegata and protoporphyria. Br J Haematol 36:171-9.

Belcher RV, Smith G, Mahler R (1969). Biliary protein-bound porphyrins in porphyria variegata. Clin Chim Acta 25:45-52.

Berglund L, Angelin B, Blomstrand R, Drummond GH, Kapas A (1988). SN-protoporphyrin lowers serum bilirubin levels, decreases biliary bilirubin output, enhances biliary heme excretion and potently inhibits hepatic heme oxygenase in normal human subjects. Hepatology 8:625-31.

Beukeveld GL, Wolthers BG, Van Saene JJ, De Haan TH, De Ruyter-Buitenhuis LW et al (1987). Patterns of porphyrin excretion in feces as determined by liquid chromatography; reference values and the effect of flora suppression. Clin Chem 33:2164-70.

Beukeveld GLL, Wolthers BG, Nordmann Y, Dybach J-C, Grandchamp B et al (1990). A retrospective study of a patient with homozygous form of acute intermittent porphyria. J Inherited Metab Dis 13:673-83.

Birnie GG, McColl KE, Thompson GG, et al (1987). Antipyrine metabolism in acute hepatic porphyria in relapse and remission. Br J Clin Pharmacol 23:358-61.

Bissbort S, Hitzeroth HW, de Wentzel DP, Van den Berg CW, Senff H et al (1988). Linkage between variegate porphyria (VP) and the alpha-1-antitrypsin (PI) genes on human chromosome 14. Hum Genet 79:289-90.

Bissell DM (1988). Treatment of acute hepatic porphyria with hematin. J Hepatol 6:1-7.

Black KS, Mirsky P, Kalina P, Greenberg RW, Drehobl KE, Sapan M, Meikle E (1995). Angiographic demonstration of reversible cerebral vasospasm in porphyric encephalopathy. Am J Neuroradiol 16:1650-2.

Bloch B (1965). *In*: J Obstets Gynaecol Brit Commonwealth 72:391.

Blom H, Andersson C, Olofsson BO, Bjerle P, Wiklund U et al (1996). Assessment of autonomic nerve function in acute intermittent porphyria: a study based on spectral analysis of heart rate variability. *J Int Med* 240:73-9.

Bloomer JR (1981). Enzyme defects in the porphyrias and their relevance to the biochemical abnormalities in these disorders. J Invest Dermatol 77: 102-6.

Bloomer JR, Logan GM, Weimer MK, Pierach CA (1990). Bile porphyrin and porphyrinogen levels in erythropoietic protoporphyria and variegate porphyria. Molec Aspects Med 11:54-5.

Blum I, Atsmon A (1976). Reduction of porphyrin excretion in porphyria variegata by propranolol. A case report. S Afr Med J 50:898-9.

Bonkovsky HL, Barnard GF (1998). Diagnosis of porphyric syndromes: a practical approach in the era of molecular biology. Semin Liv Dis 18:57-65.

Bonkovsky HL, Healey JF, Lurie AN, Gerron GG (1991). Intravenous heme-albumin in acute intermittent porphyria: evidence for repletion of hepatic hemoproteins and regulatory heme pool. Am J Gastroenterol 86:1050-6.

Bonkovsky HL, Schady W (1982). Neurologic manifestations of acute hepatic porphyria. Semin Liv Dis 2:108-24.

Bonkovsky HL, Tschudy DP, Collins A, et al (1971). Repression of the overproduction of porphyrin precursors in acute intermittent porphyria by intravenous infusion of hematin. Proc Natl Acad Sci USA 62:2725-29.

Bonkowsky HL, Bloomer JR, Ebert PS, Mahoney MJ. (1975). Heme synthetase deficiency in human protoporphyria. Demonstration of the defect in liver and cultured skin fibroblasts. J Clin Invest 56:1139-48.

Bont A, Steck AJ, Meyer UA (1996). Die akuten hepatischen Porphyrien und ihre neurologischen Sindrome. Schweize Med Wschrf 126:3-14.

Botha MC, Beighton P (1983). Inherited disorders in the Afrikaner population of southern Africa. Part I. Historical and demographic background, cardiovascular, neurological, metabolic and intestinal conditions. S Afr Med J 64:609-12.

Bottomley SS, Tanaka M, Everett MA (1975). Diminished erythroid ferrochelatase activity protoporphyria. J Lab Clin Med 86:126-31.

Bourdon R, Yonger J, Ataie M (1972). Dosage des porphyrines par spectrophotometrie absorption atomique. Ann Biol Clin (Paris) 30:427.

Bravenboer B, Erkelens DW (1989). Acute hypertension mimicking phaeochromocytoma as main presenting feature of acute intermittent porphyria. Lancet 2: 928.

Brennan MJW, Cantrill RC (1979). δ-aminolaevulinic acid is a potent agonist for GABA autoreceptors. Nature 280:514-5.

Brennan MJW, Cantrill RC (1981). δ-aminolaevulinic acid and amino acid neurotransmitters. Mol Cell Biochem 38:49-58.

Brenner DA, Bloomer JR (1977). Heme content of normal and porphyric cultured skin fibroblasts. Biochem Genetics 15:1061-70.

Brenner DA, Bloomer JR (1980). The enzymatic defect in variegate porphyria. Studies with human cultured skin fibroblasts. N Engl J Med 302:765-9.

Brodie MJ, Moore MR, Thompson GG, Goldberg A, Low RAL (1977). Pregnancy and the acute porphyrias. Br J Obstet Gynaecol 84:726-31.

Buttery JE, Carrera AM, Pannall PR (1990). Reliability of the porphobilinogen screening assay. Pathology 22: 197-8.

Bylesjo I, Forsgren L, Lithner F, Boman K (1996). Epidemiology and clinical characteristics of seizures in patients with acute intermittent porphyria. Epilepsia 37:230-5.

Calvert RJ, Rimington C (1953). Porphyria cutanea tarda: a case report. Br Med J 2:1131-4.

Camadro JM, Matringe M, Scalla R, Labbe P (1991). Kinetic studies on protoporphyhrin oxidase inhibition by diphenyl ether herbicides. Biochem J 277:17-21.

Camadro JM, Thorne F, Brouillet N, Labbe P (1994). Purification and properties of protoporphyrinogen oxidase from the yeast Saccharomyces cerevisiae. Mitochondrial location and evidence for a precursor form of the protein. J Biol Chem 269:32085-91.

Camadro J-N, Abraham NG, Levere RD (1985). Kinetic properties of the membrane-bound human liver mitochondrial protoporphyrinogen oxidase. Arch Biochem Biophys 242:206-12.

Carlson RE, Dolphin D (1976). High pressure liquid chromatographic techniques for the separation of complex mixtures of naturally occurring porphyrins. In: Doss M (ed). Porphyrins in human diseases. Basel: Karger, pp. 465-71.

Cavanagh JB, Mellick RS (1965). On the nature of peripheral nerve lesions associated with acute intermittent porphyria. J Neurol Neurosurg 28:320-7.

Chen CC, Thajeb P, Lie SK (1994). Acute intermittent porphyria: clinical analysis of nine cases. Chung Hua I Hsueh Tsa Chih Taipei 54:395-9.

Chisholm JJ, Brown DH (1975). Micro-scale photofluorometric determination of 'free erythrocyte porphyrin' (protoporphyrin IX). Clin Chem 21:1169-82.

Chogle AR, Shetty RN, Joshi VR, Shanbhag VV (1980). Acute intermittent porphyria with the syndrome of inappropriate ADH secretion (SIADH): a report of two cases. J Assoc Physicians India 28:379-82.

Christiansen L, Ged C, Hombrados I, Brons-Paulsen J, Fontanellas A, et al (1999). Screening for mutations in the uroporphyrinogen decarboxylase gene using denaturing gradient gel electrophoresis. Identification and characterization of 6 novel mutations associated with familial PCT. Hum Mutat 14:222-32.

Chu TC, Chu EJH (1962). Porphyrins from congenitally porphyric (pink-tooth) cattle. Biochem J 83:318-25.

Church SE, McColl KE, Moore MR, Youngs GR (1992). Hypertension and renal impairment as complications of acute porphyria. Nephrol Dial Transplant 7:986-90.

Coakley J, Hawkins R, Crinis N, McManus J, Blake D, et al (1990). An unusual case of variegate porphyria with possible homozygous inheritance. Aust NZ J Med 20:587-9.

Cochrane AL, Goldberg A (1968). A study of faecal porphyrin levels in a large family. Ann Hum Genet 32:195-208.

Cooper DN (1993). Human gene mutations affecting RNA processing and translation. Ann Med 25:11-7.

Cooper DN, Krawczak M (1984). The hydrophobic moment detects periodicity in protein hydrophobicity. Proc Nat Acad Sci 81:140-4.

Cooper DN, Krawczak M (1990). The mutational spectrum of single base-pair substitutions causing human genetic disease: Patterns and predictions. Hum Genet 85:55-74.

Cooper DN, Krawczak M (1993). Human gene mutation. Oxford: BIOS Scientific.

Cooper DN, Youssoufian H (1988). The CpG dinucleotide and human genetic disease. Hum Genet 78:151-5.

Copeman PWM, Cripps DJ, Summerly R (1966). Cutaneous hepatic porphyria and oestrogens. Br Med J 1:461.

Corey TJ, DeLeo VA, Christianson H, Poh-Fitzpatrick MB (1980). Variegate porphyria. Clinical and laboratory features. J Am Acad Dermatol 2:36-43.

Cormane RH, Szabo E, Tio TH (1971). Histopathology of the skin in acquired and hereditary porphyria cutanea tarda. Br J Dermatol 85: 531-9.

Corrigall AV, Hift RJ, Adams PA, Kirsch RE (1994). Inhibition of mammalian protoporphyrinogen oxidase by acifluorfen. Biochem Mol Biol Int 34:1283-9.

Corrigall AV, Hift RJ, Davids LM, Hancock V, Meissner D et al. (2000). Homozygous variegate porphyria in South Africa: Genotypic analysis in two cases. Hum Mutat 69:323-30.

Crimlisk HL (1997). The little imitator – porphyria: a neuropsychiatric disorder. J Neurol Neurosurg Psychiatry 62:319-28.

Cripps DJ, Scheuer PJ (1965). Hepatobiliary changes in erythropoietic protoporphyria. Arch Pathol 80:500-8.

Culbertson MR (1999). RNA surveillance: unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet 15:74-80.

Czuczwar SJ, Frey HH (1986). Effect of morphine and morphine-like analgesics on susceptibility to seizures in mice. Neuropharmacology 25: 465-9.

Da Silva V, Simonin S, Deybach JC, Puy H, Nordmann Y (1995). Variegate porphyria: diagnostic value of fluorometric scanning of plasma porphyrins. Clin Chim Acta 238:163-8.

Dailey HA, Dailey TA (1996b). protoporphyrinogen oxidase of Myxococcus xanthus. Expression, purification and characterisation of the cloned enzyme. J Biol Chem 271:8714-8.

Dailey HA, Dailey TA (1997). Characteristics of human protoporphyrinogen oxidase in controls and variegate porphyrias. Cell Mol Biol 43:67-73.

Dailey HA, Karr SW (1987). Purification and characterization of murine protoporphyrinogen oxidase. Biochemistry 26:2697-701.

Dailey TA, Dailey HA (1996a). Human protoporphyrinogen oxidase: expression, verification and characterisation of the cloned enzyme. Prot Sci 5:98-105.

Dailey TA, Dailey HA (1998). Identification of an FAD superfamily containing protoporphyrinogen oxidases, monoamine oxidases, and phytoene desaturase. Expression and characterization of phytoene desaturase of Myxococcus xanthus. J Biol Chem 273:13658-62.

Dailey TA, Dailey HA, Meissner PN, Prasad ARK (1995). Cloning, sequencing and expression of mouse protoporphyrinogen oxidase. Arch Biochem Biophys 324:379-84.

Dailey TA, Meissner PN, Dailey HA (1994). Expression of a cloned protoporphyrinogen oxidase gene. J Biol Chem 269:813-5.

Daily HA (1990). Conversion of coproporphyrin to protoheme in higher eukaryotes: Terminal three enzymes. In: Dailey HA (ed). Biosynthesis of haem and chlorophylls. New York: McGraw-Hill, pp. 123-61.

Darocha T, Gregor A (1971). Acute intermittent porphyria in Poland. S Afr J Lab Clin Med 45:204-13.

Day RS (1986) Variegate Porphyria. Semin Dermatol 5:138-54.

Day RS, De Salamanca RE, Eales L (1978a). Quantitation of red cell porphyrins by fluorescence scanning after thin layer chromatography. Clin Chim Acta 89:25-33.

Day RS, Eales L (1983). Porfiria variegata. Endocrinologia 2:34-57.

Day RS, Eales L, Meissner D (1982). Coexistent variegate porphyria and porphyria cutanea tarda. N Engl J Med 307:36-41.

Day RS, Pimstone NR, Eales L (1978b). The diagnostic value of blood plasma porphyrin methyl ester profiles produced by quantitative TLC. Int J Biochem 9:897-904.

De Block CE, Leeuw IH, Gaal LF (1999). Premenstrual attacks of acute intermittent porphyria: hormonal and metabolic aspects—a case report. Eur J Endocrinol 141:50-4.

De Matteis F, Ray RE (1982). Studies on cerebellar heme metabolism in the rat in vivo. J Neurochem 39:551-6.

De Matteis F, Zetterlund J, Wetterberg L (1981). Brain 5-aminolaevulinate synthase. Developmental aspects and evidence for regulator role. Biochem J 196:811-7.

De Rooij FWM, Minderman G, De Baar E, Wilson JHP, Sinke JRJ et al (1997). Six new protoporphyrinogen oxidase mutations in Dutch variegate porphyria patients and the R59W mutation in historical perspective. Acta Haematol 98 (Suppl 1):103.

De Villiers JNP, Hillermann R, Loubser L, Kotze MJ (1999). Spectrum of mutations in the HFE gene implicated in haemochromatosis and porphyria. Human Mol Gen 8:1517-22.

Deacon AC (1988). Performance of screening tests for porphyria. Ann Clin Biochem 1988: 392-7.

Deacon AC, Ledden JA (1998). Limitations of solvent fractionation methods for urinary and faecal porphyrins. Ann Clin Biochem 35: 314-6.

Dean G (1953). In: Br Med J 2:1291.

Dean G (1963). The porphyrias. A story of inheritance and environment, 1st ed. London: Pitman.

Dean G (1971). The porphyrias. A story of inheritance and environment, 2nd ed. London: Pitman.

Dean G, Barnes HD (1959). Porphyria in Sweden and South Africa. S Afr Med J 33:246-53.

Dean G, Barnes HD. (1955). The inheritance of porphyria. Br Med J 2:89.

Deeg MA, Rajamani K (1990). Normeperidine-induced seizures in hereditary coproporphyria. South Med J 83:1307-8.

DeGoeij AFPM, Christianse K, Van Steveninck I (1975). Increased haem synthetase activity in blood cells of patients with erythropoietic protoporphyria. Eur J Clin Invest 5:397-400.

Delfau MH, Picat C, De Rooij FWM, Hamer K, Bogard M et al (1990). Two different G to A point mutations in exon 10 of the porphobilinogen deaminase gene are responsible for acute intermittent porphyria. J Clin Invest 86:1511-6.

Deng WP, Nikoloff JA (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal Biochem 200:81-8.

Desnick RJ, Glass IA, Xu W, Solis C, Astrin KH (1998). Molecular genetics of congenital erythropoietic porphyria. Semin Liv Dis 18:77-84.

Deybach JC, Da Silva V, Grandchamp B, Nordmann Y (1985). The mitochondrial location of protoporphyrinogen oxidase. Eur J Biochem 149:431-5.

Deybach JC, De Verneuil H, Nordmann Y (1981). The inherited defect in porphyria variegata. Hum Genet 58:425-8.

Deybach JC, Puy H, Robreau AM, Lamoril J, Da Silva V et al (1996). Mutations in the protoporphyrinogen oxidase gene in patients with variegate porphyria. Hum Mol Genet 5:407-10.

Dhar GJ, Bossenmaier I, Cardinal R, Petryka ZJ, Watson CJ (1978). Transitory renal failure following rapid administration of a relatively large amount of hematin in a patient with acute intermittent porphyria in clinical remission. Acta Med Scand 203:437-43.

Dhar GJ, Bossenmaier I, Petryka ZJ, Cardinal R, Watson CJ. 1975). Effects of hematin in hepatic porphyria. Further studies. Ann Intern Med 83:20-30.

Di Trapani G, Casali C, Tonali P, Topi GC (1984). Peripheral nerve findings in hereditary coproporphyria. Light and ultrastructural studies in two sural nerve biopsies. Acta Neuropathol Berl 63:96-107.

Disler PB, Blekkenhorst GH, Eales L, Moore MR, Straughan J (1982). Guidelines for drug prescription with the acute porphyrias. S Afr Med J 61:656-60.

Dixon B (1997). Encephalopathy due to hyponatraemia in acute intermittent porphyria. J R Soc med 90:500-1.

Doss M (1960). In: Hoppe-Seylers Z Physiol Chem 35:490.

Doss M (1970). Analytical and preparative thin-layer chromatography of porphyrin methyl esters. Zeitschr Klin Chem Klin Biochem 8:197-207.

Doss M (1974). Porphyrins and porphyrin precursors. In: Curtis M, Roth M (eds). Clinical Biochemistry: Principles and Methods. Berlin: Walter de Gruyter, pp. 1323-71.

Doss M, Bode U (1968). Analyse der erythrocyten-porphyrine. Z Klin Chem 6:383-6.

Doss M, Sixel-Dietrich F, Verspohl F (1985). "Glucose effect" and rate limiting function of uroporphyrinogen synthase on porphyrin metabolism in hepatocyte culture: relationship with human acute porphyrias. J Clin Chem Clin Biochem 23:505-13.

Doss M, Tiepermann R, Schmisser W, Dölle W, Höcherl G et al (1977). Porphyria variegata in Deutschland. Internationales Symposium klinische Biochemie, Porphyrie und Blei-intoxication. Marburg, 1977.

Doss M, Verspohl F (1981). The "glucose effect" in acute hepatic porphyrias and in experimental porphyria. Klin Wochenscr 59:727-35.

Doss M, Von Tiepermann R, Kepp W (1984). Harderoporphyrin coproporphyria. Lancet 1: 292.

Doss M, Von Tiepermann R, Schmeisser W, Dölle W, Höcherl G et al (1978). Uroporphyrinogen-synthase in erythrocyten bei akuter intermittierender porphyrie: Neue pathobiochemische aspekte. J Clin Chem Clin Biochem 16:111-8.

Doss M, von Tiepermann R, Schneider J, et al (1979). New type of hepatic porphyria with porphobilinogen synthase defect and intermittent acute clinical manifestations. Klin Wschrf 57:1123-7.

Doss MO, Gross U, Lamoril J, Kranl C, Jacob K et al (1999). Compound heterozygous hereditary coproporphyria with fluorescing teeth. Ann Clin Biochem 36:680-2.

Douer D, Schoenfeld N, Weinberger A, Pinkhas J, Atsmon A (1978a). Favourable effect of intravenous propranolol in acute intermittent porphyria. Monogr Hum Genet 10:223-6.

Douer D, Weinberg A, Pinkhas J, Atsmon A (1978b). Treatment of acute intermittent porphyria with large doses of propranolol. JAMA 240:766-8.

Dover SB, Moore MR, Fitzsimmons EJ, Graham A, McColl KE (1993). Tin proto-porporphyrin prolongs the biochemical remission produced by heme arginate in acute hepatic porphyria. Gastroenterology 105:500-6.

Dowdle E, Mustard P, Spong N, Eales L (1968). The metabolism of (5-14C)delta-aminolaevulinic acid in normal and porphyric human subjects. Clin Sci 34:233-51.

Dowdle EB, Mustard P, Eales L (1967). δ-aminolaevulinic acid synthetase activity in normal and porphyric human livers. S Afr Med J 41:1096-8

Drummond GS (1987). Control of heme metabolism by synthetic metalloporphyrins. Ann NY Acad Sci 514:87-95.

Durosinmi MA, Adejuyigbe O, Adamolekun B, Adekile AD, Odunusi EO (1991). Variegate (mixed) porphyria in a Nigerian girl. Ann Trop Paediatr 11:95-8.

Eales L (1960). Cutaneous porphyria. Observations on 111 cases in 3 racial groups. S Afr J Lab Clin Med 6:63-86.

Eales L (1963). Porphyria as seen in Cape Town. A survey of 250 patients and some recent studies.S Afr J Lab Clin Med 9:151-62.

Eales L (1971). The acute porphyria attack. 3). Acute porphyria: the precipitating and aggravating factors. S Afr Med J Sep 25:120-5.

Eales L (1974). The porphyrias. In: Conn HF, Conn RB (eds). Current diagnosis, 4. Philadelphia: WB Saunders.

Eales L, Day RS, Blekkenhorst GH (1980). The clinical and biochemical features of variegate porphyria: an analysis of 300 cases studied at Groote Schuur hospital, Cape Town. Int J Biochem 12:837-53.

Eales L, Day RS, Pimstone NR (1978). The clinical chemistry of variegate porphyria with special reference to the identification of a new plasma marker porphyrin. In: Doss M (ed). Diagnosis and therapy of porphyrias and lead intoxication. International symposium, Clinical Biochemistry. Berlin: Springer-Verlag, pp. 51-65.

Eales L, Grosser Y, Sears WG (1975). The clinical biochemistry of the human hepatocutaneous porphyrias in the light of recent studies of newly identified intermediates and porphyrin derivatives. Ann NY Acad Sci 244: 441-71.

Eales L, Levey MJ, Sweeney GD (1966). The place of screening tests and quantitative investigations in the diagnosis of the porphyrias, with particular reference to variegate and symptomatic porphyria. S Afr Med J 40:63-71.

Edwards SR, Shanley BC, Reynoldson JA (1984a). Neuropharmacology of delta-aminolaevulinic acid. I. Effect of acute administration in rodents. Neuropharmacol 23:477-81.

Edwards SR, Shanley BC, Reynoldson JA (1984b). Neuropharmacology of delta-aminolaevulinic acid. II. Effect of chronic administration in mice. Neurosci Lett 50:169-73.

Eisenberg D (1984). Three dimensional structure of membrane and surface proteins. Ann Rev Biochem 53:595-623.

Elder GH (1972). Identification of a group of tetracarboxylate porphyrins containing one acetate and 3 proprionate β-substituents, in faeces from patients with symptomatic cutaneous

hepatic porphyria and from rats with porphyria due to hexachlorobenzene. Biochem J 126:877-91.

Elder GH (1975). Differentiation of porphyria cutanea tarda symptomatica from other types by measurement of isocoproporphyrins in feces. J Clin Pathol 26:601-7.

Elder GH (1980). The porphyrias: clinical chemistry, diagnosis and methodology. Clinics in Haematology 9:371-98.

Elder GH (1997). Hepatic porphyria in children. J Inher Metab Dis 20:237-46.

Elder GH (1998). Porphyria cutanea tarda. Semin Liv Dis 18:67-75.

Elder GH, Hift RJ, Meissner PN (1997). The acute porphyrias. Lancet 349:1613-7.

Elder GH, Magnus IA, Handa F, Doyle M (1974). Faecal X porphyrin in the hepatic porphyrias. Enzyme 17:29-38.

Elder GH, Roberts AG (1994). Uroporphyrinogen decarboxylase. J Bioenerg Biomembr 27: 207-14.

England MT, Cotton V, French JM (1962). Faecal porphyrin excretion in normal subjects and in patients with the malabsorption syndrome. Clin Sci 22:447-57.

Enriquez de Salamanca R, Sepulveda P, Moran MJ, Santos JL, Fontanellas A, Hernandez A (1993). Clinical utility of fluorometric scanning of plasma porphyrins for the diagnosis and typing of porphyrias. Clin Exp Dermatol 18:128-30.

Eriksen L (1962). In: Scand J Clin Lab Invest 14:1.

Falk JE (1964). Porphyrins and metalloporphyrins. Amsterdam: Elsevier.

Famei Li, Lim CK, Simpson KJ, Peters TJ (1989). Coproporphyrinogen oxidase, proto-porphyrinogen oxidase and ferrochelatase activities in human liver biopsies with special reference to alcoholic liver disease. J Hepatol 8:86-93.

Farese RV, Karsh SJ, Bidot-Lopez P (1979). Acute intermittent porphyria associated with inappropriate antidiuretic hormone secretion, hypokalemic alkalosis, and secondary hyperaldosteronism. South Med J 72:1201-3.

Fehr H (1891). Et par tiffaelde af sulfonalforgiftnung. Hospital-tidende 9: 1122-38.

Fernandez AA, Henry RJ, Goldenberg H (1966). Assay of urinary porphyrins. Evaluation of extraction methods and choice of instrumentation. Clin Chem 12:463-74.

Ferreira GC, Andrew TL, Karr SW, Dailey HA (1988). Organisation of the terminal two enzymes of the heme biosynthetic pathway. J Biol Chem 263:3835-9.

Ferreira GC, Dailey HA (1988). Mouse protoporphyrinogen oxidase. Kinetic parameters and demonstration of inhibition by bilirubin. Biochem. J. 250, 597-603.

Fischer H, Orth H (1934). Die Chemie des Pyrrols, 3 Bands. Akademische Verlagsgesellschaft MBH, Leipzig.

Fischer H, Zerweck W (1924). Über den harnfarbstoff bei normalen und pathologischen Verhaltnissen und seine lichtschutzende Wirkung. Zugleich einige Beitrage zur Kenntnis der Porphyrinurie. Hoppe-Seyler's Z Physiol Chem. 137:176-241.

Fischl J, Eichhorn F, Ruttenberg A, et al (1970). Conventional and preparative electrophoretic separation of some urinary porphyrins and porphyrin precursors. Clin Chem 16:331-4.

Flacks LM (1970). Propranolol in acute porphyria. Lancet 1:363.

Flügel KA, Druschky KF (1977). Electromyogram and nerve condition in patients with acute intermittent porphyria. J Neurol 214:267-79.

Fowler CJ, Ward JM (1975). Porphyria variegata provoked by contraceptive pill. Br Med J 1:663-4.

Frank J, Jugert FK, Breitkopf C, Goerz G, Merk HF, Christiano AM (1998d). Recurrent missense mutation in the protoporphyrinogen oxidase gene underlies variegate porphyria. Am J Med Genet 79:22-6.

Frank J, Jugert FK, Kalka K, Goertz G, Merk H, et al (1997). Premature termination codons in the protoporphyrin oxidase gene underlie variegate porphyria. Acta Haematol 98(suppl 1):97.

Frank J, Jugert FK, Kalka K, Goerz G, Merk HF, Christiano AM (1998a). Variegate porphyria: identification of a nonsense mutation in the protoporphyrinogen oxidase gene. J Invest Dermatol 110:449-51.

Frank J, Jugert FK, Zaider E, Goertz G, Merk HF, et al (1997). Variegate porphyria: a frameshift mutation and a missense mutation in the protoporphyrinogen oxidase gene. J Invest Dermatol 109:409.

Frank J, Lam H, Zaider E, Poh-Fitzpatrick M, Christiano AM (1998c). Molecular basis of variegate porphyria: a missense mutation in the protoporphyrinogen oxidase gene. J Med Genet 35:244-7.

Frank J, McGrath J, Lam H, Graham RM, Hawk JLM, Christiano AM (1998b). Homozygous variegate porphyria: Identification of mutations on both alleles of the protoporphyrinogen oxidase gene in a severely affected proband. J Invest Dermatol 110:452-5.

Frank J, McGrath JA, Poh-Fitzpatrick MB, Hawk JLM, Christiano AM (1999). Mutations in the translation initiation codon of the protoporphyrinogen oxidase gene underlie variegate porphyria. Clin Exp Dermatol 24:296-301.

Frank J, Zaider E, Jugert FK, Goertz G, Merk HF, et al (1997). Variegate porphyria: identification of three novel missense mutations in the protoporphyrinogen oxidase gene. Acta Haematol 98 (suppl 1):96.

Fromke VL, Bossenmaier I, Cardinal R, Watson CJ (1978). Porphyria variegata. Study of a large kindred in the United States of America. Am J Med Sci 65:80-8.

Fuhrhop JM, Smith KM (1975). Laboratory methods in porphyrin and metalloporphyrin research. Amsterdam: Elsevier, p. 243.

Gajdos A (1968). Alcohol and porphyrin metabolism. Lancet 1: 1154-5.

Galbraith RA, Drummond GS, Kappas A (1985). Sn-protoporphyrin suppresses chemically induced experimental hepatic porphyria. Potential clinical implications. J Clin Invest 76:2436-9.

Galbraith RA, Kappas A (1989). Pharmokinetics of tin-mesoporphyrin in man and the effects of tin-chelated porphyrins on hyperexcretion of heme pathway precursors in patients with acute inducible porphyria. Hepatology 9:882-8.

Gandolfo L, D'Alessandro, Macri A, Biolcati G, Griso D, Phung LN, et al (1991). Homozygous variegate porphyria: revision of a diagnostic error. Br J Dermatol 124:211.

Geill C. Sulfonal og sulfonalforgiftnung (1891). Hospitals-tidende 9: 797-812.

Gibson JB, Goldberg A (1956) The neuropathology of acute porphyria. J Pathol Bact LXXI:495-510.

Goetsch CA, Bissell DM (1986). Instability of hematin used in the treatment of acute hepatic porphyria. N Engl J Med 315:235-8.

Goldberg A (1959). Acute intermittent porphyria . A study of 50 cases. Q J Med 28:183-209.

Goldberg A, Doyle D, Yeung-Laiwah AC (1985). Relevance of cytochrome C-oxidase deficiency to the pathogenesis of acute porphyria. Q J Med 57:799.

Gorchein A (1989). δ-aminolaevulinic acid is not directly toxic to human spinal cord neurons in culture. Biochem Soc Trans 17:577-8.

Gorchein A, Webber R (1987). Delta-aminolevulinic acid in plasma, cerebrospinal fluid, saliva and erythrocytes: studies in normal, uraemic and porphyric subjects. Clin Sci 72:103-12.

Gouya L, Deybach J-C, Lamoril J, Da Silva V, Beaumont C, et al (1996). Modulation of the phenotype in dominant erythropoietic protoporphyria by a low expression of the normal ferrochelatase allele. Am J Hum Genet 58:292-9.

Grabczynska SA, McGregor JM, Hawk JLM (1996). Late onset variegate porphyria. Clin Exp Dermatol 21:353-6.

Grandchamp B (1998). Acute intermittent porphyria. Semin Liv Dis 18:17-24.

