

Clinical, Biochemical, Haematological and Molecular
Epidemiology of Erythropoietic Protoporphyrin. A United
Kingdom Cross-Sectional Study

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Dedication

I dedicate this thesis to my wife Jane
and sons Rory, Angus & Hamish

Abstract

This thesis concerns erythropoietic protoporphyria (EPP, MIM 177000), a rare photodermatosis with systemic complications which results from an inherited partial deficiency of ferrochelatase (FECH), the terminal enzyme of haem biosynthesis. Excessive formation of the substrate, protoporphyrin IX, results in protoporphyriaemia and accumulation in erythrocytes, plasma, skin and liver, prior to excretion in the bile. Protoporphyrin can absorb light, damaging surrounding tissues through the generation of free radicals, and clinically manifesting as painful photosensitivity within minutes of skin exposure to sunlight. Microcytic anaemia occurs in 20 – 60% of patients. Liver dysfunction and liver failure occurs in up to 35% and 4% of patients respectively. Management of EPP is based mainly on minimising the effects of sunlight by use of visible light sunscreens, clothing and behavioural measures to avoid direct sunlight. Despite advances in the biochemistry and genetic inheritance, there remains a lack of prospective clinical data for EPP. Previous studies have been retrospective analyses, often from tertiary centres specialising in the treatment of liver disease. Such studies typically report subjects in common, many from large family pedigrees. These limitations of ascertainment, bias and small population size have restricted accurate investigation of fundamental clinical issues relating to EPP.

This thesis describes a prospective cross-sectional study of United Kingdom patients with EPP, designed to describe in detail clinical features, assess liver and haematopoietic function and to characterise vitamin D status. Genotyping was additionally undertaken and statistical analysis of measured variables performed to identify significant associations which might predict clinical status.

The study cohort included 223 patients from 193 families (114 females, 109 males median age 34 years; range 5-87 years) most of whom were white Caucasians apart from two. Of 178 patients with ‘dominant EPP’ (dEPP), 48% of women and 33% of men were anaemic. Iron stores were decreased by two-thirds but normal serum soluble transferrin receptor-1 and iron concentrations suggested that erythropoiesis

was not limited by iron supply. Liver dysfunction was present in 25% dEPP and significantly associated with total erythrocyte protoporphyrin (TEP) concentration, male sex, age, anaemia and alcohol consumption, but not with genotype. Vitamin D deficiency and insufficiency was present in 17% and 91% of 201 subjects respectively seen over a seven-month period between January and July. Both insufficiency and deficiency were significantly associated with the TEP and inversely with the time in minutes to the onset of symptoms following sunlight exposure. A new clinical EPP subtype was identified of seasonal palmar keratoderma, characterised by low erythrocyte protoporphyrin concentrations and recessive inheritance. This condition may carry a lower risk of liver disease than other patients with recessively-inherited EPP. Investigation of individuals, clinically presenting as EPP, but in whom no *FECH* mutation was identifiable, has identified a new condition, X-linked dominant protoporphyria, which results from a gain-of-function mutation of the C-terminal of 5-amino levulinate synthase 2, the initial rate-regulating enzyme of erythroid haem biosynthesis. X-linked dominant protoporphyria causes neither anaemia nor iron overload.

Declaration of originality

I have personally designed and carried out all the clinical studies, working in association with several others who are acknowledged. I have personally seen all the subjects of this study, visiting them in hospital outpatients departments close to their homes. I also computed the results of the clinical studies with assistance from a statistician (acknowledged). The majority of laboratory data was generated by automated sample analysis. Porphyrin analysis was undertaken by Mrs Jacqueline Woolf and staff in the University Hospital of Wales Biochemistry laboratories. Genotyping was undertaken by Dr Sharon Whatley and her staff, again in the University Hospital of Wales Biochemistry laboratories.

I declare this thesis to be entirely my own work. Parts of the study have been published and presented at national and international meetings. A list and copies of resulting publications and abstracts is included at the end of this thesis.

Acknowledgements & collaborations

I am indebted to all the subjects who participated in this study.

The following colleagues are acknowledged as collaborators who have contributed to this work:

| | |
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| Professor George Elder | Emeritus Professor of Medical Biochemistry Cardiff University, School of Medicine |
| Dr Sharon Whatley | Clinical Biochemist Cardiff University, School of Medicine |

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Clinical, Biochemical, Haematological and Molecular Epidemiology of Erythropoietic Protoporphyrria. A United Kingdom Cross-Sectional Study

1. Introduction

1.1 Statement of the problem

The porphyrias are disorders associated with inherited or acquired enzyme deficiencies in the haem biosynthetic pathway. Erythropoietic protoporphyria (EPP) was first described by Magnus in 1961.¹ The condition is caused by a partial deficiency of ferrochelatase (FECH), the terminal enzyme of haem biosynthesis. This leads to accumulation of its substrate, protoporphyrin IX (PP) in erythrocytes, plasma, liver and skin. PP in the skin, through its photodynamic action, causes life-long acute photosensitivity of sun exposed areas, while its accumulation in the liver leads to potentially fatal liver failure in 1-4% of patient series.^{2,3} In most families, increased protoporphyrin formation sufficient to cause photosensitivity occurs only in individuals who have inherited a low expression *FECH* gene polymorphism (present in about 13% of the UK population)⁴ *trans* to a loss of function *FECH* mutation on the other allele that abolishes or markedly decreases FECH activity.⁵

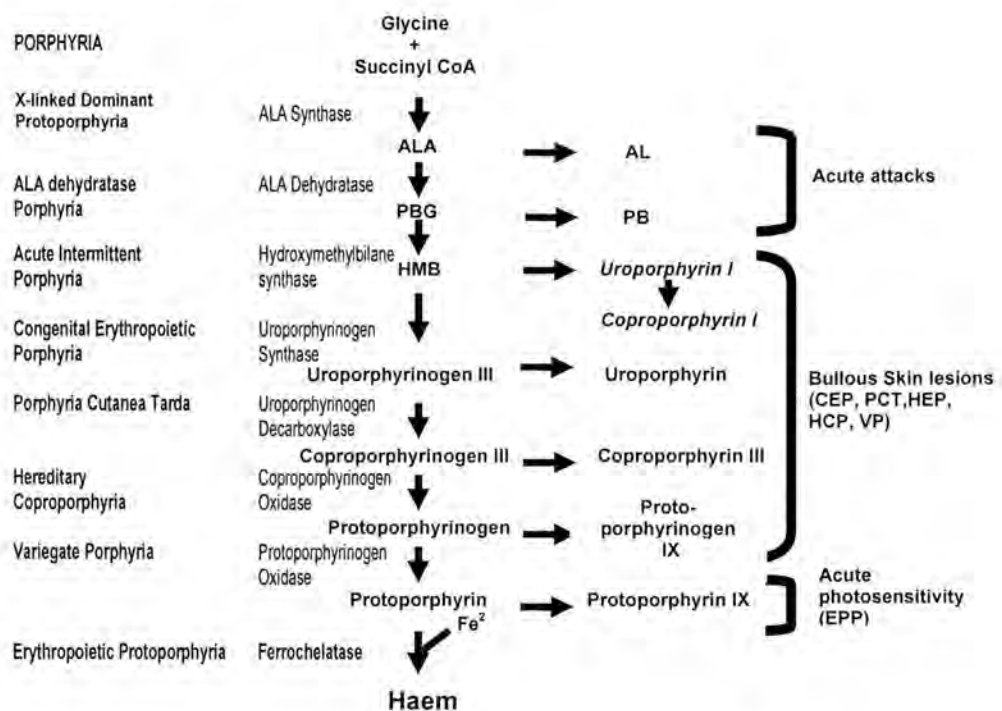
The clinical features of EPP, which are distinct from those of other cutaneous porphyrias, have been described in various case reports and small series, none containing more than 32 patients.^{6,7} Larger studies have focussed on particular aspects of the disease, such as genetics⁸ or liver disease,^{2,9} but have omitted clinical details. Information from such sources may be biased by selection and may not reflect the full clinical spectrum. In addition, no study has addressed the psychosocial consequences of EPP, despite the apparent severity of symptoms and substantial changes to lifestyle necessary to avoid episodes of acute photosensitivity.

1.2 The porphyrias

The porphyrias are a group of metabolic disorders associated with inherited or acquired enzyme deficiencies in the haem biosynthetic pathway resulting in

excessive quantities of porphyrins or their precursors. This overproduction not only results in products which are physiologically unuseful, but which are also toxic. There are eight enzymes involved in the synthesis of haem from glycine and succinyl CoA, four mitochondrial and four cytoplasmic, and in general, a specific enzyme deficiency corresponds with a specific clinical type of porphyria (figure 1). Prior to this study, the only enzyme in the synthetic chain in which deficiency had not been associated with a porphyria was the first, 5-aminolevulinate synthase (ALAS). Erythropoietic protoporphyria (EPP) is due to a relative deficiency of the final enzyme in the haem biosynthetic pathway, FECH, resulting in accumulation of its substrate, PP.

Figure 1. The haem biosynthetic pathway.



1.3 Historical perspective of the porphyrias ¹⁰

Porphyrias may have been clinically recognised for hundreds of years without appreciation of the underlying disorder. For example Hippocrates recorded what was

probably one of the first clinical descriptions of an acute porphyric attack.¹¹ Legends of werewolves, suffering from photophobia, hypertrichosis and skin mutilation were believed to be based on the features of congenital erythropoietic porphyria. The most famous suspected porphyria sufferer was King George III (1738-1820).^{12,13} The biochemical study of porphyrins commenced in the 19th century, following the demonstration by Scherer that adding concentrated sulphuric acid to dried powdered blood allowed separation of iron from the remaining material. When alcohol was subsequently added to this iron-free blood, a purple-red colour appeared which was subsequently named ‘haematin’.¹⁴⁻¹⁶ By the latter half of the 19th century further purification work of this blood derivative led to the observation of red fluorescence,¹⁷ the introduction of the term ‘hämatorphyrin’ – from the Greek for blood and purple (αἷμα - haima + πορφύρος - porphuros) – to describe the colour,¹⁸ and the demonstration of the sharp near-ultraviolet absorption band of haemoglobin.¹⁹

The first accurate clinical description of a porphyria was made in 1874, with the identification of haematin in the urine of the patient (diagnosed initially as “atypical leprosy”, but in retrospect as classical congenital erythropoietic porphyria (CEP)).²⁰ The biochemist who analysed the pigments from this first patient, perspicaciously suggested its aetiology to be an error in haemoglobin synthesis, and not secondary to anomalous haemoglobin degradation.²¹ The description in 1898 of two brothers with skin photosensitivity and urinary ‘haematoporphyrin’ excretion led to the suggestion of a close connection between the cutaneous manifestations and the urinary pigment.²² Further understanding of the link between clinical diseases and porphyrin pigments followed the introduction by the company Kast and Bayer in the late 1880s of the chemically related hypnotics *sulphonal* and *trional*. Between 5 and 10% of women treated with the drugs, developed acute porphyric attacks of abdominal pain, pareses, and port-wine discoloured urine. At the time the urinary pigment was shown to be closely related to the sulphuric acid-derived haematoporphyrin,^{23,24} and it is now recognised that these cases were acute intermittent porphyria (AIP).²⁵

Subsequently, a number of other porphyrins were chemically identified, purified and analysed. The connection of porphyrins to clinical disease was advanced by the

extensive work of Günther on his laboratory assistant Mathias Petry, who suffered from CEP.²⁶ It was Günther also first introduced the term 'hämatoporphyrin' to describe the group of diseases, and introduced a clinical classification (congenital, acute idiopathic, acute toxic and chronic), which with some modifications, is still in use today. Mathias Petry was subsequently employed by another German chemist, Hans Fischer. On Petry's death, fluorescence microscopy of his bone marrow erythropoietic cells at autopsy, revealed significantly elevated levels of porphyrin. Fischer's further work, was seminal in developing understanding of porphyrin chemistry and the haem biosynthetic pathway. He identified porphyrinogens as the synthetic substrates, and introduced the terms uroporphyrin and coproporphyrin in his description of the decarboxylation that occurs between the two intermediaries, and he is credited with first using the stand alone term 'porphyrin'. Developing work by Kämmerer and Hijmans van den Bergh in the 1920's, he described the two isomeric porphyrins and subsequently called the porphyrin described by Kämmerer protoporphyrin, demonstrating that it existed in 15 isomeric forms of which only No. IX occurs in nature. He also conducted clinical experiments into the photosensitising effects of the natural porphyrins and their excretion physiology.¹⁰

Additional work in the early 20th century led to understanding of the biosynthetic relationships, and the introduction of more modern chemical nomenclature (e.g. uro- and coproporphyrin, and the term porphyrin replacing haematoporphyrin). The demonstration that porphyrins caused photosensitisation was shown in two classic experiments: in the first, Hausmann added haematoporphyrin to cultures of the protozoa *Paramecia* – the organisms continued to grow normally in darkness, but were killed by exposure to bright daylight.²⁷ The second was the self-administration by Meyer-Betz of haematoporphyrin.²⁸ Within 30 minutes of an intra-venous infusion, he experienced liver discomfort and general pain, but no skin symptoms. Exposure to sunlight the next day resulted in an immediate pricking, burning sensation of exposed skin, followed by erythema, pain and oedema within 10-15 minutes. A photograph of his severely swollen face illustrates the photosensitivity, that lasted for at least 6 weeks (figure 2).

Later milestones included the demonstration of inheritance,²⁹ identification of porphobilinogen,^{30,31} the emergence of quantitative porphyrin analysis,^{32,33} and thin layer chromatography.³⁴ The introduction in the 1950's of the Wood's light to both biochemical and clinical practice (a source of UVA from which visible light has been excluded by a nickel oxide filter), allowed rapid screening of serum samples for fluorescence.

Figure 2. Meyer-Betz after self administration of haematoporphyrin and exposure to sunlight of the right face and left hand whilst seated on a tram.