Grandchamp B, Deybach J-C, De Verneuil H, Nordmann Y (1980). Studies of porphyrin synthesis in fibroblasts of patients with congenital erythropoietic porphyria and one patient with homozygous coproporphyria. Biochim Biophys Acta 629:577-86.

Grandchamp B, Phung N, Nordmann Y (1977). Homozygous case of hereditary coproporphyria. Lancet 2:1348-9.

Grandchamp B, Picat C, Kauppinen R, Mignotte V, Peltonen L, et al (1989). Molecular analysis of acute intermittent porphyria in a Finnish family with normal erythrocyte porphobilinogen deaminase. Eur J Clin Invest 19: 415-8.

Gray CH, Lim CK, Nicholson DC (1977). The differentiation of the porphyrias by means of high pressure liquid chromatography. Clin Chim Acta 77:167-78.

Gray CH, Rimington C, Thomson S (1948). A case of chronic porphyria associated with recurrent jaundice. Q J Med 17:123-37.

Gregor A, Kostrzweska E, Prokurat H, Pucek Z, Torbicka E (1977). Increased protoporphyrin in erythrocytes in a child with acute intermittent porphyria. Arch Dis Child 52:947-50.

Groenewald JZ, Liebenberg J, Groenewald IM, Warnich L (1998). Linkage disequilibrium analysis in a recently founded population: Evaluation of the variegate porphyria founder in South African Afrikaners. Am J Hum Genetics 62:1254-8.

Gross U, Sassa S, Jacob K, Deybach JC, Nordmann Y, et al (1998). 5-aminolevulinic acid dehydratase deficiency porphyria: a twenty year clinical and biochemical follow-up. Clin Chem 44:1892-6.

Grosser Y, Eales L (1973). Patterns of faecal porphyrin excretion in the hepatocutaneous porphyrias. S Afr Med J 47:2162-8.

Grossman ME, Bickers DR (1978). Porphyria cutanea tarda—a rare cutaneous manifestation of hepatic tumours. Cutis 21:782-4.

Grotepass W (1932). Zur Kenntnis des in Harn auftretenden Porphyrins bei Bleivegiftung. Hoppe-Seyler's Z physiol chem. 205:193-7.

Günther H (1911). Die Haematoporphyrie. Deutsche Arch Clin Med 105:89-146.

Günther H (1922). Die Bedeuting der Haematoporphyrinuriein der Physiologie und Pathologie. Ergeb allgemein Pathol Pathologisch Anat 20:608-764.

Hagmeyer KO, Mauro LS, Mauro VF (1993). Meperidine-related seizures associated with patient-controlled analgesia pumps. Ann Pharmacother 27:29-32.

Hamnström B, Haeger-Aronson B, Waldenström J, Hysing B, Molander J (1967). Three Swedish families with porphyria variegata. Br Med J 4:449-53.

Hansson M, Hederstedt L (1992). Cloning and characterization of the Bacillus subtilis hemEHY gene cluster, which encodes protoheme IX biosynthetic enzymes. J Bacteriol 174: 8081-93.

Harley V (1890). Two fatal cases of an unusual form of nerve disturbance associated with red urine, probably due to defective tissue oxidation. Br Med J 2:1169-70.

Hassoun A, Verstraeten L, Mercelis R, Markin JJ (1989). Biochemical diagnosis of an hereditary aminolaevulinate dehydratase deficiency in a 63 year old man. J Clin Chem Clin Biochem 27:781-6.

Herrick A, McColl KE, McLellan A, Moore MR, Brodie MJ, et al (1987). Effect of heme arginate therapy on porphyrin metabolism and mixed function oxygenase activity in acute hepatic porphyria. Lancet 2:1178-9.

Herrick AL, Fisher BM, Moore MR, Cathcart S, McColl KE, et al (1990). Elevation of blood lactate and pyruvate levels in acute intermittent porphyria – A reflection of heme deficiency? Clin Chim Acta 190:157-62.

Herrick AL, McColl KE, Moore MR, Brodie MJ, Adamson AR, Goldberg A (1989a). Acute intermittent porphyria in two patients on anticonvulsant therapy and with normal erythrocyte porphobilinogen deaminase activity. Br J Clin Pharmacol 27: 491-7.

Herrick AL, McColl KE, Moore MR, Cook A, Goldberg A (1989b). Controlled trial of heme arginate in acute hepatic porphyria. Lancet 1:1295-7.

Herrick AL, McColl KE, Wallace AM, Moore MR, Goldberg A (1990). LHRH analogue treatment for the prevention of premenstrual attacks of acute porphyria. Q J Med 75:355-63.

Herrick AL, Moore MR, Thompson GG, Ford GP, McColl KEL (1991). Cholelithiasis in patients with variegate porphyria. J Hepatol 12:50-3.

Hierons R (1957). Acute intermittent porphyria. Postgrad Med J 43:605.

Hift RJ, Corrigall AV, Adams PA, Kirsch RE (1994). Inhibition of mammalian protoporphyrinogen oxidase by acifluorfen. Biochem Mol Biol Int 34:1283-9.

Hift RJ, Meissner PN, Corrigall AV, Ziman MR, Petersen LA, et al (1997). Variegate porphyria in South Africa, 1688-1996—new developments in an old disease. S Afr Med J 87:722-30.

Hift RJ, Meissner PN, Todd G (1993a). Hepatoerythropoietic porphyria precipitated by viral hepatitis. Gut 34:1632-4.

Hift RJ, Meissner PN, Todd G, Kirby P, Bilsland D, et al (1993b). Homozygous variegate porphyria: an evolving clinical syndrome. Postgrad Med J 69:781-6.

Hindmarsh JT, Oliveras L, Greenway DC (1999). Plasma porphyrins in the porphyrias. Clin Chem 45:1070-6.

Holti G, Rimington C, Tate BC, Thomas G (1958). An investigation of porphyria cutanea tarda. Q J Med 27:1-17.

Hughes GS, Davis L (1983). Variegate porphyria and heavy metal poisoning from ingestion of "moonshine". Southern Med J 76:1027-9.

Hunter JAA, Khan SA, Hope E, Beattie AD, Beveridge GW, et al (1971). Hereditary coproporphyria: Photosensitivity, jaundice and neuropsychiatric manifestations associated with pregnancy. Br J Dermatol 84:301-10.

Ibrahim GW, Watson CJ (1968). Enterohepatic circulation and conversion of protoporphyrin to bile pigment in man. Proc Soc Exp Biol Med 127:890-5.

Ivanetich KM, Movsowitz C, Moore MR (1984). A rapid semi-quantitative measurement of porphyrins in urine and faeces by magnetic circular dichroism. Clin Chem 30:391-4.

Jackson AH (1977). Modern spectroscopic and chromatographic techniques for the analysis of porphyrin on a microscale. Semin Hematol 14:193-210.

Jackson AH, Elder GH, Smith SG (1978). The metabolism of coproporphyrinogen-III into protoporphyrinogen-IX. Int J Biochem 9:877-82.

Jackson AH, Kenner GW, Smith KM, Suckling CJ (1976). Pyrroles and related compounds – XXXVII. Harderoporphyrin. Tetrahedron 32:2757.

Jackson KH, Rao KR, Smith SG (1982). High-pressure liquid chromatographic analysis of tetracarboxylic porphyrins in hepatic porphyrias. Biochem J 207:599-603.

Jacobs JM, Jacobs NJ (1983). Protoporphyrinogen oxidation, an enzymatic step in haem and chlorophyll synthesis: partial characterisation of the reaction in plant organelles and comparison with mammalian and bacterial systems. Arch Biochem Biophys 229:312-9.

Jacobs JM, Jacobs NJ (1987). Oxidation and protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis. Purification and partial characterization of the enzyme from barley organelles. Biochem J 244:219-24.

Jacobs NJ, Borotz SE, Guerinot ML (1989). Protoporphyrinogen oxidation, a step in heme synthesis in soybean root nodules and free-living rhizobia. J Bacteriol 171:573-6.

Jacobs NJ, Jacobs JM (1981). Protoporphyrinogen oxidation in Rhodopseudomonas spheroides, a step in heme and bacteriochlorophyll synthesis. Arch Biochem Biophys 211: 305-11.

Jeans JB, Savik K, Gross CR, Weimer MK, Bossenmaier IC, et al (1996). Mortality in patients with acute intermittent porphyria requiring hospitalization: a United States case series. Am J Med Genet 65:269-73.

Jenkins T. 1996). The South African malady. Nature Genetics 13: 7-9.

Johnston DJ, Droz E, Rochaix J-D, Malno P (1998). Cloning and characterisation of potato cDNAs involved in tetrapyrrole biosynthesis (Accession numbers: ferrochelatase AJ005802, chloroplast protoporphyrinogen IX oxidase AJ225107, mitochondrial protoporphyrinogen IX oxidase AJ225108). Plant Physiol 118:330.

Jones KG, Sweeney GD (1979). Quantitation of urinary porphyrins by use of second derivative spectroscopy. Clin Chem 25:71-4.

Jordan P, Bagust J, Kelly M, et al (1990). A model for acute intermittent porphyria: effects of 5-aminolaevulinic acid on ventral root activity in the hemisected hamster spinal cord. Mol Asp Med 11:53-4.

Kanaan C, Veille JC, Lakin M (1989). Pregnancy and acute intermittent porphyria. Obstet Gynecol Surv 44:244-9.

Kappas A, Sassa S, Anderson KE (1989). The porphyrias. In: Stanley JB, Wyngaarden JB, Frederikson DS, Goldstein JL, Brown MS (eds). The molecular and metabolic bases of inherited disease, 7th ed. New York: McGraw-Hill, pp. 1305-65.

Kappas A, Sassa S, Galbraith RA, Nordmann Y (1995). The porphyrias. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The molecular and metabolic bases of inherited disease, 7th ed. New York: McGraw-Hill, pp. 2103-59.

Karlbling R, Craig JB, Passamanick B (1961). Urinary porphobilinogen. Arch Gen Psychiatry 5: 494-508.

Kast A (1888). Über die Art der Darreichung und Verordnung des Sulfonals. Ther Mh 11: 316-9.

Kauppinen R, Mustajoki P (1988). Acute hepatic porphyria and hepatocellular carcinoma. Br J Cancer 57:117-20.

Kauppinen R, Mustajoki P (1992). Prognosis of acute porphyria: Occurrence of acute attacks, precipitating factors and associated diseases. Medicine 71:1-13.

Kauppinen R, Mustajoki S, Pihlaja H, Peltonen L, Mustajoki P (1995). Acute intermittent porphyria in Finland: 19 mutations in the porphobilinogen deaminase gene. Hum MolecGenet 4:215-22.

Kauppinen R, Timonen K, Laitinen E, Knusisto K, Ahola H, et al (1997). Molecular genetics and clinical characteristics of variegate porphyria. Acta Haematol 98 (Suppl 1):96.

Keung YK, Chuahirlun T, Cobos E (2000). Acute intermittent porphyria with seizure and paralysis in the puerperium. J Am Board Fam Pract 13:76-9.

King PH, Bragdon AC (1991). MRI reveals multiple reversible cerebral lesions in an attack of acute intermittent porphyria. Neurology 41:1300-2.

Kirsch RE, Meissner PN, Hift RJ (1998). Variegate porphyria. Semin Liv Dis 18:33-41.

Klemm DJ, Barton LL (1987). Purification and properties of protoporphyrinogen oxidase from an aerobic bacterium, Desulfovibrio gigas. J Bacteriol 169:5209-15.

Kochar DK, Poonia A, Kumawat BL, Shubhakaran, Guptga BK (2000). Study of motor and sensory nerve conduction velocities, late responses (F-wave and H-reflex) and somatosensory evoked potential in latent phase of intermittent acute porphyria. Electromyogr Clin Neurophysiol 40:73-9.

Kordac V, Deybach JC, Martasek P, Zeman J, Da Silva V, et al (1984). Homozygous variegate porphyria. Lancet 1:851.

Kordac V, Martasek P, Zeman J, Rubin A (1985). Increased erythrocyte protoporphyrin in homozygous variegate porphyria. Photodermatology 2: 257-9.

Kotze MJ, De Villiers JN, Groenewald JZ, Rooney RN, Loubser O, et al (1998). Molecular analysis reveals a high mutation frequency in the first untranslated exon of the PPOX gene and largely excludes variegate porphyria in a subset of clinically affected Afrikaner families. Mol Cell Probes 12:293-300.

Kramer S (1980). Porphyria variegata. Clin Haematol 9:303-22.

Kuhnel A, Gross U Jacob K, Doss MO (1999). Studies of coproporphyrin isomers in urine and feces in the porphyrias. Clin Chim Acta 282:45-58.

Kupferschmidt H, Bont A, Schnorf H, Landis T, Walter E, et al (1995). Transient cortical blindness and bioccipital brain lesions in two patients with acute intermittent porphyria. Ann Intern Med 123:598-600.

Kussman BD, Sethna NF (1998). Pethidine-associated seizure in a healthy adolescent receiving pethidine for postoperative pain control. Paediatr Anaesth 8:349-52.

Lahav M, Schoenfeld N, Epstein O, Greenblat Y, Atsmon A (1984). Effect of prolonged fasting on heme metabolism in the rat. Israel J Med Sci 20: 191-6.

Lai CW, Hung TP, Lin WS (1977). Blindness of cerebral origin in acute intermittent porphyria. Report of a case and postmortem examination. Arch Neurol 34:310-2.

Lam CW, Lai CK, Chan YW (1998). Simultaneous fluorescence detection of fecal urobilins and porphyrins by reversed-phase high-performance thin-layer chromatography. Clin Chem 44:345-6.

Lam H, Dragan L, Tsou HC, Merk H, Peacocke et al (1997). Molecular basis of variegate porphyria: a de novo insertion mutation in the protoporphyrinogen oxidase gene. Hum Genet 99:126-9.

Lamon JM, Frykholm BC, Bennett M, Tschudy DP (1978). Prevention of acute porphyric attacks by intravenous haematin. Lancet 2:492-4.

Lamon JM, Frykholm BC, Hess RA, Tschudy DP (1979). Hematin therapy for acute porphyria. Medicine 58:252-69.

Lamoril J, Martasek P, Deybach J-C, Da Silva V, Grandchamp B, et al (1995). A molecular defect in coproporphyrinogen oxidase gene causing harderoporphyria, a variant form of hereditary coproporphyria. Hum Mol Genet 4:275-8.

Lamoril J, Puy H, Gouya L, Rosipal R, Da silva V, et al (1998). Neonatal hemolytic anemia due to inherited harderoporphyria: clinical characteristics and molecular basis. Blood 91:1453-7.

Lee JS, Anvret M (1991). Identification of the most common mutation within the porphobilinogen deaminase gene in Swedish patients with acute intermittent porphyria. Proc Natl Acad Sci USA 88:10912-5.

Lemberg R, Legge JW (1949). Haematin compounds and bile pigments. New York: Interscience.

Lermontova I, Kruse E, Mock HP, Grimm B (1997). Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. Proc Natl Acad Sci USA 94:8895-900.

Levene GM (1968). Porphyria cutanea tarda hereditaria. Proc R Soc Med 61:591-2.

Linet MS, Gridley G, Nyren O, Mellemkjaer L, Olsen JH, Keehn S, Adami HO, Fraumeni JF (1999). Primary liver cancer, other malignancies, and mortality risks following porphyria: a cohort study in Denmark and Sweden. Am J Epidemiol 149:1010-5.

Lipschutz DE, Reiter JM (1974). Acute intermittent porphyria with inappropriately elevated ADH secretion. JAMA 230:716-7.

Lithner F, Wetterberg L (1984). Hepatocellular carcinoma in patients with acute intermittent porphyria. Acta Medica Scand 215:271.

Llewellyn DH, Smyth SJ, Elder GH, Hutchesson AC, Rattenbury JM, et al (1992). Homozygous acute intermittent porphyria: compound heterozygosity for adjacent base transitions in the same codon of the porphobilinogen deaminase gene. Hum Genet 89:97-8.

Lockwood WH, Davies JL (1962). Paper electrophoresis of urinary porphyrins. Clin Chim Acta 7:301-9.

Logan GM, Weimer MK, Ellefson M, Pierach CA, Bloomer JR (1991). Bile porphyrin analysis in the evaluation of variegate porphyria. New Engl J Med 324:1408-11.

Long C, Smyth SJ, Woolf J, Murphy GM, Finlay AY, Newcombe RG, Elder GH (1993). Detection of latent variegate porphyria by fluorescence emission spectroscopy of plasma. Br J Dermatol 129:9-13.

Longas MO, Poh-Fitzpatrick MB (1982). A tightly bound protein porphyrin complex isolated from the plasma of a patient with variegate porphyria. Clin Chim Acta 118:219-28.

Loots JM, Becker DM, Meyer BJ, Goldstuck N, Kramer S (1975). The effect of porphyrin precursors on monosynaptic reflex activity in the isolated hemisected frog spinal cord. J Neural Trans 36:71-81.

Luo J, Lamb JH, Lim CK (1997). Analysis of urinary and faecal porphyrin excretion patterns in human porphyrias by fast atom bombardment mass spectrometry. J Pharm Biomed Anal 15: 1289-94.

MacGregor AG, Nicholls REH, Rimington C (1952). Porphyria cutanea tarda. Investigation of a case including isolation of some hitherto-undescribed porphyrins. Arch Int Med 19 483-504.

Magnus IA (1968). The cutaneous porphyrias. Semin Hematol 5:380-408.

Magnus IA, Jarrett A, Prankerd TAJ, Rimington C (1961). Erythropoietic protoporphyria. A new porphyria syndrome with solar urticaria due to protoporphyrinaemia. Lancet ii:448-51.

Maquat LE (1991). Nuclear mRNA export. Curr Opin Cell Biol 3:1004-12.

Maquat LE (1996). Defects in RNA slicing and the consequences of shortened translational reading frames. Am J Hum Genet 59:279-86.

Martásek P (1998). Hereditary coporporphyria. Semin Liv Dis 18:25-32.

Martasek P, Camadro J-M, Raman CS, et al (1997). Human coproporphyrinogen oxidase. Biochemical characterization of recombinant normal and R231W mutated enzymes expressed in E. coli as soluble, catalytically active homodimers. Cell Mol Biol 43:47-58.

Martasek P, Kordac V, Jirsa M (1983). Variegate porphyria and porphyria cutanea tarda. Arch Dermatol 119:537-8.

Martasek P, Nordmann Y, Grandchamp B (1994). Homozygous hereditary coproporphyria caused by an arginine to tryptophan substitution in coproporphyrinogen oxidase and common Intragenic polymorphisms. Hum Mol Genet 3:477-80.

Martinez CA, Milla GC (1971). Spectrofluorometric determination of porphyrins in urine. Clin Chem 17:199-205.

Matringe M, Camadro JM, Block MA, Joyard J, Scalla R, et al (1992). Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether-like herbicides. J Biol Chem 267:4646-51.

Matringe M, Camadro J-M, Labbe P, Scalla R (1989) Protoporphyrin oxidase as a molecular target for diphenyl ether herbicides. Biochem J 260:231-5.

Maytham DV, Eales L (1971). Electrodiagnostic findings in porphyria. S Afr Med J 45:99-100.

McColl KE, Thompson GG, Moore MR, Goldberg A (1980). Acute ethanol ingestion and haem biosynthesis in healthy subjects. Eur J Clin Invest 10:107-12.

McColl KEL, Moore MR, Thompson GG, Goldberg A (1981). Treatment with haematin in acute hepatic porphyria. Q J Med L198:161-74.

McColl KEL, Thompson GG, Moore MR, Goldberg A, Church SE, et al (1985). Chester porphyria: biochemical studies of a new form of acute porphyria. Lancet 2:796-9.

McEneany D, Hawkins S, Trimble E, et al (1993). Porphyric neuropathy – a rare and often neglected differential diagnosis of Guillain-Barre syndrome. J Neurol Sci 114:231-3.

McEwan R, Lawn J, Jonas CT (1972). A survey of porphyria amongst psychiatry patients. Med J Australia 2:302-6.

McGrath H, Taaffe JA, Gilsenan D, Cunnane K (1984). An Irish family with variegate porphyria. Clin Exp Dermatol 9:583-590.

McHugh GJ (1999). Norpethidine accumulation and generalized seizure during pethidine patient-controlled analgesia. Anaesth Intensive Care 27:289-91.

McIntyre H, Pearson AJ, Allan GJ, Craske S, West GML et al (1971). Hepatic δ-aminolaevulinic acid synthetase in an attack of hereditary coproporphyria and urine remission. Lancet 1:560-4.

McManus JF, Begley CG, Sassa S, Ratnaike S (1996). Five new mutations in the uroporphyrinogen decarboxylase gene identified in families with cutaneous porphyria. Blood 88:3586-600.

McManus JF, Begley CG, Sassa S, Ratnaike S (1999). Three new mutations in the uroporphyrinogen decarboxylase gene in familial porphyria cutanea tarda. Mutation in brief No. 237 online. Hum Mutat 13: 412.

Medenica R, Lazovic G, Long P, Corbitt W, Powell D (1997). Plasmapheresis combined with somatostatin is a successful treatment of porphyrias. Ther Apher 1:159-64.

Meguro K, Fujita H, Ishida N, et al (1994). Molecular defects of uroporphyrinogen decarboxylase in a patient with mild hepatoerythropoietic porphyria. J Invest Dermatol 102:681-5.

Meissner P, Adams P, Kirsch RE (1993). Allosteric inhibition of human lymphoblast and purified porphobilinogen deaminase by protoporphyrinogen and coproporphyrinogen. J Clin Invest 91:1436-44.

Meissner PN, Dailey TA, Hift RJ, Ziman M, Corrigall AV, et al (1996). A R59W mutation in human protoporphyrinogen oxidase results in decreased enzyme activity and is prevalent in South Africans with variegate porphyria. Nature Genetics 13:95-7.

Meissner PN, Day RS, Moore MR, Disler PB, Harley E (1986). Protoporphyrinogen oxidase and porphobilinogen deaminase in variegate porphyria. Eur J Clin Invest 16:257-61.

Mendez M, Sorkin L, Rossetti MV, Astrin KH, Del C Battle AM, et al (1998). Familial porphyria cutanea tarda: characterisation of 7 novel uroporphyrinogen decarboxylase mutations and frequency of common hemochromatosis alleles. Am J Hum Genet 63:1363-75.

Meyer UA, Schuurmans MM, Lindberg RLP (1998). Acute porphyrias: pathogenesis of neurological manifestations. Semin Liv Dis 18:43-52.

Meyer UA, Strand LJ, Doss M, Reese AC, Marver HS (1972). Intermittent acute porphyria. Demonstration of a genetic defect in porphobilinogen metabolism. N Engl J Med 286:1277-82.

Mgone CS, Lanyon WG, Moore MR, Louie GV, Connor JM (1994). Identification of five novel mutations in the porphobilinogen deaminase gene. Hum Molec Genet 3:809-11.

Miller G, Lipman M (1973). Release of infectious Epstein-Barr virus by transformed pharmacy leucocytes. Proc Natl Acad Sci USA 70:190-4.

Milpetz F, Argos P, Persson B (1995). TMAP: a new email and WWW service for membrane-protein structural predicitions. TIBS 270:204-5.

Moore MR, Disler PB (1985). Biochemical diagnosis of the porphyrias. Clinics in Dermatology 3:25-40.

Moore MR, McColl KE, Goldberg A (1984). The effects of alcohol on porphyrin biosynthesis and metabolism. Contemp Issues Clin Biochem 1:161-87.

Moore MR, McColl KE, Rimington C, Goldberg A (1987). Disorders of porphyrin metabolism. New York: Plenum, pp. 1-374.

Moore MR, McColle KE, Goldberg A (1980). The activities of the enzymes of haem biosynthesis in the porphyrias and during treatment of acute intermittent porphyrias. Int J Biochem 12:941-6.

Moore MR, Thompson GG, Allen BR, Hunter JAA, Parker S (1973). Plasma porphyrin concentrations in porphyria cutanea tarda. Clin Sci Molec Med 45:711-4.

Moore MR, Thompson GG, Goldberg A (1972). Amounts of faecal porphyrin peptide conjugates in the porphyrias. Clin Sci 43:299-302.

Moran-Jimenez MJ, Ged C, Romana M, Enriquez de Salamanca R, Taieb A, et al (1996). Uroporphyrinogen decarboxylase: complete gene sequence and molecular study of three families with hepatoerythropoietic porphyria. Am J Hum Genet 58:712-21.

Muhlbauer JE, Pathak MA, Tishler PV, Fitzpatrick TB (1982). Variegate porphyria in New England. JAMA 247:3095-102.

Müller WE, Snyder SH (1977). δ-aminolevulinic acid: influences on synaptic GABA receptor binding may explain CNS symptoms of porphyria. Ann Neurol 2:340-2.

Muraoka A, Suehiro I, Fujii M, Murakami K (1995). Delta-aminolevulinic acid dehydratase deficiency in porphyria (ADP) with syndrome of inappropriate secretion of antidiuretic hormone (SIADH) in a 69 year old woman. Kobe J Med Sci 41:23-31.

Murphy GM, Magnus IA, Barrett DF, Elder GH, Hawk JLM, et al (1986). Homozygous variegate porphyria: two similar cases in unrelated families. J Royal Soc Med 79: 361-3.

Mustajoki P (1976). Red cell uroporphyrinogen I synthetase in acute intermittent porphyria. Ann Clin Res 8:133-8.

Mustajoki P (1978). Variegate porphyria. Ann Intern Med 89:238-44.

Mustajoki P (1980). Variegate porphyria. 12 years experience in Finland. Q J Med 49:191-203.

Mustajoki P (1985). Prevention and treatment of acute porphyric attacks. Ann Clin Res 17:289-91.

Mustajoki P, Heinonen J (1980). General anaesthesia in inducible porphyrias. Anaesthesiology 53:15-20.

Mustajoki P, Himberg JJ, Tokola O, Tenhunen R (1992). Rapid normalization of antipyrine oxidation by heme in variegate porphyria. Clin Pharmacol Ther 51:320-4.

Mustajoki P, Kauppinen R, Lannfelt L, et al (1992). Frequency of low erythrocyte porphobilinogen deaminase activity in Finland. J Int Med 231:389-95. WAS THIS USED IN SECTION 2??

Mustajoki P, Koskelo P (1976). Hereditary hepatic porphyrias in Finland. Acta Med Scand 200:171-8.

Mustajoki P, Mustajoki S, Rautio A, Arvela P, Pelkonen O (1994). Effects of heme arginate on cytochrome P450-mediated metabolism of drugs in patients with variegate porphyria and in healthy men. Clin Pharmacol Ther 56:9-13.

Mustajoki P, Nordmann Y (1993). Early administration of heme arginate for acute porphyric attacks. Arch Intern Med 153:2004-8.

Mustajoki P, Seppalainen AM (1975). Neuropathy in latent hereditary hepatic porphyria. Br Med J 2:310-2.

Mustajoki P, Tenhunen R, Niemi KM, Nordmann Y, Kaariainen H, et al (1987). Homozygous variegate porphyria: a severe skin disease of infancy. Clin Genetics 32:300-5.

Mustajoki P, Tenhunen R, Tokola O, Gothoni G (1986). Heme arginate in the treatment of acute hepatic porphyrias. Br Med J 293:538-9.

Mustard P, Spoug N, et al (1968). The metabolism of [5-14C]-δ-aminolevulinic acid in normal and porphyric human subjects. Clin Chem 354:233-51.

Nakau K, Wada O, Kitamura T, Vono K, Urata G (1966). Activity of amino-laevulinic acid synthetase in normal and porphyric human livers. Nature 210: 838-9.

Narita S, Tanaka R, Ito T, Okada K, Taketani S, et al (1996). Molecular cloning and characterization of a cDNA that encodes protoporphyrinogen oxidase of Arabidopsis thaliana. Gene 182:169-75.

Neilson DR, Neilson RP (1958). In: Western J Surgery, Obstetrics Gynaecol 66: 134.

Nicoll RA (1976). The interaction of porphyrin precursors with GABA receptors in the isolated frog spinal cord. Life Sci 19:521-6.

Nishimura K, Taketani S, Inokuchi H (1995). Cloning of a cDNA for protoporphyrinogen oxidase by complementation in vivo on a hemG mutant of Escherichia coli. J Biol Chem 270:8076-80.

Nordmann Y, Grandchamp B, de Verneuil ,H Phung L (1983). Harderoporphyria: a variant hereditary coproporphyria. J Clin Invest 72: 1139-49.

Norris PG, Elder GH, Hawk JLM (1990). Homozygous variegate porphyria: a case report. Br J Dermatol 122:253-7.

Ostrowski J, Kostrzewska E, Michalak T, Zawirska B (1983). Abnormalities in liver function and morphology and impaired aminopyrine metabolism in hereditary hepatic porphyrias. Gastroenterology 85:1131-7.

Parsons JL, Sahn EE, Holden KR, Pai GS (1994). Neurological disease in a child with hepatoerythropoietic porphyria. Pediatr Dermatol 11:216-21.

Parzer S, Mannholter C (1991). A rapid method for the isolation of genomic DNA from citrated whole blood. Biochem J 273:229-31.

Patience DA, Blackwood DH, McColl KE, Moore MR (1994). Acute intermittent porphyria and mental illness – a family study. Acta Psychiatr Scand 89:262-7.

Percy VA, Lamm MCL, Taljaard JJF (1981). Delta-aminolaevulinic acid uptake, neurotoxicity and effect on [14C] delta-aminobutyric acid uptake into neurons andglia in culture. J Neurochem 36:69-76.

Percy VA, Shanley BC (1977). Porphyrin precursors in blood, urine and cerebrospinal fluid in acute porphyria. S Afr Med J 52:219-22.

Perrot H, Boucherat , Thivolet J (1978). Faecal porphyrin extraction in various types of porphyria. Thin layer chromatography study. Arch Derm Res 263:67-73.

Petryka ZJ, Watson CJ (1978). A new rapid method for isolation of naturally occurring porphyrins and their quantitation after high performance liquid chromatography. Anal Biochem 84:133-78.

Picat C, Delfau MJ, De Rooij FWM, Beukeveld GJJ, Wolthers BG et al (1990). Identification of the mutations in the parents of a patient with a putative compound heterozygosity for acute intermittent porphyria. J Inherited Metabol Dis 13: 684-6.

Pierach CA (1982). Hematin therapy for the porphyric attack. Semin Liv Dis 2:125-31.

Pierach CA (1986). Treatment of the acute porphyric attack. In: Mustajoki P, ed. New therapeutic approach to hepatic porphyrias. Basel: Vammalan kirjapaino Oy, pp. 30-5.

Pierach CA, Bossenmaier I, Cardinal R, Weimer M, Watson CJ (1980). Hematin therapy in porphyric attacks. Klin Wochenschr 58: 829-32.

Pierach CA, Cardinal R, Bossenmaier J, Watson CJ (1977). Comparison of the Hoesch and the Watson-Schwartz tests for urinary porphobilinogen. Clin Chem 23:1666-8.

Pierach CA, Edwards PS (1978). Neurotoxicity of δ-aminolevulinic acid and porphobilinogen. Exp Neurol 62:810-4.

Pimstone NR, Gandhi N, Mukerji S (1987). Therapeutic efficacy of oral charcoal in congenital erythropoietic porphyria. N Engl J Med 316:390-3.

Pimstone NR, Gandhi SN, Mukerji SK, Blekkenhorst G, Day R (1982). Entero-hepatic circulation of porphyrins in man: does this exist? Hepatol 2:718.

Plewinska M, Thunnell S, Holmberg L, Wetnur JG, Desnick RJ (1991). Delta-aminolevulinate dehydratase deficient porphyria: identification of the molecular lesions in a severely affected homozygote. Am J Hum Genet 49:167-74.

Poh-Fitzpatrick MB (1980). A plasma porphyrin fluorescence marker for variegate porphyria. Arch Dermatol 116:543-7.

Poh-Fitzpatrick MB, Lamola AA (1976). Direct spectrofluorometry of diluted erythrocytes and plasma: a rapid diagnostic method in primary and secondary porphyrinemias. J Lab Clin Med 87:362-70.

Porra RJ, Falk JE (1961). In: Biochem Biophys Res Commun 5:179-84.

Porra RJ, Falk JE (1964). The enzymic conversion of coproporphyrinogen III in protoporphyrin IX. Biochem J 90:69-75.

Poulson R (1976). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX in mammalian mitochondria. J Biol Chem. 251:3730-3.

Poulson R, Polglase WJ (1974). Site of glucose repression of heme biosynthesis. FEBS letters 40: 258-60.

Poulson R, Polglase WJ (1975). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX. J Biol Chem 250:1269-74.

Price JM, Brown RR, Peters HA (1959). Tryptophan metabolism in porphyria schizophrenia and a variety of neurologic and psychiatric diseases. Neurology 9:456-68.

Proulx KL, Dailey HA (1992). Characteristics of murine protoporphyrinogen oxidase. Protein Sci 1:801-9.

Pudek MR, Schreiber WE, Jamani A (1991). Quantitative fluorometric screening test for fecal porphyrins. Clin Chem 37:826-31.

Puy H, Deybach J-C, Baudry P, Callebert J, Touitou Y, et al (1993). Decreased nocturnal plasma melatonin levels in patients with recurrent acute intermittent porphyria attacks. Life Sci 53:621-7.

Puy H, Deybach J-C, Bogdan A, Callebert J, Baumgartner M, et al (1996b). Increased δ-aminolevulinic acid and decreased pineal melatonin production. J Clin Invest 97:104-10.

Puy H, Deybach JC, Lamoril J, Robreau AM, Da Silva V et al (1997). Molecular epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. Am J Hum Genet 60:1373-83.

Puy H, Robreau AM, Rosipal R, Nordmann Y, Deybach JC (1996a). Protoporphyrinogen oxidase: Complete genomic sequence and polymorphisms in the human gene. Biochem Biophys Res Comm 226:226-30.

Qadiri MR, Church SE, McColl KEL, Moore MR, Youngs GR (1986). Chester porphyria: a clinical study of a new form of acute porphyria. Br Med J 292:455-9.

Ranking JE, Pardington GL (1890). Two cases of haematoporphyrin in the urine. Lancet 2: 607-9.

Rimington C (1958). Investigation of porhyrin/porphyria. Broadsheet 20 (reprinted 1962). Association of Clinical Pathologists.

Rimington C (1971). Quantitative determination of porphobilinogen and porphyrins in urine and porphyrins in faeces and erythrocytes. Broadsheet No. 70 (Rev Broadsheet No. 36), Association of Clinical Pathologists. London, Br Med Assoc.

Rimington C, Belcher RV (1967). Separation of porphyrins on sephadex dextran gels. J Chromatogr 28:112-6.

Rimington C, Lockwood WH, Belcher RV (1968). The excretion of porphyrin peptide conjugates in porphyria variegata. Clin Sci 35:211-47.

Rimington C, Lockwood WH. (1968). Unusual hydrophilic porphyrins in a case of porphyria. Pan Minerva Med 8: 325-7.

Rimington C, Magnus IA, Ryan EA, Cripps DJ (1967). Porphyria and photosensitivity. Q J Med 36:29-57.

Rimington C, Sveinsson SL (1950). The spectrophotometric determination of uroporphyrin. Scand J Clin Lab Invest 2:209.

Roberts AG, Elder GH, De Salamanca RE, Mascaro JM (1995b). A mutation (G281E) of the human oroporphyrinogen decarboxylase gene causes both hepatoerythropoietic porphyria and overt familial porphyria cutanea tarda. J Invest Dermatol 104:500-2.

Roberts AG, Puy H, Dailey TA, Morgan RR, Whatley SD, et al (1998). Molecular characterization of homozygous variegate porphyria. Hum Mol Genet 7: 1921-5.

Roberts AG, Whatley SD, Daniels J, Holmans P, Fenton I, et al (1995a). Partial characterisation and assignment of the gene for protoporphyrinogen oxidase and variegate porphyria to human chromosome 1q23. Hum Mol Genet 4:2387-90.

Rogers PD (1997). Cimetidine in the treatment of acute intermittent porphyria. Ann Pharmacother 31:3655-7.

Rose JA, Hellmann ES, Tschudy DP (1961). Effect of diet on induction of experimental porphyria. Metabolism 10:514-21.

Rosipal R, Lamoril J, Puy H, Da Silva V, Gouya L et al (1999). Systematic analysis of coproporphyrin oxidase gene defects in hereditary coproporphyria and mutation update. Hum Mut 13:44-53.

Rosipal R, Lamoril J, Puy H, Da silva V, Gouya L, et al (1999). Systematic analysis of coproporphyrinogen oxidase gene defects in hereditary coproporphyria and mutation update. Hum Mutat 13:44-53.

Runge W, Watson CJ (1962). Experimental production of skin lesions in human cutaneous porphyria. Proc Soc Exp Biol Med 109:809-11.

Sadeh M, Blatt I, Martonovits G, Karni A, Goldhammer Y (1991). Treatment of porphyric convulsions with magnesium sulfate. Epilepsia 32:712-5.

Sagen E, Romslo I (1985). Determination of porphyrins by high-performance liquid chromatography: fluorescence detection compared to absorbance detection. Scand J Clin Lab Invest 45:309-14.

Saksena HC, Panwar RB, Rajvanshi P, Sabir M, Suri M (1991). Alcohol and Indian porphyrics. Postgrad Med J 67:823-4.

Sano S, Granick S (1961). Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J Biol Chem 236:1173-80.

Sarkany RPE, Alexander GJM, Cox TM (1994). Recessive inheritance of erythropoietic protoporphyria with liver failure. Lancet 343:1394-6.

Sassa S (1998). ALAD porphyria. Semin Liv Dis 18:95-101.

Sassa S, Furuyama K (1998). How genetic defects are identified. Clin Dermatol 16:235-43.

Sassa S, Granick S, Kappas A (1975a). Effect of lead and genetic factors on heme biosynthesis in the human red cell. Ann NY Acad Sci 244:419-39.

Sassa S, Solish G, Levere RD, Kappas A (1975b). Studies in porphyria IX. Expression of the gene defect of acute intermittent porphyria in cultured human skin fibroblasts and amniotic cells. Prenatal diagnosis of the porphyric trait. J Exp Med 142:722-31.

Savage MW, Reed P, Orrman-Rossiter SL, Weinkove C, Anderson DC (1992). Acute intermittent porphyria treated by testosterone implant. Postgrad Med J 68:479-81.

Schermuly E, Doss M (1975). Separation of a coproporphyrin isomers I and III by thin-layer chromatography. Z Klin Chem Klin Biochem 13:299-304.

Schreiber WE, Jamani A, Pudek MR (1989). Screening tests for porphobilinogen are insensitive. The problem and its solution. Am J Clin Pathol 92:644-9.

Schwark WS, Frey HH, Czuczwar SJ (1986). Effect of opiates on the parameters of seizures in rats with full amygdaloid-kindled convulsions. Neuropharmacology 25:839-44.

Schwartz SM, Berg M, Bossenmaier I, Dinsmore M (1960). Determination of porphyrins in biological materials. In: Glick D (ed). Methods of biochemical analysis. New York: Interscience, Vol. 8: p. 221.

Schwartz SM, Edmondson P, Stephenson B, Sarkar D, Freyholtz M (1976). Direct spectrofluorometric determination of porphyrin in diluted urine. Ann Clin Res 8:156-61.

Sears WG, Darocha T, Eales L (1974). Thin-layer chromatographic behaviour of the coproporphyrin isomers in relation to porphyrin P(1). Enzyme 17:69-75.

Seppalainen AM, Hernberg S (1972). Sensitive technique for detecting subclinical local neuropathy. Br J Industrial Medicine 29:443.

Shanley BC, Percy VA, Neethling AC (1976). Neurochemistry of acute porphyria. In: Doss M (ed). Porphyrins in human diseases. Basel: Karger.

Shimizu Y, Ida S, Naruto H, Urata G (1978). Excretion of porphyrins in urine and bile after the administration of delta-aminolevulinic acid. J Lab Clin Med 32:795-802.

Siepker L, McCulloch J, Kramer S (1986). Protoporphyrinogen oxidase in porphyria variegata. A report of the findings in 7 families. S Afr Med J 70:819-22.

Siepker LJ, Ford M, De Kock R, Kramer S (1987). Purification of bovine protoporphyrinogen oxidase: immunological cross-reactivity and structural relationship to ferrochelatase. Biochim Biophys Acta 913:349-58.

Siepker LJ, Kramer S (1985). Protoporphyrin accumulation by mitogen stimulated lymphocytes and protoporphyrinogen oxidase activity in patients with porphyria variegata and erythropoietic protoporphyria: evidence for deficiency of protoporphyrinogen oxidase and ferrochelatase in both diseases. Br J Haematol 60:65-74.

Simpson JA (1962). In: Pinelli P, Buchthal F, Thiebaut F. Progress in electromyography. Amsterdam, Elsevier, p. 36.

Smith KM (1975). Porphyrins and metalloporphyrins. Amsterdam: Elsevier.

Smith SG (1978). The use of thin layer chromatography in the separation of free porphyrins and porphyrin methyl esters. Br J Dermatol 93:291-5.

Smith SG (1986). Hepatoerythropoietic porphyria. Semin Dermatol 5:125-37.

Sobel C, Cano C, Thiers RE (1974). Separation and quantitation of coproporphyrinogen and uroporphyrinogen in urine. Clin Chem 20:1397-1402.

Solis JA, Betancor P, Campos R, Enriquez de Salamanca R, Rojo P, et al (1982). Association of porphyria cutanea tarda and primary liver cancer: report of ten cases. J Dermatol (Tokyo) 9:131-7.

Sorensen AW, With TK (1971). Persistent paresis after porphyric attacks. Acta Med Scand 200:171-8.

Stathers GM (1966). Porphyrin-binding effect of cholestyramine. Lancet 2:780-3.

Stewart PM, Hensley WJ (1981). An acute attack of variegate porphyria complicated by severe autonomic neuropathy. Aust NZ J Med 11:82-3.

Stokvis BJ (1889). Over twee zeldsame kleurstoffen in urine van zicken. Ned Tijdschr Geneeskunde 13:409-17.

Strand LJ, Felsher BF, Redeker AG, Marver HS (1970). Heme biosynthesis in intermittent acute porphyria. Decreased hepatic conversion of porphobilinogen to porphyrins and increased delta-aminolevulinic acid synthetase activity. Proc Nat Acad Sci USA 67:1315-20.

Sturrock ED, Meissner PN, Maeder DL, Kirsch RE (1989). Uroporphyrinogen decarboxylase and protoporphyrinogen oxidase in dual porphyria. S Afr Med J 76:405-8.

Susa S, Daimon M, Morita Y, Kitagawa M, Hirata A, et al (1999). Acute intermittent porphyria with central pontine myelinolysis and cortical laminar necrosis. Neuroradiology 41:835-9.

Sweeney GD (1963). Patterns of porphyrin excretion in South African porphyric patients. S Afr J Lab Clin Med 9:182-98.

Taddeini L, Watson CJ (1968). The clinical porphyrias. Semin Hematol 5:335-69.

Taketani S, Inazawa J, Abe T, Furukawa T, Kobno H et al (1995). the human protoporphyrin oxidase gene (PPOX), organisation and location to chromosome 1. Genomics 29:698-703.

Tan CH, Yeow YK (1988). Acute intermittent porphyria (AIP) – an unusual cause of acute confusional state. A case report. Ann Acad Med Singapore 17:451-3.

Taylor RL (1981). Magnesium sulfate for AIP seizures. Neurology 31:1371-2.

Tchernitchko D, Lamoril J, Puy H, Robreau AM, Bogard C et al (1999). Evaluation of mutation screening by heteroduplex analysis in acute intermittent porphyria: comparison with denaturing gradient gel electrophoresis. Clin Chim Acta 279:133-43.

Te Velde K, Noordhoek KH (1970). Two Dutch families with variegate porphyria. Ned Tijdschr Geneeskd 114:409-12.

Te Velde K, Noordhoek KHN, Wilson JHP, De Rooy FWM (1989). Een familie-onderzoek bij de eerste Nederlandse patiëten met porfyrie. Ned Tijdschr Geneeskd 133:2547-52.

Tenhunen R, Tokola O, Linden IB (1987). Heme arginate: a new stable heme compound. J Pharmacol 39:780-6.

Thorner PS, Bilbao JM, Sima AA, Briggs S (1981). Porphyric neuropathy: an ultrastructural and quantitative case study. Can J Neurol Sci 8:281-7.

Thunell S, Andersson D, Harper P, Henrichson A, Floderus Y, Lindh U (1997). Effects of administration of antioxidants in acute intermittent porphyria. Eur J Clin Chem Clin Biochem 35:427-33.

Thunell S, Floderus Y, Henrichson A, Moore MR, Meissner PN, Sinclair J (1992). Alcoholic beverages in acute porphyria. J Studies on Alcohol 53:272-6.

Thunnell S, Holmberg L, Lundgreen J (1987). Aminolevulinate dehydratase porphyria in infancy. A clinical and biochemical study. J Clin Chem Clin Biochem 25:5-14.

Tian H, Yu L, Mather MW, Yu CA (1998). The flexibility of the neck region of the rieske iron sulfur protein is functionally important in the cytochrome bc1 complex. J Biol Chem 273:27953-9.

Tidman MJ, Higgins EM, Elder GH, MacDonald DM (1989). Variegate porphyria associated with hepatocellular carcinoma. Br J Dermatol 121:503-5.

Timonen K, Mustajoki P, Tenhunen R, Lauharanta J (1990a). Effects of heme arginate on variegate porphyria. Br J Dermatol 123:381-7.

Timonen K, Niemi KM, Mustajoki P, Tenhunen R (1990). Skin changes in variegate porphyria. Clinical, histopathological and ultrastructural study. Arch Dermatol Res 282:108-14.

Tio TH (1958). Porphyria cutanea tarda. Arch Dermatol 77:568-75.

Tishler PV, Woodward B, O'Connor J, Holbrook DA, Seidman J,et al.1985). High prevalence of acute intermittent porphyria in a psychiatric patient population. Am J Psychiatry 142:1430-36.

Tokola O, Linden IB, Tenhunen R (1987). The effects of heme arginate and hemeatin upon the allylisopropylacetamide induced experimental porphyria in rats. Pharmacol Toxicol 61:75-8.

Tokola O, Mustajoki P, Himberg J-J (1988). Haem arginate improves hepatic oxidative metabolism in variegate porphyria. Br J Clin Pharmacol 26:753-7.

Treece GL, Magnussen CR, Patterson JR, Tschudy DP (1976). Exacerbation of porphyria during treatment of pulmonary tuberculosis. 113:233-7.

Tschudy DP, Lamon JM (1980). Porphyrin metabolism and the porphyrias. In: Bondy PK, Rosenberg LE (eds). Metabolic control and disease. Philadelphia: WB Saunders p. 939.

Tschudy DP, Perlroth MG, Marver HS, Collins A, Hunter G Junior et al (1965). Acute intermittent porphyria: The first "overproduction disease" localised to a specific enzyme. Proc Natl Acad Sci USA 53:841-7.

Usalan C, Erdem Y, Altun B, Gursoy M, Celik I, et al (1996). Severe hyponatremia due to SIADH provoked by acute intermittent porphyria. Clin Nephrol 45:418.

Van den Bergh AAH, Grotepass W (1937). Ein bemerkenswerter Fall von Porphyrie. Wien Klin Wochenschr 50:830-1.

Van der Sar A, Den Ouden A (1976). Porphyria variegata in a curacao negroid female. Neth J Med 19:19-23.

Viljoen DJ, Cayanis E, Becker DM, Kramer S, Dawson B, et al (1979). Reduced ferrochelatase activity in fibroblasts from patients with porphyria variegata. Am J Hematol 6:185-90.

Volin L, Rasi V, Vahtera E, Tenhunen R (1988). Heme arginate: effects on hemeostasis. Blood 71:625-8.

Waldenström J (1937). Studien über porphyrie. Acta Med Scand (Suppl) 82:1-254.

Waldenström J (1957). The porphyria as inborn errors of metabolism. Am J Med 22:758-73.

Warnich L, Kotze MJ, Groenewald IM, Groenewald JZ, van Brakel MG, et al (1996b). Identification of three mutations and associated haplotypes in the protoporphyrinogen oxidase gene in South African families with variegate porphyria. Hum Mol Genetics 5:981-4.

Warnich L, Meissner PN, Hift RJ, Louw JH, Van Heerden CJ, et al (1996a). Mapping of the variegate porphyria (VP) gene: contradictory evidence for linkage between VP and microsatellite markers at chromosome 14q32. Hum Genet 97:690-2.

Watson CJ (1960). The problem of porphyria—some facts and questions. N Eng J Med 263:1205-15.

Watson CJ (1975). Hematin and porphyria. N Engl J Med 293:605-7.

Wells GC, Rimington C (1953). Studies on a case of porphyria cutanea tarda. Br J Dermatol 65:337-51.

Wenger S, Meisinger V, Brucke T, Deecke L (1998). Acute porphyric neuropathy during pregnancy – effect of haematin therapy. Eur Neurol 39:187-8.

Westerhof W, Bebelaar D, Cormane RH, Langelaar J, Hup JM (1981). The protective role of epidermal melanin in a patient with porphyria variegata and vitiligo. Acta Derm Venereol 61:23-8.

Westerhof W, Smit EM (1981). Porphyria variegata: een familie-onderzoek. Ned T Geneeskd 125:1235-41.

Wetterburg L (1967). A neuropsychiatric and genetic investigation of acute intermittent porphyria. PhD dissertation, Stockholm: Scandinavian University Books, pp. 1-87.

Whatley SD, Puy H, Morgan RR, Robreau AM, Roberts AG, et al (1999). Variegate porphyria in Western Europe: Identification of PPOX gene mutations in 104 families, extent of allelic heterogeneity, and absence of correlation between phenotype and type of mutation. Am J Hum Genet 65:984-994.

Wierenga RK, Terpstra P, Hol WGJ (1986). Prediction of the occurrence of the ADP binding bab-fold in proteins, using an amino acid fingerprint. J Biol Chem 187:101-7.

Willstätter R, Mieg W (1906). Untersuchungen über das Chlorophyll. Liebigs Ann Chem 350:1-47.

Willstätter R, Stoll A (1913). Untersuchungen über Chlorophyll. Berlin: Springer.

Wilson JMP, Van de Berg JWO, Edixhoren-Bosdijk A, Van Gastel-Quist LHM (1978). Preparation of porphyrin methyl esters for high pressure liquid chromatography. Clin Chim Acta 89:165-7.

Windebank AJ, Bonkovsky Hl (1992). Porphyric neuropathy. In: Dyck RJ, Thomas SK (eds). Peripheric neuropathy. Philadelphia: Saunders.

With TK (1969). Hereditary hepatic porphyrias. Gene penetration, drug sensitivity and subdivision in the light of systematic family studies. Acta Med Scand 186:117-24.

With TK (1973). On porphyrin esterification and ester hydrolysis. Scand J Clin Lab Invest (suppl) 32:134.

With TK (1975). The clinical use of porphyrin ester chromatography. Dan Med Bull 22:74-80.

With TK (1976). Use and abuse of the Rimington-Sveinsson correction. In: Doss M (ed). Porphyrins in human diseases. International Meeting, Freiburg 1975. Report of Discussions. Basel: Karger, pp. 279-280.

With TK (1977). Porphyrins in erythrocytes and blood plasma: preliminary studies with qualitative thin-layer chromatography. Clin Sci Molec Med 52:463-7.

With TK (1978). Clinical porphyrin analyses: indications and interpretations. Scand J Clin Lab Invest 38:501-5.

With TK (1983). Hereditary coproporphyria and variegate porphyria in Denmark. Danish Medical Bulletin 30:106-12.

With TK, Pedersen JS (1977). Molar absorption coefficients of porphyrin esters in chloroform determined by copper tritiation. Biochem J 161:425-9.

Wranne L (1960). Free erythrocyte copro- and protoporphyrin. Acta Paediatr Suppl 214.

Xu W, Solis C, Bermejo AM et al (1995). Clinical and molecular delineation of severe homozygous dominant acute intermittent porphyria. Pediatr Res 37:A155.

Yamamori I, Asai M, Tanaka F, Muramoto A, Haseggawa H (1999). Prevention of premenstrual exacerbation of hereditary coproporphyria by gonadotropin-releasing hormone analogue. Intern Med 38:365-8.

Yasunobu KT, Tan A (1985). In: Mondovi B (ed). Advances in monoamine oxidase enzymology. Structure and functions of amine oxidases. Boca Raton, FL: CRC Press, pp. 209-17.

Yeung-Laiwah AC, Macphee G, Boyle P, et al (1987). Autonomic neuropathy in acute intermittent porphyria. J Neurol Neurosurg PS 48:1025-30.

Yeung-Laiwah AC, MacPhee GJ, Boyle P, Moore MR, Goldberg A (1985). Autonomic neuropathy in acute intermittent porphyria. J Neurol Neurosurg Psychiatry 48:1025-30.

APPENDIX 1:

BIOCHEMICAL METHODS EMPLOYED IN THE UCT PORPHYRIA DIAGNOSTIC LABORATORY

INDEX

1.1 WATSON-SCHWARTZ SCREENING TEST FOR PBG

Reagents

- Ehrlich's aldehyde (4-dimethylaminobenzaldehyde 2 g made up to 100 ml with 6N HCl)
- Chloroform.

Method

Approximately 1 ml of Ehrlich's aldehyde is added to 1 ml of urine in a test tube. The resulting mixture is inspected for the development of a pink to red colour. The result is subjectively reported on a scale negative, trace, +, ++. In the event of a positive result , 2 ml chloroform are added and mixed; the tube is allowed to stand or gently spun until the aqueous and organic phases have separated. Urobilinogen will extract into the chloroform layer; PBG into the aqueous layer. If necessary, the aqueous layer is pipetted off, mixed with a further equal volume of chloroform and the process continued until there is compete partition of the red colour into one or other layer.

1.2 SCREENING TEST FOR URINARY PORPHYRINS

Reagents

- Dean's solution: equal volumes of glacial acetic acid, diethyl ether and amyl alcohol.

Method

4.5 ml of urine are mixed with 2 ml of Dean's solution; shaken and separated by gentle centrifugation. The supernatant is observed under ultraviolet light.

1.3 SCREENING TEST FOR STOOL PORPHYRINS

Reagents

- Dean's solution
- Hydrochloric acid.

Method

A small volume of stool, approximately pea-sized, is carefully mixed and 4.5 ml of Dean's solution are added. The mixture is shaken and separated by gentle centrifugation. The supernatant is observed under ultraviolet light. The supernatant is poured off and 2 ml 1.5N hydrochloric acid are added; the tube is shaken and allowed to separate. The lower phase is examined under ultraviolet light.

1.4 THIN-LAYER CHROMATOGRAPHY

Equipment

- Fluoroscanning photodensitometer
- Peak integrator and chart recorder

Reagents

- 5% sulphuric acid/methanol solution
- 17% ammonia solution
- Chloroform
- Separating solvent: carbon tetrachloride, dichloromethane, ethyl acetate, ethyl propionate (2:2:1:1, v/v/v/v)
- Enhancing solvent: chloroform, dodecane, hexadecane (18:1:1, v/v/v)
- Porphyrin standards, containing equal proportions of uroporphyrin, C7, C6, C5, coproporphyrin and mesoporphyrin methyl esters (Porphyrin Products, Logan, Utah, USA).

Preparation of specimens

Urine porphyrins

A volume of 3 ml of urine is esterified in 30 ml of a 5% sulphuric acid/methanol solution overnight at room temperature in the dark. The resulting mixture is transferred into a separating funnel and the pH neutralised with 17% ammonia solution. The solution is then extracted into a total volume of 20 ml of chloroform. Where large amounts of porphyrin are present, more chloroform is used. All volumes used are noted.

Stool porphyrins

Approximately 300 mg of stool is esterified in 30 ml of a 5% sulphuric acid/methanol solution at room temperature in the dark overnight. The wet weight of the stool sample is determined and noted; a further aliquot is weighed, dried overnight at 60°C and its dry weight determined: the wet:dry weight ratio of the sample can therefore be calculated. The stool esterification mixture is centrifuged at 800 g. for 50 min and the supernatant is transferred

into a separating funnel. The pH is neutralised with 17% ammonia solution. The porphyrins are then extracted into a total volume of 45 ml of chloroform. Where large amounts of porphyrin are present, more chloroform is used. All volumes used are noted.

Plasma porphyrins

3 ml of plasma is esterified in 30 ml of a 5% sulphuric acid/methanol solution at room temperature in the dark overnight. The esterification mixture is centrifuged at 800 g. for 15 min. The supernatant is transferred into a separating funnel. The pH is neutralised with 17% ammonia solution. The porphyrins are then extracted into a total volume of 45 ml of chloroform and transferred to a small glass bottle. This is left open and the sample is allowed to evaporate to dryness. Immediately prior to chromatography, 1 ml of chloroform is added and the container gently agitated to redissolve the porphyrins. All volumes used are noted.

Quantitative thin layer chromatography

Immediately prior to chromatography, the volume of the porphyrin extract in chloroform is noted. Using a Hamilton microsyringe, precise aliquots of each extract are applied as a spot on Merck silica gel- 60 TLC plates. 30 to 50 µl of stool extract, 100 to 300 µl of urine extract and 50 to 100 µl of plasma extract are applied to the plate. The precise volume applied is predicted by reference to the initial screening results with Dean's solution; in the presence of large amounts of porphyrin, smaller volumes are applied so as to reduce the quenching of fluorescence. The volume applied is noted. A constant stream of warmed air is blown over the working area to aid evaporation of the chloroform during application to the plate. On each plate, two lanes are reserved for porphyrin methyl ester standards. Samples are run in a further nine lanes.

Plates are allowed to stand upright in a bath containing a small volume of separating solvent. The plate is removed when the solvent front approaches the top of the plate, typically after 45 to 60 min, and is then dipped in an enhancing system in order to increase the yield of fluorescence.

The plates are then scanned on a fluoroscanning photodensitometer connected to an integrator and recorder unit. Excitation and emission wavelengths of 399 nm and 620 nm are employed. Individual porphyrin esters are identified by direct comparison of the retention times with those of the standards. The area under the curve for each peak of fluorescence is calculated by the integrator.

All appropriate data, including the fluorescence detected, the fluorescence associated with each standard, the volume of sample applied to the plate and the volume or mass of initial sample are all entered into the computerised porphyrin database, which thereafter automatically calculates the concentrations of each individual porphyrin in the individual samples according to the following formulae.

Calculation of porphyrin concentration

Urine

Porphyrin concentration (nmol/10 mmol creatinine) = [(SA/SV)/(PA/PV)] * [VF*C/IV] * 10/UC
(The concentrations of urine porphyrins are corrected for the degree of urinary concentration by reference to the urinary creatinine concentration.)

Stool

Porphyrin concentration (nmol/g dry weight) = [SA/SV)/(PA/PV)] * [VF*C)**(WW/DW)/WS]

Plasma

Porphyrin concentration (nmol/litre) = [(SA/SV)/(PA/PV)] * [VF*C/IV]

Where

SA = peak area of peak of sample porphyrin
PA = peak area of relevant standard porphyrin
VF = volume of sample applied to TLC plate (μl)
PV = volume of relevant porphyrin standard applied to TLC plate (μl)
VF = final volume of chloroform extract from sample (ml)
IV = initial sample volume of urine (ml)
IW = initial sample wet weight of stool (g)
C = relevant porphyrin ester standard concentration (nmol/l)
WW = wet weight of stool sample (g)
DW = dry weight of stool sample (g)
SW = wet weight of stool sample esterified (g).
UC = measured urinary creatinine concentration (mmol/l)

1.5 PLASMA PORPHYRIN FLUOROSCANNING

Equipment

Spectrofluorometer (Hitachi 650-109 Fluorescence spectrophotometer, Hitachi Koki Co. Ltd, Japan) equipped with red-sensitive photomultiplier and chart recorder.

Reagents

- Phosphate-buffered saline: 0.9% saline, 0.01 M sodium phosphate, pH 7.4.

Method

Plasma is separated from blood collected into heparin or EDTA by centrifugation. 1ml of plasma is diluted with 9 ml of phosphate-buffered saline. Samples are transferred to 1 cm path-length optical glass cuvettes for spectrofluorometry. The fluorescence spectrophotometer is zeroed against phosphate-buffered saline at an excitation wavelength of 405 nm and emission wavelength of 620 nm, with the monochromator slit width set at 5 nm. The emission spectrum is then scanned from 580 nm to 650 nm with the excitation monochromator set at 405 nm.

APPENDIX 2:

PREPARATION OF EBV-TRANSFORMED LYMPHOBLASTS

Transformation medium

Fresh Ham F10 medium with 15% fetal calf serum is added to semi-confluent cultures of the EBV-producing marmoset cell line B95/8 (Miller and Lipman 1973). The Ham F10 medium is removed after four days, and cells are removed by centrifugation and filtering through a 0.45 μm membrane filter. The filtrate is then added to further equal volume of Ham F of 10 medium with 15% fetal calf serum. This medium is effective for at least three months when stored at 4°C.