During the 20th century the individual clinical conditions were more clearly delineated and classified. For example, Günther's chronic porphyria was renamed porphyria cutanea tarda (PCT). New entities described included acute intermittent porphyria (AIP),³⁵ hereditary coproporphyria (HCP),³⁶ and variegate porphyria (VP).³⁷ In 1926, the first known case of EPP was described as "hydroa aestivale without haematoporphyrinuria",³⁸ although a formal diagnosis of EPP in this individual was only confirmed in the early 1960s.³⁸ In 1953, a similar case of a 12-year-old male was reported again with "atypical hydroa aestivale" without excessive urinary porphyrin, but with elevated erythrocyte protoporphyrin and faecal protoporphyrin³⁹ probably also representing EPP, although some of the biochemical results identified at the time were atypical for the condition. It was not until 1961 that the condition was clearly delineated, in a report of a 35-year-old man with light-induced urticaria and maximal photosensitivity to short wavelength visible light at 400-410nm, by Magnus et al.¹ They demonstrated that it was the red cell component of blood in which the majority of the elevated protoporphyrin was detected. Significant numbers of other cases were quickly reported in the years that followed.^{6,40-42} A case of liver dysfunction in EPP was reported shortly after the initial description,⁴³ followed by other similar cases of protoporphyrin liver disease, some of which were fatal.^{44,45}

Table 1. Historical description of porphyrias

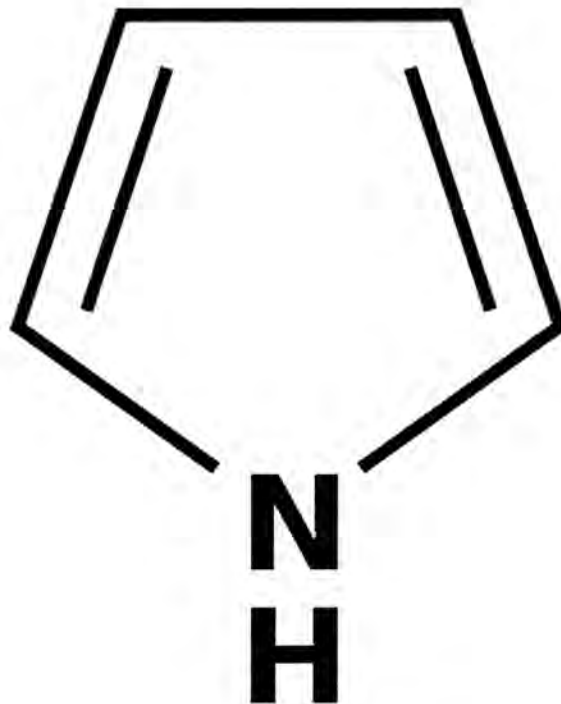
| Porphyria | Date of Description | First Author |
|--------------------------------------|----------------------------|----------------------------|
| X-linked dominant protoporphyrinuria | 2008 | Whatley ⁴⁶ |
| ALA dehydratase porphyria | 1983 | Doss ⁴⁷ |
| Acute intermittent porphyria | 1951 | Watson ³⁵ |
| Congenital erythropoietic porphyria | 1874 | Schultz ²⁰ |
| Porphyria cutanea tarda | 1911 | Günther ²⁶ |
| Hepatoerythropoietic porphyria | 1969 | Piñol-Aguadé ⁴⁸ |
| Hereditary coproporphyria | 1955 | Berger ³⁶ |
| Variegate porphyria | 1963 | Dean ³⁷ |
| Erythropoietic protoporphyrinuria | 1961 | Magnus ¹ |

1.4 Biochemistry of porphyrias and haem

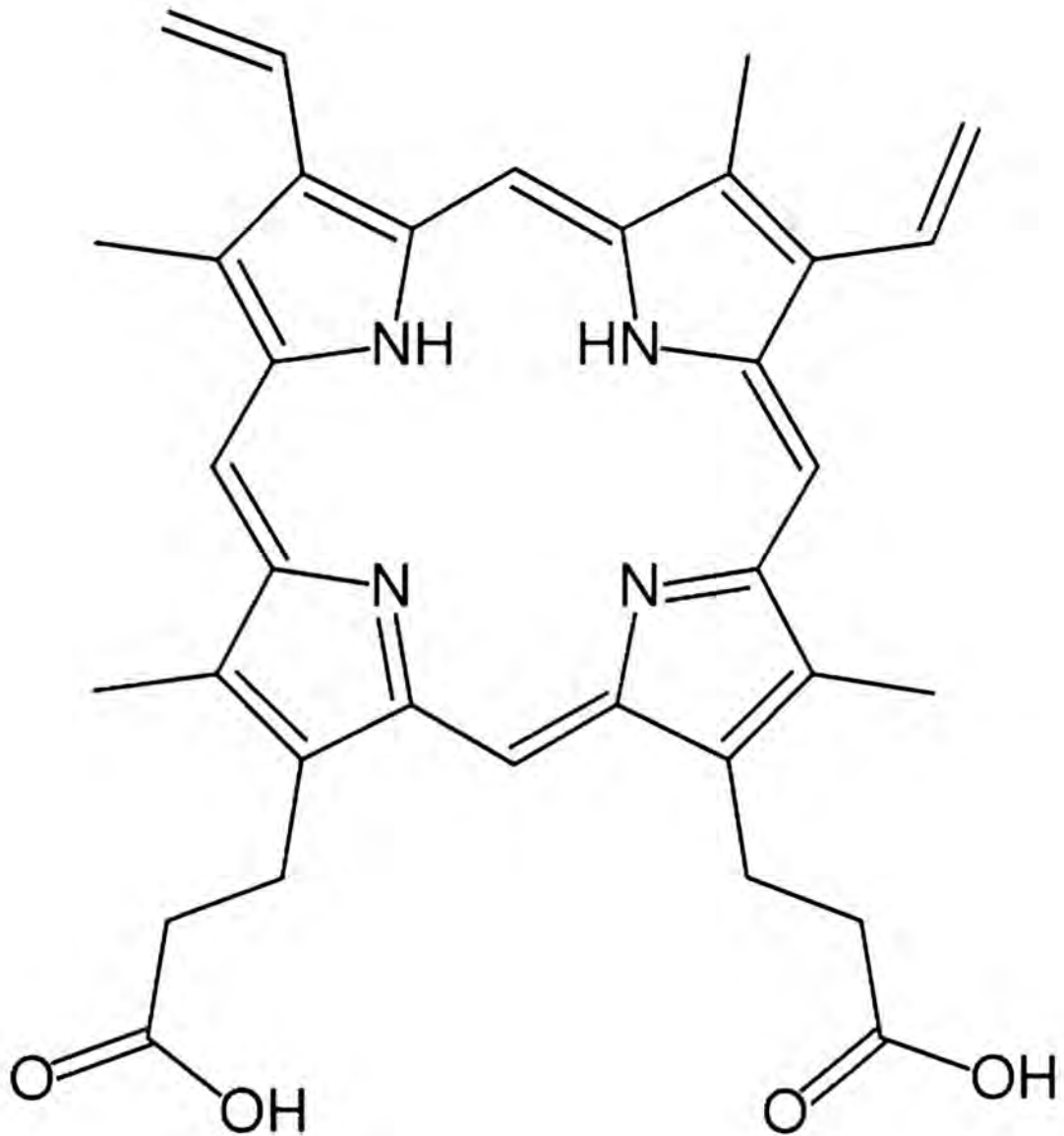
Pyrroles are 5-membered aromatic compounds with a basic formula of C_4H_4NH (figure 3a), formed by the condensation of glycine and succinyl-CoA. Porphyrins are composed of four pyrrole molecules joined to produce a tetrapyrrole ring (figure 3b). Substitution of side chains to the basic pyrrole molecule alters the biochemical and physical properties such as lipid solubility and absorption spectra. During haem biosynthesis, other than protoporphyrin IX (a porphyrin), the actual substrates for the enzymes catalysing haem biosynthesis are porphyrinogens, reduced forms of porphyrins. Porphyrinogens are highly unstable molecules that rapidly oxidise to porphyrins resulting in their irreversible removal from the biosynthetic pathway.

Figure 3.

a) A pyrrole ring.



b) A tetrapyrrole ring consisting of a large ring composed of four pyrroles, each a ring structure. All porphyrins are tetrapyrroles.



Porphyrins and metalloporphyrins are found in almost all living organisms and have a range of functions. Their ring structure confers enhanced molecular stability, a variety of redox levels for electron transfer, and acts as a powerful site for the

chelation of transitional metals, greatly enhancing their catalytic capacity. This is particularly evident when a metalloporphyrin binds to proteins, as in the catalase enzymes. Porphyrins are sometimes described as the pigments of life, providing through photosynthesis, a “free” energy source. Haem is involved in electron transport systems and the metabolic utilisation of photosynthetic energy and energy from food, and oxygen.⁴⁹ Haem is a cofactor in the reduction of sulphite and nitrites, and may participate in the oxidization of deleterious substances. The few organisms which do not have porphyrins rely on complex organic molecules (themselves produced by photosynthesis), or inorganic redox pairs such as hydrogen and sulphate ions, where the hydrogen most commonly results from the action of other bacteria on organic molecules. Thus porphyrin molecules, through their ability to provide energy gradients, have been central to evolution as we know it, although whether photosynthesis was a requirement for the origin of self-replicating life is less clear.

The Granick Hypothesis for the evolutionary development of porphyrin biosynthesis suggested that the pathway was built in a forward direction.⁵⁰ It states that each pathway product had a function in it's time, but through mutations or chance events, was superseded by the next more efficient or more useful molecule.⁵⁰ This supports the Oparin-Haldane hypothesis of life beginning in the oceans as the start of the pathway consists of ionic, highly water-soluble simple molecules and finishes with lipid-soluble complex molecules. Thus the location of each step in the biosynthetic pathway recapitulates the sequence of evolution: ALAS is found in the mitochondria, adjacent to the tricarboxylic acid cycle, the origin of succinyl CoA. The next four enzymes are all in the cytosol, and their substrates are highly water-soluble. The final three, where the substrates and products are progressively less water-soluble, are in the lipid mitochondrial membranes. The negatively charged earlier soluble products will not bind to the negatively charged cytosolic proteins, whilst keeping the insoluble substrates close to their enzymes in the compartments of the mitochondria prevents their loss through binding to hydrophobic proteins and cell membranes.

The haem biosynthetic pathway is essentially the sequential formation of a cyclical iron metalloprotein capable of electron transfer, from less complex precursors, catalysed by eight enzymes. It starts with the condensation of glycine and succinyl Coenzyme A (CoA) to form 5-aminolevulinate (ALA) under the control of the mitochondrial enzyme ALAS. For each molecule of porphyrin produced, eight molecules of glycine and succinyl CoA are required. A series of enzymes (the next four intra-cytoplasmic, and the remaining three within the mitochondrion) control the conversion of ALA first to porphobilinogen (PBG), a pyrrole, hydroxymethylbilane, a linear tetrapyrrole and then uroporphyrinogen III, the first cyclical tetrapyrrole in the pathway. The first of two alternative biosynthetic forks occurs from uroporphyrinogen III: subsequent formation of corrins, tetrapyrroles with two pyrrolic rings joined directly, leads to synthesis of hydroxycobalamin (vitamin B₁₂), and the siderohaems and nickel complexes found in phylogenetically early bacteria. The second biosynthetic fork occurs at the level of protoporphyrin, branching to produce either haem or chlorophyll. Insertion of magnesium into the protoporphyrin ring, catalysed by magnesium chelatase, followed by additional light-dependent formation of a fifth ring from propionic acid side chains (cyclization), reduction of a double bond in one of the other rings, and attachment of a phytol tail, convert the molecule into chlorophyll. Non-photosynthetic organisms have lost this ability to insert magnesium, and instead iron is inserted by ferrochelatase to produce haem. In mammals, haem biosynthesis is most active in erythropoietic tissue, where it is required for haemoglobin synthesis, and in hepatic tissue, where it forms the basis for various haem-containing enzymes such as cytochrome P450, the catalases and cytochrome oxidase. Thus the haem biosynthetic pathway is one of the most highly conserved metabolic pathways, with identical steps in all cells of primitive bacteria, plants and mammals. This high degree of conservation reflects the importance of the pathway in producing critical components of living systems.

1.5 Protoporphyrin IX and ferrochelatase

Protoporphyrin IX (PP), the terminal porphyrin in the haem biosynthetic pathway, is the substrate for the enzyme ferrochelatase (FECH). This enzyme is found on the inner mitochondrial membrane and catalyses the insertion of ferrous iron into the

centre of the molecule to form haem. The enzyme is synthesised in the cytosol as a larger precursor form, and imported into the mitochondria, dependant on the membrane potential. The human FECH maps to chromosome 18 q21.3 and spans 45kb over a total of 11 exons (a nucleic acid sequence that is represented in the mature form of an RNA molecule after portions of a precursor RNA (introns) have been removed).⁵¹ The complementary DNA (cDNA - DNA synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase) has an open reading frame of 1269 base pairs, which encodes a protein of 423 amino acid residues. The leader sequence of 62 amino acids appears important for the mitochondrial targeting;⁵² removal of which yields the mature enzyme of 369 amino acids. The transcripts are identical in both erythroid and non-erythroid cells: in all tissues there are two mRNA of different lengths, both of which generate an identical FECH protein, and are produced by two different polyadenylation sites (site for addition of a tail composed of multiple adenosine monophosphates - important for the nuclear export, translation and stability of the mRNA).

The description of the three-dimensional crystal structure of FECH (figure 4) enhanced understanding of the catalytic mechanism and allowed predictions of the effects of mutations on enzymatic activity.⁵³⁻⁵⁵ The human FECH enzyme is a homodimer (a protein composed of two identical polypeptide chains) metalloenzyme, which in contrast to prokaryotic FECH, has an additional 30-50 amino acid residues at the carboxyl terminus. This may function by providing three cysteine residues to co-ordinate with an iron-sulphur (2Fe-2S) cluster which acts as an essential co-factor.⁵⁶ Iron-sulphur clusters are found in a variety of metalloenzymes involved in oxidation-reduction reactions, particularly mitochondrial electron transfer. Although FECH activity is dependant on the presence of this cluster, its exact function in FECH has yet to be clearly elucidated, as it apparently does not provide a direct catalytic role: it has been postulated to mediate electron transfer or play a structural role by maintaining the correct conformation.⁵⁷ In erythropoietic protoporphyria (EPP), FECH activity is less than 30% that of normal subjects,⁵⁸ which leads to accumulation of it's substrate, PP. The enzyme is active in

cells producing haem, including erythroid precursors in the bone marrow and hepatocytes.^{59,60}

Radio-labelling studies suggest that in EPP the majority of PP (approximately 80%) originates from the bone marrow, with most of the remainder generated by the liver.⁶¹ This is evidenced post-liver transplantation in EPP by the demonstrated reduction in excess protoporphyrin and improvement in symptoms in the presence of comparable raised faecal protoporphyrin levels, suggesting increased excretion but similar levels of production.^{62,63} In contrast, those EPP individuals who have successfully received bone marrow transplantation have normal porphyrin concentrations in erythrocytes, plasma and faeces.^{64,65}

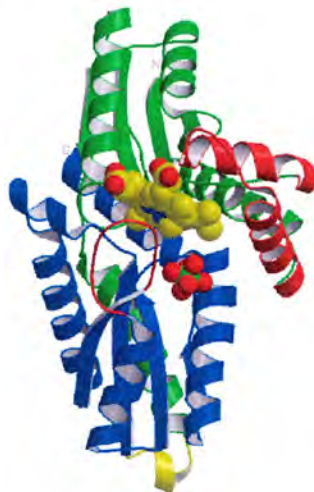
The concentration of PP in circulating erythrocytes falls as it diffuses across the cell membrane into the plasma. Exposure of erythrocytes to light results in increased release.⁶⁶ As the PP molecule is relatively hydrophobic, due to the presence of only two carboxylic side chains, the majority is bound to albumin, haemopexin and other proteins, including lipoproteins, with only a low level being unbound.⁶⁷ As a result, PP is not excreted in the urine: it is excreted in bile, although a proportion is re-absorbed through an enterohepatic circulation.⁶⁸ Due to physical proximity, free and protein-bound PP is taken up by endothelial cells membranes either through a concentration gradient, membrane lipoprotein receptors or by direct contact with erythrocyte membranes, resulting in a higher concentration than elsewhere in the dermis.⁶⁹ The lipoproteins may also be important in the uptake of PP by the liver, through their specific receptors.⁶⁹

Figure 4. The three dimensional crystal structure of *Bacillus subtilis* ferrochelatase showing an elongated shape of approximately 60 Å. The protein has two main approximately symmetrical domains (green and blue) consisting of a central four-stranded β sheet flanked by α helices. The parts of the chain in red build up the walls of a hydrophobic cleft containing conserved amino acids – the presumed catalytic site. (reproduced with permission from Al-Karadaghi et al.)⁵³

(a) empty structure.