Transformation of lymphoblasts

A 20 ml sample of heparinised blood is taken from the subject and diluted with a one-third volume of Ham F10 nutrient medium. Lymphocytes are isolated by layering 10 ml aliquots of the diluted blood on to 5 ml of Histopaque solution (Sigma) followed by centrifugation at 1500 g for 20 min. The leukocyte band at the interface is gently removed by pipette, ished twice in Ham F10 and is then re-suspended in 3 ml of the transformation medium. The cells are then incubated at 37°C in a 5% carbon dioxide environment in a 25 cc tissue culture flask. Volumes of 0.5-1 ml of medium are added on alternate days until rapid multiplication of transformed lymphoblasts allows passage into new flasks. This generally requires 7-21 days. Secondary culture can be maintained as a suspension in Ham F10 containing 10% fetal calf serum at densities of $1\text{-}5x10^6$ cells/ml. A proportion of transformed lymphoblasts are frozen to -180°C and stored in liquid nitrogen. Frozen lymphoblasts cell lines appear to remain viable indefinitely.

APPENDIX 3:

PROTOPORPHYRINOGEN OXIDASE ASSAY

Our assay method (Meissner et al 1986) relies on the quantitation of the fluorescent product (protoporphyrinogen) resulting from the oxidation of the non-fluorescent substrate (protoporphyrinogen) by the enzyme PPO and is based on the assay described by Brenner and Bloomer (1980) and Jacobs and Jacobs (1982).

3.1 EQUIPMENT

1. Hitachi 650-10 S fluorescence spectrophotometer (Hitachi Koki Co. Ltd, Japan).
2. Hitachi U-1100 UV/VIS spectrophotometer (Hitachi Koki Co. Ltd, Japan).
3. Waterbath (Memmert, Laboratory & Scientific, Cape Town, South Africa).
4. Reaction vessel: a round-bottomed flask with a long side arm.
5. PH meter
6. Ultraviolet light source.

3.2 REAGENTS

7. Protoporphyrin IX (Porphyrin Products, Logan, UT, USA)
8. Assay buffer:
   100mM Tris/HCl, 3 mM dithiothreitol, 1 mM EDTA, 1% Tween 20, pH 8.1
9. Cuvette buffer
   As for assay buffer but without Tween 20
10. Protoporphyrin IX (Porphyrin Products, Logan, UT, USA).

3.3 METHOD

Preparation of substrate

1. Dissolve 12 mg protoporphyrin IX in 30 ml 40 mM KOH in 20% ethanol (freshly prepared) by stirring in the dark.
2. Filter protoporphyrin stock through 0.45 μm filter. Determine concentration of protoporphyrin by measuring absorbance of a 1/100 dilution of the solution in 2.7 N HCl at 408 nM and using the extinction coefficient of 262:
   protoporphyrin concentration (μM) = $OD_{408}/262 \times 10^5$
3. Dilute protoporphyrin stock to obtain 20 ml of 100-125 μM protoporphyrin and use it to calibrate the fluorometer.
4. Prepare a 4% sodium-mercury amalgam for the reduction of protoporphyrin to protoporphyrinogen. Weigh out 1.8 g Na and 43.2 g Hg. Cut mercury into small pieces and place the pieces in the reaction vessel. Run a continuous flow of nitrogen through the side arm. Add sodium piece by piece. The reaction may occur spontaneously. If not, heat the vessel gently over a burner. Once all sodium is added, continue heating until the

amalgam is completely liquid. Pour the amalgam into a mortar and chop it is with a spatula until it solidifies. Grind the pieces with a mortar into small granules.

5. Reduce the protoporphyrin to protoporphyrinogen. Working in the dark, place 20 ml of diluted protoporphyrin in a boiling tube with a glass stopper. Add half the amalgam. Shake well. Allow gas to escape from time to time. Add the rest of the amalgam. Shake well and check fluorescence under ultraviolet light. Continue shaking until there is no further decrease in fluorescence. Filter the protoporphyrinogen solution through a 0.45 μm filter.
6. Immediately the correct the pH of the protoporphyrinogen solution to 8.1 by the addition, drop-wise, of 2 M MOPS.
7. Immediately start assay reaction by adding 100 μl of this to each tube.

Assay procedure

1. Add 25 to 100 μl of sample as necessary to each assay to and add assay buffer to a total vol of 900 μl.
2. Prepare a corresponding blank for each assay to by substituting the sample with an equal Volume of sample heated to 80°C for 10 min.
3. Prepare cuvette tubes containing 900 μl of cuvette buffer: 1 tube per sample, including blanks, per time point.
4. Pre-incubate samples in assay tubes in the waterbath at 30°C for 20 min.
5. Calibrate fluorometer. Zero against water. Make 1/10 dilution of proto stock in assay buffer. Make a further 1/250 dilution of this and prepare tubes for a standard curve as follows. Read fluorescence.

1/250 dilution stock buffer (μl)	Cuvette buffer (μl)
200	800
400	600
600	400
800	200
1000	0

6. Add substrate (100 μl) to assay tubes. Mix and allow to stand for 10 min at 30°C before taking first reading.
7. At each time point transfer 100 μl from the assay tube into a cuvette tube and read fluorescence. Use 4 time intervals up to 40 min.

Determination of activity

8. Use the customised spreadsheet (Lotus 123) to calculate kinetic activity. (subtract the rate of autooxidation, measured by the change in fluorescence in the blank tubes, from the rate of oxidation observed in each sample, corrects this value for dilution factors and converts the value to nmol/ml/h comparison with the standard curve.
9. Determine the protein concentration in the initial samples using the Bio-Rad assay.
10. Express the final value in nmol/g/h.

APPENDIX 4:

ANALYTICAL METHODS FOR THE DETECTION OF MUTATIONS

INDEX

4.1 EXTRACTION OF RNA FROM LYMPHOBLASTS

Equipment

1. Jouan KR422 centrifuge, Saint-Herblain, France
2. Microfuge Force 14, Denver Instruments, Laboratory & Scientific, Cape Town, SA.

Reagents

1. Diethylpyrocarbonate (DEPC) H2O (0.05%)
2. Stock solution (autoclaved)

Guanidinium thiocynate	50 g
Distilled H_2O (DEPC treated)	58.6 ml
0.75 M Na citrate, pH7	3.5 ml
10% sarkosyl	5.3 ml

3. Solution A
   Add 72 µl mercaptoethanol to 10 ml of the above stock solution.
4. Water saturated phenol
   Melt solid phenol. Add DEPC H2O to top of phenol and shake. Allow to settle and discard water. Repeat three times. Leave water on top and colour with small amount of β-

hydroxyquinaline. This imparts a straw colour and allows one to differentiate between the layers.

Method

1. Gloves are worn and RNAse-free pipette tips used at all times. All glassware and solutions must be treated with 0.05% DEPC—an RNAse inhibitor—and are autoclaved before use.
2. Transfer cell culture to 50 ml sterile capped centrifuge tubes.
3. Spin at 400 g for 10 min at 4°C.
4. Pour off supernatant and drain tube by standing on clean paper towel for a few min.
5. Add 10 ml Hanks Balanced Salt Solution (HBSS), re-suspend pellets and transfer to 1.5 ml microfuge tubes.
6. Respin at 2000 g for 10 min and discard saline, once again draining off as much fluid as possible.
7. Add 1 ml of solution A. Lyse the cells by drawing up and down 6 times through a 1 ml disposable pipette tip. Transfer and divide sample between two 1.5 ml microfuge tubes.
8. Add the following reagents, mixing after each addition:
   60 µl 2M Na acetate
   600 µl H2O saturated phenol
   120 µl chloroform/isoamyl alcohol (49:1 v/v).
9. Shake vigorously for 10 seconds and cool on ice for 15 min.
10. Microfuge at 14000 g for 15 min at 4°C.
11. Remove aqueous phase (DNA and protein are in interphase and phenol layer) and add 600 µl isopropanol (stored at -20°C) and leave overnight at -20°C.
12. Pellet by microfuging at 14000 g for 20 min at 4°C.
13. Discard supernatant and drain tube by inverting on paper towel. Ish pellet with 1 ml 70% cold ethanol.
14. Pellet RNA by microfuging at 14000 g for 20 min.
15. Add 500 µl of 70% ethanol and freeze sample at –70°C in 10 aliquots.
16. When required for RT-PCR, spin down precipitate from an aliquot, discard the supernatant and dissolve the dried precipitate in 10 µl H_2O. Use 1 µl for RT PCR (1-2 µg).

4.2 REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT–PCR)

Equipment

1. Hybrid Omnigene thermal cycler, Teddington, UK.
2. Force 14 microfuge, Denver Instruments, Laboratory & Scientific, Cape Town, SA.

Reagents

1. Reverse transcriptase (Maloney-murine Leukemia virus) 200 units/µl, (Stratagene, La Jolla, USA)
2. RNAse inhibitor (40 units/µl) (GibcoBRL, Paisley, UK)

3. 10x concentrated PCR buffer solution:
   500 mM KCl, 10 mM Tris, pH 8.3 (Stratagene, La Jolla, USA)
4. Deoxynucleotide Triphosphates (dNTPs), (Promega, Madison, USA).

Method

1. Take equal volumes of dATP, dCTP, dGTP and dTTP (all 100 mM) and dilute and combine to make a stock solution containing 2.5 mM of each dNTP.
2. In a sterile microfuge tube on ice, prepare the following mixture to a total volume of 20 µl:

10x buffer	2 µl
DTT 10x	2 µl
dNTP	4 µl
Oligo (dT) 100 mM	2 µl
RNAsin	1 µl
MMLV-RT	0.25µl (50 units)
RNA (~3 µg)	1 µl
Distilled H_2O	7.75 µl

3. Perform the PCR on the Omnigene thermocycler using the following temperature profile:

Temperature	Time	No. of cycles
37°C	60 min	1
95°C	5 min	
5°C	5 min in	

4. Check that RT-PCR product is specific on a 6% polyacrylamide gel (see Appendix 4. 4).

4.3 POLYMERASE CHAIN REACTION (PCR) OF cDNA

Reagents

1. Primers (Genosys Biotechnologies (Europe) Ltd, Pampisford, Cambridgeshire, UK) were designed to cover the entire cDNA PPO sequence (Nishimura 1995; Dailey H personal communication) using Primer Designer for Windows, Soft Packaging version 2, Scientific and Education Software.

Fragment 1. (533 bp)	PF_1 CATCATGGTATGGCTATGCC PR_1 AGACTGTCCATGGCTAGAGA
Fragment 2. (410 bp)	PF_2 CTGCATGCCCTACCCACTG PR_2 TTCAAGGCCTGAGGCAACA
Fragment 3. (389 bp)	PF_3 ACTTCGTGGAGGTCTAGAGA PR_3 CCGTCCTGCTCAGGGAAAGCAAC
Fragment 4. (455 bp)	PF_4 GTGCCATCTTCAGAAGATCC PR_4 TCAGCTGTTAGGTTCTGTGC

2. Taq DNA polymerase, Promega, Madison, USA.
3. DNTPs, Promega, Madison, USA.

Methods

1. In a sterile 1.5 ml microfuge tube make the following mixture (scale up according to the number of PCR reactions required. Allow sufficient to include a blank).
2. Using disposable sterile filtered tips, prepare the following PCR reaction mixture to a total volume of 47 µl:

	Volume (µl)	Final concentration
10 x buffer	5	1 x
Mg Chloride solution (25 mM)	5	1 x
DNTP mix (2.5 mM)	2	100 µM
Primer forward	2	1 µM
Primer reverse	2	1 µM
Taq DNA polymerase	0.2	1 unit
Sterile H_2O	30.8	

3. Add 47 µl of the mixture to 0.6 ml microfuge tubes on ice. Add 3 µl DNA (RT-PCR product) to each tube. Vortex tubes. Microfuge briefly then overlay with mineral oil.
4. Place tubes in thermal cycler and start the PCR reaction. The temperature profiles are as shown below.

	Temperature (°C)	**Time (min)**	**No. of cycles**
Initial Denaturation	94	1	1
Denaturation	94	1	32
Annealing (see below)	51–65	0.5	
Extension	72	1	
Final extension	72	5	1

Fragment	**Annealing temperatures (°C)**
1	51
2	56
3	65
4	51

5. Analyse the PCR product on 6% acrylamide gel to assess purity (see Appendix 4.4).
6. Run product on MS8 (2%) agarose (see Appendix 4.5) and extract the PCR product using the Qiaex II agarose gel extraction protocol described below.

4.4 6% NON-DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Equipment

1. SE600 vertical slab gel electrophoresis unit, (Hoefer Scientific Instruments Pharmacia Biotech, Cambridge, UK).
2. PS1200 DC power supply, (Hoefer Scientific Instruments Pharmacia Biotech, Cambridge, UK).
3. Uvitec gel documentation system, (Uvitec, Cambridge, UK).

Reagents

1. 10 x TBE (Tris/Borate/EDTA), 1L
   890 mM Tris base
   890 mM boric acid
   20 mM EDTA, pH 8.0
2. Ethidium Bromide:
   Stock solution 1 mg/ml, stored in the dark. For use add 200 µl of stock solution to 200 ml H_2O.
3. Sucrose sample solution: 30 g Sucrose
   10 mg bromophenol blue
   5 ml 0.5M Na_2 EDTA
   Make up to 50 ml with water.
4. 100 bp DNA ladder, (Promega Corporation, Southampton, UK).

Method

1. To a 100 ml beaker add:
   a) 5 ml TBE 10 x
   b) 10 ml A-Bis-A solution (30% acrylamide, 0.8% bisacrylamide)
   c) 500 µl ammonium persulphate (10%)
   d) 50 µl TEMED
   e) Make up to 50 ml with distilled water.
2. Pour into the space between the clamped 2 glass gel plates (1.5 mm spacers) mounted in the gel casting stand.
3. Insert a 20 sample bay spacer comb and allow to set.
4. Assemble upper buffer chamber on top of gel plate. Fill upper buffer chamber with 500 ml 1x TBE.
5. Fill lower buffer chamber with 2 L of 1x TBE.
6. Load PCR producted diluted 1:1 with sucrose sample solution into sample bays using a Hamilton syringe.
7. Load 10 µl of DNA marker (diluted 1:1 with sucrose sample solution) in a bay (100 bp DNA ladder).
8. Run at 150 V for ± 2 h.
9. Stain gel in ethidium bromide for 7 min. Rinse in water and then visualize under UV light.
10. Photograph gel using gel documentation system.

4.5 QIAEX II AGAROSE GEL EXTRACTION PROTOCOL

Reagents

1. Qiaex II Gel extraction kit, Southern Cross Biotechnology, Cape Town, South Africa.

Method

1. Place gel slice in 1.5 ml microfuge tube.
2. Add 3 volumes of buffer QX1 to 1 vol of gel.
3. Resuspend the Qiaex II silica particles by vortexing for 30 sec.
4. Add 10 µl Qiaex II to tube.
5. Incubate at 50°C for 30 min with vortexing every 2 min to keep Qiaex in suspension (this solubilizes the agarose and binds the DNA).
6. Centrifuge samples for 1 min in microfuge (14000 x g) and remove supernatant.
7. Ish pellet with 500 µl of buffer QX1 (vortex to suspend pellet).
8. Centrifuge for 1 min in microfuge.
9. Ish pellet twice with 500 µl of buffer PE (vortex to suspend pellet and centrifuge for 1 min).
10. Remove supernatant after each ish.
11. Air dry pellet for ± 15 min until white.
12. Elute DNA from pellet with 20 µl of sterile H_2O. (after adding water, vortex and allow to stand at RT for 30 min).
13. Centrifuge for 1 min. Carefully remove supernatant which contains purified DNA.
14. Quantitate DNA on Gene Quant.

4.6 MANUAL SEQUENCING OF PCR PRODUCT

Equipment

1. Gel sequencing apparatus, Whitehead Scientific, Brackenfell, South Africa
2. LKB 2197 power supply, LKB/Pharmacia Biotech, Cambridge, UK
3. Gel drier SE1160, Hoefer/Pharmacia Biotech, Cambridge, UK.

Reagents

1. Sequenase PCR product sequencing kit (United States Biochemical, Cleveland, Ohio, USA).
2. Gel slick (FMC Bioproducts, Rockland, USA).
3. Medical X-ray film (Cronex 10 g 354 x 430 mM), Protea Electro-Medical Services, South Africa under licence from E.I. du Pont de Nemours Co. Inc.
4. Cronex Xtra life intensifying screens (Protea Electro-Medical Services, Cape Town, South Africa).
5. [α^{32}p]ATP 10 mCi/ml, Amersham, Buckinghamshire, UK.

Method

Casting of sequencing gel

1. Clean plates with alcohol followed by acetone.
2. Coat upper plate with 1 ml gel slick. Allow to dry for 5 min then wipe off with tissue.
3. Assemble plates.
4. Prepare gel solution:
   21 g urea, 5 ml 10x TBE, 10 ml (30% acrylamide/0.8% bis) in 50 ml. H_2O. Filter through 0.8 µm filter. Add 500 µl 10% ammonium persulphate and 50 µl TEMED.
5. Pour gel into space between plates and insert comb (ensure no air bubbles are present).
6. Allow to set and stand for at least 2 h prior to use.

Enzymic pre-treatment of PCR product

(Exonuclease 1 removes residual single-stranded primers and extraneous single-stranded DNA produced by the PCR and shrimp alkaline phosphatase the remaining dNTPs).

1. To 5 µl (± 2-5 pmol) of PCR amplification mixture in a 0.6 ml microfuge tube add 1 µl of exonuclease I (1 unit/µl). Mix and incubate for 15 min at 37°C.
2. Heat at 80°C for 15 min to inactivate the exonuclease. Spin on ice and microfuge briefly.
3. Add 1 µl Shrimp alkaline phosphatase. Mix and incubate for 15 min at 37°C.
4. Heat at 80°C for 15 min to inactivate phosphatase. Microfuge briefly and cool on ice.
5. To a 0.6 microcentrifuge tube add the following:

Treated PCR product	5 µl (2-5 pmol)
Primer (either forward or reverse)	1 µl (5 pmol)
Distilled H_2O	4 µl

6. Mix, cover with drop of oil and microfuge briefly.
7. Denature by heating at 99.9°C for 3 min in a thermocycler. Immediately place in an ice/water bath for 5 min. Store on ice.
8. Label and fill 4 tubes with 2.5 µl of each dideoxy termination mixture (G,A,T & C) for dGTP sequencing.
9. Dilute the labelling mixture 1:5 in distilled H_2O (i.e. 2 µl + 8 µl H_2O).
10. Pre-warm the 4 termination tubes at 37°C for 1 min.
11. Prepare the labelling reaction. To a 0.6 ml chilled microfuge tube add:

Chilled annealed DNA mixture	10 µl
Reaction buffer	2 µl
0.1 M DTT	1 µl
Diluted labelling mix (1/5)	2 µl
[αP32]dATP	0.5 µl
Sequenase DNA polymerase	2 µl

12. Vortex and incubate at room temperature for 5 min.
13. To each of the 4 termination tubes add 3.5 µl of the labelling reaction. Vortex and incubate at 37°C for 10 min in the thermocycler.
14. Add 4 µl of stop solution to each of the reaction tubes. Mix and place on ice.

15. Heat to 75°C for 2 min immediately before loading on to a assembled sequencing gel (in the order G, A, T and C). Load 3 µl of reaction mixture per lane.
16. Pre-run sequencing gel in 1xTBE for ± ½ h at 55°C. (One can run 1 or 2 loadings at different time intervals). Run at 60 watt (constant), 2000 volts for approximately 1.5 hs till dye front reaches the bottom of the gel.
17. Remove the gel from the apparatus and remove the top plate. Remove spacers and cover gel with blotting paper. Press blotting paper onto the gel and then lift. The gel should peel off on to the blotting paper.
18. Dry the gel immediately on a gel drier (cover surface of gel with plastic wrap).
19. Place the gel in the radiographic casette with intensifying screen. Place photographic film on top and expose at -70°C for 48 hs about 1 week prior to development.

4.7 GENOMIC DNA EXTRACTION (PARZER AND MANNHOLTER, 1991)

Equipment

1. Hitachi himac centrifuge SCR 20 BA, Hitachi Koki Co. Ltd, Japan.
2. Waterbath, Memmert, Laboratory & Scientific, Cape Town, South Africa
3. Gene Quant, Pharmacia Biotech, Cambridge, UK

Reagents

1. Cell lysis buffer:
   109.5 g sucrose
   5 ml 1M MgCl
   10 ml 1M Tris pH 7.6
   10 ml Triton X 100
   Make up to 1 litre with H_20. Store in fridge.
2. Freeze mix:
   17.8 g Trisodium citrate
   2.4 g NaH_2 PO4
   2.8 g Na2 HPO4
   1 g Na azide
   400 ml glycerol
   Make up to 1 litre with H_20. Store in fridge.
3. Ish buffer:
   10 mM NaCl, 10 mM EDTA pH 8.0. Store in fridge.
4. Sarcosyl solution:
   20% laurylsarcosinate. Store in aliquots in freezer.
5. Ammonium acetate solution:
   7.5 M. Store in aliquots in freezer.
6. Proteinase K solution:
   10 mg/ml. Store in aliquots in freezer.
7. DNA storage buffer (TE):
   1 M Tris/HCl
   100 mM EDTA, pH 8.0

8. Ice cold ethanol:
   (Store in fridge)

Method

1. Collect blood in 2x5 ml EDTA tubes. Spin at 1500 x g for 10 min. Remove buffy layer and add to 2 ml freeze mix solution. Mix well by inversion. Store in freezer.
2. Thaw sample in freeze mix at 37°C for 10 min.
3. Add sample to 50 ml cell lysis buffer and incubate on ice for 15 min.
4. Spin at 6800 g for 10 min. Discard supernatant.
5. Resuspend in 50 ml ish buffer.
6. Spin at 6300 g for 10 min. Remove supernatant and discard.
7. To pellet add:
   350 µl sarcosyl solution
   250 µl ammonium acetate
   3.5 ml guanidinium HC
8. Mix, then add 125 µl proteinase K and incubate at 60°C for 15 min.
9. Remove and cool on ice.
10. Add 10 ml ice cold ethanol. Invert and leave on ice for a minimum of 10 min.
11. Remove DNA with glass hooks made from a pasteur pipette and place in 1 ml Tris EDTA (TE) buffer.
12. Incubate at 37°C for 1 h.

To test purity:

Make a 1 in 20 dilution of DNA in H_20. Read OD at 260 and 280 nM. The OD ratio 260:280 should be approximately 1.8.

4.8 DNA PURIFICATION PROCEDURE

Equipment

1. Force 14 microfuge, Denver Instruments, Laboratory & Scientific, Cape Town, South Africa.

Method

1. Briefly vortex 100 µl extracted DNA.
2. Add 100 µl 1:49 v/v isoamyl alcohol : chloroform (chilled to -20°C).
3. Vortex briefly.
4. Spin in microfuge at 14 000 g for 10 min at 4°C.
5. Transfer top layer to new tube.
6. Add 250 µl 100% ethanol (chilled to -20°C) + 10 µl 3M Na acetate, pH 5.2.
7. Stand at -70°C for 30 min.

8. Spin in microfuge at 14 000 g for 10 min at 4°C.
9. Carefully decant supernatant without disturbing precipitate.
10. Ish precipitate with 70% ethanol (chilled to -20°C).
11. Spin in microfuge at 14 000 g for 10 min.
12. Invert on paper towel and allow precipitate to dry completely.
13. Reconstitute in 50 µl distilled H_2O.

4.9 MUTATIONAL ANALYSIS

Genomic DNA is amplified by the polymerase chain reaction (PCR), using exon specific primers. Primers were designed with the Primer Designer for Windows software package (Soft Packaging version 2, Scientific & Education Software), and the PPO genomic sequence retrieved from Genbank (accession No. X99450). The primers used for the amplification of the 13 PPO exons are as follows. All primers were obtained from Genosys Biotechnologies (Europe) Ltd, Pampisford, Cambridgeshire, UK.

Exon 1.	DF_1	5'CCGCCAATCCAGATGTAGG
	DR_1	5'AACTAAGTGTGCACGGATGG
Exon 2.	DF_2	5'TCTGCCTGTCCATATCGC
	DR_2	5'ATTAAATGAAGCTCCCTC
Exon 3.	DF_3	5'GAATATGCCTCTTCCCCTCCCC
	DR_3	5'CACAACCTCTCCTAGACATCCC
Exon 4.	SF_4	5'CCTCTTCTGAGGGCATGTGG
	SR_4	5'GAGGGCACAGTAAAAGGAGC
Exon 5.	SF_5	5'GAGGTATGTCAGGAGCTTCC
	SR_5	5'GATTTGAACAGGGAGCTCTG
Exon 6.	DF_6	5'TATCCCACCCTCATTCCCTACCA
	DR_6	5'ATTGAATAGCACCCCTTGTCC
Exon 7.	SF_7	5'TGTGAGCCACTGCATCCAG
	SR_7	5'CAGGTTCATTACACTCCAGG
Exon 8.	SF_8	5'CTCATCAAATTCTCAGGTTCTGG
	SR_8	5'TGTGGTCCTGCTGACCCAGG
Exon 9.	SF_9	5'CCTTCTGAGTCAGGCCTCTGC
	SR_9	5'GGATTACAGGTGTGAGCCACCA
Exon 10.	SF_{10}	5'AGAGCCCTTTCCTTCTGACGCATG *
	SR_{10}	5'TGGCCTTGCCTACAATGGAGCAC
Exon 11.	SF_{11}	5'GTGGCATTTCCAGAGGGCTCC
	SR_{11}	5'GGAGAGCTGAGGGAAGTTTATCC
Exon 12-13	SF_{12-13}	5'CTGGATCCTCTCCTCTCTTC
	SR_{12-13}	5'TAGAACAGCCAGACCAAGCC

* Note: SR_{10} must be heat denatured (1 min at 95°C, chill on ice 2 min) immediately before use as it has a stable secondary structure.

Dissolve each primer in 1 ml sterile distilled H_2O. Make 25 µM stocks of each primer. Freeze in small aliquots and store at -20°C.

4.10 POLYMERASE CHAIN REACTION OF GENOMIC DNA

Equipment

1. Sterile eppendorf tubes, sterile filtered tips and sterile water must be used for the PCR reaction.
2. Robocycler gradient 40 (temperature cycler), Stratagene, Cambridge, UK.
3. or Hybaid Omnigene thermal cycler, Teddington, UK.
4. Force 14 microfuge, Denver Instruments, Laboratory & Scientific, Cape Town, South Africa.

Reagents

1. Deoxynucleotide Triphosphates (dNTP's), Promega Corporation, Southampton, UK.
2. dNTP stock solution:
   Take equal volumes of dATP, dCTP, dGTP and dTTP (all 100 mM), dilute and combine to make a stock solution containing 2.5 mM of each dNTP.
3. Forward primer and reverse primer (both 25 µM).
4. Polymerase solution:
   a) Taq DNA polymerase (5 units/µl)
   b) Thermophilic DNA polymerase 10 x buffer, magnesium free.
   c) Magnesium chloride solution, 25 mM (Promega Corporation, Southampton, UK).
5. Sterile water
6. Mineral oil

Method

1. Prepare the following reaction mixture In a sterile 1.5 ml microfuge tube on ice, using sterile filter tips.

		Final concentration
10 x buffer	5 µl	1 x
Magnesium chloride solution (25 mM)	5 µl	1 x
dNTP mix of 4 dinucleotide triphosphates (2.5 mM each)	1 µl	50 µM (each)
Primer forward (25 µM)	1 µl	0.5 µM
Primer reverse (25 µM)	1 µl	0.5 µM
Taq DNA polymerase	0.2 µl	1 unit/50 µl
Sterile water	31.8 µl	

Scale these volumes up to make sufficient mixture to allow for 45 µl for each PCR required in addition to sufficient volume for a blank.

2. Aliquot 45 µl of the mixture into 0.6 ml microfuge tubes on ice. Add 5 µl of DNA (± 200 ng/µl). (5µl of H_20 is added to the blank). Briefly vortex tubes, centrifuge briefly to collect the sample at the bottom of the tube.
3. Overlay with drop of mineral oil. Place tubes in thermocycler and start PCR reaction, using the temperature profiles shown in the following table (Table A) for exons 1,2,3,6,7,8,9,10,11,12-13.

Table A:

	Temperature	Time	No. of cycles
Initial denaturation	95°C	1 min	1
Denaturation Annealing Extension	95°C 53-68°C (see Table B) 72°C	30 sec 30 sec 30 sec	35
Final extension	72°C	7	1

Table B:

Exon	Annealing temperature °C	BP size
1	57	365
2	65	167
3	65	252
4	65	180
5	65	247
6	65	317
7	55	272
8	55	197
9	68	202
10	65	223
11	65	257
12-13	53	333

Use the temperature profiles shown in the following table (Table C) for exons 4 and 5.

Table C:

	Temperature	Time	No. of cycles
Initial denaturation	**95°C**	**1 min**	**1**
Denaturation Annealing Extension	95°C 65°C 72°C	30 sec 45 sec 30 sec	10
Denaturation Annealing Extension	95°C 60°C 72°C	30 sec 45 sec 30 sec	30
Final extension	72°C	8 min	1

4. Analyse the PCR products for all 13 exons on a 6% acrylamide gel. It is essential that a pure PCR product is obtained (single band) for SSCP/heteroduplex analysis. Check that the PCR product is the correct size by comparison with a DNA marker.

4.11 SINGLE STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) AND HETERODUPLEX (HD) ANALYSIS

Equipment

1. SE600 vertical slab gel electrophoresis unit, PS1500 DC power supply, Hoeffer Scientific Instruments/Pharmacia Biotech, Cambridge, UK.
2. Techni dri block BD-2D, Laboratory & Scientific, Cape Town, South Africa.
3. Hybaid Omnigene thermal cycler, Teddington, UK.

Reagents

1. MDE gel solution, FMC BioProducts, Rockland Maine, USA.
2. Sample loading buffer:
   95% formamide
   10 mM NaOH
   20 mM EDTA pH 8.0
   0.02% bromophenol blue
   0.02% xylenecyanol

Method

Casting of MDE gel

3. Clamp 2 glass gel plates (using 1 mm spacers) and mount in the gel casting stand.
4. Prepare MDE solution as below:
   15 ml 2x MDE
   1.8 ml 10x TBE (final concentration=0.6x TBE)
   3 ml glycerol (final concentration=10%; for gels without glycerol replace the 3ml glycerol with H_2O)
   Add distilled water to a final volume of 30 ml.
5. Mix well.

6. Cast approximately 1 cm of fast-setting gel. Take 5 ml of the MDE solution and add 20 µl 10% ammonium persulphate and 20 µl TEMED. Mix and pour between 2 clamped plates, using 1 mm spacers.
7. Allow to set for approximately 10 min.
8. To the remainder of the MDE solution, add 175 µl 10% ammonium persulphate and 17.5 µl TEMED.
9. Pour on top of the gel apparatus plug until the space between glass plates is almost filled.
10. Insert a 1 mm comb and allow to set for 1 h.
11. Once set, remove the comb and mount the upper tank chamber, fill with 500 ml 0.6x TBE.
12. Fill lower tank chamber with 4.5 l of 0.6x TBE.
13. Prepare samples for loading as below:
    a) 5 µl PCR product + 5 µl sample loading buffer.
    b) Denature for 5 min at 95°C in thermocycler.
    c) Chill on ice for 5 min.
14. Load samples immediately.
15. Run at room temperature between 150 and 400 V depending on the size of the PCR product. It is vital that good separation is achieved, otherwise aberrant mobility shifts may be missed.
16. In addition, run gels in the absence of glycerol (10%). Halve the voltage in the absence of glycerol.
17. Stain gels as follows.

Silver staining

1. Remove MDE gel from between the 2 gel plates and rinse in tray in distilled H_2O.
2. Rinse with 0.1% $AgNO_3$ and then gently agitate in 0.1% $AgNO_3$ for 20 min.
3. Rinse gel well in distilled H_2O.
4. Agitate in the following solution in a fume cupboard for for 20 min:
   30 g NaOH
   0.2 g Na borohydride
   20 ml 15% formaldehyde.
5. Rinse in enhancer (7.5 g $NaCO_3$ /l distilled H_2O).
6. Rinse in distilled H_2O and visualize on light box. Seal in clear plastic. (These gels cannot be dried as they crack.)

4.12 DIRECT SEQUENCING OF PCR PRODUCT

Run PCR product on 1.5% agarose gel as described below.

Equipment

1. Horizontal mini-gel systems (Mgu-202T), (CBS Scientific Company Inc., Del Mar, USA).
2. HSI PS1500 DC power supply (Hoefer/Pharmacia Biotech, Cambridge, UK).

Reagents

1. MS-8 Agarose, Whitehead Scientific, Brackenfell, South Africa.

Method

1. Insert gel tray between tapered baffles.
2. Microwave agarose (0.75 g / 50 ml TBE) for approximately 1 min. Mix well. Add 100 µl of ethidium bromide (1 mg/ml). Mix and pour into gel tray and insert comb.
3. Allow to set for 1 h.
4. Remove comb and tapered baffles.
5. Add 250 ml 1x TBE to fill both reservoirs of gel tank and to cover surface of gel by ± 3 mm.
6. Load PCR product diluted 1:0.5 (product/sample solution v/v).
7. Place safety cover on unit.
8. Run at 100 V for ± 1.5 hs.
9. Rinse gel in distilled H_2O and visualize under UV light. Excise the DNA band from the gel with a sterile scalpel blade, taking care to minimise time of exposure of DNA to UV light).
10. Extract DNA from agarose using a Qiaex II gel extraction kit as described in Appendix 4.5.

For sequencing

Use 5 µl of primer solution (1.1 pmol/µl; 30 ng/µl).

Direct sequencing is performed with a Big Dye terminator cycle sequencing kit on an ABI prism 377 DNA sequencer, by the Core DNA sequencing facility of the University of Stellenbosch.

4.13 RESTRICTION ANALYSES

Equipment

1. Techni dry block BD-2D, Laboratory & Scientific, Cape Town, South Africa.
2. Force 14 microfuge, Denver Instruments, Laboratory & Scientific, Cape Town, South Africa.

Further equipment as for the 6% non-denaturing gel electrophoresis (Appendix 4.4).

Method

All volumes quoted below are for a single digestion reaction. These volumes should be scaled up to allow for the number of digests required. Include a positive and negative control in each run. Analyse pre & post-digestion products on 6% polyacrylamide gels, or in the case of MvaI 1% agarose.

Ava I

(Promega Corporation, Madison, USA). This is used for identification of R59W in exon 3 and recognises the following sequence:

5' C' ↓(T/C) CG(A/G)G3'
3' G (A/G) G C (T/C) ↑C5'

Wild type: 252 bp product, 1 cutting site: digestion products of 168 bp and 84bp.
Mutant: the cutting site is abolished.

1. Prepare the digest as follows:

Buffer B 10x	2.0 µl
Bovine serum albumin (BSA) 10 mg/ml	0.20 µl
Distilled H_2O	7.3 µl
Ava I (10 units/µl)	0.5 µl
Total	10 µl

2. Mix and microfuge briefly. In a 0.6 ml microfuge tube add 10 µl of the above mixture and 10 µl PCR product (1 – 1.5 µg) . Mix. Layer mineral oil on top of mixture and microfuge briefly. Incubate at 37°C for 4 h.

EaeI

(Roche Diagnostics Pty Ltd, Randburg, South Africa). This is used for the identification of the L15F mutation in exon 2 and recognises the following sequence:

5' Py↓GGCCPu 3' Py (C/T)
3' Pu CCGG↑Py 5' Pu (A/G)

Wild type: 167 bp, 1 cutting site: digestion products of 85 bp and 82 bp.
Mutant: the cutting site is abolished.

1. Prepare the digest as follows:

Buffer A (10x)	2 µl
Distilled H_2O	14.5 µl
EAE 10 units/µl	0.5 µl
Total	17 µl

2. To the above, add 3 µl PCR product (300 ng – 450 ng). Layer with oil. Mix. Microfuge briefly. Incubate at 37°C for 5 h.

Hpa II

(Promega Corporation, Madison, USA). This is used for the identification of the c769delG; $770^{T\rightarrow A}$ mutation in exon 7 and recognises the following sequence:

5' C↓CGG 3'
3' GGC↑C 5'

Wild type: 272 bp, 1 cutting site: digestion products of 200 bp and 72 bp.
Mutant: the cutting site is abolished.

1. Prepare the digest as follows:

Buffer A (10x)2 µl
BSA (10 mg/ml) 0.2 µl
Distilled H_2O 7.55 µl
Hpa II (10 units/µl) 0.25 µl
Total 10 µl

2. To the above add 10 µl PCR product (1–1.5 µg). Layer with oil, mix and microfuge briefly. Incubate at 37°C for 3 hs.

Mae III

(Roche Diagnostics Pty Ltd, Randburg, South Africa). This is used for the identification of the Y348C mutation in exon 10 and the Q375X mutation in exon 11 and recognises the following sequence:

5' ↓GTNAC 3' [N = Pu or Py]
3' CANTG 5'

Y348C

Wild type: 223 bp, 1 cutting site: digestion products of 146 bp and 77 bp.
Mutant: an additional cutting site is created: digestion produc ts of 96 bp, 50 bp and 77 bp.

Q375X

Wild type: 257 bp, 1 cutting site: digestion products of 161 bp and 96 bp.
Mutant: the cutting site is abolished.

1. Prepare digest as described below.

Incubation buffer (2x)	10 µl
Distilled H_2O	2.25 µl
Mae III (2 units/µl)	0.25 µl
Total	12.5 µl

2. To the above add 7.5 µl PCR product (750 ng – 1125 ng).

Mix, layer mineral oil on top of mixture. Microfuge briefly. Incubate at 55°C for 4 hs.

BsaJI

(New England Biolabs, Beverly, MA, USA). This is used for the identification of the R168C mutation in exon 6 and recognises the following sequence:

5' C↓CNNGG 3'
3' GGNNC↑C 5'

Wild type: 317 bp, 2 cutting sites: digestion products of 195 bp, 106 bp and 16 bp.
Mutant: 1 cutting site is abolished: products of 106 bp and 211 bp.

1. Prepare digest as described below.

NE buffer (10x)	2 µl
Distilled H_2O	7 µl
BsaJI 2.5 units/µl	1 µl
Total	10 µl

2. To the above 10µl, add 10 µl PCR product (1 – 1.5 µg). Incubate at 60°C for 3 hs.

Mva I

(Roche Diagnostics Pty Ltd, Randburg, South Africa). This is used for the identification of the 537del AT mutation in exon 6 and recognises the following sequence:

5' CC↓(A/T)GG 3'

3' GG(T/A)↑CC 5'

Wild type: 317 bp, no cutting site. Undigested product remains.
Mutant: a cutting site is created: products of 158 and 157 bp.

1. Prepare digest as described below.

Buffer H (10x)	2.0 µl
Distilled H_2O	7.5 µl
Mva I 10 units/µl	0.5 µl
Total	10 µl

2. To the above add 10 µl PCR product (1 – 1.5 µg). Mix. Overlay with oil. Microfuge briefly. Incubate at 37°C for 3 h.
3. These digestion products are best visualised on 1% agarose. On 6% polyacrylamide, electrophoresis produces aberrant bands running above the predigested product, apparently as a result of the deletion.

BsiYI

(Roche Diagnostics Pty Ltd, Randburg, South Africa). This is used for the identification of the V290M mutation in exon 8 and recognises the following sequence:

5' CCNNNNN↓NNGG 3'

3' GGNN↑NNNNNCC 5'

Wild type: 197 bp, 1 cutting site: digestion products of 107 bp and 90 bp.
Mutant: the cutting site is abolished.

1. Prepare digest as below.

Buffer M	2 µl
Distilled H_2O	7.75 µl
$BsiY_1$ (10 units/µl)	0.25 µl
Total	10 µl

2. To 5 µl of the above, add 10 µl PCR product (1 – 1.5 µg). Mix. Cover with mineral oil. Briefly microfuge then incubate for 3 hs at 55°C.

APPENDIX 5:

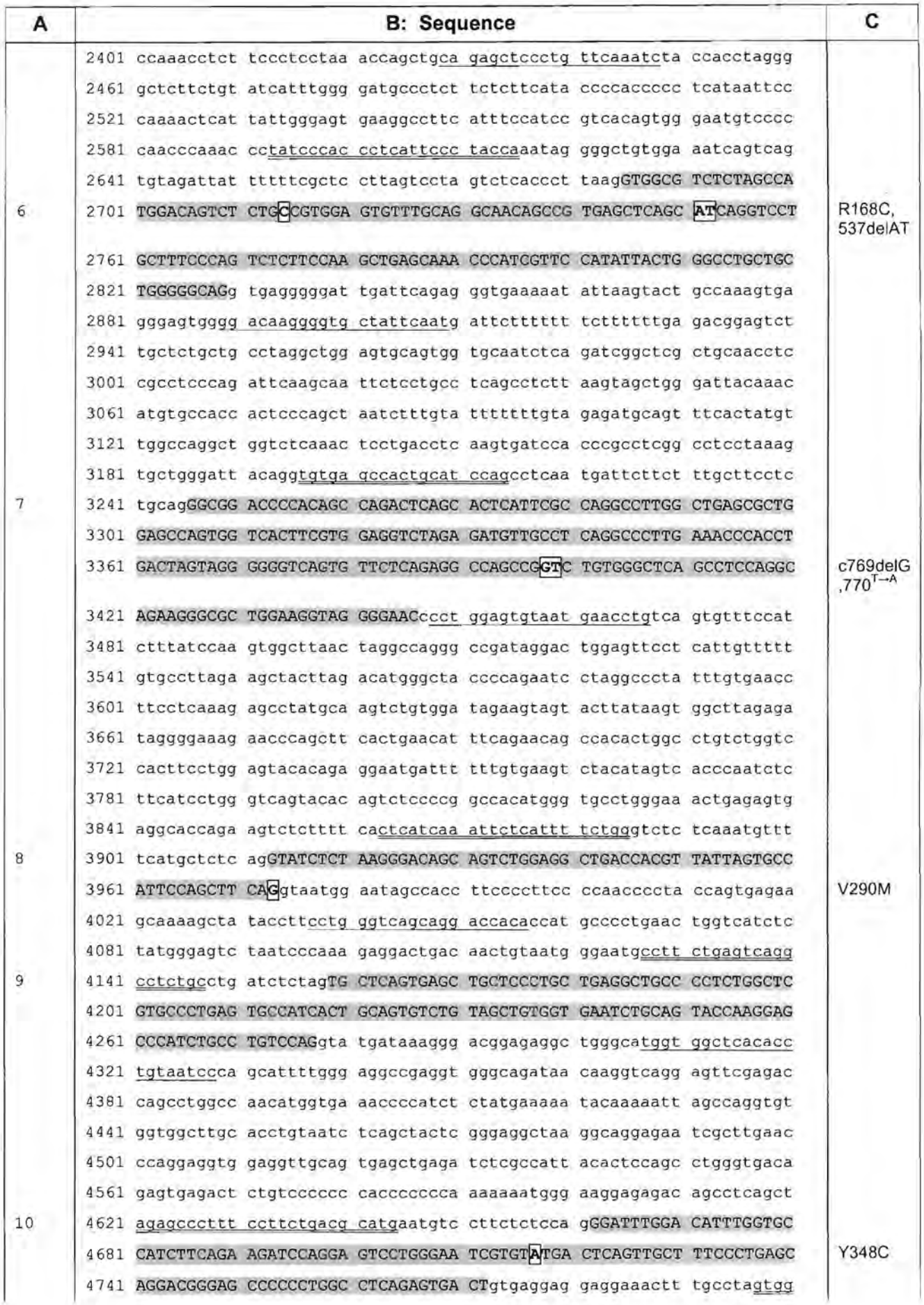
COMPLETE GENOMIC SEQUENCE FOR HUMAN PPO, PRIMERS FOR DNA ANALYSIS AND SITE OF MUTATIONS

The table on the following page contains the genomic sequence for human PPO (Puy H, Robreau AM, Rosipal R, Nordmann Y, Deybach J-C). The comments are drawn from Puy et al (Genbank accession No X 99450), Puy et al (1996), Taketani et al (1995), Nishimura et al (1995),:

- Intronic sequences are denoted by lower case letters.
- Exonic sequences are denoted by upper case letters and are shaded, and the exon number appears in Column A.
- Sequences recognised by the forward (sense) primers used in these studies are denoted by a double underline and precede the relevant exon.
- Sequences complementary to the reverse (antisense) primers are denoted by a single underline following the relevant exon.
- Mutated or deleted bases are printed in bold type and are outlined by a box: the relevant name appears in Column C.
- Areas of interest are printed in bold italic type, and are idenitified by a number preceded by # in Column C, as follows:
  - #1 GGGCCGG signal, which interacts with the transcriptional factor Sp1 (Kadonaga et al 1986).
  - #2 Sequence inverted to TGATAG which corresponds to the binding site of erythroid cell-specific transcriptional factor GATA-1.
  - #3 Sequence inverted to CCAAT (overlapping #3).
  - #4 Sequence inverted to GGGCCGG signal .
  - #5 CCAAT box (overlapping #4).
  - #6 Transcription initiation site.
  - #7 TGA stop codon.
  - #8 Polyadenylation (Poly A) signal.
  - #9 Polyadenylation tail.

  Areas #1 to #5 are all potential regulatory elements within the promoter region.
  Note the conserved consensus dinucleotides *GT* and *AG* respectively at the 5' and 3' boundaries of each intron.

A	B: Sequence	C
	1 taagaggtga tagagaactg gcccaaaatt ggagtcttct tgggacccaa gttccctctc	
	61 taacaggtgg gggcgctctc agagccgagg gggaaggttt cggggatggt gctggacccg	
	121 gacctgtgtc ccccagcagt gaaggttatg tactgggagg aaaagaggaa aggcatactt	
	181 gagtgaggct gcgtgcaggc tctggggcga ctggtttggt cggcgatggg ggagtgccac	
	241 tgttgacaaa cactgggtac gttgc***cgggc ggcc***ctagct cccgtaggcc cggtctgttg	#1
	301 ctaacatcaa ggagctgttt atggagccgc ctccccgtgg actggcctta agtgtc***ccta***	#2
	361 ***tct***attctcc ctacgaccaa agctcctgcg gccttccctc tagggttgtc accctagctg	
	421 gacctggtaa gcttgtgctg gggaacgcta actccagcat taagcgctcg ccgcctcggt	
	481 cctgaggagg gcagtgacgg gtacggccgc tcactcccag ctcggcgcta acacggttaa	
	541 cctccagctc ttacagctgc cgtcgctccg cctctgccag ttcaatgttt t***attgg***tgaa	#3
	601 cgtgatccgg ggccttccaa g***tcccgccaa t***ccagatgta ggagaggtag ggttaggcgc	#4, #5
1	661 GTGCCGCGAG AACAGAGTGG ACGGAGAGTA GGAGAGACCG AAAAGGCTGG GGGTGGGAGT	
	721 AGCGGATTTG AAGCACTTGT TGGCCTACAG AGGTGTGGCA AGCAGAGCAC CTCAGAACTC	
	781 AGGCGTACTG CCCGCCGCCC GAGCCCTGCG AGGGCCGATA GCGAGGGTGT GGCCCTTATC	
	841 TGCACCCAGC AGAGCGCCGG CGGGGTACGG TCTTAGGACC TCGATCTCCT TCTCCCTCAT	
	901 TTTCTCTCAT CCCTACCTAT TGTGGgtgag tcctggcccc tggacgggga cctcgctgtt	
	961 ccaccagccc atccgtgcac acttagtttc ccctaaagca gtgagtggcc gggatagaac	
	1021 tcaaaaccgg cggggcttct ggagcgcagg ttgtccccgg tctgcctgtc catatcgccc	
2	1081 cctttccccc agGTTTCCGC ***ATG***GGCCGGA CCGTGGTCGT GCTGGGCGGA GGCATCAGCG	#6, L15F
	1141 GCTTGGCCGC CAGTTACCAC CTGAGCCGGG CCCCCTGCCC CCCTAAGgtg agtgctccac	H20P
	1201 ttgtgccaga gggagcttca tttaatgctc ttcccatttc catcaaaagc tagatggatc	
	1261 ctggccctct gaatatgcct cttacccctc cctactgacc tctcggcggc ggctacaggc	
3	1321 ggtgcgtgca gtgtctctcc ctcttgtcgc cagGTGGTCC TAGTGGAGAG CAGTGAGCGT	
	1381 CTGGGAGGCT GGATTCGCTC CGTTCGAGGC CCTAATGGTG CTATCTTTGA GCTTGGACCT	
	1441 CGGGGAATTA GGCCAGCGGG AGCCCTAGGG GCCCGGACCT TGCTCCTGgt gagaggcttg	R59W
	1501 tgggatgtct aggagaggtt gtggaggggg cttccattgg ggaatagagt ttaggggagg	
	1561 aagtatgttt ggtgggtcag atcttccctt agtttctcct cttctgaggg catgtggaga	
4	1621 gcagGTTTCT GAGCTTGGCT TGGATTCAGA AGTGCTGCCT GTCCGGGGAG ACCACCCAGC	
	1681 TGCCCAGAAC AGGTTCCTCT ACGTGGGCGG TGCCCTGCAT GCCCTACCCA CTGGCCTCAG	
	1741 gtaacaccag cacctccgct ccttttactg tgccctcatc ctcatatgcc ttccatttct	
	1801 ttcttctttt cttttttttt ttttttttt ttttttgag acggagtatc gctctcgccc	
	1861 aggatggagt gcaatggcgc gatcttggct cactgcaact tccgcctttc gggttcaagc	
	1921 gattctcctg cctcagcctc ccgagtagct gggactacag gtgcctacca ccacgtctgg	
	1981 ctagtttttg tatttttagt agagatgggg tttcaccata ttggccaggc tggtcttgaa	
	2041 ctcctgacct tgtgatccgc ccgcctcggc ctgccaaagt gctgggatta caggcgtgag	
	2101 ccaccacgcc cgacctgcct tccatttctt catctccctg tcagccttcc cagcaaaagg	
	2161 aagccaaatg agtggaaatg accccagcgc ctggagctgg ggaggtatgt caggagcttc	
5	2221 ccctcacta tgcctttctc catgcagGGG GCTACTCCGC CCTTCACCCC CCTTCTCCAA	
	2281 ACCTCTGTTT TGGGCTGGGC TGAGGGAGCT GACCAAGCCC CGGGGCAAAG AGCCTGATGA	R138P
	2341 GACTGTGCAC AGTTTTGCCC AGCGCCGCCT TGGACCTGAG gtgacacttg cccagaggcc	



A	B: Sequence	C
	2401 ccaaacctct tccctcctaa accagctgca gagctccctg ttcaaatcta ccacctaggg	
	2461 gctcttctgt atcatttggg gatgccctct tctcttcata ccccaccccc tcataattcc	
	2521 caaaactcat tattgggagt gaaggccttc atttccatcc gtcacagtgg gaatgtcccc	
	2581 caacccaaac cctatcccac cctcattccc taccaaatag gggctgtgga aatcagtcag	
	2641 tgtagattat tttttcgctc cttagtccta gtctcaccct taagGTGGCG TCTCTAGCCA	
6	2701 TGGACAGTCT CTGCCGTGGA GTGTTTGCAG GCAACAGCCG TGAGCTCAGC ATCAGGTCCT	R168C, 537delAT
	2761 GCTTTCCCAG TCTCTTCCAA GCTGAGCAAA CCCATCGTTC CATATTACTG GGCCTGCTGC	
	2821 TGGGGGCAGg tgaggggat tgattcagag ggtgaaaaat attaagtact gccaaagtga	
	2881 gggagtgggg acaaggggtg ctattcaatg attctttttt tctttttga gacggagtct	
	2941 tgctctgctg cctaggctgg agtgcagtgg tgcaatctca gatcggctcg ctgcaacctc	
	3001 cgcctcccag attcaagcaa ttctcctgcc tcagcctctt aagtagctgg gattacaaac	
	3061 atgtgccacc actcccagct aatctttgta ttttttgta gagatgcagt ttcactatgt	
	3121 tggccaggct ggtctcaaac tcctgacctc aagtgatcca cccgcctcgg cctcctaaag	
	3181 tgctgggatt acaggtgtga gccactgcat ccagcctcaa tgattcttct ttgcttcctc	
7	3241 tgcagGGCGG ACCCCACAGC CAGACTCAGC ACTCATTCGC CAGGCCTTGG CTGAGCGCTG	
	3301 GAGCCAGTGG TCACTTCGTG GAGGTCTAGA GATGTTGCCT CAGGCCCTTG AAACCCACCT	
	3361 GACTAGTAGG GGGGTCAGTG TTCTCAGAGG CCAGCCGGTC TGTGGGCTCA GCCTCCAGGC	c769delG ,770T→A
	3421 AGAAGGGCGC TGGAAGGTAG GGGAACccct ggagtgtaat gaacctgtca gtgtttccat	
	3481 ctttatccaa gtggcttaac taggccaggg ccgataggac tggagttcct cattgttttt	
	3541 gtgccttaga agctacttag acatgggcta ccccagaatc ctaggcccta tttgtgaacc	
	3601 ttcctcaaag agcctatgca agtctgtgga tagaagtagt acttataagt ggcttagaga	
	3661 taggggaaag aacccagctt cactgaacat ttcagaacag ccacactggc ctgtctggtc	
	3721 cacttcctgg agtacacaga ggaatgattt tttgtgaagt ctacatagtc acccaatctc	
	3781 ttcatcctgg gtcagtacac agtctccccg gccacatggg tgcctgggaa actgagagtg	
	3841 aggcaccaga agtctctttt cactcatcaa attctcattt tctgggtctc tcaaatgttt	
8	3901 tcatgctctc agGTATCTCT AAGGGACAGC AGTCTGGAGG CTGACCACGT TATTAGTGCC	
	3961 ATTCCAGCTT CAGgtaatgg aatagccacc ttccccttcc ccaaccccta ccagtgagaa	V290M
	4021 gcaaaagcta taccttcctg ggtcagcagg accacaccat gcccctgaac tggtcatctc	
	4081 tatgggagtc taatcccaaa gaggactgac aactgtaatg ggaatgcctt ctgagtcagg	
9	4141 cctctgcctg atctctagTG CTCAGTGAGC TGCTCCCTGC TGAGGCTGCC CCTCTGGCTC	
	4201 GTGCCCTGAG TGCCATCACT GCAGTGTCTG TAGCTGTGGT GAATCTGCAG TACCAAGGAG	
	4261 CCCATCTGCC TGTCCAGgta tgataaaggg acggagaggc tgggcatggt ggctcacacc	
	4321 tgtaatccca gcattttggg aggccgaggt gggcagataa caaggtcagg agttcgagac	
	4381 cagcctggcc aacatggtga aaccccatct ctatgaaaaa tacaaaaatt agccaggtgt	
	4441 ggtggcttgc acctgtaatc tcagctactc gggaggctaa ggcaggagaa tcgcttgaac	
	4501 ccaggaggtg gaggttgcag tgagctgaga tctcgccatt acactccagc ctgggtgaca	
	4561 gagtgagact ctgtcccccc cacccccca aaaaaatggg aaggagagac agcctcagct	
10	4621 agagcccttt ccttctgacg catgaatgtc cttctctcca gGGATTTGGA CATTTGGTGC	
	4681 CATCTTCAGA AGATCCAGGA GTCCTGGGAA TCGTGTATGA CTCAGTTGCT TTCCCTGAGC	Y348C
	4741 AGGACGGGAG CCCCCCTGGC CTCAGAGTGA CTgtgaggag gaggaaactt tgcctagtgg	

A	B: Sequence	C
	4801 catttccaga ggctcctct gtgctccatt gtaggcaagg ccagactgat cagtgctata	
11	4861 ttccctcctt agGTGATGCT GGGAGGTTCC TGGTTACAGA CACTGGAGGC TAGTGGCTGT	Q375X
	4921 GTCTTATCTC AGGAGCTGTT TCAACAGCGG GCCCAGGAAG CAGCTGCTAC ACAATTAGGA	
	4981 CTGAAGGAGA TGCCGAGCCA CTGCTTGGTC CATCTACACA AGgtaagttg ggataaactt	
	5041 ccctcagctc tccactgaag gccttgaaga cagagactgg aacatttgtc actgtatgtc	
	5101 agccaaggcc taggacatca ataataaact tttcctgca tcctctcctc tcttctcagA	
12	5161 ACTGCATTCC CCAGTATACA CTAGGTCACT GGCAAAAACT AGgtaagttg ggaaaacagc	
	5221 tgggctgagg agggccaagg acatcagacc cccagctaaa acattccttt catcctttcc	
13	5281 ttccagAGTC AGCTAGGCAA TTCCTGACTG CTCACAGGTT GCCCCTGACT CTGGCTGGAG	
	5341 CCTCCTATGA GGGAGTTGCT GTTAATGACT GTATAGAGAG TGGGCGCCAG GCAGCAGTCA	
	5401 GTGTCCTGGG CACAGAACCT AACAGC***TGA***T CCCCAACTCT CATTCATGAA ***AATAAA***AATT	#7, #8
	5461 GCTTGGCTTG GCTTGgtctg gctgttct***aa aaaaaaaaaa aaaaaa***	#9

APPENDIX 6:

GUIDELINES RECOMMENDED BY THE UCT PORPHYRIA DIAGNOSTIC LABORATORY TO SOUTH AFRICAN DOCTORS AND LABORATORIES FOR THE DIAGNOSIS OF VP

A RATIONAL APPROACH TO THE DIAGNOSIS OF PORPHYRIA IN SOUTH AFRICA

RJ HIFT, D MEISSNER, PN MEISSNER

1 INTRODUCTION

Since the publication in May 1996, of the R59W mutation prevalent in South Africans with variegate porphyria, there have been numerous enquiries about the relevance of this to the diagnosis of VP. The DNA based test which is now available can identify beyond doubt at any age, from newborn, the specific gene defect for VP in the majority of South African VP subjects.

However, the advent of this DNA test has served to highlight the immediate need to reassess our diagnostic approach to the porphyrias and to make it as rational as possible. It is essential to realise that DNA based testing will be no more than an adjunct, albeit an important one, to conventional biochemical testing and can replace it **only** in appropriate circumstances.

2 INDICATIONS FOR R59W TESTING

The R59W test is *only appropriate as the single test under two circumstances*:

1. For detection of VP carriage in a family known to carry the R59W mutation
2. If result is positive in a patient with characteristic skin disease.

In all other circumstances, R59W DNA testing must either be preceded, or followed, by *accurate biochemical quantitation* of porphyrins. Any laboratory which merely provides a negative R59W result for VP when asked to exclude porphyria and does not proceed to the porphyrin quantitation essential for excluding **all** types of porphyria, is opening itself to legal action.

3 ACCEPTABLE BIOCHEMICAL METHODS FOR PORPHYRIA DIAGNOSIS

The only acceptable method of porphyrin diagnosis is by porphyrin quantitation using **high-performance liquid chromatograpy** or **thin-layer chromatography coupled with fluorometric detection and quantitation**. Qualitative methods and solvent extraction **are not acceptable** except, perhaps, where results are strongly and unequivocally positive. Negative, doubtful and low-level positive results are unacceptable and must be followed by chromatographic confirmation. No patient must under any circumstances be labelled as porphyric or not porphyric on the basis of less accurate tests.

Only if this is accepted will we correct the present shambles where large numbers of people have been incorrectly labelled as having VP on the basis of inadequate testing.

4 IMPORTANCE OF FAMILY STUDIES

R59W testing is most appropriate when used for the detection of latent R59W-positive VP in family studies. A window of opportunity has now opened for the effective management of porphyria in South Africa and it is now essential that testing is consistently applied.

5 CHOICE OF DIAGNOSTIC INVESTIGATION

The decision as to which test should be employed first depends on the following questions:

1. Is the patient symptomatic?

2. Is there a *proven* family history of porphyria?

5.1. If the patient is being investigated for symptoms suggestive of porphyria; i.e. skin disease, abdominal pain or neuropathy.

It is axiomatic that patients who are symptomatic from porphyria *always have disturbed porphyrin biochemistry.* Under these circumstances, biochemical testing is the appropriate first investigation as it will:

1. **Confirm porphyria**
2. **Indicate the type of porphyria present (VP, AIP, HCP, PCT or the rarer erythropoietic porphyrias)**
3. **Indicate the degree of activity of the porphyria (quiescent, active, acute phase etc)**

The R59W test *does no more than indicate* the presence or absence of a specific mutation. In particular

1. A positive result **does not prove** that a patient's symptoms are caused by porphyria
2. A negative result **does not exclude** a diagnosis of porphyria, whether VP or otherwise.