(b) containing the protoporphyrin IX - iron complex



Excess PP in the skin and cutaneous blood vessels absorbs light maximally at 400-410 nm (the Soret Band – an absorption band in the blue short wavelength region of the optical absorption spectrum). Visible light of this wavelength passes through window glass and penetrates deeply into the skin. Following absorption of light, PP generates excited electrons with the capacity to transfer their energy to oxygen, or generate free radicals and singlet oxygen. Subsequent tissue damage ensues from complement activation, peroxidation of lipids and protein cross-linking in cellular membranes.⁷⁰⁻⁷⁵

1.6 Regulation of haem biosynthesis

The chief regulatory step in haem production is ALAS,^{76,77} upon which haem exerts a negative feedback, although factors that limit each of the other enzyme steps in haem production have been described; for example a secondary control lies at the level of PBG deaminase (PBGD), which is inhibited by both copro- and protoporphyrinogen. ALAS is encoded by two different genes: *ALAS2* on the X-chromosome (Xp11.21) is expressed only in erythroid cells, and mutations of this gene cause certain types of X-linked hereditary sideroblastic anaemia. *ALAS1* is located on chromosome 3 (3p21.1) and encodes an ubiquitous enzyme which is readily induced by drugs and other compounds associated with acute attacks of porphyria.⁷⁸ Due to relative ease of tissue sampling, the majority of porphyrin research has focused on hepatic tissue where 15% of the daily haem production is based, mainly for use in cytochromes such as P450. Production here is much more sensitive to intracellular haem levels, and is able to respond more rapidly. Erythroid production of haem is linked to tissue differentiation and has a much slower turnover than hepatic haem.

Haem regulates ALAS in three different ways. Firstly, synthesis of new enzyme may be repressed; secondly, the transfer of the newly synthesised enzyme to the mitochondria may be limited; thirdly, haem decreases the stability of mRNA for ALAS.^{79,80} In hepatic cells, synthesis of ALA is inhibited by haem,⁷⁶ through the post-transcriptional reduction of ALAS mRNA half-life and blocking the

translocation of ALAS into the mitochondrion. The transcription rate does not appear to be affected.⁸⁰

ALAS2 mRNA contains an area in its 5' untranslated region termed the iron responsive element (IRE). This RNA sequence allows specific binding of two iron regulatory proteins (IRP1 and IRP2), which inhibit *ALAS2* mRNA translation.^{81,82} IRP 1 and 2 are activated by iron deficiency whereas high intracellular iron availability results in post-translational modification of IRP-1 and degradation of IRP-2.⁸³ This demonstration of a direct molecular mechanism for iron availability to influence haem biosynthesis, and may be particularly relevant in the case of EPP where oral iron supplementation can lead to differing clinical outcomes. Similar IRE have been shown in the mRNA of ferritin and transferrin,⁸⁴ indicating the probability of multiple mechanisms through which control of porphyrin production is coordinated and regulated.

Each of the other enzymes in the haem biosynthetic pathway is coded by a single gene, although most have features that allow enhanced expression during erythroid differentiation. For example, the *FECH* gene in both erythroid and non-erythroid cell lines transcription is facilitated by a single promoter (the region of DNA regulating gene transcription, located 'upstream' of a gene, containing specific DNA sequences and response elements that provide a binding site for RNA polymerase and transcription factor proteins that recruit RNA polymerase). Two *FECH* polyadenylation sites can produce two mRNAs of different length, both of which are believed to be transcribed to functional FECH.⁸⁵ However binding sites in the promoter region, for example to the transcription factor Sp1, appear to confer preferential erythroid expression.⁸⁶ These probably interact with other erythroid-specific transcription factors such as GATA-1 and NF-E2, whose binding sites have also been identified on the FECH promoter region, permitting tissue-specific expression of FECH in erythroid cells. Furthermore, these transcription factors have binding sites in the promoter regions of other genes such as β -globulin, allowing coordinated production of haem and globin during erythroid cell maturation.

Deficiency of one of the biosynthetic enzymes (excepting ALAS) or failure to produce sufficient haem results in loss of negative feedback, and a consequent enhanced production of porphyrins, particularly the deficient enzyme's substrate. Accumulation of porphyrins leads to the clinical presentation of the specific porphyria. The degree of water solubility of the substrates dictates the excretion pattern: more water-soluble porphyrin precursors in the urine, while lipid soluble compounds are mainly excreted via the bile in the faeces. Acute porphyrias overproduce all the porphyrins and precursors proximal to the deficiency. In contrast, the non-acute porphyrias are characterised by accumulation of porphyrins immediately proximal to the defect in the pathway. The reason for this is not clear, but may be due to a compensatory increase in PBGD and ALAS.

It is believed that the pathogenesis of all the inherited porphyrias has now been defined at a molecular level, with observed genetic heterogeneity within each group. However, some individuals have more than one haem biosynthetic enzyme affected, presenting either as two forms of porphyria in one family, or two forms in one patient. Examples of this concurrent porphyria include the Chester porphyria (AIP and VP), and dual porphyria (VP and PCT).⁸⁷

1.7 Porphyrin-induced photosensitivity

All the porphyrias, except AIP and ALA-dehydratase deficiency, have skin photosensitivity as a clinical feature. Porphyrins absorb light energy in the blue spectrum (400-410 nm: the Soret range), raising electrons into an excited triplet state. Energy can be dissipated to the singlet and ground states by emission, either in the form of light in the red spectrum (600-660 nm), or in the presence of oxygen, through the formation of free radicals (chemically highly reactive molecules due to unpaired electrons) with the potential for tissue damage. Photoactivated protoporphyrin IX (PP) and uroporphyrin (URO) in erythrocytes have been shown to oxidise cellular membrane amino acids, leading to cross-linking of proteins. The resulting inhibition of membrane-bound enzymes, increased membrane permeability, and loss of structural flexibility cause loss of function, damage and cell destruction, particularly from osmotic haemolysis^{74,88} In EPP the major source of cutaneous PP

is the erythrocyte, and the concentration declines over the lifetime of the cells.⁶⁹ Irradiation of erythrocytes increases the release of PP,⁶⁶ probably through photodynamic damage to porphyrin binding sites on globin resulting in lower affinity and release through the plasma membrane. *In vivo*, erythrocytes are exposed to intermittent Soret-band irradiation every time they pass through light-exposed dermal capillaries. *In vitro* simulation of this has shown release of most of the PP and only minimal amounts of haemoglobin, suggesting the cells remain intact.⁸⁹ PP binds to several plasma carriers, such as albumin, low density lipoprotein, high density lipoprotein,^{67,90} allowing PP to be internalised into cell lysosomes through the LDL receptor, or transported to the liver via HDL, akin to cholesterol metabolism. It is not known which out of the carriers or erythrocytes is the most important for the delivery of PP to endothelial cells in the superficial cutaneous blood vessels, but probably all contribute, either through direct proximity or specific receptor-mediated uptake, resulting in a higher PP load in light exposed vessel endothelium. Studies suggest that localisation of PP in plasma membranes results in photo-oxidation of membrane-bound proteins with inhibition of cation transport as an initial event, leading to cell lysis with lipid peroxidation as a later process.⁹¹ The observation of reduced light tolerance on days following an initial exposure to intense visible light is termed the “priming phenomenon”, and probably reflects the photodynamic release of PP on the first day, with subsequent uptake by endothelium and higher local concentrations on subsequent days.^{89,92,93}

In contrast to URO, photoactivated PP also induces calcium and protein kinase-independent release of histamine and serotonin from cutaneous mast cells (present in perivascular locations in the dermis) and loss of their membrane integrity *in vitro*.^{72,94} This is markedly inhibited by catalase, a hydrogen peroxide scavenger, suggesting peroxidation as a mechanism.⁹⁴ It is likely that mast cells release other inflammatory mediators at the same time as the histamine and serotonin, and that other cell types are similarly damaged by photo-activated protoporphyrin to release pro-inflammatory cytokines. Thus while some aspects of photosensitivity experienced by patients with PCT and EPP share common mechanisms, the EPP-specific symptoms of burning, stinging, erythema and oedema may be explained by a direct

effect of singlet oxygen or PP on sensory neurones and mast cells.⁷² Furthermore, the relatively hydrophobic nature of PP and hydrophilic nature of URO may result in differential tissue distribution and cellular damage.

Activation of complement is another important mechanism for photoactivated porphyrin cellular damage. *In vitro* and *in vivo* irradiation of serum from patients with PCT and EPP results in a decrease of both C3 and C5, generating the C5b-9 membrane attack complex and anaphylatoxins, chemotactic for leucocytes.⁹⁵ This finding is not seen in individuals with acquired photodermatoses such as solar urticaria and polymorphic light eruption.^{71,95} The exact mechanism of light-induced complement activation by PP remains to be elucidated, but complement activation is central to the inflammatory response, through the induction of chemotaxis, increased vascular permeability, immunomodulation of T cells and the opsonisation and lysis of cells.

These experimental studies are supported by ultrastructural studies of irradiated skin from individuals with EPP showing degeneration of upper dermal vasculature, with loss of the endothelial cell cohesion,⁹⁶ degranulation of mast cells, and later release of other organelle-bound mediators such as lysosomal acid hydrolases,⁹⁷ in addition to chemotaxis of polymorphonuclear leucocytes.⁹⁵

1.8 Classification and diagnosis of porphyrias

Historically classification of porphyrias has been based either on the major site of excess production (hepatic: liver, or erythropoietic: bone marrow – the tissue specificity resulting from a tissue-specific control of the haem pathway gene expression) or in terms of the presentation: acute porphyrias characterised by central and peripheral nervous system dysfunction (AIP, ADP); non-acute or cutaneous porphyrias, characterised solely by cutaneous photosensitivity (PCT, EPP, CEP); and a third group, mixed porphyrias, in which both acute attacks and photosensitivity are present (VP, HCT).

Formal diagnosis is made on the characteristic porphyrin profiles evident in serum, erythrocytes, stool ⁹⁸ and urine ⁹⁹⁻¹⁰¹ (table 2). A defective enzyme (either due to genetic or environmental factors) will not catalyse its respective substrate effectively leading to accumulation of this substrate and its precursors.

Table 2. Diagnostic features of the porphyrias

| Porphyria | Erythrocytes | Plasma | Urine | Faeces |
|--|--------------------------------------|-------------------------------|---|-----------------------|
| X-linked dominant protoporphyria | ↑ PP ↑ Zn PP | ↑ PP | - | - |
| ALA dehydratase porphyria | ↑ zinc PP ↓ ALAD activity | - | ALA coproporphyrin III | - |
| Acute intermittent porphyria | ↓ PBGD activity | ↑ porphyrin during attacks | ↑ ALA, PBG, URO I | - |
| Congenital erythropoietic protoporphyria | ↑ UPO, zinc PP ↓ UROS activity | ↑ URO I, CP I | ↑ URO I, CP I | ↑ URO I, CP I |
| Porphyria cutanea tarda | - | ↑ URO | ↑ URO I, ISCP, 7-carboxyl porphyrin III | ISCP |
| Hereditary coproporphyria | ↑ zinc PP | ↑ URO | ISCP/CP ratio | ISCP/CP ratio |
| Variegate porphyria | | porphyrin- protein complex | during attack: ↑ CP III, PBG, ALA | PP > CP III > CP I |
| Erythropoietic protoporphyria | ↑ free PP | ↑ PP | - | PP > CP |

1.8.1 Qualitative measures

The neuropathic porphyrias tend to be associated with excessive urinary porphyrin precursor excretion in the urine during an acute attack. Urinary PBG and ALA are

increased in AIP, VP and HCP, with increased ALA alone in ALADP and lead poisoning. Urine, faeces and blood, after appropriate processing if necessary, can be screened for porphyrins by exposure to a Wood's light looking for characteristic red fluorescence. Urine can also be screened for PBG using Ehrlich's reagent (1% sodium *p*-dimethylaminobenzaldehyde in hydrochloric acid, a strong electrophile, which reacts with the electron-rich α -carbon of indole/pyrrole rings to form a blue-coloured adduct, allowing the detection of some indoles, pyrroles and related nitrogen-containing compounds).

Plasma fluorescence scanning is used as the main simple qualitative diagnostic technique to screen for individual porphyrins. The method relies upon the capacity of porphyrins to fluoresce when irradiated with wavelengths near to 400nm, producing characteristic emission spectra. The plasma sample is placed in optical glass cuvettes for spectrofluorometry, and a fluorescence spectrophotometer is zeroed against any buffering dilutant at an excitation wavelength of 405 nm (the Soret range) and emission wavelength of 620 nm. The emission spectrum is then scanned from 580 to 650 nm with the excitation monochromator set at 405 nm. The plasma porphyrin fluorescence peaks can be recorded and can be characteristic for individual porphyrias, for example a maximum at 634-636nm is characteristic for free PP and 587 for zinc PP. The intensity of this peak may vary between individuals and within individuals depending on disease activity and treatment. Thin layer chromatography and cytofluorometry can also be used for screening samples.

1.8.2 Quantitative assays

Quantitation involves specific measurement of the porphyrin concentration in the sample. Spectrophotometry and fluorometry are the current standard methods, and as previously discussed, rely on the characteristic absorption and emission spectra of the different porphyrins. The porphyrin in the plasma or urinary sample is extracted using high performance liquid chromatography (HPLC), a highly improved form of column chromatography. The solvent, instead of being allowed to drip through a column under gravity, is forced through under high pressures of up to 400 atmospheres, making the process faster and allowing the use a very much smaller

particle size for the column packing material. This greater surface area facilitates interactions between the stationary phase and the molecules flowing past it, resulting in better separation of the components of the mixture. The difference between the porphyrins that allows separation on an HPLC system is the number of carboxyl groups and hence water solubility. This affects their ability to interact with the column particles, and the more soluble elute earlier from the column. The detection methods in HPLC are highly automated and sensitive. In the detection of porphyrins, the eluate being assessed is excited with Soret range light at 405nm, and fluorescence monitored at 620nm. As discussed above, the fluorescence emission peaks are characteristic for individual porphyrins, allowing individual porphyrins to be identified from a sample, gives a qualitative diagnostic result. The amount of light produced will depend on the amount of a particular compound that is passing through the beam at the time, thus quantitation of porphyrins in a sample can be performed against a known concentration of a reference standard.¹⁰²

Similar spectrophotometry against a known concentration of a reference standard is used to quantify the total red cell PP levels, but first the PP must be extracted from the erythrocytes first using ethyl acetate/acetic acid mixture and then hydrochloric acid.

Quantitation of urinary porphyrin precursors can be performed by extraction and separation using ion-exchange resins, formation of a coloured compound using Ehrlich's reagent and assessment of this product colorimetrically. Erythrocyte porphyrins are abnormally increased in EPP, CEP and HEP.

1.8.3 Enzyme assays

Enzyme activity studies can be performed using erythrocytes, leucocytes, transformed lymphoblasts skin fibroblasts, or recombinant *Escherichia coli*, usually employing a spectrophotometric or fluorometric analysis of an enzyme reaction product.^{103,104} Although the presence of sub-normal enzyme activity is not always associated with clinical expression, these investigations are particularly useful in the investigations of families where an index case has been identified.

1.8.4 Potential pitfalls in diagnosis

It is recommended that analysis for porphyria is carried out in a laboratory accustomed to the appropriate technical methods and quality control. Failure to adhere to this will inevitably lead to false negative diagnoses (which may also arise from deterioration of samples in transit). Ideally samples should include plasma and whole blood EDTA samples (more stable than serum samples), stored at 4°C before testing and protected from light to prevent light-induced porphyrin degradation.¹⁰⁵

Increased urinary ALA is seen in hereditary tyrosinaemia. Excess urinary coproporphyrin occurs in toxic syndromes or hepatic conditions. High erythrocyte zinc protoporphyrin may be found in lead poisoning, iron deficiency and sideroblastic anaemia; due to its inability to diffuse through the erythrocyte membrane, plasma porphyrin levels are usually normal. Use of a qualitative fluorescent scan prior to quantitative analysis helps to avoid false-positive results from fluorescent drug metabolites with emission peaks near to those characteristic for porphyrins. Mildly elevated levels of plasma porphyrins are also seen in some disorders such as renal failure, cholestasis and the acquired immune deficiency syndrome. In most of these free erythrocyte porphyrin concentration is usually normal.

1.8.5 Diagnosis of erythropoietic protoporphyria

The most important diagnostic feature is elevation (often 50-100 fold) of protoporphyrin (PP) in erythrocytes and plasma. A useful screen is the demonstration of orange-red fluorescence in samples irradiated with violet light during fluorescence microscopy or in a spectrophotometer. Increased levels of PP are demonstrable in plasma, bile and faeces, but not in urine due to its hydrophobic nature. However where liver disease is manifest, decreased excretion into the faeces leads to lower faecal concentrations, elevated plasma levels and urinary excretion of coproporphyrin. *In vivo* phototesting of skin in suspected cases shows swelling and erythema to high doses of monochromatic violet light at 405 nm, but normal responses to UVA and UVB.¹⁰⁶ This response is maximal at between 6-12 hours, unlike the more usual 24 of other photodermatoses.

1.9 Clinical features of erythropoietic protoporphyria

1.9.1 Acute cutaneous manifestations of EPP

EPP is characterised by mild to moderate photosensitivity, with symptoms usually present from early childhood. Patients present with intense itching, painful burning or a stinging sensation following minutes to hours of sunlight exposure. In young children these features may be accompanied by inconsolable crying or screaming. Symptoms usually occur in spring and summer but some patients are also symptomatic in the winter,¹⁰⁷ and experience exacerbation by cold winds.^{106,108}

The initial skin discomfort may be followed by cutaneous signs. These tend to be subtle, to the degree that affected individuals may be considered to have a psychiatric disorder.^{7,109} The lack of clinical signs or a relatively mild phenotype can lead to a significant delay before diagnosis is reached.¹¹⁰⁻¹¹⁴ Unlike other cutaneous porphyrias which show blistering, EPP is characterised by delayed skin redness and oedema.⁴⁴ Petechiae, purpura, crusting and blistering are only evident following prolonged sunlight exposure (figure 5). Skin fragility, vesiculation, crusting and hypertrichosis of other porphyrias are seldom seen in EPP. Some report the signs coincident with the onset of the symptoms,¹¹⁵ whilst others have noted them occurring later.⁷ The sensitivity tends to affect only sun-exposed skin, including finger nails, and can also occur following exposure through normal window glass.⁷ The frequency of acute urticated erythema is reported to be a rare sign. Subsequent purpura or bullae are dependant on the dose of light sustained and the individual sensitivity.¹⁰⁶ The localisation of the photosensitivity eruption is significantly affected by clothing: lesions are most commonly observed on the face, neck and upper chest, lower arms and hands.¹⁰⁶

Figure 5. Acute skin changes in EPP

a) Subtle purpura on radial aspect of an index finger of a 12-year-old.



b) Crusting over tip of nose and upper lip in an eight-year-old.



Cutaneous tolerance to sunlight is markedly reduced for several days after sun exposure, even if the initial exposure did not progress to the oedematous or petechial stage. This sensitivity has been termed the ‘priming phenomena’ and can be explained by the photodynamic release of PP from erythrocytes in the dermal

vasculature to vascular endothelial cells in response to relatively small repetitive sunlight exposure.^{66,93} One paper reported white nails, onycholysis and occasional nail shedding concomitant with acute skin changes.¹¹⁵ These have not been recorded elsewhere.

1.9.2 Chronic cutaneous manifestations of EPP

Episodes of photosensitivity often commence during infancy before the child is able to communicate effectively. The paucity of signs and limited awareness of EPP among healthcare professionals typically result in misinterpretation of the cause of the child's symptoms and result in delay in diagnosis. Thus repeated acute photosensitivity reactions may occur, leading to the development of chronic skin changes, characteristically restricted to the light-exposed skin of the nose, forehead, cheeks and dorsae of the hands. Waxy or leathery scars particularly on the face or hyperkeratotic thickening of skin is described, particularly over the knuckles (in one series evident in 27%).^{106,116} Small shallow depressed scars may be evident on the face, whilst stellate scarring may be evident, particularly over the hands. Radial grooves (pseudorhagades) are seen on the lips and course wrinkles may be evident, particularly around the eyes (figure 6).¹¹⁵ In some individuals, chronic changes are so subtle that the skin appears normal.^{7,70,106,115} Two early case reports suggested hypertrichosis was a feature of the condition;^{117,118} this has not been supported by later EPP patient series.⁷

Improvement of symptoms has been reported in up to 45% of individuals with increasing age, possibly through efforts to avoid sunlight exposure,⁴⁴ although none have resolve spontaneously.^{115,119} Late onset of EPP is rare. Only 13 patients have been reported in whom symptoms started after the age of 40 years. In the majority, this was due to acquired haematopoietic *FECH* mutations in the context of myelodysplastic and myeloproliferative disorders.¹²⁰⁻¹²² There is however a report of late-onset with a genotype known to cause EPP and a close family history: In this case the authors postulate that this was due to the *FECH* mutation contributing residual activity such that overall *FECH* activity was similar to that of asymptomatic carriers.¹²³

Figure 6. Chronic skin changes in EPP

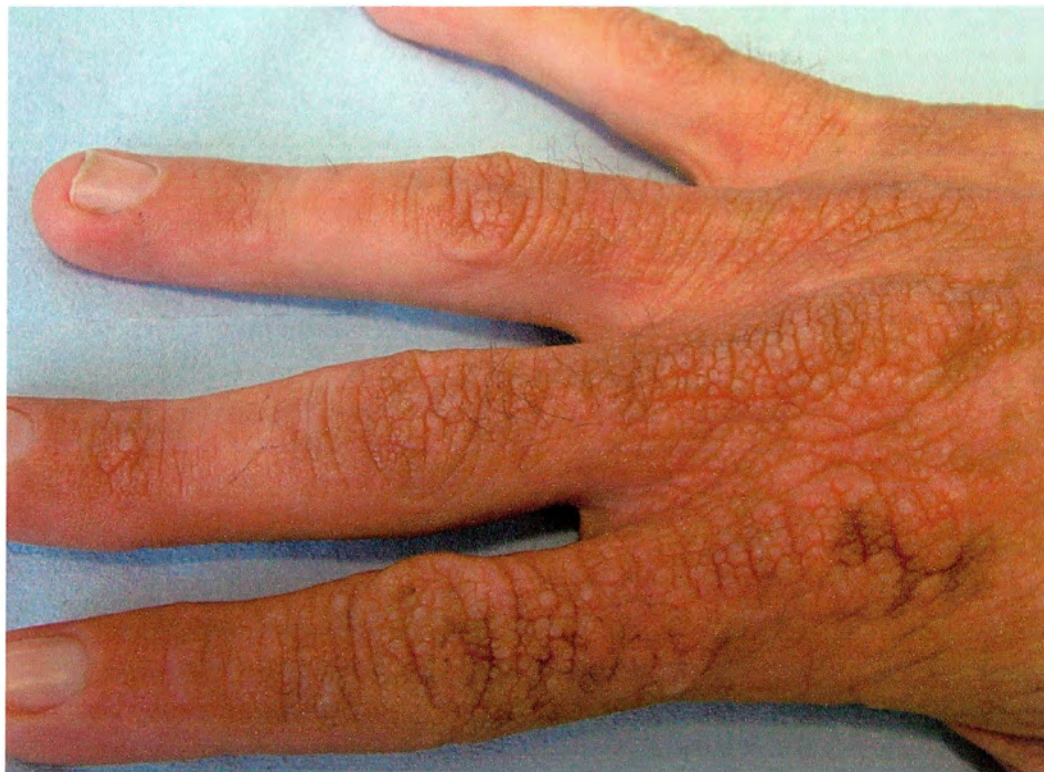
a) Waxy thickening and linear scarring on a 36-year-old man's nose.



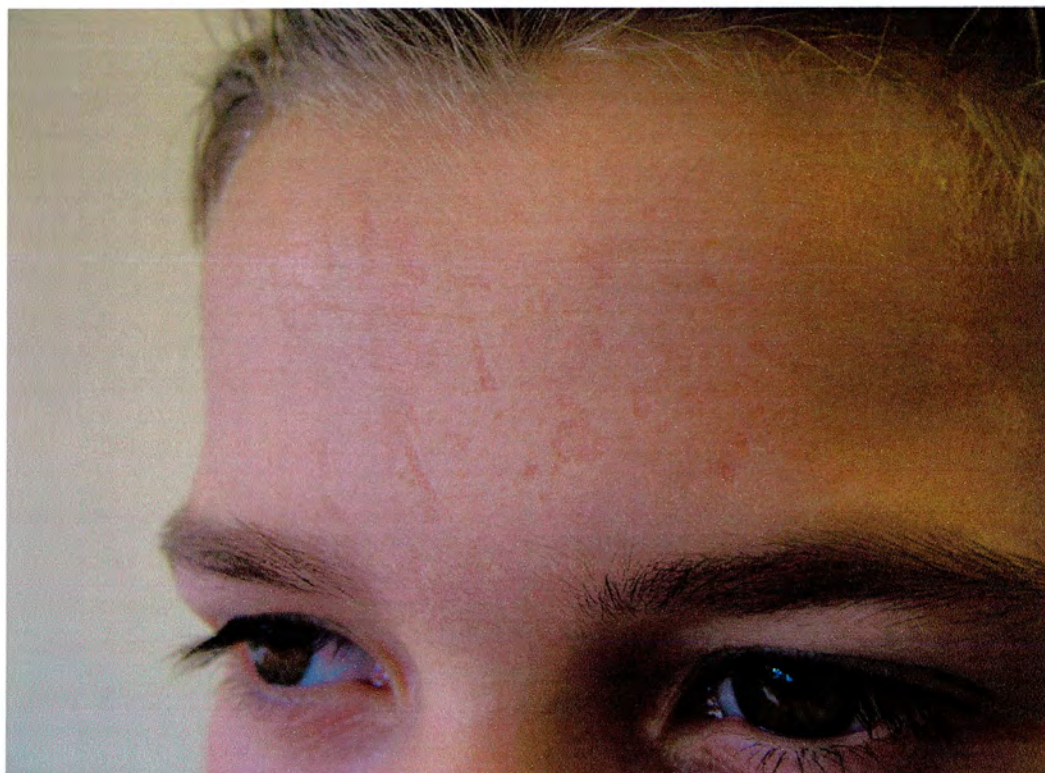
b) Hyperkeratotic thickening over knuckles of a 53-year-old gardener.



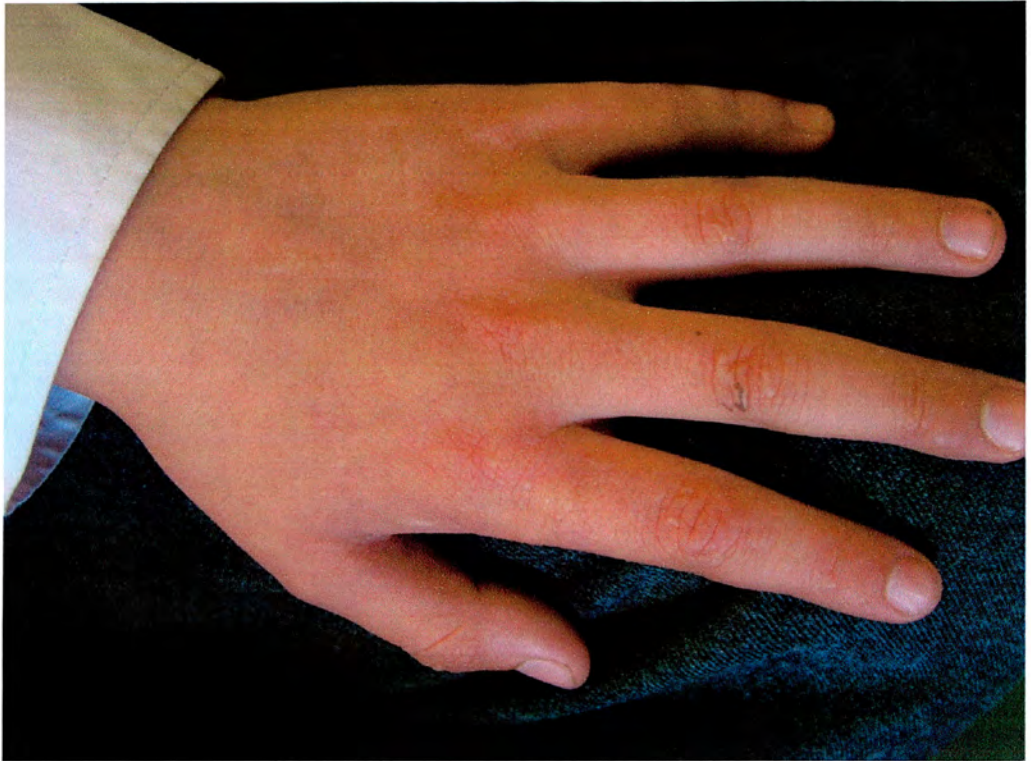
- c) Pseudolichenified hyperkeratotic thickening over the dorsum of a 35-year-old man's hand.**



- d) Shallow depressed pox-like scars over a 10-year-old's forehead.**



e) Subtle macular scarring on the hand dorsum (same patient as (d)).