However, it may under some circumstances be appropriate to perform the R59W test as a screening test, provided that it is **always** supplemented by biochemical testing where appropriate, as suggested below. Additionally, where a review of the patient's symptoms suggests that they are not compatible with porphyria, then it may not be necessary to test further, or at all.

5.1.1 Where a patient is investigated for cutaneous symptoms

Alternative A:

Perform urine, stool and plasma porphyrin estimation. This will reveal the characteristic changes of VP, HCP, PCT, EPP or CEP.

Negative biochemistry:	Porphyria is excluded as a cause of the skin disease.
Positive biochemistry:	A proper diagnosis can be made and the patient treated or counseled further.

Alternative B:

Perform the R59W DNA-based assay.

Negative R59W:	Porphyria is **NOT** excluded. Perform urine stool and plasma biochemistry
Positive R59W:	VP is confirmed. It is not necessary to check biochemistry if skin disease is typical.

5.1.2 Where a patient is investigated for "acute" symptoms such as abdominal pain or neuropathy

Biochemical testing is **mandatory**, as even a positive R59W result does not prove that a patient's symptoms are due to porphyria, and a negative result **does not exclude** the acute attack of AIP, HCP or non-R59W VP.

5.1.2.1 For a suspected acute attack

Perform an **urgent Watson-Schwartz reaction** with Ehrlich's aldehyde, and follow up with quantitation of ALA and PBG. Thereafter confirm the type of porphyria present by either biochemical porphyrin quantitation, or by a R59W test followed by porphyrin quantitation if R59W negative.

5.1.2.2 For a history of recurrent abdominal pain, neuropathy etc

Perform urine, stool and plasma porphyrin estimation. This will reveal the characteristic changes of VP, AIP or HCP.

Negative biochemistry:	Porphyria is excluded as a cause of the symptoms
Positive biochemistry:	A proper diagnosis can be made and the patient treated or counseled further.

5.2. Where patients are asymptomatic, but are investigated because of a postive family history of porphyria.

Approximately 50% of blood relatives of a proven index case of variegate porphyria should be gene carriers. Many of these will however be latent. Some will be detectable by biochemical porphyrin estimation where others will be entirely silent. Here DNA-based testing is entirely appropriate as a first line of investigation. Results are interpreted as follows:

5.2.1 If an index case within that family is known with 100% confidence to carry the R59W mutation

Perform the R59W test.

Negative R59W	The patient is not a carrier, the disease is absent and their children and subsequent descendants will not need testing.
Positive R59W	Carriage of the VP gene is confirmed. The patient should be counselled and managed as appropriate thereafter. Supplemental biochemical testing is not needed except to assess symptoms or for research purposes.

5.2.2 Where VP in the family is proven or suspected, but the nature of the mutation is not satisfactorally established.

Perform the R59W DNA-based assay.

Negative R59W:	Porphyria is **NOT** excluded. Follow up with urine, stool and plasma biochemistry, including ALA and PBG, and **preferably test the original proband** to confirm porphyria. Patients with confirmed VP but who are R59W negative should be referred to us for mutational analysis.
Positive R59W:	VP is confirmed. The family should be labelled as a "R59W positive" family.

5.2.3 Where the family is known to have a porphyria other than R59W-positive VP

R59W testing is unnecessary. Perform urine, stool and plasma porphyrin estimation. This will reveal the characteristic changes of VP, AIP, HCP, PCT or the rarer erythropoietic porphyrias, but may fail to detect true latent porphyria. More sensitive testing may be possible in some families where a mutation other than R59W has been identified, or by enzyme assays.

6 SPECIMENS

For DNA testing: 10ml EDTA blood. This must NOT be frozen. It should reach our laboratory within 48 hours from Monday to Thursdays and not on a weekend.

For biochemical testing: Stool 10g, Urine 30 ml, Blood (heparinised) 10 ml. All in sealed, light protected containers.

APPENDIX 7:

RESULT SHEET ISSUED BY THE UCT DIAGNOSTIC PORPHYRIA LABORATORY

An example of the result form issued by the UCT Porphyria Diagnostic Laboratory appears on the following page. This is generated automatically by the database software directly from the thin-layer chromatographic readings and was programmed by the author personally. As shown, the database also holds the results of plasma fluoroscanning and DNA analysis.

PORPHYRIA LABORATORY

MRC/UCT LIVER RESEARCH CENTRE

NO CHARGE

Lab Number 97/397 DE V

Patient: DE VILLIERS, JP JACOBA
Sex: F
Age: 73
Born: 20/07/1923

Medical Aid
Hospital: DSP
Number: 677021886
Ward:

Address FITZROYSTR 25
GOODWOOD

Doctor:

Tel 5912539

Tel Bleep

Specimen Date 26/05/1997
Clinical Data: family project

Send Report To DOREEN MEISSNER

---------- PORPHYRIN PLASMA SCAN ----------

Plasma scan **Peak present: 625nm = High Positive**

---------- PORPHYRIN BIOCHEMISTRY ----------

	URINE	STOOL	PLASMA	RBC
Porphobilinogen	**NEG**			
Urobilinogen	**N**			
Screen	**NEG**	**++**		**NEG**
Bacteriochlorophyll		**Y**		
Creatinine	5.3 mmol/l			
ALA	11.1 (<45) umol/10 mmol creat			
PBG	3.0 (<16) umol/10 mmol creat			
Uroporphyrin	0.0 (<20)	0.0 (<1.7)		
7-COOH Porphyrin	0.0 (<1.5)	0.0		
6-COOH Porphyrin	0.0	0.0		
Pseudo 5-COOH Porphyrin		0.0		
5-COOH Porphyrin	0.0	0.0		
Isocoproporphyrin/PU		0.0		
Coproporphyrin	155.0 (<240)	75.9 (<50)		
3-COOH Porphyrin		0.0		
Protoporphyrin		387.3 (<200)		
Units	nmol/ 10 mmol creat	nmol/ g dry mass		

Porphyrin diagnosis: 11 **Variegate porphyria**

---------- DNA ASSAY ----------

VP DNA Study: **R59W POSITIVE**

Genetic Diagnosis: 3 **R59W positive: biochemically expressed variegate porphyria**

---------- COMMENTS ----------

Lab Comments: **Urine porphyrins may have been obscured by pigments.**

Interpretation: **This confirms the presence of the R59W gene mutation, the common defect reponsible for Variegate Porphyria in South Africa.**

APPENDIX 8:

SPECIMEN VALUES USED FOR THE DETERMINATION OF THE AGREEMENT BETWEEN EXPERT OBSERVERS.

Shown in the table on the following page are the sets of porphyrin results presented to the three observers (A, B and C) and the diagnoses they assigned to each set as described in Chapter 8. These specimen sets represent genuine values determined on routine specimens by the diagnostic laboratory.

The diagnostic codes are as follows:

For screening results

1	The screenings suggest a **normal result**.
2	The screenings are **equivocal** – could be either normal or VP
3	The screenings suggest **either VP or PCT**
4	The screenings **strongly suggest VP**.

For TLC results

There is no doubt about the biochemical diagnosis – either VP or normal.	
1	The trace is unequivocally normal
4	The trace is unequivocally diagnostic of VP
There is an element of doubt about the biochemical diagnosis as results are not unequivocally diagnostic.	
2	There is a suspicion that VP may be present, but the probability is low.
3	There is a suspicion that VP may be present, but the probability is high.

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
1	1	1	1	1	1	1	NEG	NEG			7	0	0	0	102.4	1.3	0	0	0	0	19	0	151.6	
2	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	9	0	56	
3	2	1	2	1	1	1		+								1.1	0	0	0	0	25	0	157.7	
4	1	1	1	1	1	1	NEG	NEG			4	0	0	0	98.9	0	0	0	0	0	9.1	0	28.2	
5	1	1	1	1	1	1	NEG	NEG			16	0	0	0	88.4	0.9	0	0	0	0	5.4	0	49.2	
6	2	1	1	1	1	1	+/-	NEG			10	0	0	0	65.5	0	0	0	0	0	6.9	0	51	
7	2	1	3	3	3	1	+	+			0	0	0	0	86.5	0	0	0	8.6	6.1	71.6	0	129.6	
8	2	1	1	2	1	1	NEG	+/-								0	0	0	0	0	7	0	241.3	
9	3	2	4	4	2	2	NEG	++			10	0	0	0	180.5	1.3	0	0	0.7	0	17.7	0	418.1	
10	1	1	1	1	1	1	+/-	NEG			3	0	0	0	78.8	0	0	0	0	0	9.3	0	54.1	
11	2	1	2	4	4	4	+/-	+			34	11.9	0	20.3	69.4	0	0	0	78.3	22.3	200.4	0	369.7	
12	4	4	3	4	4	4	+++	+++	66	23.4	14	0	0	0	557.7	0	0	0	23	7.2	344.2	0	429.3	POS
13	3	2	4	4	4	1	NEG	++			5	0	0	0	35.2	0	0	0	16.1	3.7	59	0	238	LOW
14	2	1	2	2	1	1	NEG	+	8.6	1.3	4	0	0	0	21.8	0	0	0	0	0	20.9	0	267.3	NEG
15	2	1	1	4	3	4	NEG	+/-			0	0	0	0	37.5	1.6	0	0	3.6	4.6	34.3	0	619.5	
16	1	1	1	1	1	1	NEG	NEG								0.5	0	0	0	0	12.3	0	81.6	
17	2	1	2	1	1	1	+	NEG	7.3	0.3	1	0	0	0	51.6	0	0	0	0	0	4.2	0	54.7	
18	1	1	1	1	1	1	NEG	NEG								1.2	0	0	0	0	9	0	84.1	
19	2	1	1	1	1	1	NEG	+/-			5	0	0	0	20.1	1.7	0	0	0	0	18.7	0	157.8	
20	2	1	2	4	1	2	+/-	+	10.9	3.9	1	0	0	0	30.1	0	0	0	3	3.4	29.7	0	356.5	
21	2	1	1	1	1	1	NEG	+/-	9.2	2.4	4	0	0	0	21.5	0	0	0	0	0	9.8	0	101.6	
22	3	2	4	4	4	4	+/-	++	19.8	1.7	22	0	0	0	72.8	0	0	0	16	3.8	79.8	0	332	POS
23	2	1	1	1	1	1	+/-	+/-			7	0	0	0	54.1	0	0	0	0	0	8.3	0	71.1	
25	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	7	0	26.1	
26	1	1	1	1	1	1	NEG	NEG	6.9	1.1	0	0	0	0	19.2	0	0	0	0	0	3.2	0	69.6	
27	3	4	4	2	2	3	NEG	+++			0	0	0	0	17	1.3	0	0	0	0	12.6	0	427.9	
28	3	2	3	3	4	4	+/-	++			21	0	0	0	363.5	0	0	0	0	0	93.9	0	298.7	
31	2	1	1	1	1	1	+	NEG	9.1	3.8	4	0	0	0	41.3	0	0	0	0	0	5.3	0	62.5	
32	2	1	1	1	1	1	+/-	+/-	9.6	1.9	4	0	0	0	37	0	0	0	0	0	9.1	0	87.9	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
34	4	4	4	4	4	4	++	+++	65.6	40.8	120	36.1	9.6	56.4	240	12.5	0	0	40.8	0	160.1	0	633.6	
35	1	1	1	1	1	1	NEG	NEG			6	0	0	0	81.2	0	0	0	0	0	4.4	0	14.8	
37	3	2	2	4	4	3	+/-	++	29.4	8.2	8	0	0	0	27.1	0	0	0	18.4	8.6	40.2	0	405.5	
38	3	4	4	4	4	4		+++								0	0	0	15.9	0	82.3	0	616.3	
39	2	1	1	1	1	1	NEG	+			8	0	0	0	13.6	0	0	0	0	0	7.4	0	79.1	
40	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	10.5	0	21.4	
41	1	1	1	1	1	1		NEG								0.7	0	0	0	0	8.4	0	76.6	
42	2	1	1	3	1	1	NEG	+/-			5	0	0	0	131	0	0	0	1	1	21.8	0	239.2	
43	3	4	4	4	4	4	NEG	+++	12.9	2.5	0	0	0	0	58.8	0	0	0	13.9	0	39.5	0	940.2	
44	4	4	4	4	4	4	++	+++	30.7	14.1	22	0	0	0	176.7	0	0	0	42.1	6.6	158.7	0	797.5	
45	1	1	1	1	1	1	NEG	NEG								0.6	0	0	0	0	4.5	0	35.8	
46	3	2	2	4	1	1	+/-	++			5	0.8	0	0	24	0.7	0	0	3.3	3.4	25.6	0	222.9	
47	2	1	2	1	1	1	+/-	+	11.4	1.2	0	0	0	0	106.4	0	0	0	0	0	21	0	129.1	NEG
48	2	1	1	1	1	1	+/-	NEG	10.8	2.1	8	0	0	0	51.9	0	0	0	0	0	22.8	0	70.8	
49	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	5.9	0	54.5	
50	2	1	1	1	1	1	NEG	+/-			17	0	0	0	268.8	4.6	0	0	0	0	9	0	185.7	
52	2	1	1	4	3	4	NEG	+	16.5	4	0	0	0	0	312.6	1.6	0	0	8.3	5.9	46.7	0	458.8	NEG
53	2	1	1	1	1	1	+/-	NEG			6	0	0	0	118.4	0.8	0	0	0	0	16.3	0	34.2	
54	2	1	1	1	1	1	+/-	NEG			14	0	0	0	311.1	1.3	0	0	0	0	16	0	72.2	
55	2	1	1	2	2	1	+/-	NEG			49	0	0	0	340.7	0	0	0	0	0	55.5	0	98.7	
57	1	1	1	1	1	1		NEG								0.2	0	0	0	0	10.1	0	41.8	
58	1	1	1	1	1	1	NEG	NEG			8	0	0	0	26.5	0	0	0	0	0	18.1	0	98.4	
59	2	1	1	1	1	1	NEG	+/-	12.2	4.4	14	0	0	0	333.3	2.2	0	0	0	0	19	0	202.3	
60	1	1	1	1	1	1	NEG	NEG	8.3	1	4	0	0	0	24.3	0	0	0	0	0	7.1	0	85.4	
61	3	2	2	4	4	4	+	++	32.5	9.7	3	0	0	0	121.2	0	0	0	51.7	13.6	113.9	0	540.1	NEG
62	1	1	1	1	1	1	NEG	NEG	10	3.2	16	0	0	0	111.2	0	0	0	0	0	11.6	0	73.2	NEG
63	2	1	1	2	1	2	NEG	+	3.5	2.1	8	0	0	0	91	0	0	0	0	0	32.2	0	263.5	NEG
64	2	1	1	1	1	1	+/-	+/-			12	0	0	0	121.3	0	0	0	0	0	16.5	0	103.5	
65	3	4	4	4	4	4	+	+++	15.7	5	16	0	0	0	210.7	0	0	0	123.1	36	96	0	1713	POS

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
66	3	2	2	4	2	3	NEG	++			0	0	0	0	45.8	0	0	0	0	1.3	29.5	0	379.9	
67	3	2	2	4	4	4	NEG	++			28	6.2	0	0	73.8	0	0	0	39	7	168.2	0	722.3	NEG
68	2	1	2	1	1	1	NEG	+	3.2	1.9	0	0	0	0	36.3	0	0	0	0	0	22.6	0	205.2	NEG
69	2	1	2	2	1	3	NEG	+	4.3	3.5	13	0	0	0	133.4	0	0	0	0	0	22.5	0	380.5	NEG
70	2	1	1	1	1	1	NEG	+/-	5.9	1.8	24	0	0	0	102.1	0	0	0	0	0	14.4	0	121.2	NEG
71	2	1	2	2	1	1	NEG	+	7.8	3.1	0	0	0	0	110.2	0	0	0	0	0	27.3	0	231.5	NEG
72	2	1	1	2	1	1	+/-	+/-			0	0	0	0	21.1	0	0	0	0	0	35.8	0	191.7	
73	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	23.3	0	41	
74	2	1	1	1	1	1	NEG	+/-			0	0	0	0	28.8	0	0	0	0	0	25.2	0	191.8	NEG
75	2	1	1	2	1	1	NEG	+/-			4	0	0	0	236.7	0	0	0	0	0	39.7	0	204.7	NEG
76	1	1	1	1	1	1	NEG	NEG			12	0	0	0	89.4	0	0	0	0	0	7.2	0	49.6	NEG
77	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	23.3	0	41	
78	2	1	1	1	1	1	NEG	+/-			0	0	0	0	28.8	0	0	0	0	0	25.2	0	191.8	NEG
79	2	1	1	2	1	1	NEG	+/-			4	0	0	0	236.7	0	0	0	0	0	39.7	0	204.7	NEG
80	1	1	1	4	1	1	NEG	NEG			7	0	0	0	81	0.6	0	0	1.9	2.3	9.6	0	142.6	
81	2	1	1	4	4	4	NEG	+			14	2.5	0	0	211.1	4.1	0	0	21.6	7.9	105.1	0	497.5	
82	1	1	1	1	1	1	NEG	NEG			0	0	0	0	30.1	0	0	0	0	0	14.1	0	58	
84	3	2	2	4	2	3	NEG	++	4.2	2	9	0	0	0	133.7	0	0	0	11.3	0	21.6	0	922.7	
85	2	1	1	1	1	1		+/-								0	0	0	0	0	11.9	0	47.3	
86	4	4	4	4	4	4	++	+++			354	156.6	37.9	200.6	721.3	0	0	0	51.1	0	221.2	0	615.2	
87	2	1	1	1	1	1	NEG	+/-			7	2.1	0	0	33.3	0	0	0	0	0	14.2	0	116.3	
88	1	1	1	1	1	1	NEG	NEG			6	0	0	0	13.8	0	0	0	0	0	11.3	0	34.6	
89	2	1	2	2	1	2	NEG	+			38	0	0	0	226.4	2.1	0	0	0	0	13.9	0	479	NEG
90	2	1	2	4	1	2	NEG	+								1.7	0	0	1.8	3.2	18.6	0	167.9	
91	2	1	2	3	1	2	NEG	+								3	0	0	0	0	33.9	0	388.9	
92	4	4	4	4	4	4	+	+++	20.9	4.1	29	9.6	2	6.3	203	0	0	0	54.5	20.9	120.9	0	1074	POS
93	2	1	1	1	1	1	+	NEG			11	0	0	0	134.6	0	0	0	0	0	4.6	0	137	
94	2	1	1	1	1	1	+/-	+/-			3	0	0	0	22.5	1	0	0	0	0	3	0	44.1	
95	2	1	2	4	1	2	+/-	+			0	0	0	0	9.6	0	0	0	3.9	1.1	34.8	0	266.6	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
96	1	1	1	3	1	1	NEG	NEG			0	0	0	0	95.6	0	0	0	1.5	3	29.1	0	106.3	
97	1	1	1	2	1	1	NEG	NEG			13	3.1	0	0	147.8	1	0	0	0	0	19.3	0	244.9	
98	3	4	4	4	4	4	NEG	+++	23.8	0.7	14	0	0	0	20.3	0	0	0	56.1	19.5	236.4	0	1604	
99	3	4	4	4	3	4	NEG	+++								0	0	0	9.3	2.3	22.2	0	903.1	
100	2	1	2	2	1	2		+								0	0	0	0	0	17.2	0	318.4	
101	4	4	3	4	4	4	+++	+++	16.3	3.7	568	219.2	17.7	43	268.5	0	0	0	200.4	28.2	400.4	0	2559	
102	3	2	2	1	1	1	++	NEG	19.8	4.2	16	2.5	0	10.9	131.4	0	0	0	0	0	26.5	0	27.3	
103	2	1	1	1	1	1	+/-	+/-			10	0	0	0	19.4	0.6	0	0	0	0	19.8	0	63.2	
104	4	4	4	4	4	4	++	+++	39.5	20.5	12	0	0	0	164.7	0	0	0	17.9	5.5	114.3	0	382.8	
105	2	1	1	1	1	1	NEG	+/-			5	1.8	0	0	57.1	0.5	0	0	0	0	19.6	0	108.1	
107	1	1	1	3				NEG								7	56.7	20.7	0	30.2	12.1	0	31.6	
109	3	2	2	1	1	1	+/-	++	8.2	1.9	0	0	0	0	19.5	0	0	0	0	0	3.9	0	139.1	
110	1	1	1	1	1	1		NEG			29	0	0	0	247.5	0	0	0	0	0	30.1	0	81.5	
112	3	2	2	4	3	3	+	++	13.4	2.9	14	6.3	0	1.5	18.9	1.3	1.5	0	0	0	71.7	0	267.1	
113	3	2	2	4	2	3	NEG	++	8.1	1.8	5	0	0	0	50.8	1.2	0	0	2.3	3	37.4	0	278.6	
114	3	2	2	4	3	3	NEG	++	7	1.4	3	0	0	0	69.6	0	0	0	3.4	2	22.8	0	546.6	
115	1	1	1	1	1	1	NEG	NEG	10.6	2.1	6	0	0	0	27.6	0	0	0	0	0	9.8	0	33	
116	1	1	1	1	1	1	NEG	NEG	18.7	1.9	0	0	0	0	17.2	0	0	0	0	0	9	0	143.9	
117	3	4	4	4	4	4	NEG	+++	36.3	7.1	21	0	0	0	199.4	0	0	0	89.4	29.8	305.1	0	2354	
118	2	1	2	1	1	1	NEG	+	10.5	0.8	33	0	0	0	83	0	0	0	0	0	12.6	0	197.3	
119	1	1	1	1	1	1	NEG	NEG			0	0	0	0	78.6	1.4	0	0	0	0	16.7	0	86.7	
120	1	1	1	1	1	1	NEG	NEG	17.8	1.2	1	0	0	0	19.4	0	0	0	0	0	4.1	0	17.6	
121	1	1	1	1	1	1	NEG	NEG	28.9	8.9	12	0	0	0	306.5	0	0	0	0	0	8.7	0	30.5	
122	2	1	1	1	1	1	+	NEG	24.4	4.9	11	0	0	0	85.6	0	0	0	0	0	14.8	0	81.3	
123	1	1	1	1	1	1	NEG	NEG	16.5	3.6	5	0	0	0	47.7	0	0	0	0	0	11.7	0	71.7	
124	2	1	1	1	1	1	+/-	NEG	12.4	4.5	14	0	0	0	210.7	0	0	0	0	0	12.2	0	12	
125	2	1	2	4	4	4	NEG	+	17.6	3.8	19	3.5	0	0	69.7	0	0	0	46.8	17.6	217.7	0	662.2	
126	3	2	2	4	4	4	NEG	++	11.1	3	0	0	0	0	155	0	0	0	0	0	75.9	0	387.3	
127	2	1	1	1	1	1	NEG	+/-	8.2	1.8	2	0	0	0	15.7	0.6	0	0	0	0	6.3	0	115.6	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
128	1	1	1	1	1	1	NEG	NEG	9.4	1.5	0	0	0	0	38.4	0	0	0	0	0	8.4	0	29	
130	1	1	1	1	1	1	NEG	NEG	9	1.1	7	0.8	0	1.6	44.3	0	0	0	0	0	13.3	0	19.4	
131	2	1	1	4	3	2	NEG	+/-	6.5	1.1	5	0	0	0	35.8	0	0	0	25.9	7.3	29.1	0	249.1	
132	2	1	1	1	1	1	NEG	+/-			0	0	0	0	15.1	0	0	0	0	0	5.4	0	96.9	
133	1	1	1	2	1	2	NEG	NEG			4	0	0	0	61.1	0	0	0	0	0	15	0	367.8	
134	2	1	1	2	3	3	NEG	+/-			15	0	0	0	265.2	1.6	0	0	0	0	102	0	205.4	
135	3	4	4	4	4	4	NEG	+++								0	0	0	45.8	0	160.3	0	697.7	
136	1	1	1	1	1	1	NEG	NEG			2	0	0	0	19.5	0	0	0	0	0	10.2	0	103.6	
137	1	1	1	1	1	1	NEG	NEG			4	0	0	0	16	0	0	0	0	0	3.8	0	11.8	
138	2	1	1	1	1	1	+	NEG			11	0	0	0	134.6	0	0	0	0	0	4.6	0	137	
139	2	1	1	1	1	1	+/-	+/-			3	0	0	0	22.5	1	0	0	0	0	3	0	44.1	
140	1	1	1	1	1	1	NEG	NEG			7	0	0	0	51.3	0	0	0	0	0	12.2	0	52.7	
141	2	1	1	2	1	1	NEG	+/-			6	0	0	0	21.2	0	0	0	0	0	24.8	0	224.1	
142	1	1	1	1	1	1	NEG	NEG			14	0	0	0	51.5	0	0	0	0	0	26	0	204.4	
143	2	1	1	4	4	4	NEG	+			25	0	0	0	218	0	0	0	85.9	27.1	239	0	1054	NEG
144	2	1	1	1	1	1	+/-	NEG			11	0	0	0	98.4	0	0	0	0	0	18.2	0	129.9	
146	4	4	4	4	4	4	+	+++			7	0	0	0	38.7	0	0	0	75.5	14.1	357.7	0	2620	
148	2	1	2	4	3	3		+	11.7	2.2	0	0	0	0	41.7	0	0	0	1.8	2	27.9	0	482.4	NEG
150	3	3	3	4	4	4	+++	++	55.4	51.3	71	35.9	0	104.9	309	0	0	0	15	16.9	244.1	0	492.6	
153	2	1	2	3	1	2		+	10.7	1.7	20	0	0	0	52.6	0	0	0	6.7	6.7	38.8	0	131	NEG
154	3	2	2	4	4	4		++	8.8	1.5	49	15.8	3.9	7.9	83.3	0	0	0	8.6	9.9	62.9	0	270.2	NEG
155	2	1	1	4	4	4		+/-	9.6	0.6	66	28.7	2.6	29.6	56.3	0	0	0	11.4	11.4	52.1	0	269.2	NEG
156	3	2	2	3	3	3		++	9.5	1	10	0	0	0	123.4	5	0	0	0	0	46.2	0	482.4	NEG
157	1	1	1	1	1	1		NEG	14.3	1.4	31	0	0	0	29.2	0	0	0	0	0	13.3	0	57.2	NEG
158	3	4	4	4	4	4		+++	24.9	6.1	11	0	0	0	172.6	0	0	0	55.1	34.8	368	0	2058	POS
159	2	1	2	2	1	1		+	17.1	1.5	10	0	0	0	50.6	0	0	0	0	0	25.5	0	217	NEG
161	3	4	4	4	4	4	NEG	+++	6.4	0.7	0	0	0	0	99.1	0	0	0	33.9	0	189.3	0	1602	
163	3		2	3	1	1	++	NEG								1.7	13.2	10.5	0	10	11.8	0	24.6	
165	2	1	1	1	1	1	NEG	+/-			8	0	0	0	80.3	0	0	0	0	0	16.7	0	76.1	NEG