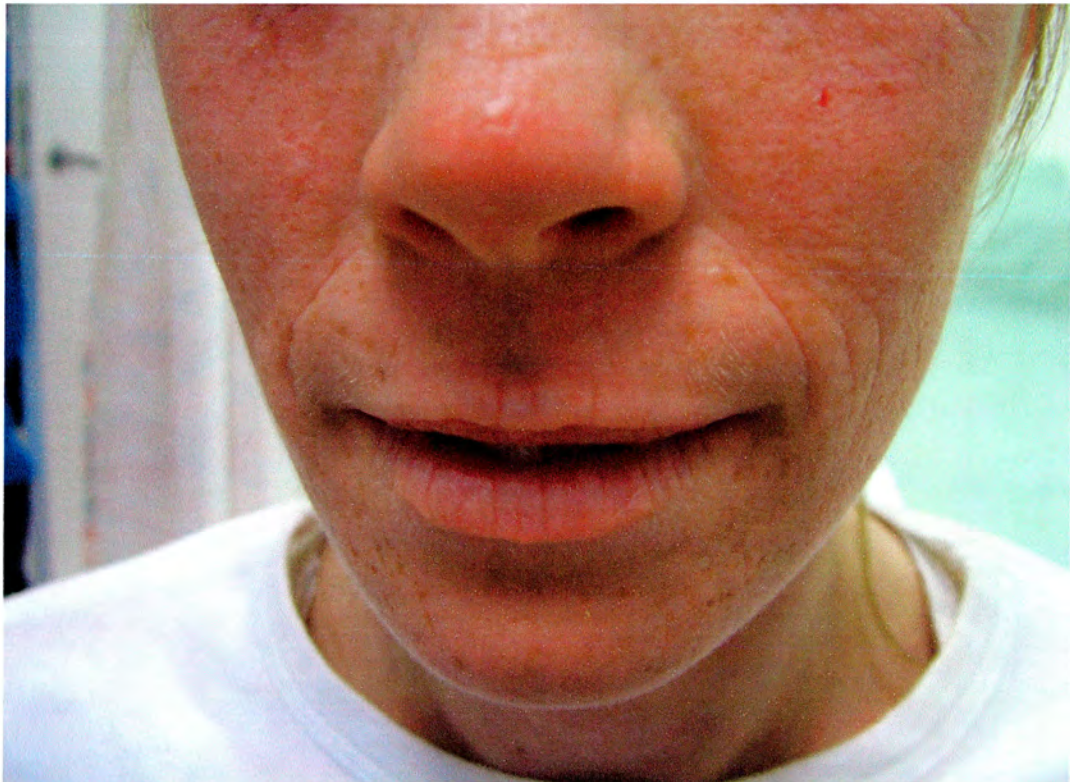
f) More pronounced macular scarring on the right cheek and neck of a 49-year-old lorry driver.



g) Course wrinkles evident over lateral cheeks (same patient as (a)).



h) Course pseudorhagades in a 32-year-old teacher. Scarring is also evident over the tip of the nose.



compared to experimental models. Furthermore, application may miss certain sites such as neck, temples and ears, and shedding due to rubbing, sweat or contact with water or clothing requires reapplication every 2 to 3 hours.²³⁸ The topical cosmetic tanning agent, dihydroxyacetone (DHA) can provide a degree of protection to wavelengths greater than 370 nm and has been suggested as a potential adjunct to sunscreen therapy.²³⁹

Figure 7. Use of Dundee sunscreen (beige colour) and clothing to protect against sunlight exposure.



Normal clear window glass absorbs wavelengths below 320nm (UVB). Lamination provides partial protection against UVA(320-380nm),²⁴⁰ but does not protect against the Soret band.²³⁴ Within houses, curtains and blinds in houses provide a cheap and pragmatic solution for EPP photoprotection, but motor vehicles provide more of a problem as current UK legislation requires 75% of visible light to come through front and back windows and 70% visible light to come through side windows. Thus dark tinted glass is illegal for windscreens and driver's side windows. A compromise can be reached by applying window films such as CLS200XSR (Madico) or Dermagard (Bonwyck) which block light up to 400nm, and allow visible light over 420nm through. Within the 400-420nm range, there is an approximate 50% reduction in passage of light. Yellow tinted museum films, such as TA81 XSR (Madico) or

1.9.3 Haemopoietic effects of EPP

Microcytic anaemia has been reported in 20 – 60% of EPP patient series.^{7,106,115,124} In contrast to other inherited disorders of erythroid haem biosynthesis,¹²⁵ the anaemia is not dyserythropoietic (the erythroid cells are differentiating normally), there is no iron overload, and there is evidence for iron-deficiency, with low serum ferritin and serum iron levels,^{7,106,126,127} without iron loss.¹²⁷ A mouse model of EPP, the homozygous *Fech*^{m1Pas} mutant, develops a similar microcytic anemia.^{128,129} At an ultra-structural level there is consistent pathological iron deposits in the mitochondria of bone marrow erythroblasts.¹²⁷ Although it is probable that the anaemia of EPP reflects limitation of haem formation by FECH deficiency, its mechanism and relationship to disordered iron metabolism remains unclear.

1.9.4 Hepatic manifestations of EPP

In contrast to non-cutaneous porphyrias, acute abdominal and neurological attacks do not occur, nor are drugs known to exacerbate EPP. The major non-cutaneous clinical manifestation of EPP is hepatobiliary disease, caused by accumulation of PP in the liver.^{130,131} Hepatic PP deposition manifests progressively as cholestasis, cirrhosis, rapid decompensation and death.¹³²⁻¹³⁴ The exact mechanism of the PP toxicity in the absence of light, ‘dark toxicity’, is unclear. There may be a direct effect of PP crystal deposition in the liver: Canalicular secretion of PP appears to be the rate-limiting step in its transport from plasma to hepatocytes and subsequently to bile and faeces,¹³⁵ and when this excretory capacity is saturated, rising intrahepatic concentration of PP results in aggregation and deposition of solid pigment and crystalline PP both within the hepatocytes and canaliculae, causing cholestasis, accelerating PP deposition, and subsequent liver cirrhosis.¹³⁶ However this may not be the initial event in PP liver damage, as ultrastructural studies show abnormalities of hepatocytes even in patients with clinically mild disease and normal hepatic architecture.¹³⁷ Furthermore, in rat models, PP appears to be toxic to the liver when it is in a soluble form, decreasing bile flow, and histologically resulting in canalicular distortion, dilatation and loss of canalicular cell microvillae in the absence of PP deposition.¹³⁸ Thus it was proposed that the cirrhosis and fibrosis may coincide with,

or precipitate, the cholestasis. Studies show hepatocyte cell membrane ATPase enzyme activities are significantly reduced in livers infused with soluble PP, perhaps explaining the decreased excretory capacity of the liver in EPP, and the degree of this reduction appears related to the PP concentration within the infusion.¹³⁹ A mechanism might be the hydrophobic nature of the PP molecule and its lipid solubility, resulting in change to the physico-chemical properties of hepatocyte and canalicular secretory membrane lipid bilayers. The resulting effacement of canalicular microvillae may additionally reduce the functional excretory area, resulting in the cholestasis, which in the presence of an apparent unsaturable hepatic uptake of PP, leads to increasing intracellular PP concentrations deposition and fibrosis.¹³⁵

The dark toxicity of PP may also be through its relation with hydrogen peroxide. PP has been demonstrated to be associated with increased intracellular hydrogen peroxide concentrations (perhaps reflecting increased oxidative stress on the cell due to the mechanisms described above) and inhibition of hepatocellular proliferation, both in a dose-dependant manner.¹⁴² Increased intracellular hydrogen peroxide may exert a direct toxic effect on hepatocytes.¹⁴³ However PP's generation of hydrogen peroxide, in the presence of iron, also permits a Fenton reaction.^{140,141} The Fenton reaction describes the oxidation of ferrous (II) iron by hydrogen peroxide to ferric (III) iron, a hydroxyl radical and a hydroxyl anion ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}\cdot + \text{OH}^-$), during which the presence of iron is catalytic. These generated reactive molecules can result in lipid peroxidation and disruption of the structure and function of cellular membranes and their associated enzyme systems.¹⁴⁴

Clinically, by the time jaundice is clinically evident it is likely that hepatic damage is advanced. At this stage the clinical presentation is said to resemble an acute porphyria: in addition to significantly increased photosensitivity, neurological dysfunction (motor neuropathy), abdominal symptoms, tachycardia and hypertension may develop. Indeed, it may be this deteriorating photosensitivity which brings some EPP sufferers into contact with the medical profession for the first time.^{9,112}

It is unclear why some individuals with EPP develop hepatic dysfunction whilst others do not. The features of liver dysfunction in EPP have yet to be clearly established, including its age of onset, with reports from children aged 6, 11 and 13 years.^{43,145,146} There appears to be a genetic basis in some cases with increased incidence in AR inheritance,¹⁴⁷⁻¹⁴⁹ and in those with liver failure in affected siblings.¹⁵⁰⁻¹⁵² In one case, alcohol has been reported to be contributory to EPP related liver disease,¹⁵³ while in another, serum γ -glutamyl transferase (GGT) appeared to parallel levels of TEP.¹⁵⁴ Because the onset of liver disease cannot yet be predicted, patients should be monitored with regular liver function tests (LFTs). No large-scale epidemiological studies of unrelated EPP patients have been done to assess the frequency of hepatic dysfunction in EPP. Results of previous studies are difficult to interpret due to retrospective analysis, small sample size or selection bias (table 3), however the incidence of abnormal LFTs in EPP has variously been estimated to be between 0 and 36%.^{6,115,137}

Data from these same studies has estimated rates for liver failure to be about 2-4%.^{9,70,130} The majority of individuals with this complication were aged 30 years and over,^{45,155,156} although it has also been reported in children.^{7,9,145,146} Once jaundice is apparent, hepatic disease is usually so advanced that transplantation is required to prevent death. There is also uncertainty about risk factors for liver disease in EPP. Most studies on this topic consist of reports of one or a few patients with liver failure and include the following: alcohol intake,^{153,157} hepatitis,⁹ haemolytic anaemia, disturbed iron metabolism,^{124,158,159} and high erythrocyte and plasma protoporphyrin concentrations,^{9,130,160} (probably secondary to developing cholestasis^{130,158}).

In small series previous cholecystectomy in EPP, as a proxy for symptomatic gallstones, have been reported to have a prevalence of between 6 and 12%, which may be increased above that of normal populations.^{7,115,162} In white Caucasian populations the prevalence of gallstones is variously reported as between 4 and 6%, with a cholecystectomy prevalence of 0.6% in Italy.^{163,164} The use of cholecystectomy rates for symptomatic gallstone disease by these studies allow only an estimation of increase: Assessment of the true prevalence of gallstones in a large

EPP series has not been conducted, and could prove difficult by objective means, as ultrasonic diagnosis is required and asymptomatic gallstones are common. The gallstones have also been reported to present at a younger age than would be expected. However neither of these two apparent observations have been statistically proven due to the low prevalence of EPP and the variable incidence of symptomatic gallstones.⁷⁰ Those gallstones which have been analysed have high levels of PP (310-479µg/g compared to 0.14-4.06µg/g in normal individuals) with no apparent difference in composition between the nucleus and the outer layers.^{7,44} This suggests the stones to be initiated by a precipitate of PP, rather than by any alternative compound such as cholesterol.

Table 3. Liver dysfunction in EPP patient series.

| Year | No. of subjects | Definition Abnormal LFT | Number Abnormal LFT | Percentage Abnormal LFT | Virology | Comment | Reference |
|------|-----------------|------------------------------------|---------------------|-------------------------|-------------------------------|---|-----------|
| 2005 | 204 | ↑ AST/ALT | 70 | 34% (25% dEPP) | Reported: no active infection | Current study | - |
| 1991 | 90 | ↑ Bi AST/ALT | 22 | 24% | Not reported | Retrospective Some patients previously reported ⁹ | 2 |
| 1990 | 11 | ↑ AST/ALT | 4 | 36% | Not reported | Retrospective, small sample size | 137 |
| 1989 | 55 | Not defined | 19 | 35% | Not reported | Retrospective analysis. Biochemistry not all performed at same centre | 9 |
| 1986 | 9 | ↑ Alk Phos, GGT, AST, ALT, LDH, Bi | 2 | 22% | Not reported | Small sample size | 161 |
| 1976 | 32 | ↑ AST Bi Alk Phos | 1 | 3% | Not reported | retrospective | 7 |
| 1974 | 29 | ↑ AST Bi Alk Phos | 0 | 0 | Not reported | Biochemistry not performed in all cases | 115 |
| 1965 | 4 | ↑ Bi | 0 | 0 | Not reported | 3 other cases biochemistry not performed | 6 |

1.9.5 EPP & pregnancy

Some women report significant improvement of their symptoms in pregnancy,^{8,115,165} apparently through a pregnancy-related reduction in erythrocyte PP levels.^{166,167} The mechanism for this remains to be elucidated, but could result from a hormonal influence on *FECH* expression, the dilutional effects on the vasculature of pregnancy, or haemodynamic or hormonal changes causing diminished protoporphyrin production, decreased efflux from erythrocytes, or enhanced biliary excretion. Furthermore, as PP crosses the human placenta in a dose-dependant manner, maternal PP may act as a substrate for the foetal *FECH*, leading to suppression of foetal *FECH* and sequestration of maternal PP.¹⁶⁸

1.10 Histopathology of EPP

The histopathological features of EPP affect only sun-exposed skin. Within the first few days following sunlight exposure an acute inflammatory response is observed with intercellular oedema in the epidermis and dermis and a dermal leucocytoclastic neutrophil-rich infiltrate. Superficial cutaneous vessels appear selectively damaged, with vacuolation, swollen organelles, cytolysis and loss of intercellular contact.⁹⁶ Red cell extravasation may be seen within vessel walls and perivascularly. Involvement of epidermal cells, fibroblasts and other dermal elements appears to be a later event,⁹⁶ suggesting that endothelial cells represent the initial target of the photodynamic reaction.⁶⁹ This observation has been explained by the relatively high concentration of PP within endothelial cells and presence of high partial pressure of oxygen, facilitating oxidative photodynamic damage.¹⁶⁹

The most common chronic changes evident are epidermal hyperkeratosis, acanthosis, but also sometimes atrophy. In the dermis, amorphous hyalin-like PAS-positive deposits are observed, particularly around superficial vessel walls and papillary dermis, caused by concentric reduplication of the basal lamina. These are thought to represent carbohydrate-protein complexes from repeated acute damage and repair of blood vessel basement membrane.^{170,171} Immunohistochemically there is IgG deposition (with occasional IgM) at the dermo-epidermal zone and around blood vessels. Electron microscopy confirms extensive reduplication of the basal lamina of

small upper dermal blood vessels from light-exposed skin, with occasional clumps of dense amorphous material within the reduplication.¹⁷²

Within the liver, most individuals, even those without serological evidence of dysfunction, will show evidence of vacuoles containing characteristic needle-like PP crystals.¹⁷³ The histopathological changes range from essentially normal liver architecture with PP pigment deposition within hepatocytes, Kupffer cells and canaliculi, some widening of portal tracts and mild periportal fibrosis, to more advanced cases demonstrate larger collections of PP crystals within hepatocytes, Kupffer cells and free in canaliculi, where they appear to plug the lumen. Changes include hepatocyte degeneration and necrosis, micronodular cirrhosis, bile duct proliferation, cholestasis and porphyrastasis. Under polarised light, large quantities of PP exhibit a 'Maltese Cross' appearance, while smaller deposits appear as brilliant reflections.¹⁷³

1.11 Prevalence of EPP

EPP is not specific for sex.⁸ The prevalence of the IVS3-48C low expression allele shows clear differences between ethnic populations,¹⁷⁴ with low levels in Afro-Caribbeans (<1%) and north Africans (2.7%), and high levels in Japanese populations (43%) compared to white Europeans (11%).^{175,176} It has been proposed that the prevalence of symptomatic EPP in a population would be determined by the frequency of the IVS3-48C polymorphism within the population.¹⁷⁶ However the true prevalence of the clinical condition is difficult to predict in the absence of large-scale randomised population screening. Previous estimates have been based on patients known to physicians, and probably significantly underestimate the true incidence due to patients failing to seek medical advice, failure of physicians to recognise the condition, or false negative investigations. Studies have estimated prevalences between 1:200,000 (Sweden)¹⁷⁷ to higher rates of 1:79,000 (Northern Ireland)⁷⁰ and 1:75,000 (Netherlands)¹⁰⁶ for more well-defined populations. In view of the overall underestimation of cases of EPP, these latter figures are probably more accurate, although a founder effect is possible in these smaller populations, as demonstrated among Swiss EPP families.¹⁷⁸

1.12 Genetic inheritance of EPP

Early case reports of EPP patients described between 10 and 25% of red blood cells showing a transient red fluorescence when viewed under a fluorescence microscope. Analysis for these 'fluorocytes' provided a simple qualitative screen for the condition using light-protected dried or wet blood samples (with a differential diagnosis of iron deficiency anaemia, lead poisoning, HCP and CEP).¹⁷⁹ However asymptomatic individuals from EPP families were also shown to have peripheral blood fluorocytes and that this asymptomatic group with fluorocytes outnumbered those clinically with EPP.¹⁷⁹ Studies of peripheral blood fluorocytes showed no gender predilection, that the asymptomatic group with fluorocytes had normal or slightly elevated PP, significantly lower percentage of fluorocytes than relations with EPP, and that they occurred in half of the children and siblings of affected individuals following an apparent autosomal dominant pattern.^{8,41} The EPP affected individuals appear to represent a quarter of children in these families suggesting recessive inheritance. Further pedigree and biochemical studies suggested that the genetics of the condition were complex, as clear autosomal dominant parent-child transmission of the clinical condition occurs in only 8% families,^{8,147,180} and FECH activities in overt EPP are 10-30% of normal enzyme activity: significantly less than the 50% predicted by haploinsufficiency (where a gene is mutant or absent on one diploid copy).^{5,58,60,181} Furthermore, FECH activities in asymptomatic siblings and offspring of EPP individuals are decreased below normal, although not to the 30% level seen in symptomatic cases.¹⁸⁰ To explain these observations, a three allele hypothesis was proposed comprising a normal allele (*f*), an allele which led to the presence of occasional fluorocytes in unaffected parents (*F*⁺) and a third allele with a high population frequency (*F*⁻).⁸

The cloning, sequencing and characterisation of the human *FECH* gene, located on the long arm of chromosome 18 and containing 11 exons spread over 45kb, allowed examination of the genetic defects underlying the condition.^{51,103,182} A French EPP family where the asymptomatic father had a deleterious *FECH* mutation which reduced his FECH activity to 50% was studied. One of his children was

symptomatic with EPP and had FECH activity of 25%. The mother was shown to be heterozygous for an intronic single nucleotide polymorphism, IVS3-48T/C, and had FECH activity of 75%. The affected son had received the C allele, while his asymptomatic brother, who had also received the deleterious mutation from his father, received the T allele and had FECH activities of 50%. Two sisters were phenotypically and genotypically normal.¹⁸³ This co-inheritance of a mutation which considerably affects or abolishes FECH activity, *trans* to a wild-type hypomorphic (reduced activity) allele causing a mild deficit in FECH activity causing symptomatic EPP, confirmed the postulated 3-allele hypothesis: in this family the father is F^+f , the mother Ff , the daughters ff , the asymptomatic brother F^+f and the symptomatic brother F^+F . This hypomorphic IVS3-48C allele (F) was subsequently shown to result in aberrant splicing of the *FECH* mRNA (where RNA is modified by the removal of introns and joining of exons), inserting a premature stop codon into the *FECH* mRNA which is then more rapidly degraded than normal mRNA (by the process of nonsense-mediated mRNA decay), resulting in decreased levels of FECH.¹⁸⁴ It is present in approximately 10% of white Caucasian populations,^{5,175} and in an unpublished study, the porphyria group in Cardiff have shown that IVS3-48C allele is present in 108 of 111 unrelated EPP patients without liver disease. A *FECH* mutation on both alleles explains the small subgroup of families in whom EPP inheritance appears autosomal recessive (estimated to have a prevalence in the UK of about 3% all EPP individuals).^{4,180,185-187} Reports of individuals with liver failure in this subgroup of EPP subjects have led to the suggestion that autosomal recessive inheritance may put individuals at particular risk of more severe clinical features.^{147,148,152} Rarely, other mechanisms must be involved in the aetiology of EPP as occasional patients are described in whom FECH activity appears normal.¹⁸⁸ Furthermore, late-onset EPP has been intermittently reported.¹⁸⁹ In some individuals this has been recognised to be in association with aplastic anaemia or myeloproliferative disorders, resulting from FECH mutations in erythroid progenitor cells.^{122,190,191}

To date over 60 disease-specific deleterious *FECH* mutations have been reported, with the majority being family-specific.^{9,192-194} A small minority of families have

mutations that are shared by a limited number of other EPP families (possibly representing a founder effect).^{178,195} The majority of identified mutations are so-called “null-allele” mutations, where the mutation leads to the formation of a truncated, and therefore inactive enzyme completely lacking its normal function, and includes nonsense and frameshift mutations.¹⁹⁶ In contrast, point mutations, in which a single nucleotide is changed, resulting in a codon that codes for a different amino acid, which have been identified in approximately 20% of EPP individuals. They do not necessarily inhibit enzymatic activity, but, for example, may result in a reduction of activity, or affect other characteristics such as thermal lability, a so-called “missense mutation”.¹⁰⁴ *FECH* mutations in the central iron-sulphur cluster seem to be of particular importance in affecting *FECH* activity.¹⁹⁶

The significance of the different *FECH* mutations on clinical phenotype has not been established, but while there is considerable genetic heterogeneity in those with liver complications, all appear to have “null-allele” mutations which produce major structural alterations in *FECH*,^{158,160,197} and this association has been reported to be statistically significant.¹⁹⁸ These observations suggest that there may be a correlation between genotype and protoporphyria-induced liver disease with severe mutations predisposing to this complication, although because the same mutations are found in asymptomatic family members, other factors must also be critical to the development of this severe phenotype. *FECH* activities are said to be particularly low in autosomal recessive EPP, predisposing to severe liver disease;¹⁴⁷ although homozygous missense mutations do not appear to be associated with this complication.¹⁸⁶

These genetic models of *FECH* activity are further complicated by the observation that *FECH* exists as a dimeric molecule. Theoretically, the amounts of wild-type/wild type, mutant/wild-type and mutant/mutant dimers in EPP individuals will be 25, 50 and 25% in the same cell respectively. The observation that a majority of EPP individuals exhibit less than 30% normal *FECH* activity could be explained by *FECH* only being active when the dimer contains two wild-type subunits. Heterodimers consisting of wild-type and mutated subunits have been shown to

exhibit significant activity,¹⁹⁹ however these molecules are inherently less stable, and this explanation might explain the lower than expected FECH activity.²⁰⁰ No strict correlations have been identified between genetic defects and either the erythrocyte protoporphyrin levels, FECH activities or disease severity.

1.13 EPP & surgery

During surgery, EPP individuals are at risk of burn injuries to skin and intra-abdominal organs exposed to operating and other strong light sources emitting wavelengths around 400nm (operating theatre lights' normal emission spectra is 300-750nm), and circulating erythrocytes are more susceptible to photolysis, causing haemolytic anaemia.^{201,202} This is particularly the case in the setting of liver failure when excess accumulation of protoporphyrin results in extreme photosensitivity, massive haemolysis, and with at least one death attributed to such complications.^{203,204} A number of measures can be implemented to reduce risks of surgery: Firstly PP levels can be reduced by perioperative whole blood transfusions to reduce haem synthesis and thereby PP synthesis, or omitting to use own-blood re-circulation cell savers. Secondly, all lamps in all rooms can be shielded using yellow-coloured acrylate filter, such as TA81 XSR (Madico) or Bexfilm U (Summerside Blinds), to cut off wavelength transmission below about 530nm and give almost complete protection from UV and visible violet light.²⁰⁵ Likewise, windows adjacent to patient areas should have filters applied. The yellow filter can, however, cause problems for both anaesthetists and surgeons, as blue objects appear black, and other colours are perceived with a yellow hue.¹³⁰ With a few notable exceptions,²⁰⁶ the requirement to avoid anaesthetic agents is a fallacy in both the anaesthetic and surgical literature (unlike the acute porphyrias, EPP is not associated with drug-induced crises).²⁰⁷⁻²⁰⁹

Massive intra-operative haemolysis and severe polyneuropathy are two significant peri-operative complications of surgery in EPP patients with liver failure. The haemolysis, due to the photoactivation of PP by theatre light sources, releases further PP from erythrocytes, perhaps exacerbating the risk of subsequent neuropathy.^{203,210} The severe polyneuropathy, usually motor and which is seen both in the pre and post

transplant period, clinically resembles Guillain-Barré syndrome.^{202,210,211} Elevated PP has been postulated to cause a neuronal haem deficiency or may be directly neurotoxic. The neuropathy does recover slowly; peri-operative haematin infusion in combination with plasmapheresis or dialysis with molecular adsorption (such as MARS or Prometheus systems) may help to prevent its development.²¹²⁻²¹⁴

1.14 Vitamin D and porphyria

Vitamin D is an essential fat-soluble hormone required for bone integrity and calcium homeostasis.²¹⁵ Approximately 90% of requisite vitamin D is formed within the skin as a result of sunlight photolysis of 7-dehydrocholesterol by ultra-violet B (UVB) radiation, before a temperature-dependent isomerisation to cholecalciferol.²¹⁶ White populations in the northern hemisphere tend to develop physiological vitamin D insufficiency in winter and early spring.²¹⁷ This propensity can be exacerbated by a number of factors including increased ethnic skin pigmentation, occlusive clothing, institutional residency and decreased synthetic capacity with increasing age.²¹⁸⁻²²⁰ Although sunscreen use does appear to cause some reduction in 25-hydroxyvitamin D levels,^{221,222} there is no equivocal evidence of sunscreen use causing insufficiency or deficiency (in cases where this has been reported, other risk factors such as ethnic skin pigmentation, institutional residency, age or occlusive clothing, have been present).²²¹⁻²²³ Increased incidence of various conditions such as diabetes mellitus, hypertension, tuberculosis and some malignancies within populations at increasing latitudes have led to suggestions that it may also protect against their development.²²⁴⁻²²⁷

Previous studies of photosensitive patient cohorts with xeroderma pigmentosum (XP) and Smith-Lemli-Opitz syndrome (SLOS) who actively avoid sunlight have failed to show convincing evidence of vitamin D insufficiency.^{228,229} There are case reports and small series of individuals with photodermatoses such as oculocutaneous albinism, coproporphyrinuria, chronic actinic dermatitis (a case report of an elderly Pakistani man resident in the UK, with several risk factors for insufficiency) and cutaneous lupus erythematosus which make such an association.²³⁰⁻²³² (Jury CS, personal communication) Following a report in 1955 of a boy with coproporphyrinuria

and rickets there have been no further reported studies or cases commenting on vitamin D status in individuals with porphyria.³⁶

1.15 Therapeutic approaches in EPP

1.15.1 Patient information

One of the most helpful measures for EPP patients and their families is a detailed explanation of the condition provided by a well-informed expert and complimented by the provision of accurate and up-to-date written information. A wealth of information can be found on the internet about porphyrias, but some of this is of poor quality and most is focussed on porphyrias with acute symptoms. For example, the excellent European Porphyria Association site (<http://www.porphyrria-europe.org/>) currently has nothing in the patient section about non-acute porphyrias, and has only four lines of text describing EPP in the clinicians' section. Likewise, national support organisations are available for porphyrias as a group of conditions (e.g. British Porphyria Association <http://www.porphyrria.org.uk/>), but tend to focus on the acute and mixed group of porphyrias, with only a few providing quality information and support for the cutaneous porphyrias such as EPP (<http://www.porphyrriafoundation.com/>). The Cardiff Porphyria Service maintains an accurate website (<http://www.cardiff-porphyrria.org/>), but this is directed mainly at clinicians rather than patients. Using 'erythropoietic protoporphyria' as a search term does return more specific information, but this ranges considerably in quality from the good (<http://www.netdoctor.co.uk/diseases/facts/erythropoietic.htm>) to the inaccurate (<http://www.britannica.com/>). Prior to this project, the New Zealand Dermatology Society was the only major professional association to host a patient information sheet about the condition (<http://dermnetnz.org/systemic/erythropoietic-protoporphyria.html>). There are currently no English-language EPP-specific patient support groups, although such groups do exist in Germany and the Netherlands (<http://www.epp-deutschland.de/> and <http://www.epp.info/>).

1.15.2 General management

The cutaneous symptoms of EPP result from the interaction between visible light in the Soret range (400-410nm), and protoporphyrin IX (PP). Hence, avoidance of

strong light sources, particularly sunlight, through behaviour modification and photoprotection should form the mainstay of treatment. This can be achieved using clothing or sunscreens that include protection against the visible spectrum, particularly the longer wavelengths. Avoidance of outdoor activities is recommended, particularly in the middle of the day, and career guidance should advocate an indoor occupation.²³³ Clothing such as hats, long-sleeved tops and long trousers provide excellent photoprotection, particularly for closed-weaved, dark-coloured fabrics.²³⁴ Thus socks and shoes should be recommended over bare feet or open footwear such as sandals, and summer clothing avoided as this tends to have insufficient protection.²³⁵ Some clothing manufacturers advertise their products using a UV protection factor (UPF) rating, which unlike the SPF which only measures protection against UVB, measures protection from clothing against UVB and UVA. The UPF rating for a fabric/textile is the ratio of UV measured without the protection of the fabric compared to with protection of the fabric. For example, a fabric rated UPF 30 means that if 30 units of UV fall on the fabric only 1 unit will pass through; thus by blocking or absorbing 29 out of 30 units of UV it is blocking 96.7% of UV. It has been suggested that photosensitive individuals wear fabrics with a UPF > 30, as this has been shown to protect against UV-induced erythema and the development of pre-malignant lesions in animal models.²³⁶ While it does not automatically follow that UV-opaqueness equates to visible-light opaqueness, in practice this is likely to be the case.

As the symptoms of photosensitivity in EPP are triggered by visible light in the Soret range, sunscreens specifically effective against UVB (280-320nm) or UVA (400-320 nm) are inappropriate and ineffective protection. Opaque sunscreens containing titanium dioxide, zinc or iron oxide may be effective theoretically,²³⁷ but a thick coating is necessary to achieve a sufficient degree of reflection (figure 7), and the white appearance and greasy formulation are usually cosmetically unacceptable for most patients. Decreasing the particle size into micronised form (10-50nm, compared to 500nm of non-micronised form) increases cosmetic acceptability but shifts protection towards shorter wavelengths.²³⁴ In practice, sunscreen tends not to be applied in sufficient quantities or frequency, resulting in reduced photoprotection

compared to experimental models. Furthermore, application may miss certain sites such as neck, temples and ears, and shedding due to rubbing, sweat or contact with water or clothing requires reapplication every 2 to 3 hours.²³⁸ The topical cosmetic tanning agent, dihydroxyacetone (DHA) can provide a degree of protection to wavelengths greater than 370 nm and has been suggested as a potential adjunct to sunscreen therapy.²³⁹

Figure 7. Use of Dundee sunscreen (beige colour) and clothing to protect against sunlight exposure.