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
166	2	1	1	2	1	2	+/-	NEG			9	0	0	0	132.8	0.9	0	0	0	0	21.1	0	245.9	
167	2	1	1	1	1	1	+/-	NEG			3	0	0	0	37	1.7	0	0	0	0	13.2	0	94.5	
168	3	4	2	1	1	1	+	++								0	0	0	0	0	4.7	0	57.6	
169	2	1	1	1	1	1	+/-	NEG			12	0	0	0	118.6	0	0	0	0	0	9.9	0	32.6	
170	2	1	1	1	1	1	+	NEG			9	0	0	0	131	2.9	0	0	0	0	36.3	0	54.9	
171	2	1	2	4	4	4	NEG	+			14	2.5	0	0	211.1	4.1	0	0	21.6	7.9	105.1	0	497.5	
172	1	1	1	1	1	1	NEG	NEG			0	0	0	0	30.1	0	0	0	0	0	14.1	0	58	
173	1	1	1	1	1	1	NEG	NEG			2	0	0	0	26.5	0	0	0	0	0	25	0	171.1	
174	1	1	1	2	1	2	NEG	NEG	7.3	2.3	10	0	0	0	131.4	0	0	0	0	0	11.9	0	274.4	
175	2	1	1	1	1	1		+/-								0	0	0	0	0	31.3	0	149	
176	3	4	4	3	3	3	NEG	+++	7.2	0.7	8	0	0	0	25.2	0	0	0	0	0	34.7	0	966.7	
177	2	1	1	2	2	2	NEG	+/-			28	0	0	0	225.6	0	0	0	0	0	8.6	0	419.7	
178	2	2	2	3	3		+	+	17.5	2.9	0	0	0	0	358.3	1.2	6.5	3.1	0	29.8	30	0	121.1	NEG
179	2	1	1	3			+/-	+/-			329	178.2	0	0	148.3	0.6	6.5	7.7	0	18.3	19.4	0	117	NEG
180	1	1	1	2	2	1	NEG	NEG			10	0	0	0	134.6	0	0	0	0	0	47.3	0	198.7	NEG
181	1	1	1	1	1	1	NEG	NEG			6	0	0	0	136.4	0	0	0	0	0	22.4	0	146.7	
182	2	1	1	3	2	2	NEG	+								0	0	0	0	0	36	0	457.9	
184	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	4.9	0	29.7	
188	2	1	1	1	1	1	+/-	+/-			14	0	0	0	106.4	0	0	0	0	0	16.4	0	75	
189	2	1	1	3	1	2	+/-	+			21	4.4	0	0	105.6	0	0	0	0	0	31	0	407.8	
190	2	1	1	1	1	1	NEG	+/-			0	0	0	0	141.3	5.6	0	0	0	0	38.3	0	74.4	
191	2	1	1	2	1	2		+			7	0	0	0	134.4	2.3	0	0	0	0	39.8	0	248.8	
192	2	2	2	3	3		+	+	25.4	7.9	36	11.5	0	17.9	148.7	0	0	0	0	0	112.8	0	204.6	
193	3	2	2	4	4	4	+/-	++	17	3.3	0	0	0	0	146.9	0	0	0	0	0	60.3	0	333.3	
195	3		1	3	1		++	NEG								1.7	13.2	10.5	0	10	11.8	0	24.6	
197	2	1	2	4	4	2	+/-	+			0	0	0	0	279.4	0	0	0	53.7	16.3	87.5	0	425.1	NEG
202	2	1	1	3	1	1	+/-	+/-	7.8	1.5	7	1.9	0	1.3	72.5	1	0	0	1	0.9	31.7	0	194.1	
203	4	4	3	4	4	4	+++	+++	72.1	13	32	7.7	0	40.1	597.8	0	0	0	77.9	64.9	557.2	0	1106	POS
206	2	1	1	3	3	4	NEG	+/-								0	0	0	0	0	105	0	243.7	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
207	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	21.1	0	183.4	
210	2	1	1	1	1	1	+/-	+/-			5	0	0	0	34.7	1	0	0	0	0.7	7.2	0	102.8	
211	3	2	2	4	3	3	NEG	++			4	0	0	0	23.9	2.6	0	0	2.1	2.4	16	0	647.2	
212	2	1	1	1	1	1	+/-	+			6	0	0	0	285.6	0	0	0	0	0	36.2	0	91.9	
213	2	1	2	3	3	3	+	+			0	0	0	0	217.8	0	0	0	6.1	0	103.6	0	144.9	
214	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	16.6	0	32.5	
215	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	21.3	0	23.6	
216	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	19.9	0	42.2	
217	2	1	1	1	1	1	+/-	NEG	8.6	3.9	2	0	0	0	98.7	0	0	0	0	0	23.7	0	40.1	
218	2	1	2	3	3	3	+/-	+			1	0	0	0	58	0	0	0	0	0	44.5	0	465	
219	3	2	2	4	4	4	+/-	++								0	0	0	52	15.3	415.8	0	1081	
221	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	16.7	0	47.2	
222	2	1	1	1	1	1	+/-	+/-			0	0	0	0	159.7	0	0	0	0	0	34.8	0	186.4	
223	2	1	1	1	1	1	+/-	NEG			5	0	0	0	38.3	0.3	0	0	0	0	7.1	0	11.6	
224	3	2	2	4	4	4	+/-	++			0	0	0	0	52.4	0	0	0	17.8	8.1	111.2	0	315.5	
225	2	1	1	1	1	1	+/-	+/-			9	0	0	0	71.4	0	0	0	0	0	32.6	0	139.4	
226	2	1	2	2	2	1	+/-	+			2	0	0	0	140.1	0	0	0	0	0	47.9	0	195	
227	4	4	4	4	4	4	++	+++								0	0	0	100.3	21.5	318.9	0	360.6	
228	1	1	1	1	1	1	NEG	NEG								1.2	0	0	0	0	7.6	0	33.7	
230	3	2	2	3	3	3	+/-	++			3	0	0	0	147.1	0	0	0	0	0	63.9	0	223.8	
232	2	1	1	1	1	1	NEG	+								0	0	0	0	0	4.6	0	71.1	
233	3	2	2	4	4	4	+	++			0	0	0	0	305.1	0	0	0	124.8	40.8	340.5	0	2156	
236	2	1	1	1	1	1	+/-	NEG			12	0	0	0	184.3	1.6	0	0	0	0	3.1	0	26.6	
238	2	1	1	4	1	2		+/-								2.9	0	0	4	3.2	36.5	0	265.6	
239	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	26.7	0	108	
241	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	32.2	0	105.3	
244	2	1	1	2	2	1	NEG	+/-								0	0	0	0	0	53.5	0	173.7	
248	4	4	3	4	4	3	+++	+++			347	281.5	19.7	14.4	26.2	7.6	48.6	17.3	0	40.9	33.4	0	531.7	
251	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	4.9	0	29.7	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
252	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	32.2	0	101	
253	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	10.4	0	60.9	
256	3	2	2	2	1	2	+/-	++	9.9	4	5	0	0	0	39.8	0	0	0	1.5	0	17.4	0	343.2	
257	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	27.5	0	161.5	
260	2	1	2	4	4	4	+/-	+	24.7	2.8	227	159.6	32.9	38.2	373.2	0	0	0	16.2	8.1	209.8	0	235.8	
262	2	1	1	1	1	1	NEG	+/-	14.6	2.5	0	0	0	0	107	0	0	0	0	0	28.7	0	166.3	
263	1	1	1	1	1	1	NEG	NEG	32.4	11.9	0	0	0	0	130.4	0	0	0	0	0	21.7	0	19.5	
266	1	1	1	1	1	1	NEG	NEG	18.8	7.3	37	0	0	0	215.3	1.6	0	0	0	0	29.1	0	28.7	
267	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	18.5	0	70.4	
269	3	2	2	4	4	4	+/-	++			0	0	0	0	209.8	0	0	0	51.1	21.9	335.6	0	1109	
270	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	15.8	0	101.2	
274	2	1	2	3		1	+/-	+			341	135.1	18.4	18.8	38.7	2.6	17.9	9.5	0	14	15.4	0	36.5	
275	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	32.9	0	147.4	
277	1	1	1	3	1	1	NEG	NEG								0	0	0	0.9	0	14.7	0	224.8	
278	2	1	2	3	1	2	+/-	+			2	0	0	0	81.4	0	0	0	1.7	1.3	12.8	0	204	NEG
280	2	1	1	1	1	1	+/-	+/-			6	0	0	0	71	0.1	0	0	0	0	11.7	0	124.4	NEG
282	2	1	2	4	4	4	+/-	+			23	27.3	0	9.7	177.1	0	0	0	19.4	7.8	115.2	0	326.1	
284	2	1	2	4	4	4	NEG	+								0	0	0	35.3	6.3	55.3	0	295.6	
287	3	4	3	4	4	4	++	++								0	0	0	61.3	18.4	77.6	0	296.1	
288	3		2	3			++	+/-			260	110	8.7	25.8	84.5	0.7	2.4	2.4	0	3.5	20.8	0	18.7	
289	2	1	2	2	2	3	NEG	+			7	0	0	0	95.3	0	0	0	0	0	23.9	0	706.6	
291	1	1	1	1	1	1	NEG	NEG			2	0	0	0	0.6	0	0	0	0	0	9.3	0	43.1	NEG
292	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	7.4	0	70.6	
293	3		2	3	4		+++	+	331.2	0.4	1329	469.8	381.8	431.5	556.6	9.6	0	0	0	8	87.7	0	114.7	
295	3	4	2	4	4	4	+++	++	27.8	3.8	31	0	0	0	369.7	0	0	0	16.6	6.6	146	0	361	
296	3	4	4	4	4	4	NEG	+++			10	0	0	0	425.5	0	0	0	82	20.1	131.9	0	1153	POS
297	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	32.8	0	141.2	
299	2	1	1	2	3	3	NEG	+/-			22	0	0	0	834.5	0	0	0	0	0	26.6	0	417.1	
301	1	1	1	1	1	1	NEG	NEG			5	0	0	0	12.3	0	0	0	0	0	30.5	0	41.4	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
302	2	1	2	2	1	1	NEG	+			0	0	0	0	42.3	0	0	0	0	0	45.3	0	88.4	
303	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	27.9	0	140	
304	3	2	2	4	4	4	+/-	++			11	0	0	0	424.4	0	0	0	70.1	18	86.6	0	259.7	
305	2	1	2	1	1	1	NEG	+			15	3.7	8.5	1.6	39.8	1	0	0	0	0	7.2	0	118.9	
306	3	2	2	2	2	1	NEG	++			35	20	0	0	28	0	0	0	0	0	17.5	0	247	
307	1	1	1	1	1	1		NEG								0	0	0	0	0	8.1	0	33.4	
308	3	2	2	4	4	4	NEG	++			172	245.3	146.2	174.4	300.3	7.1	36.7	47.5	0	42.1	71.5	0	461.5	
309	2	1	1	3	1	1	+/-	+/-			15	0	0	0	74.7	0	0	0	3.7	1.4	26.5	0	34.5	
310	2	1	1	4	1	1		+/-								2.1	0	0	3.4	3.7	19.5	0	290.1	
313	3		2	3			+++	NEG	17.6	3.3	670	295.5	42.7	115.4	117.2	6.7	21.3	12.3	0	36.7	18.7	0	30.2	
315	3	2	2	4	4	4	NEG	++								0	0	0	57.3	23.6	198.9	0	267.5	
317	3	2	2	4	4	4	+/-	++	31	15.7	18	0	0	0	97.3	0	0	0	31.1	9	165.1	0	413.2	
318	2	1	2	3	1	2	NEG	+								0	0	0	14.6	5.9	34.3	0	105.8	
320	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	16.7	0	47.2	
321	1	1	1	1	1	1	NEG	NEG	27.4	5.7	22	0	0	0	162.7	0	0	0	0	0	33.7	0	185.1	
324	2	1	1	1	1	1	+/-	NEG	9.5	0.8	4	0	0	0	45.6	0	0	0	0	0	17	0	40.5	
326	1	1	1	1	1	1	NEG	NEG	9.1	0.9	0	0	0	0	218.7	1	0	0	0	0	26.1	0	92.2	
332	1	1	1	2	1	1	NEG	NEG								1.1	0	0	0.6	0	19.3	0	75.3	
335	1	1	1	2	1	1	NEG	NEG								1.1	0	0	0.6	0	19.3	0	75.3	
336	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	11.7	0	59.7	
340	2	1	2	4	4	4		+								7.4	29.7	22.6	0	66.6	60.8	0	389.5	
342	2	1	2	2	2	2	NEG	+								4.4	0	0	0	0	43.8	0	274.6	
343	3	2	2	3	2	3		++								0.7	0	0	7.8	0	6.7	0	676	
344	2	1	1	4	2	2	+/-	+/-			5	0	0	0	102.5	1.2	0	0	0	2.1	31.1	0	380	
345	3	1	2	4	1	2	+/-	+	6.4	2.6	6	0	0	0	108.4	0	0	0	0.9	0.7	26.7	0	260.4	NEG
348	1	1	1	1	1	1	NEG	NEG								0	0	0	0	0	9.9	0	42	
351	3	2	2	4	4	4	+	++	16.7	2.8	46	6.2	0	0	93.6	0	0	0	51.5	14	242.1	0	397.3	
352	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	35.7	0	106.4	
356	3		2	3		1	+++	+								18.4	56.7	35.3	0	43.5	31.9	0	98.6	

Key	Diagnosis Screening			Diagnosis TLC			Screen		Urine							Stool								Plasma
	A	B	C	A	B	C	Urine	Stool	ALA	PBG	Uro	C7	C6	C5	Copro	Uro	C7	C6	Ps5	C5	Copro	C3	Proto	
359	2	1	1	4	1	1	+/-	+/-			5	0	0	0	101.5	1.1	0	0	2.1	1.2	25.2	0	200.5	
360	2	1	1	2	1	1	NEG	+/-	19.4	5.7						0	0	0	5.5	1.7	7.1	0	69.5	
363	2	1	1	1	1	1	NEG	+/-								0	0	0	0	0	22.6	0	158.7	

APPENDIX 9:

DRUG SAFETY DATABASE

The UCT Porphyria Centre maintains an extensive and comprehensive drug-safety database. The author has been principally responsible for the creation and software programming of this computerised database, as well as for the evaluation of the clinical and laboratory data on which the recommendations are based. An innovation introduced by the author has been the replacement of the traditional *safe, contentious, unsafe* categories with those which recommend directly whether they should be used, avoided or used with caution. A major function of our Centre is the provision of drug safety information to patients, doctors and pharmacists. A list of drugs with their recommendations for use appears on the following pages.

DRUG SAFETY IN PORPHYRIA

INTRODUCTION

Drugs are the most common factor precipitating the acute attack of porphyria, a potentially fatal condition. Thus it is essential to identify those drugs which may precipitate the acute crisis and avoid prescribing them to porphyrics, except where no safer alternative exists and the indication outweighs the risks.

This list which follows contains most drugs available in South Africa at present. Note that new drugs are constantly being introduced, and the absence of a drug from this list does not mean that its safety can be assumed. Drugs are listed by generic name and trade names must be cross-checked against these. The information presented here has been obtained by examining data from the literature (itself based on clinical experience as well as animal and in vitro experiments), from personal experience and by the examination of drugs for structure, structural similarity and routes of metabolism. *Such information cannot be guaranteed to prove valid under all circumstances, and the list should serve as a guide only.*

Responsibility cannot be taken for errors or for any adverse experience that may be encountered. The Lennon Porphyria Information Centre of the MRC/UCT Liver Research Centre, University of Cape Town, offers advice on all aspects of the diagnosis and management of porphyria, as well as maintaining a database of drug safety. It may be contacted at:

- Phone (021) 4066332
- Fax (021) 4486815

USE OF THESE LISTS

As a general rule, all topical preparations are safe. Thus any agent may be prescribed for external dermatological use or for instillation into the nose, ears or eyes, for gargling or, in the case of asthma, for administration by nebulisation or metered-dose inhaler. Caution should however be exercised when the risk of systemic absorption is high, as in dermatological preparations applied to large areas of denuded skin. The following list omits all agents used only by these routes, and comprises only those intended for systemic use. The following are the status terms used in the list.

Use	Likely to be safe and may be used freely.
UWC	*Use with caution:* Though safety is not established beyond doubt, the evidence suggests that the drug is unlikely to prove unsafe in practice.
UWECO	*Use with extreme caution only:* There is evidence to suggest that the drug may yet prove unsafe in practice, or grounds to suspect this may be so, or too little evidence to suggest that it may be safe. Such drugs should only be used if the expected benefits strongly outweigh the risks, and an adverse outcome must be anticipated.
Avoid	There is evidence that such drugs have precipitated acute attacks in patients, or other grounds for believing that the risk of an acute attack is high.
ND/Avoid	*No data/Avoid:* There is too little evidence to draw a conclusion, and it is wisest to regard the drug as potentially hazardous.

Note that the description *Use with extreme caution only (UWECO)* is **not** an invitation to use the drug; it is a warning that it should only be used with great reluctance, and is potentially unsafe.

CHOICE OF DRUG FOR A PORPHYRIC

No porphyric patient should receive any medication unless it is felt to be absolutely vital. Many drug-related problems involve medication which was not strictly necessary in the first place. Beware of compound medications: particularly analgesics: though the paracetamol and codeine in them may be acceptable, often one of the "lesser" ingredients, such as *meprobamate*, is not.

When a prescription is necessary, select drugs in the order; *Use, UWC, UWECO, ND/Avoid, Avoid.* Before using any drug labelled *UWECO, Avoid or ND/Avoid*, doctors must satisfy themselves that a very real need for the drug exists and that no safer alternative is possible; they should inform the patient fully of the potential danger of the drug and get the patient's consent, preferably in writing; they should advise the patient on what symptoms to expect should the porphyria become more active (for practical purposes, the development of any abdominal pain) and should advise the patient to stop the drug immediately under these circumstances. Darkening of urine may indicate a rise in porphyrin precursors and hence an incipient acute attack.

Where the risk is particularly great, such as with anticonvulsants and antituberculous therapy, the patient should be monitored biochemically during the early stages of treatment. This is best performed by daily estimations of urinary ALA and PBG by a quantitative technique, or failing this, urine PBG screening with Ehrlich's aldehyde. Any rise in these precursors is strong presumptive evidence of an incipient acute attack, and treatment should be ceased forthwith.

Information on the outcome of drug treatment in porphyric patients is always welcome and will assist with the improvement of the drug safety database. It may be communicated to the address given above.

Anaesthesia

Alcuronium	UWC
Atracurium	UWC
Atropine	Use
Enflurane	UWC
Etomidate	Avoid
Flumazenil	Use
Glycopyrronium	Use
Halothane	Use
Isoflurane	UWC
Mivacurium	UWC
Naloxone	Use
Neostigmine	Use
Nitrous oxide	Use
Pancuronium	Use
Propofol	Use
Rocuronium	UWC
Sevoflurane	UWC
Suxamethonium	Use
Thiopental	Avoid
Vecuronium	UWC

Local anaesthetics

Bupivacaine	Use
Lidocaine	Use
Mepivacaine	UWC
Oxybuprocaine	Use
Prilocaine	Use
Procaine	Use
Tetracaine	Use

Analgesics and anti-inflammatories

Alclofenac	UWC
Alfentanil	Use
Aspirin	Use
Azapropazone	UWECO
Buprenorphine	Use
Codeine	Use
Dextropropoxyphene	Use
Diclofenac	UWECO
Diflunisal	Use
Dihydrocodeine	Use
Dipipanone	Use
Fenbufen	Use
Fenoprofen	Use
Fentanyl	Use
Flurbiprofen	Use
Ibuprofen	Use
Indomethacin	Use
Ketoprofen	UWC
Ketorolac	UWECO
Meclofenamic acid	UWC
Mefenamic acid	UWC
Meloxicam	UWC
Methadone	Use
Morphine	Use
Nabumetone	ND/Avoid
Nalbuphine	Use
Naproxen	Use
Oxaprozin	UWC
Oxyphenbutazone	UWECO
Paracetamol	Use
Pentazocine	Avoid
Pethidine	Use
Phenylbutazone	Avoid
Piroxicam	UWC
Pyrazolone	Avoid
Sufentanil	Use
Sulindac	Use
Tenoxicam	UWC
Tiaprofenic acid	Use
Tilidine	UWECO
Tolmetin	ND/Avoid
Tramadol	UWC

Arthritis and gout

Allopurinol	Use
Auranofin	UWECO
Aurothiomalate	UWECO
Benzbromarone	UWECO
Colchicine	Use
Gold	UWECO
Penicillamine	Use
Probenecid	UWECO
Sulfasalazine	Avoid
Sulfinpyrazone	UWECO

Antibiotics: aminoglycosides

Amikacin	Use
Aminoglycosides	Use
Gentamicin	Use
Kanamycin	Use
Netilmicin	Use
Streptomycin	Use
Tobramycin	Use

Antibiotics: penicillins

Amoxycillin	Use
Ampicillin	Use
Benzathine benzylpenicillin	Use
Benzylpenicillin	Use
Clavulanate	Use
Cloxacillin	Use
Flucloxacillin	Use
Penicillin	Use
Phenoxymethylpenicillin	Use
Piperacillin	Use
Pivampicillin	UWECO
Procaine penicillin	Use
Talampicillin	Use
Tazobactam	ND/Avoid

Antibiotics: cephalosporins

Cefaclor	UWC
Cefadroxil	UWC
Cefalexin	UWC
Cefalotin	UWC
Cefamandole	UWC
Cefazolin	UWC
Cefepime	UWC
Cefixime	UWC
Cefotaxime	UWC
Cefoxitin	UWC
Cefpodoxime	UWC
Cefradine	UWC
Ceftazidime	UWC
Ceftibuten	UWC
Ceftriaxone	UWC
Cefuroxime	UWC
Cephalexin	UWC
Cephalothin	UWC
Cephradine	UWC

Antibiotics: macrolides and tetracyclines

Azithromycin	UWECO
Chlortetracycline	UWECO
Clarithromycin	UWC

Clindamycin	Avoid
Dirithromycin	UWECO
Doxycycline	Avoid
Erythromycin	Avoid
Lincomycin	UWECO
Lymecycline	UWECO
Minocycline	UWECO
Oxytetracycline	UWECO
Rolitetracycline	UWECO
Roxithromycin	UWECO
Tetracycline	UWECO
Troleandomycin	UWECO

Antibiotics: quinolones

Cinoxacin	UWECO
Ciprofloxacin	UWECO
Enoxacin	UWECO
Lomefloxacin	UWECO
Norfloxacin	UWECO
Ofloxacin	UWECO
Sparfloxacin	UWECO

Antibiotics: sulphonamides

Sulfadimethoxine	Avoid
Sulfadimidine	Avoid
Sulfadoxine	Avoid
Sulfafurazole	Avoid
Sulfamerazine	Avoid
Sulfamethizole	Avoid
Sulfamethoxazole	Avoid
Sulfamoxole	Avoid
Sulfathiazole	Avoid

Antibiotics: other

Chloramphenicol	Avoid
Co-trimoxazole	Avoid
Fosfomycin	ND/Avoid
Fusidic acid	Use
Imipenem	ND/Avoid
Loracarbef	UWECO
Mandelamine	Use
Methenamine	Use
Nalidixic acid	Avoid
Nitrofurantoin	UWECO
Nitroxoline	ND/Avoid
Phenazopyridine	ND/Avoid
Pipemidic acid	UWECO
Spectinomycin	UWC
Trimethoprim	UWECO
Vancomycin	Use

Antibiotics: tuberculosis and leprosy

Clofazimine	ND/Avoid
Dapsone	Avoid
Ethambutol	Use
Ethamsylate	Avoid
Ethionamide	Avoid
Isoniazid	UWECO
Pyrazinamide	Avoid
Rifabutin	UWECO
Rifampicin	UWECO
Terizidone	ND/Avoid

Antibiotics: malaria and protozoal infections

Atovaquone	ND/Avoid
Chloroquine	Use
Halofantrine	ND/Avoid
Mefloquine	UWC
Melarsoprol	ND/Avoid
Metronidazole	UWECO
Nimorazole	UWECO
Pentamidine	Use
Primaquine	Use
Proguanil	Use
Pyrimethamine	Use
Quinine	Use
Sodium stilbogluconate	ND/Avoid
Suramin	UWECO
Tinidazole	UWECO

Antifungals

Amphotericin	UWC
Econazole	UWECO
Fluconazole	UWECO
Flucytosine	Use
Griseofulvin	Avoid
Itraconazole	UWECO
Ketoconazole	Avoid
Miconazole	Avoid
Terbinafine	UWECO

Anthelmintics

Albendazole	UWC
Diethylcarbamazine	ND/Avoid
Ivermectin	ND/Avoid
Levamisole	ND/Avoid
Mebendazole	UWC
Metrifonate	ND/Avoid
Niclosamide	ND/Avoid
Oxamniquine	ND/Avoid
Piperazine	ND/Avoid
Praziquantel	UWECO
Pyrantel	ND/Avoid
Tiabendazole	UWC

Antivirals

Aciclovir	Use
Didanosine	ND/Avoid
Famciclovir	Use
Foscarnet	ND/Avoid
Ganciclovir	Use
Indinavir	UWECO
Lamivudine	UWECO
Ritonavir	UWECO
Saqinavir	ND/Avoid
Valaciclovir	Use
Zalcitabine	Use
Zidovudine	UWECO

Antiarrhythmics

Amiodarone	Avoid
Bretylium	ND/Avoid
Disopyramide	UWC
Flecainide	ND/Avoid
Mexiletine	ND/Avoid
Procainamide	Use
Propafenone	ND/Avoid
Quinidine	Use

Diuretics

Amiloride	Use
Bendroflumethiazide	UWC
Bumetanide	Use
Chlortalidone	UWC
Cyclopenthiazide	Use
Furosemide	UWC
Hydrochlorothiazide	Use
Indapamide	UWECO
Metolazone	UWECO
Piretanide	ND/Avoid
Spironolactone	UWC
Torasemide	ND/Avoid
Triamterene	Use
Xipamide	UWECO

Hypertension and angina

Acebutolol	Use
Amlodipine	UWECO
Atenolol	Use
Benazepril	UWC
Captopril	UWC
Carteolol	Use
Carvedilol	Use
Cilazapril	UWC
Diazoxide	Use

Dihydralazine	Avoid
Diltiazem	UWC
Doxazosin	UWC
Enalapril	UWC
Esmolol	Use
Felodipine	UWECO
Fosinopril	UWC
Glyceryl trinitrate	Use
Hydralazine	UWECO
Indoramin	ND/Avoid
Isosorbide	UWC
Isradipine	UWECO
Labetalol	Use
Lacidipine	UWECO
Lisinopril	UWC
Losartan	ND/Avoid
Methyldopa	Avoid
Metoprolol	Use
Minoxidil	UWECO
Nadolol	Use
Nifedipine	UWECO
Nitrendipine	UWECO
Nitroprusside	UWC
Oxprenolol	Use
Oxyprenolol	Use
Pentaerythrityl tetranitrate	UWC
Perindopril	UWC
Pindolol	Use
Prazosin	Use
Propranolol	Use
Quinapril	UWC
Ramipril	UWC
Reserpine	Use
Sotalol	Use
Terazosin	UWC
Timolol	Use
Trandolapril	UWC
Verapamil	UWC

Lipid lowering agents

Acipimox	UWC
Bezafibrate	UWC
Clofibrate	UWC
Colestipol	Use
Colestyramine	Use
Fenofibrate	UWECO
Fluvastatin	UWECO
Gemfibrozil	UWC
Nicotinic acid	UWC
Pravastatin	UWECO
Probucol	Use
Simvastatin	UWECO

Other cardiovascular drugs

Digoxin	Use
Dobutamine	Use
Dopamine	Use
Isoprenaline	UWC
Lanatoside	Use
Pentifylline	ND/Avoid
Pentoxifylline	UWECO
Phentolamine	Use
Proscillaridin	ND/Avoid

Anticoagulants, fibrinolytics and haemostatics

Alteplase	UWC
Aminocaproic acid	Use
Anistreplase	UWC
Antithrombin iii	Use
Aprotinin	ND/Avoid
Dalteparin	Use
Dipyridamole	Use
Enoxaparin	Use
Heparin	Use
Pentosan polysulphate sodium	UWC
Phytomenadione	Use
Plasminogen activator	Use
Streptokinase	Use
Ticlopidine	ND/Avoid
Tinzaparin	Use
Tranexamic acid	Use
Urokinase	Use
Warfarin	Use

Blood components and plasma substitutes

Albumin	Use
Blood coagulation factors	Use
Dextran	Use
Hetastarch	Use
Polygeline	Use

Antihistamines and anti-allergics

Acrivastine	Nil yet
Alimemazine	UWC
Astemizole	UWECO
Azatadine	ND/Avoid
Cetirizine	UWC
Chlorphenamine	Use
Clemastine	Avoid
Cromoglicic acid	Use
Cyproheptadine	ND/Avoid
Dexchlorpheniramine	Use
Diphenhydramine	Use
Doxylamine	ND/Avoid
Epinephrine	Use
Hydroxyzine	UWECO
Loratadine	UWC
Mebhydrolin	ND/Avoid
Mepyramine	UWC
Oxatomide	ND/Avoid
Promethazine	Use
Terfenadine	UWECO
Triprolidine	UWC

Asthma

Aminophylline	Avoid
Etofylline	Avoid
Ketotifen	UWC
Oxtriphylline	Avoid
Salbutamol	Use
Theophylline	UWECO

Corticosteroids

Betamethasone	UWC
Cortisone	Use
Dexamethasone	Use
Hydrocortisone	Use
Methylprednisolone	Use
Prednisolone	Use
Prednisone	Use
Triamcinolone	UWC

Coughs and colds

Carbocisteine	ND/Avoid
Clobutinol	ND/Avoid
Dextromethorphan	Use
Fedrilate	ND/Avoid
Guaifenesin	UWECO
Isoaminile	ND/Avoid
Noscapine	UWC
Pholcodine	Use
Pseudoephedrine	Use

Ulcers and dyspepsia

Alginic acid	Use
Aluminium salts	Use
Bismuth	Use
Calcium carbonate	Use
Cimetidine	UWC
Cisapride	ND/Avoid
Dicycloverine	Use
Dimethicone	Use
Famotidine	UWC
Lansoprazole	UWECO
Magaldrate	Use
Magnesium salts	Use

Misoprostol	UWECO
Nizatidine	UWC
Omeprazole	UWECO
Oxethazaine	ND/Avoid
Pantoprazole	UWC
Pirenzepine	Use
Ranitidine	UWC
Roxatidine	UWC
Simethicone	Use
Sodium bicarbonate	Use
Sodium lauryl sulphate	Use
Sucralfate	Use

Antiemetics

Betahistine	UWECO
Buclizine	ND/Avoid
Cinnarizine	UWECO
Cyclizine	Use
Dimenhydrinate	Avoid
Domperidone	Use
Granisetron	ND/Avoid
Metoclopramide	UWC
Ondansetron	ND/Avoid
Prochlorperazine	Use
Scopolamine	Use
Tropisetron	ND/Avoid

Constipation, diarrhoea and inflammatory bowel disease

Bisacodyl	UWC
Codeine phosphate	Use
Difenoxin	Use
Diphenoxylate	Use
Ispaghula husk	Use
Kaolin	Use
Lactitol	Use
Lactulose	Use
Loperamide	Use
Mesalazine	Use
Methylcellulose	Use
Olsalazine	UWC
Pectin	Use
Polyethylene glycol	UWC
Psyllium	Use
Senna	Use
Sodium lauryl sulphoacetate	Use
Sodium phosphate	Use
Sodium sulphate	Use
Sorbic acid	Use
Sorbitol	Use
Sterculia	Use

Antispasmodics

Belladonna	Avoid
Dipyrone	Avoid
Hyoscine	UWECO
Hyoscine butylbromide	Avoid
Mebeverine	UWECO
Propantheline	Use

Sedatives and hypnotics

Alprazolam	UWC
Bromazepam	UWC
Buspirone	ND/Avoid
Chlordiazepoxide	UWECO
Clobazam	UWC
Clomethiazole	UWC
Clorazepate	UWC
Diazepam	UWC
Flunitrazepam	UWC
Flurazepam	UWECO
Ketazolam	UWC
Loprazolam	UWC
Lorazepam	Use
Lormetazepam	UWC
Meprobamate	Avoid
Midazolam	UWC
Nitrazepam	UWECO
Oxazepam	UWC
Prazepam	UWC
Quazepam	UWC
Temazepam	Use
Triazolam	Use
Triclofos	UWC
Zopiclone	ND/Avoid

Antidepressants

Amitriptyline	UWECO
Citalopram	UWC
Clomipramine	UWC
Desipramine	UWECO
Dibenzepin	UWECO
Dosulepin	UWECO
Fluoxetine	UWC
Fluvoxamine	UWECO
Imipramine	UWECO
Lofepramine	UWECO
Maprotiline	UWC
Mianserin	Use
Moclobemide	ND/Avoid
Nefazodone	UWECO
Nortriptyline	UWECO
Paroxetine	UWC
Phenelzine	UWECO
Sertraline	ND/Avoid
Tranylcypromine	UWECO
Trazodone	UWECO
Trimipramine	UWECO
Venlafaxine	UWECO

Antipsychotics

Chlorpromazine	Use
Clotiapine	ND/Avoid
Clozapine	UWC
Droperidol	Use
Flupentixol	UWECO
Fluphenazine	Use
Haloperidol	Use
Lithium	Use
Periciazine	UWC
Perphenazine	UWC
Pimozide	ND/Avoid
Pipotiazine	Use
Risperidone	ND/Avoid
Sulpiride	UWECO
Thioridazine	UWC
Trifluoperazine	Use
Zuclopenthixol	UWECO

Epilepsy

Carbamazepine	Avoid
Clonazepam	UWC
Ethosuximide	UWECO
Gabapentin	Use
Lamotrigine	ND/Avoid
Mesuximide	Avoid
Oxcarbazepine	Avoid
Phenobarbital	Avoid
*Phenytoin	Avoid
Primidone	Avoid
Topiramate	ND/Avoid
Valproic acid	Avoid
Vigabatrin	UWC