Normal clear window glass absorbs wavelengths below 320nm (UVB). Lamination provides partial protection against UVA(320-380nm),²⁴⁰ but does not protect against the Soret band.²³⁴ Within houses, curtains and blinds in houses provide a cheap and pragmatic solution for EPP photoprotection, but motor vehicles provide more of a problem as current UK legislation requires 75% of visible light to come through front and back windows and 70% visible light to come through side windows. Thus dark tinted glass is illegal for windscreens and driver's side windows. A compromise can be reached by applying window films such as CLS200XSR (Madico) or Dermagard (Bonwyck) which block light up to 400nm, and allow visible light over 420nm through. Within the 400-420nm range, there is an approximate 50% reduction in passage of light. Yellow tinted museum films, such as TA81 XSR (Madico) or

Bexfilm U (Summerside Blinds), give almost complete protection from UV and visible violet light but are not legal on UK motor vehicles. Newer, non-tinted propriety glass films (e.g. 3M prestige) designed to reduce UV transmission also claim to absorb up to 99% light up to wavelengths of 410nm, and may prove useful for school and home windows.

Individuals' symptom management has been well described in previous small series, consisting of attempts to relieve the pain by cooling the skin using wet cloths or towels, and scratching or rubbing to relieve the itch.¹⁰⁶ Antihistamines are commonly used first line agents for the pruritus and to reduce weal and flare.²⁴¹

Because of the small unpredictable proportion of EPP individuals at risk of life-threatening liver disease, it is good practice for affected individuals to avoid or minimise exposure to factors which could exacerbate this propensity, such as medication, excess alcohol and risk behaviour for hepatitis virus infection.

1.15.3 Interventional measures to reduce solar sensitivity

There are a number of suggested therapies for EPP, but for the majority, it is difficult to assess efficacy due to lack of objective measurement.²⁴² The main symptom of EPP is pain; thus, assessment of severity is subjective and prone to placebo effect.²⁴³ In contrast, skin phototesting objectively induces skin erythema; however skin erythema does not necessarily correlate with individuals' self-assessment of pain.²⁴⁴

Antioxidants are molecules capable of removing free radical intermediates and inhibiting other oxidation reactions, through donation of hydrogen, formation of a complex between the radical and the antioxidant (free radical acceptor), loss of electrons or an increase in their oxidation state. The resulting antioxidant free radical is generally stable and does not initiate another free radical due to the stabilisation of and delocalisation of radical electron (resonance stabilised) within an aromatic molecule, or through conformational change of the molecule. This 'quenching' of reactive oxygen species keeps them at an optimum levels for biological functions, whilst at the same time preventing oxidative damage to cellular components such as

DNA, proteins and lipids. Antioxidants have been shown to have an inhibitory effect on porphyrin-induced cellular phototoxicity *in vitro*,²⁴⁵ and one such compound, β -carotene, is a standard therapeutic agent used in EPP. There is sound theory for its use, as photosynthetic organelles in plants utilise carotenoids to quench the excited singlet oxygen formed by the porphyrin chlorophyll during rapid energy and electron transfers.²⁴⁶ It also absorbs light maximally at 450-470nm, and may therefore, act as a chromophore, reducing some of the light that would activate PP.²⁴⁷ Clinical trials in bacterial and animal models suggested benefit; β -carotene was approved for use in EPP by the United States Food and Drug Administration in 1975.²⁴⁸ The initial uncontrolled trial found an increased sunlight tolerance in 84% of subjects,²⁴⁹ however discolouration of the skin at therapeutic doses makes blinded assessment difficult and subsequent trials have found a similarly high response rate to placebo.²⁴³ The only controlled cross-over trial of β -carotene (which assessed subject's recordings of time spent outside during placebo and β -carotene treatments) did not show a significant therapeutic effect.²⁵⁰ The small numbers used in the trial (13) could have resulted in failure to detect a small effect, and there was no objective measurement used of exposure to sunlight. Furthermore, following publication of the trial, the treatment's main proponent, Dr Mathews-Roth, suggested that doses higher than the 100mg daily use in the trial (and which she had initially proposed) were lower than optimal. Unfortunately the preparation has not been tested again at these higher doses. Thus if β -carotene has any effect at lower doses, this is probably small, and has not been proven to be effective at higher doses. The maximum daily oral dose is age-related (1-4 years: 60-90 mg, 5-8 years: 90-120mg, 9-12 years: 120-150mg, 13-16 years: 150-180mg, age 16 and over: 180mg), although a maximum of 300mg/day has been used safely. Approximately 80% of individuals on maximal dose β -carotene report significant prolongation in the time they can spend outside before developing the symptoms of EPP, although one to two months may elapse before this benefit is noticed. Approximately 20% of EPP individuals did not find β -carotene helpful, and if there is no improved tolerance to sunlight by 3 months at maximal dose, it can be concluded that β -carotene is not effective in that individual.^{248,251} The main side effects are carotenaemia, manifesting as striking yellow-orange discolouration of the skin, most marked on the palms and soles. Less

commonly, gastro-intestinal side effects such as nausea, abdominal discomfort and diarrhoea occur. There are conflicting reports of β -carotene and the risk of systemic malignancy. Epidemiological studies of β -carotene dietary supplementation of individuals at high risk of lung cancer (smokers and asbestos workers) found more cancers in the intervention group compared with placebo,^{252,253} while another looking at all cancers found neither an increase or a protective effect.²⁵⁴ The currently recommended formulation of β -carotene supplementation is a 'beadlet' preparation (Lumitene) which has superior bioavailability over crystalline formulations. Increased intake of carotenoid-containing foods would not attain the serum levels recommended, and could result in toxicity from other food constituents. Previous use of preparations containing the carotenoid pigment canthoxanthine in combination with β -carotene is no longer recommended due to lack of photoprotection, and risk of retinal deposition of canthoxanthine granules.

Vitamin E (α -tocopherol), similar to β -carotene, shares the ability to quench singlet oxygen. Case reports have suggested possible efficacy,^{156,157} but *in vivo* studies have failed to show clinical benefit as a systemic photoprotective agent.^{7,255} N-acetylcysteine (NAC) is an agent that increases levels of glutathione, an endogenous antioxidant. The mechanism of action is unknown, but may involve the quenching (a process which decreases the intensity of a reaction through charge or energy transfer reactions) of radical oxygen molecules, or increased levels of glutathione to attenuate porphyrinogen oxidation. Case reports suggest efficacy in reducing the cutaneous symptoms,²⁵⁶⁻²⁵⁸ however a small cross-over double blinded trial in EPP showed no benefit.²⁴³ The chemically similar L-cysteine has been suggested to have a beneficial effect in EPP; again the mechanism is thought to be an antioxidant function.²⁵⁹

Subjective and objective increased tolerance to sunlight of EPP individuals can be induced using narrow-band UVB (TL-01) phototherapy in spring to 'harden' the skin over the summer period.^{260,261} The mechanism of action is not fully understood but may result from UV-induced skin thickening and melanogenesis. Long-term risks of phototherapy are a disadvantage, but are probably more than offset by the cumulative minimal UV exposure of EPP individuals due to their expert and rigorous sun-

avoidance behaviour. PUVA has also been used successfully,²⁶¹ but as the wavelengths of UVA more closely approach those of visible violet light, and therefore increase the risk precipitating EPP symptoms, UVB is probably to be preferred.

Subcutaneous administration of synthetic α -melanocyte stimulating hormone (MSH) induces skin tanning without excess UV exposure.^{262,263} An open-label trial in five EPP patients has suggested significantly increased tolerance to artificial light and increased cutaneous melanin density.²⁶⁴

Case reports suggest utility or possible utility of a number of other agents such as pyridoxine,²⁶⁵ zinc (as zinc protoporphyrin is less phototoxic than metal-free protoporphyrin),²⁶⁶ and dietary fish oils (the mechanism is unknown although the high omega-3 polyunsaturated fatty acids may act as an antioxidant).²⁶⁷ Other non-anti-oxidant treatment approaches have focussed on trying to reduce the enterohepatic circulation of PP excreted by the liver but then subsequently reabsorbed. In the context of incipient or actual liver decompensation, when levels of PP are significantly elevated, cholestyramine has been reported to reduce blood levels of PP and reduce photosensitivity. Treatment may also be associated with histological evidence of a reduction in hepatic crystalline deposits of PP.²⁶⁸

1.15.4 Suppression of haematopoiesis

Haematological investigations of EPP patients often suggest an iron-deficient picture,¹²⁷ although the mechanism remains uncertain, as no studies have examined the aetiology. It seems unlikely that individuals with EPP are losing iron; the mechanism probably involves either failure to adequately absorb iron from the gut, or failure to utilise it (suggested by electron microscopic demonstration of iron deposition in EPP erythroblast mitochondria).¹²⁷ The role of iron supplementation in EPP is controversial and the literature limited, consisting mainly of small series and case reports (table 4). Clinically iron has been reported to normalise liver function and erythrocyte protoporphyrin levels²⁶⁹⁻²⁷² however others report symptomatic and biochemical deterioration of EPP following supplementation^{159,273} with improvement following the discontinuation of treatment. Theories for the beneficial effects of iron

supplementation in EPP include reduced enterohepatic circulation due to chelation with protoporphyrin in the intestine, increased non-enzymatic intracellular chelation with protoporphyrin, or facilitated chelation by residual active ferrochelatase due to increased intracellular iron concentrations.²⁶⁹ Conversely deterioration following supplementation may result from stimulation of haematopoiesis and increased production of protoporphyrin, interaction with other products such as the oral contraceptive, which predispose to cholestasis, or induction of photodamage by acting as a catalyst in oxygen free radical formation.²⁷³

A clinical observation of decreased photosensitivity following transfusions with washed packed red blood cells, led to the observation that this was accompanied by a decline in free erythrocyte PP levels in EPP individuals.^{270,271} Transfusions (usually in the setting of decompensated cirrhosis) of packed red cells or haematin have been reported to reduce both photosensitivity and biochemical markers of the condition such as free PP and TEP,²⁷⁰⁻²⁷² however others have reported there is no benefit in preventing progressive hepatic deterioration²¹² and of symptomatic deterioration following such treatment.²⁷⁴ The mechanism for action of packed red cell transfusions may be through suppression of endogenous bone marrow erythropoiesis and PP synthesis, as has been demonstrated in sickle cell anaemia and thalassaemia, or through a dilutional effect.^{275,276} Improvement in symptoms logically results from the reduced free PP and TEP levels, although a direct link between these parameters and degree of photosensitivity has not previously been demonstrated. However this therapeutic approach carries significant risk of transmission of blood-borne viral and prion infections, is not generally used, except in the setting of fulminant protoporphyrinic liver disease.

Table 4. Summary of the effects of oral iron supplementation in EPP.

| Year of paper | Number of subjects | Haematological investigations | Treatment | Results of oral iron therapy | Reference |
|---------------|--------------------|---|------------------------------|--|----------------|
| 2006 | 1 | low Hb, serum ferritin raised TIBC | oral iron | normalisation ferritin and TIBC, improved Hb no change LFT or TEP reduced photosensitivity | current report |
| 1990 | 1 | low Hb, MCV, serum ferritin | oral iron | increased photosensitivity raised LFT | 273 |
| 1988 | 4 | a) iron deficiency, low Hb b) low Hb c) iron deficiency, low Hb d) iron deficiency, low Hb | oral iron | a) increased photosensitise b) increased photosensitivity c) increased photosensitivity raised TEP d) increased photosensitivity raised TEP | 159 |
| 1986 | 1 | low Hb, MCV, iron, serum ferritin raised TIBC, LFT | oral iron | reduced TEP improved LFT photosensitivity effect not reported | 269 |
| 1970 | 1 | intrahepatic porphyrastasis, iron deficiency anaemia | oral iron and cholestyramine | reduced photosensitivity reduced pp liver deposition | 268 |

1.15.5 Gene therapy treatment for EPP

Since the underlying abnormality in EPP is a reduced level of ferrochelatase activity, mainly in the bone marrow, the definitive treatment for the condition would be to augment FECH levels to 50% or above that of the wild-type population. Human *FECH* has been transferred using a retrovirus into the haematopoietic stem cells of a missense *FECH* EPP mouse model. Transplantation into irradiated recipient EPP-model mice, resulted in elevated erythrocyte protoporphyrin levels falling to within the normal range, and clinically correction of the skin photosensitivity, compared to non-transplanted mice, although pre-existing liver dysfunction and cirrhosis was not reversed.²⁷⁷ As greater than 50% of haematopoietic stem cells required correcting to reverse the EPP phenotype, and there was no substantial *in vivo* selection of the transfected cells compared to FECH-deficient cells, *ex-vivo* preselection of a high proportion of transfected cells is necessary, for the current time making this technique impractical in EPP. Unfortunately human experiments of gene therapy using retroviral vectors for haemophilia B detected vector in participants' semen, raising concern about germline modifications, and those in children with severe combined immunodeficiency (SCID) had a higher than expected level of leukaemia in the recipients. Thus human gene therapy is currently facing a period of further experimentation, particularly to find alternative vectors, before additional *in vivo* trials can be conducted.²⁷⁸

The identification of both the mutant *FECH* and the low activity wild-type *FECH* has enabled useful genetic counselling for the first time. If the partner does not possess the low-expression variant, the chances of children having the condition is very low, whilst if the variant is present, the probability is 25% for each conception. Potential parents who previously might have opted not to have children or experienced considerable anxiety prenatally can now make an informed choice.

1.15.6 Treatment of hepatic failure

For EPP patients who progress to cirrhosis or acute liver failure, liver transplantation has been established as a successful treatment.²⁷⁹ By 2007, sixteen patients in Europe and 20 in the USA had received transplantation.⁶⁴ However the underlying

metabolic aetiology is not corrected and liver disease may recur in the graft.²⁷⁹ Complications of this technique include perioperative light-induced tissue damage and motor neuropathy. Furthermore rapid decompensation of liver function may occur in those developing fulminant hepatic failure, manifesting as an EPP crisis, where the acute decline in hepatic function is accompanied by a marked increase in erythrocyte and plasma PP, with severe abdominal and back pain. Light-induced tissue damage is prevented by screening all light sources with a filter to remove wavelengths of 400-410 nm.²⁸⁰

It is standard practice to use a number of therapeutic interventions simultaneously for EPP patients with decompensating liver disease; clinical or biochemical improvement can be difficult to ascribe to any one therapy.²⁸¹ Haematin, usually combined with plasmapheresis, has been used to treat crises and neuropathy by reducing PP levels.^{272,282} In acute porphyrias there is evidence that haematin reduces excess production of porphyrin by the bone marrow.²⁸³ Plasmapheresis decreases free plasma PP by plasma exchange, but does not treat protein-bound PP. Replacement with extracorporeal albumin dialysis (molecular adsorbents recirculating system (MARS) and the Prometheus system) increase the proportion of PP removed.^{214,284}

Oral cholestyramine has been reported to be successful in reducing TEP, normalising serum transaminases and improving hepatic architecture on biopsy, both as monotherapy and in combination with oral iron or vitamin E.^{156,268} These effects are probably related to its demonstrated effect on increasing faecal PP excretion, probably through interruption of the enterohepatic PP circulation.²⁸⁵

A case of oral iron supplementation has been reported to improve the biochemical parameters of liver dysfunction, possibly through enhanced conversion of excess protoporphyrin to haem.²⁸⁶ However, others have reported deterioration both biochemically and symptomatically when this approach has been tried for other patients.¹⁵⁹ The mechanism for these observations has yet to be elucidated.