Migraine

Clonidine	Avoid
Dihydroergotamine	Avoid
Ergotamine	Avoid
Flunarizine	Avoid
Methysergide	Avoid
Nimodipine	UWECO
Pizotifen	ND/Avoid
Sumatriptan	UWC

Parkinson's disease

Amantadine	Use
Benserazide	ND/Avoid
Biperiden	ND/Avoid

Bromocriptine	UWECO
Carbidopa	ND/Avoid
Levodopa	UWC
Orphenadrine	Avoid
Pergolide	ND/Avoid
Selegiline	UWECO
Trihexyphenidyl	Avoid

Central muscle relaxants and cholinesterase inhibitors

Baclofen	UWECO
Chlormezanone	UWECO
Chlorzoxazone	ND/Avoid
Dantrolene	UWECO
Methocarbamol	ND/Avoid
Methylphenidate	Use
Pemoline	UWECO
Pyridostigmine	UWC

Diabetes

Acarbose	ND/Avoid
Acetohexamide	UWECO
Chlorpropamide	UWECO
Glibenclamide	UWECO
Gliclazide	UWECO
Glipizide	UWC
Insulin	Use
Metformin	Use
Tolbutamide	Avoid

Endocrine

Cabergoline	Avoid
Carbimazole	Use
Corticotropin	Use
Danazol	Avoid
Desmopressin	UWC
Fludrocortisone	UWC
Glucagon	Use
Levothyroxine	Use
Liothyronine	Use
Metergoline	Avoid
Octreotide	Use
Ornipressin	UWC
Propylthiouracil	Use
Somatostatin	Use
Somatropin	UWC
Tetracosactide	Use
Vasopressin	Use

Gynaecology, obstetrics and sex hormones

Androgens	UWECO
Buserelin	Use
Clomiphene	Use
Cyproterone	UWC
Dinoprost	UWECO
Dinoprostone	UWECO
Dydrogesterone	UWECO
Ergometrine	Avoid
Estradiol	UWECO
Estriol	UWECO
Estrone	UWECO
Ethinylestradiol	UWECO
Ethylestrenol	ND/Avoid
Etynodiol	UWECO
Fluoxymesterone	ND/Avoid
Gestodene	UWECO
Gestrinone	ND/Avoid
Gnrh agonists	Use
Gonadorelin	Use
Goserelin	Use
Hexoprenaline	UWC
Hydroxyprogesterone	UWECO
Leuprorelin	Use
Levonorgestrel	UWECO
Lhrh	Use
Lynestrenol	UWECO
Medrogestone	UWECO
Medroxyprogesterone	UWECO
Mesterolone	UWECO
Mestranol	UWECO
Metenolone	ND/Avoid
Methyltestosterone	UWECO
Nafarelin	Use
Nandrolone	ND/Avoid
Norethisterone	Avoid
Norethynodrel	UWECO
Norgestrel	UWECO
Oestrogens	UWECO
Oral contraceptives	UWECO
Oxymethalone	ND/Avoid
Oxytocin	Use
Progesterone	UWECO
Ritodrine	ND/Avoid
Testosterone	UWECO
Tibolone	UWECO

Osteoporosis

Alendronic acid	UWC
Calcitonin	Use
Clodronic acid	UWC
Etidronic acid	UWC
Pamidronic acid	UWC
Sodium acid phosphate	Use

Chemotherapy and immunosuppression

Aminoglutethimide	Avoid
Antilymphocyte immunoglobulin	Use
Asparaginase	UWC
Azathioprine	Use
Bleomycin	ND/Avoid
Busulfan	UWECO
Carboplatin	UWC
Carmustine	ND/Avoid
Chlorambucil	Avoid
Ciclosporin	UWECO
Cisplatin	Use
Cyclophosphamide	UWECO
Cytarabine	ND/Avoid
Dacarbazine	ND/Avoid
Dactinomycin	Use
Daunorubicin	UWC
Docataxel	UWECO
Doxorubicin	UWC
Epirubicin	UWC
Estramustine	ND/Avoid
Etoposide	ND/Avoid
Fluorouracil	UWC
Flutamide	UWECO
Formestane	UWECO
Fosfestrol	UWECO
Gemcitabine	ND/Avoid
Hydroxycarbamide	UWECO
Idarubicin	UWECO
Ifosfamide	UWECO
Interferon	UWC
Lomustine	ND/Avoid
Melphalan	UWC
Mercaptopurine	UWECO
Methotrexate	UWC
Mitomycin	ND/Avoid
Mitoxantrone	ND/Avoid
Paclitaxel	ND/Avoid
Procarbazine	UWECO
Tamoxifen	UWECO
Teniposide	ND/Avoid
Tioguanine	UWECO
Vinblastine	UWECO
Vincristine	UWC
Vindesine	UWECO

Dermatology

Acitretin	UWECO
Etretinate	Avoid
Isotretinoin	UWECO
Methoxsalen	ND/Avoid
Trioxysalen	ND/Avoid

Radiology

Amidotrizoate	Use
Barium sulphate	Use
Iotalamic acid	UWC

Urology

Bethanechol	ND/Avoid
Distigmine	Use
Finasteride	UWECO
Flavoxate	ND/Avoid
Oxybutynin	UWECO

Obesity

Amfepramone	Avoid
Amphetamines	UWECO
Cathine	Nil yet
Dexfenfluramine	Avoid
Fenfluramine	UWECO
Mazindol	ND/Avoid
Phendimetrazine	UWECO
Phentermine	UWECO

Miscellaneous

Acetazolamide	Use
Acetylcysteine	Use
Calcium carbimide	ND/Avoid
Cannabis	Avoid
Cyanidanol-3	ND/Avoid
Demeclocycline	UWECO
Desferrioxamine	Use
Dextrose	Use
Dimercaprol	Use
Disulfiram	ND/Avoid
Epoietin alfa	Use
Fluorescein	Use
Fluoride	Use
Glucose	Use
Immunoglobulins (all)	Use
Ipecacuanha	ND/Avoid
Iron salts	Use
Methaqualone	UWECO
Obidoxime	ND/Avoid
Pyridoxine	Use
Silymarin	ND/Avoid
Sodium fluoride	Use
Thiamine	Use
Ursodeoxycholate	Use
Vaccines (all)	Use
Vitamins	Use

ALPHABETICAL LISTING

Acarbose	ND/Avoid
Acebutolol	Use
Acetazolamide	Use
Acetohexamide	UWECO
Acetylcysteine	Use
Aciclovir	Use
Acipimox	UWC
Acitretin	UWECO
Acrivastine	Nil yet
Albendazole	UWC
Albumin	Use
Alclofenac	UWC
Alcuronium	UWC
Alendronic acid	UWC
Alfentanil	Use
Alginic acid	Use
Alimemazine	UWC
Allopurinol	Use
Alprazolam	UWC
Alteplase	UWC
Aluminium salts	Use
Amantadine	Use
Amfepramone	Avoid
Amidotrizoate	Use
Amikacin	Use
Amiloride	Use
Aminocaproic acid	Use
Aminoglutethimide	Avoid
Aminoglycosides	Use
Aminophylline	Avoid
Amiodarone	Avoid
Amitriptyline	UWECO
Amlodipine	UWECO
Amoxycillin	Use
Amphetamines	UWECO
Amphotericin	UWC
Ampicillin	Use
Androgens	UWECO
Anistreplase	UWC
Antilymphocyte immunoglobulin	Use
Antithrombin iii	Use
Aprotinin	ND/Avoid
Asparaginase	UWC
Aspirin	Use
Astemizole	UWECO
Atenolol	Use
Atovaquone	ND/Avoid
Atracurium	UWC
Atropine	Use
Auranofin	UWECO
Aurothiomalate	UWECO
Azapropazone	UWECO
Azatadine	ND/Avoid
Azathioprine	Use
Azithromycin	UWECO
Baclofen	UWECO
Barium sulphate	Use
Belladonna	Avoid
Benazepril	UWC
Bendroflumethiazide	UWC
Benserazide	ND/Avoid
Benzathine benzylpenicillin	Use
Benzbromarone	UWECO
Benzylpenicillin	Use
Betahistine	UWECO
Betamethasone	UWC
Bethanechol	ND/Avoid
Bezafibrate	UWC
Biperiden	ND/Avoid
Bisacodyl	UWC
Bismuth	Use
Bleomycin	ND/Avoid
Blood coagulation factors	Use
Bretylium	ND/Avoid
Bromazepam	UWC
Bromocriptine	UWECO
Buclizine	ND/Avoid
Bumetanide	Use
Bupivacaine	Use
Buprenorphine	Use
Buserelin	Use
Buspirone	ND/Avoid
Busulfan	UWECO
Cabergoline	Avoid
Calcitonin	Use
Calcium carbimide	ND/Avoid
Calcium carbonate	Use
Cannabis	Avoid
Captopril	UWC
Carbamazepine	Avoid
Carbidopa	ND/Avoid
Carbimazole	Use
Carbocisteine	ND/Avoid
Carboplatin	UWC
Carmustine	ND/Avoid
Carteolol	Use
Carvedilol	Use
Cathine	Nil yet
Cefaclor	UWC
Cefadroxil	UWC
Cefalexin	UWC
Cefalotin	UWC
Cefamandole	UWC
Cefazolin	UWC
Cefepime	UWC
Cefixime	UWC
Cefotaxime	UWC
Cefoxitin	UWC
Cefpodoxime	UWC
Cefradine	UWC
Ceftazidime	UWC
Ceftibuten	UWC
Ceftriaxone	UWC
Cefuroxime	UWC
Cephalexin	UWC
Cephalothin	UWC
Cephradine	UWC
Cetirizine	UWC
Chlorambucil	Avoid
Chloramphenicol	Avoid
Chlordiazepoxide	UWECO
Chlormezanone	UWECO
Chloroquine	Use
Chlorphenamine	Use
Chlorpromazine	Use
Chlorpropamide	UWECO
Chlortalidone	UWC
Chlortetracycline	UWECO
Chlorzoxazone	ND/Avoid
Ciclosporin	UWECO
Cilazapril	UWC
Cimetidine	UWC
Cinnarizine	UWECO
Cinoxacin	UWECO
Ciprofloxacin	UWECO
Cisapride	ND/Avoid
Cisplatin	Use
Citalopram	UWC
Clarithromycin	UWC
Clavulanate	Use
Clemastine	Avoid
Clindamycin	Avoid
Clobazam	UWC
Clobutinol	ND/Avoid
Clodronic acid	UWC
Clofazimine	ND/Avoid
Clofibrate	UWC
Clomethiazole	UWC
Clomiphene	Use
Clomipramine	UWC
Clonazepam	UWC

Clonidine	Avoid	Dibenzepin	UWECO	Enoxaparin	Use	Flunarizine	Avoid
Clorazepate	UWC	Diclofenac	UWECO	Epinephrine	Use	Flunitrazepam	UWC
Clotiapine	ND/Avoid	Dicycloverine	Use	Epirubicin	UWC	Fluorescein	Use
Cloxacillin	Use	Didanosine	ND/Avoid	Epoietin alfa	Use	Fluoride	Use
Clozapine	UWC	Diethylcarbamazine	ND/Avoid	Ergometrine	Avoid	Fluorouracil	UWC
Co-trimoxazole	Avoid	Difenoxin	Use	Ergotamine	Avoid	Fluoxetine	UWC
Codeine	Use	Diflunisal	Use	Erythromycin	Avoid	Fluoxymesterone	ND/Avoid
Codeine phosphate	Use	Digoxin	Use	Esmolol	Use	Flupentixol	UWECO
Colchicine	Use	Dihydralazine	Avoid	Estradiol	UWECO	Fluphenazine	Use
Colestipol	Use	Dihydrocodeine	Use	Estramustine	ND/Avoid	Flurazepam	UWECO
Colestyramine	Use	Dihydroergotamine	Avoid	Estriol	UWECO	Flurbiprofen	Use
Corticotropin	Use	Diltiazem	UWC	Estrone	UWECO	Flutamide	UWECO
Cortisone	Use	Dimenhydrinate	Avoid	Ethambutol	Use	Fluvastatin	UWECO
Cromoglicic acid	Use	Dimercaprol	Use	Ethamsylate	Avoid	Fluvoxamine	UWECO
Cyanidanol-3	ND/Avoid	Dimethicone	Use	Ethinylestradiol	UWECO	Formestane	UWECO
Cyclizine	Use	Dinoprost	UWECO	Ethionamide	Avoid	Foscarnet	ND/Avoid
Cyclopenthiazide	Use	Dinoprostone	UWECO	Ethosuximide	UWECO	Fosfestrol	UWECO
Cyclophosphamide	UWECO	Diphenhydramine	Use	Ethylestrenol	ND/Avoid	Fosfomycin	ND/Avoid
Cyproheptadine	ND/Avoid	Diphenoxylate	Use	Etidronic acid	UWC	Fosinopril	UWC
Cyproterone	UWC	Dipipanone	Use	Etofylline	Avoid	Furosemide	UWC
Cytarabine	ND/Avoid	Dipyridamole	Use	Etomidate	Avoid	Fusidic acid	Use
Dacarbazine	ND/Avoid	Dipyrone	Avoid	Etoposide	ND/Avoid	Gabapentin	Use
Dactinomycin	Use	Dirithromycin	UWECO	Etretinate	Avoid	Ganciclovir	Use
Dalteparin	Use	Disopyramide	UWC	Etynodiol	UWECO	Gemcitabine	ND/Avoid
Danazol	Avoid	Distigmine	Use	Famciclovir	Use	Gemfibrozil	UWC
Dantrolene	UWECO	Disulfiram	ND/Avoid	Famotidine	UWC	Gentamicin	Use
Dapsone	Avoid	Dobutamine	Use	Fedrilate	ND/Avoid	Gestodene	UWECO
Daunorubicin	UWC	Docataxel	UWECO	Felodipine	UWECO	Gestrinone	ND/Avoid
Demeclocycline	UWECO	Domperidone	Use	Fenbufen	Use	Glibenclamide	UWECO
Desferrioxamine	Use	Dopamine	Use	Fenfluramine	UWECO	Gliclazide	UWECO
Desipramine	UWECO	Dosulepin	UWECO	Fenofibrate	UWECO	Glipizide	UWC
Desmopressin	UWC	Doxazosin	UWC	Fenoprofen	Use	Glucagon	Use
Dexamethasone	Use	Doxorubicin	UWC	Fentanyl	Use	Glucose	Use
Dexchlorpheniramine	Use	Doxycycline	Avoid	Finasteride	UWECO	Glyceryl trinitrate	Use
Dexfenfluramine	Avoid	Doxylamine	ND/Avoid	Flavoxate	ND/Avoid	Glycopyrronium	Use
Dextran	Use	Droperidol	Use	Flecainide	ND/Avoid	Gnrh agonists	Use
Dextromethorphan	Use	Dydrogesterone	UWECO	Flucloxacillin	Use	Gold	UWECO
Dextropropoxyphene	Use	Econazole	UWECO	Fluconazole	UWECO	Gonadorelin	Use
Dextrose	Use	Enalapril	UWC	Flucytosine	Use	Goserelin	Use
Diazepam	UWC	Enflurane	UWC	Fludrocortisone	UWC	Granisetron	ND/Avoid
Diazoxide	Use	Enoxacin	UWECO	Flumazenil	Use	Griseofulvin	Avoid

Guaifenesin	UWECO	Kaolin	Use	Mazindol	ND/Avoid	Mianserin	Use
Halofantrine	ND/Avoid	Ketazolam	UWC	Mebendazole	UWC	Miconazole	Avoid
Haloperidol	Use	Ketoconazole	Avoid	Mebeverine	UWECO	Midazolam	UWC
Halothane	Use	Ketoprofen	UWC	Mebhydrolin	ND/Avoid	Minocycline	UWECO
Heparin	Use	Ketorolac	UWECO	Meclofenamic acid	UWC	Minoxidil	UWECO
Hetastarch	Use	Ketotifen	UWC	Medrogestone	UWECO	Misoprostol	UWECO
Hexoprenaline	UWC	Labetalol	Use	Medroxyprogesterone	UWECO	Mitomycin	ND/Avoid
Hydralazine	UWECO	Lacidipine	UWECO	Mefenamic acid	UWC	Mitoxantrone	ND/Avoid
Hydrochlorothiazide	Use	Lactitol	Use	Mefloquine	UWC	Mivacurium	UWC
Hydrocortisone	Use	Lactulose	Use	Melarsoprol	ND/Avoid	Moclobemide	ND/Avoid
Hydroxycarbamide	UWECO	Lamivudine	UWECO	Meloxicam	UWC	Morphine	Use
Hydroxyprogesterone	UWECO	Lamotrigine	ND/Avoid	Melphalan	UWC	Nabumetone	ND/Avoid
Hydroxyzine	UWECO	Lanatoside	Use	Mepivacaine	UWC	Nadolol	Use
Hyoscine	UWECO	Lansoprazole	UWECO	Meprobamate	Avoid	Nafarelin	Use
Hyoscine butylbromide	Avoid	Leuprorelin	Use	Mepyramine	UWC	Nalbuphine	Use
Ibuprofen	Use	Levamisole	ND/Avoid	Mercaptopurine	UWECO	Nalidixic acid	Avoid
Idarubicin	UWECO	Levodopa	UWC	Mesalazine	Use	Naloxone	Use
Ifosfamide	UWECO	Levonorgestrel	UWECO	Mesterolone	UWECO	Nandrolone	ND/Avoid
Imipenem	ND/Avoid	Levothyroxine	Use	Mestranol	UWECO	Naproxen	Use
Imipramine	UWECO	Lhrh	Use	Mesuximide	Avoid	Nefazodone	UWECO
Immunoglobulins	Use	Lidocaine	Use	Metenolone	ND/Avoid	Neostigmine	Use
Indapamide	UWECO	Lincomycin	UWECO	Metergoline	Avoid	Netilmicin	Use
Indinavir	UWECO	Liothyronine	Use	Metformin	Use	Niclosamide	ND/Avoid
Indomethacin	Use	Lisinopril	UWC	Methadone	Use	Nicotinic acid	UWC
Indoramin	ND/Avoid	Lithium	Use	Methaqualone	UWECO	Nifedipine	UWECO
Insulin	Use	Lofepramine	UWECO	Methenamine	Use	Nimodipine	UWECO
Interferon	UWC	Lomefloxacin	UWECO	Methocarbamol	ND/Avoid	Nimorazole	UWECO
Iotalamic acid	UWC	Lomustine	ND/Avoid	Methotrexate	UWC	Nitrazepam	UWECO
Ipecacuanha	ND/Avoid	Loperamide	Use	Methoxsalen	ND/Avoid	Nitrendipine	UWECO
Iron salts	Use	Loprazolam	UWC	Methylcellulose	Use	Nitrofurantoin	UWECO
Isoaminile	ND/Avoid	Loracarbef	UWECO	Methyldopa	Avoid	Nitroprusside	UWC
Isoflurane	UWC	Loratadine	UWC	Methylphenidate	Use	Nitrous oxide	Use
Isoniazid	UWECO	Lorazepam	Use	Methylprednisolone	Use	Nitroxoline	ND/Avoid
Isoprenaline	UWC	Lormetazepam	UWC	Methyltestosterone	UWECO	Nizatidine	UWC
Isosorbide	UWC	Losartan	ND/Avoid	Methysergide	Avoid	Norethisterone	Avoid
Isotretinoin	UWECO	Lymecycline	UWECO	Metoclopramide	UWC	Norethynodrel	UWECO
Ispaghula husk	Use	Lynestrenol	UWECO	Metolazone	UWECO	Norfloxacin	UWECO
Isradipine	UWECO	Magaldrate	Use	Metoprolol	Use	Norgestrel	UWECO
Itraconazole	UWECO	Magnesium salts	Use	Metrifonate	ND/Avoid	Nortriptyline	UWECO
Ivermectin	ND/Avoid	Mandelamine	Use	Metronidazole	UWECO	Noscapine	UWC
Kanamycin	Use	Maprotiline	UWC	Mexiletine	ND/Avoid	Obidoxime	ND/Avoid

Octreotide	Use
Oestrogens	UWECO
Ofloxacin	UWECO
Olsalazine	UWC
Omeprazole	UWECO
Ondansetron	ND/Avoid
Oral contraceptives	UWECO
Ornipressin	UWC
Orphenadrine	Avoid
Oxamniquine	ND/Avoid
Oxaprozin	UWC
Oxatomide	ND/Avoid
Oxazepam	UWC
Oxcarbazepine	Avoid
Oxethazaine	ND/Avoid
Oxprenolol	Use
Oxtriphylline	Avoid
Oxybuprocaine	Use
Oxybutynin	UWECO
Oxymethalone	ND/Avoid
Oxyphenbutazone	UWECO
Oxyprenolol	Use
Oxytetracycline	UWECO
Oxytocin	Use
Paclitaxel	ND/Avoid
Pamidronic acid	UWC
Pancuronium	Use
Pantoprazole	UWC
Paracetamol	Use
Paroxetine	UWC
Pectin	Use
Pemoline	UWECO
Penicillamine	Use
Penicillin	Use
Pentaerythrityl tetranitrate	UWC
Pentamidine	Use
Pentazocine	Avoid
Pentifylline	ND/Avoid
Pentosan polysulphate sodium	UWC
Pentoxifylline	UWECO
Pergolide	ND/Avoid
Periciazine	UWC
Perindopril	UWC
Perphenazine	UWC
Pethidine	Use
Phenazopyridine	ND/Avoid
Phendimetrazine	UWECO
Phenelzine	UWECO
Phenobarbital	Avoid
Phenoxymethylpenicillin	Use
Phentermine	UWECO
Phentolamine	Use
Phenylbutazone	Avoid
Phenytoin	Avoid
Pholcodine	Use
Phytomenadione	Use
Pimozide	ND/Avoid
Pindolol	Use
Pipemidic acid	UWECO
Piperacillin	Use
Piperazine	ND/Avoid
Pipotiazine	Use
Pirenzepine	Use
Piretanide	ND/Avoid
Piroxicam	UWC
Pivampicillin	UWECO
Pizotifen	ND/Avoid
Plasminogen activator	Use
Polyethylene glycol	UWC
Polygeline	Use
Pravastatin	UWECO
Prazepam	UWC
Praziquantel	UWECO
Prazosin	Use
Prednisolone	Use
Prednisone	Use
Prilocaine	Use
Primaquine	Use
Primidone	Avoid
Probenecid	UWECO
Probucol	Use
Procainamide	Use
Procaine	Use
Procaine penicillin	Use
Procarbazine	UWECO
Prochlorperazine	Use
Progesterone	UWECO
Proguanil	Use
Promethazine	Use
Propafenone	ND/Avoid
Propantheline	Use
Propofol	Use
Propranolol	Use
Propylthiouracil	Use
Proscillaridin	ND/Avoid
Pseudoephedrine	Use
Psyllium	Use
Pyrantel	ND/Avoid
Pyrazinamide	Avoid
Pyrazolone	Avoid
Pyridostigmine	UWC
Pyridoxine	Use
Pyrimethamine	Use
Quazepam	UWC
Quinapril	UWC
Quinidine	Use
Quinine	Use
Ramipril	UWC
Ranitidine	UWC
Reserpine	Use
Rifabutin	UWECO
Rifampicin	UWECO
Risperidone	ND/Avoid
Ritodrine	ND/Avoid
Ritonavir	UWECO
Rocuronium	UWC
Rolitetracycline	UWECO
Roxatidine	UWC
Roxithromycin	UWECO
Salbutamol	Use
Saqinavir	ND/Avoid
Scopolamine	Use
Selegiline	UWECO
Senna	Use
Sertraline	ND/Avoid
Sevoflurane	UWC
Silymarin	ND/Avoid
Simethicone	Use
Simvastatin	UWECO
Sodium acid phosphate	Use
Sodium bicarbonate	Use
Sodium fluoride	Use
Sodium lauryl sulphate	Use
Sodium lauryl sulphoacetate	Use
Sodium phosphate	Use
Sodium stilbogluconate	ND/Avoid
Sodium sulphate	Use
Somatostatin	Use
Somatropin	UWC
Sorbic acid	Use
Sorbitol	Use
Sotalol	Use
Sparfloxacin	UWECO
Spectinomycin	UWC
Spironolactone	UWC
Sterculia	Use
Streptokinase	Use
Streptomycin	Use
Sucralfate	Use
Sufentanil	Use
Sulfadimethoxine	Avoid
Sulfadimidine	Avoid
Sulfadoxine	Avoid
Sulfafurazole	Avoid
Sulfamerazine	Avoid
Sulfamethizole	Avoid
Sulfamethoxazole	Avoid
Sulfamoxole	Avoid
Sulfasalazine	Avoid
Sulfathiazole	Avoid
Sulfinpyrazone	UWECO
Sulindac	Use

Sulpiride	UWECO	Thiamine	Use	Tranexamic acid	Use	Vancomycin	Use
Sumatriptan	UWC	Thiopental	Avoid	Tranylcypromine	UWECO	Vasopressin	Use
Suramin	UWECO	Thioridazine	UWC	Trazodone	UWECO	Vecuronium	UWC
Suxamethonium	Use	Tiabendazole	UWC	Triamcinolone	UWC	Venlafaxine	UWECO
Talampicillin	Use	Tiaprofenic acid	Use	Triamterene	Use	Verapamil	UWC
Tamoxifen	UWECO	Tibolone	UWECO	Triazolam	Use	Vigabatrin	UWC
Tazobactam	ND/Avoid	Ticlopidine	ND/Avoid	Triclofos	UWC	Vinblastine	UWECO
Temazepam	Use	Tilidine	UWECO	Trifluoperazine	Use	Vincristine	UWC
Teniposide	ND/Avoid	Timolol	Use	Trihexyphenidyl	Avoid	Vindesine	UWECO
Tenoxicam	UWC	Tinidazole	UWECO	Trimethoprim	UWECO	Vitamins	Use
Terazosin	UWC	Tinzaparin	Use	Trimipramine	UWECO	Warfarin	Use
Terbinafine	UWECO	Tioguanine	UWECO	Trioxysalen	ND/Avoid	Xipamide	UWECO
Terfenadine	UWECO	Tobramycin	Use	Triprolidine	UWC	Zalcitabine	Use
Terizidone	ND/Avoid	Tolbutamide	Avoid	Troleandomycin	UWECO	Zidovudine	UWECO
Testosterone	UWECO	Tolmetin	ND/Avoid	Tropisetron	ND/Avoid	Zopiclone	ND/Avoid
Tetracaine	Use	Topiramate	ND/Avoid	Urokinase	Use	Zuclopenthixol	UWECO_
Tetracosactide	Use	Torasemide	ND/Avoid	Ursodeoxycholate	Use		
Tetracycline	UWECO	Tramadol	UWC	Valaciclovir	Use		
Theophylline	UWECO	Trandolapril	UWC	Valproic acid	Avoid		

APPENDIX 10:

PORPHYRIA QUESTIONNAIRE

The questionnaire completed by the members of the large R59W-positive family described in Chapter 17 is reproduced on the following pages.

Form A. For those testing positive.

QUESTIONNAIRE

The questions which follow are designed to allow us to determine more about the effect that porphyria has on our patients. Please be kind enough to answer it fully and truthfully. The information is strictly confidential, and will not be shown to anyone other than the investigators.

In the case of children, it is perfectly acceptable for the parent to complete the form on behalf of the child.

The first set of questions allows us to check that we have your records correct and lets us find out more about you.	
1. What is your name?	
2. How old are you now?	
3. When were you born?	
4. Are you male or female?	
5. What is your father's name?	
6. What is your mother's name?	
7. What work do you do?	

The following questions tell us more about a diagnosis and how it was made.	
8. Had you had porphyria tests before the ones done by us as part of this study?	
If yes, answer these questions 9. Where were they done?	
10. Were they positive or negative?	
11. Did you think you had porphyria before you received your gene test result?	
If so, answer these questions 12. You assumed you had porphyria because there was "porphyria in your family"	
13. Because you had been told previously that tests were positive	

14. Because you were experiencing symptoms which you believed were due to porphyria	
If so..... 15. Describe these symptoms.	

The following questions tell us more about your complaints

Acute attacks

16. Do you believe you have ever had an acute attack?	
If so, for each attack, provide the following details 17. At what age did you have yor first attack?	
18. Write down the year of each attack.	
19. Describe the symptoms you experienced.	
20. Did you notice whether you had dark urine?	
21. Were you in hospital?	
22. Did you develop paralysis?	
23. How long did the attack last?	
24. Can you write down the names of any medicines that you believe have made your porphyria worse?	
If so, answer this question 25. Give brief details of each.	

Skin disease

26. Do you believe you suffer from porphyric skin disease?	
If so, answer these questions 27. Have you at any stage experienced the "typical" skin lesions, that is blisters and open sores which heal very slowly and a fragile skin?	

28. Is your face affected?	
29. Are your hands affected?	
30. Are your forearms affected?	
31. Are your feet affected?	
32. How old were you when you first became aware of skin disease?	
33. Have you developed any other form of skin problem which you believe is related to porphyria?	
If so…… 34. Describe it.	
35. Do you suffer from any other skin disease?	
If so….. 36. Name it or describe it.	

If you are more than 40 years old.

37. Have you noticed any change in your porphyria as you get older?	
If so…… 38. Please describe the changes.	

The following questions are for females only.

39. Are you old enough to have started menstruating?	
If so, answer these questions 40. Did you notice any change in your porphyria after puberty?	
If so…… 41. Describe the changes.	
42. Do you believe that your periods have or had any effect on your porphyria?	
If so, answer this question	

43. Describe the changes.	
44. Have you experienced the menopause ("change of life")?	
If so, answer question 45 45. Has there been any change in your porphyria since then?	
If so....: 46. Please describe the effect.	

The following questions are for males only.

47. Have you undergone puberty?	
If so, answer this question 48. Did you notice any change in your porphyria after puberty?	
If so..... 49. Describe the changes.	

The following questions tell us more about any other illnesses you may have.

50. Do you suffer from hypertension (high blood pressure)?	
51. Do you suffer from heart disease?	
If so..... 52. Please give more details.	
53. Have you ever been treated for anxiety, depression or any other emotional or mental illness?	
If so..... 54. Give more details.	
55. Have you ever consulted a doctor about abdominal pain, whether due to porphyria or not?	
If so..... 56. Give more details.	

Please tell us how to contact you with a view to arranging to see you in October.	
57. Phone: (home)?	
58. Phone (work)?	
59. Fax?	
60. Email?	
61. Other?	
62. Address?