Chenodeoxycholic acid was reported to lower erythrocyte and plasma protoporphyrin and reduce faecal excretion,^{137,287} perhaps through a reduction in synthesis or assisted conversion to haem.²⁸⁸ Rat studies have suggested that ferrochelatase activity in hepatocytes could be altered through dietary manipulation with fatty acids, particularly those diets rich in triolein oil,²⁸⁹ or dietary fish oils.²⁶⁷

Thus, the most effective treatment for liver failure is currently liver transplantation, through the removal of the hepatic contribution to excess protoporphyrin production, and reinstatement of efficient protoporphyrin excretion.^{146,203} As the underlying bone marrow ferrochelatase deficiency remains, transplanted patients are at risk of developing damage in the new liver. In such a situation, some advocate chronic intravenous haem-albumin and plasmaphoresis to support the transplanted organ.²⁸²

Bone marrow is primarily responsible for protoporphyrin over-production and the resulting hepatic complications. Bone marrow transplantation (BMT) has been shown to be successful in an EPP mouse model,^{277,290} and as an unintentional effect in a human following transplantation for acute myelogenous leukaemia.⁶⁵ One further individual successfully received bone marrow, rather than liver, transplantation, following the development of severe progressive protoporphyric hepatic failure, with resolution of his serum liver biochemistry and improvement in histological liver architecture.⁶⁴ A further case of successful liver transplantation followed by bone marrow transplantation has recently been reported, with the rationale of protecting the engrafted organ and preventing recurrent liver disease.²⁹¹ However because of the significant associated risks, experience of the therapeutic use of BMT in EPP remains limited.

1.15.7 Follow-up for EPP patients

Evidence upon which to base recommendations for the management of EPP individuals, particularly with regard to predicting and treating liver disease, is lacking. This is made more difficult by the widely varying incidences for liver disease given by previous studies, and no proven evidence for liver function tests being a useful tool to identify significant liver disease. Unsurprisingly therefore,

recommendations for surveillance to identify liver disease vary. Mathews-Roth recommended liver biopsy in any patient with abnormal liver function, even when this was minimal, or in those with markedly raised erythrocyte or plasma porphyrin levels (TEP > 2000 µg/dl; plasma protoporphyrin > 50 µg/dl).²⁹² Mooyaart suggested annual measurement of serum liver function before the age of 20, and biennial subsequently, with liver biopsies at a minimum of 5-year intervals.¹⁶¹ Sarkany and Norris measure liver function at six monthly intervals, on the basis of the speed at which deterioration can progress.²⁹³ Thunell advocates annual follow-up with more frequent visits for those with increasing TEP > 30 µg/dl and referral to a hepatologist if there is evidence of abnormal liver function.¹⁰⁸ Harper recommends an annual follow-up including vaccination for hepatitis A and B, monitoring liver function and porphyrin levels, and liver biopsy in the presence of elevated results.²⁹⁴

A recent review has proposed the following programme for non invasive monitoring of liver function, albeit without a suggested frequency:¹³¹

1. Investigations to exclude other causes of hepatic dysfunction (viral hepatitis, haemochromatosis).
2. Liver function tests (including AST, ALT, Alk Phos and GGT).
3. Red cell (TEP) and plasma protoporphyrin levels.
4. Non-specific serum markers for hepatic fibrosis (YKL-40, Fibrotest, Fibroscan and serum aminoterminal propeptide of type III procollagen).
5. Ultrasound scan for gallstones.
6. CT and MRI studies of the liver.

They also suggest indications for liver biopsy in the following EPP patients:

1. Those with null mutations or autosomal recessive disease in countries where *FECH* genotyping studies are available.
2. In the presence of a family history of EPP-related liver disease.
3. Those with other risk factors for liver disease (viral hepatitis, haemochromatosis, alcohol excess, non-alcoholic fatty liver disease).
4. Abnormal liver function tests.

5. Evidence for hepatic decompensation (worsening of photosensitivity, liver function tests, rising protoporphyrin levels).
6. Patient anxiety or preference.

2. Methods

2.1 Ethical committee approval

The study was conducted in accord with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments. Multicentre research ethics committee (MREC) approval was obtained from the north-west MREC, Manchester (MREC No. 03/8/065 – 13th October 2003) for a ‘no local researcher’ study. Following their instructions, 84 local research ethics committees for areas in which study subjects were resident were informed of the study, the name of the subjects’ local consultant, and sent a copy of the MREC approval letter.

2.2 Statistics and sample size

A minimum sample size of 150 patients was selected following discussion with the Department of Epidemiology, Statistics and Public Health, University of Wales College of Medicine (Mr R. Newcombe, Reader). At prevalences of 20% for abnormal LFTs and 20-30% for missense mutations, estimated statistical power is about 88%. Statistical analysis was performed by the study investigator using an SPSS package version 12 under the guidance of this department. All independent factors were analysed using Spearman’s correlation coefficient for continuous quantitative data, or the Mann-Whitney test for nominal data, to identify significant associations (where significance was taken to be $p < 0.05$), or where the association approached significance ($p < 0.1$). Linear regression analysis was subsequently used to investigate associations between these factors.

For statistical analysis of liver function test status, results were graded both into a binary normal/abnormal and into a 4-category ordinal variable (normal, A (elevated γ GT, isolated increase in serum alkaline phosphatase, bilirubin < 34 U/L), B (elevated AST ≤ 68 , ALT ≤ 60) and C (AST > 68 , ALT > 60). As the majority of variables had skewed distribution, non-parametric data analysis was performed: a Kruskal-Wallis test (KWT) for one-way analysis of concordance and the Mann-Whitney test (MWT) for independent groups.

2.3 Identification of cases of erythropoietic protoporphyria

All available EPP patients were identified from databases or patient records held in 5 British supra-regional referral centres for photodermatoses:

1. Department of Medical Biochemistry and Immunology, University Hospital of Wales, Cardiff.
2. St John's Institute of Dermatology, St Thomas' Hospital, London.
3. Departments of Biochemistry and Dermatology, Hope Hospital, Salford.
4. Department of Biochemistry, The General Infirmary, Leeds.
5. Department of Dermatology, Ninewells Hospital, Dundee.

Inclusion criteria

- Age at least 5 years
- Biochemical diagnosis of EPP

Exclusion criteria

- Age less than 5 years
- Haematological or hepatic malignancy

The referring consultant for each patient was identified, either from the records, or by telephoning the referring hospital or department. Each of these physicians was sent details of the study, informing them of the ethics approval and asking if they would allow their patients to be contacted and invited to take part in the study. Consultants were asked to reply to the dermatology department in Cardiff using a postage-paid envelope, confirming the current contact details of their patients. To minimise travel by patients, the consultants were also asked if they would allow the use of their local out-patients department for the study investigator to see their patients. In situations where patients had moved areas, participating consultants were asked if they minded their patients being seen at a different hospital. Following permission, patients were sent a postal study invitation consisting of a covering letter (stating that their local consultant's and the local ethical committee permission had been sought), a study information sheet, a reply form to indicate whether they did or did not wish to

participate and a stamp addressed envelope. If the individual's consultant felt this would be perceived as 'cold-calling' and might cause distress, the patient invitation letter was sent to the consultant to allow them to insert a personalised covering letter.

2.4 Organisation of patient visits

With secretarial help provided by the Department of Dermatology, University of Wales College of Medicine, groups of patients in each locality with available first degree family members, were asked to attend the local NHS out-patient facility, following the prior agreement of the unit. Patients were allocated appointments at 30 minute intervals. With the prior agreement of the local biochemistry department, serum blood samples were centrifuged prior to transportation back to Cardiff for analysis.

2.5 History taking

History taking for details of photosensitivity was standardised by use of a proforma (appendix III). The questionnaire was constructed for completion by the investigator, as part of an interview with the subject (or subject and their parents, if subjects were children). The same investigator saw all the study subjects to minimise inter-observer variability. The questions were constructed to be clear and concise. Some questions were relatively focused, with a yes/no format, or with a limited number of choices. Other questions were deliberately open-ended to ensure that clinical information of relevance was not overlooked, and all comments made during the interview relating to EPP were recorded. Every effort was made to avoid ambiguous or leading questions.

The clinical proforma was divided into four main sections:

- General questions relating to the photosensitivity and the appearance of any associated eruption.
- Questions relating to photoprotective measures and treatments taken to prevent the photosensitivity.
- Questions concerning the individual's previous medical history.
- Questions about their family history and social background.

2.5.1 Photosensitivity

- i. *How old were you when you first experienced problems with EPP?*
- ii. *What were these initial symptoms?*
crying / screaming / burning / itching / tingling / other
- iii. *How old were you when the diagnosis was made?*
- iv. *Who made diagnosis? GP / dermatologist / paediatrician / gastroenterologist / other doctor / other person*
- v. *What symptoms do you have now after sun exposure?*
nil / crying / screaming / burning / itching / tingling / other
- vi. *How long does it take for the symptoms to start after sunlight exposure?*
- vii. *How long does it take for the symptoms to settle?*
- viii. *Does your skin have any visible changes immediately after sunlight exposure?*
nil / redness / swelling / eczema / bruising / blistering / crusting / other
Are there any visible changes later on?
nil / redness / swelling / eczema / bruising / blistering / crusting / other
- ix. *Apart from the skin symptoms, do you feel unwell in any other way after sunlight exposure?*
nil / fever / generally unwell / can't sleep / feel down / irritable / other
- x. *On the second day of sun exposure, are the symptoms the same, better or worse?*
- xi. *Is your skin sensitive to sunlight through window glass?*
- xii. *Is your skin more sensitive on windy days? Y / N*
- xiii. *Do your nails ever get affected by the sunlight? Y / N*
- xiv. *Is your skin more fragile / difficult to heal than others? Y / N*
- xv. *Do you think your skin sensitivity changes over the summer or stays about the same?*
- xvi. *Do you think there has been any change in sensitivity as you've got older? Y / N*
If yes, improved / deteriorated, and any thoughts why?
- xvii. *Is your skin ever sensitive over winter (November – February)? Y / N*
If yes, how often / circumstances etc

xviii. Does your sunlight sensitivity interfere with your daily activities in summer?

Y/N

The age of onset of a photosensitivity reaction is important to establish. Onset in the first year of life and associated with first exposure to sunlight, would suggest a congenital photosensitivity syndrome. In contrast, onset later in life suggests an acquired form of photosensitivity (e.g. porphyria cutanea tarda) although there have been reports of late-onset or late presentations of EPP.

Because of the relative lack of obvious physical signs, problems with diagnostic samples being insufficiently light protected and a general lack of knowledge of the condition, there is a perception that individuals with EPP may suffer a delay in obtaining a formal diagnosis. By asking about the age at presentation and diagnosis and by whom the diagnosis was made, we sought to identify diagnostic delay and whether a perceived lack of knowledge amongst health professionals is borne out by the initial diagnosis being mainly by secondary care professionals, particularly dermatologists.

To determine the interval between going into direct sunlight and the onset of the eruption, patients were told to imagine a day in June with little cloud cover. According to the majority of dermatology textbooks, EPP is a condition in which there is a paucity of visible signs, particularly acutely, although there are reports of individuals who experienced one or more presentations from redness, swelling, eczema, bruising, blistering or crusting. We sought to investigate the veracity of these statements. The list of possible signs was not read out to the participants, but used to assist with the recording of replies. As the majority of previous clinical descriptions have focused on the acute cutaneous symptoms, we also sought to discover if individuals experienced any other symptoms when acutely affected, the degree to which the reported priming phenomenon is experience,¹¹⁹ and other aspects of the sun sensitivity such as sensitivity to sunlight through glass (glass filters out UVB, and photosensitive eruptions triggered by sunlight transmitted through glass are therefore usually a manifestation of sensitivity to UVA or visible light) and the

effect of wind (once briefly mentioned in a previously report,¹⁰⁶ and spontaneously by a number of the EPP individuals on whom the proforma was piloted). This effect may well be the cooling effect of wind on the skin, reducing the burning perception and allowing a longer exposure to sunlight.

A single case of onycholysis in an EPP patient following sun-exposure has been reported,¹¹⁵ so a proforma question aimed to discover if this had been a chance finding, or was an experienced shared by other individuals with the condition.

Activation of protoporphyrin in the skin by light results in the formation of reactive oxygen species, and damage to surrounding structures such as vascular endothelium and beyond that, the dermis. One might expect such damage to result in skin fragility, as in other cutaneous porphyrias, although this has been said to be uncommon by a major dermatology textbook.²⁹⁵

Some photosensitivity syndromes improve with repeated exposure to sunlight (the so-called “hardening response”), and this forms part of the rationale for their treatment with phototherapy. We sought to establish the proportion of individuals with EPP who had noticed this phenomenon.

Again to check to veracity of a statement in a major dermatology textbook that the natural history of the photosensitivity in EPP “often (improves) spontaneously after the age of 10 or 11 years”, the proforma asked for participants’ personal experience.²⁹⁵

Over the winter months in the UK, the lower angle of the sun in the sky results in a greater proportion of sunlight being absorbed in the atmosphere. Combined with the shorter day lengths, it would be reasonable to speculate that photosensitivity is not a significant problem at this time of year. An open question sought to test this hypothesis.

A final rather non-specific question was asked about whether the condition interferes with daily activity in summer. The purpose of this question was to provide an overall global picture of the impact of EPP and whether the results of this question would correlate with and provide a degree of validation for the score from the quality of life questionnaires.

2.5.2 Photoprotection

- xx. *Do you regularly try to avoid sunlight? Y / N*
- xxi. *What kind of things do you do?*
stay inside / seek shade/ go out in evening / at night/ other
- xxii. *Do you regularly wear special clothes to go out in sunlight? Y / N*
hat / high collar / long sleeves / trousers / gloves / other
- xxiii. *Do you use sunscreen? Y / N*
- xxiv. *What type and sun protection factor (SpF)?*
- xxv. *How often? daily / at weekends / less often*
all year / in any sunny weather / over summer only / abroad only / other
- xxvi. *Are you aware what the star ratings for sunprotection mean? Y / N*
- xxvii. *Have you tried any of the following, and were they helpful?*
- | | | |
|-------------------------|---------------------------------------|------------------------------|
| <i>antihistamines</i> | <i>currently / previously / never</i> | <i>helpful / not helpful</i> |
| <i>β-carotene</i> | <i>currently / previously / never</i> | <i>helpful / not helpful</i> |
| <i>Dundee sunscreen</i> | <i>currently / previously / never</i> | <i>helpful / not helpful</i> |
| <i>cysteine</i> | <i>currently / previously / never</i> | <i>helpful /not helpful</i> |
| <i>phototherapy</i> | <i>currently / previously / never</i> | <i>helpful / not helpful</i> |
| <i>sunbeds</i> | <i>currently / previously / never</i> | <i>helpful / not helpful</i> |
| <i>fake tan</i> | <i>currently / previously / never</i> | <i>helpful / not helpful</i> |
| <i>other treatments</i> | <hr/> | |
- xxviii. *Who is involved in the care of your skin problem?*
No-one / GP / dermatologist / paediatrician / gastroenterologist / other
How often does each of these people see you?

Clothing is an important aspect of photoprotective measures, and is a helpful indicator of how severe a photosensitive eruption has been in the past and the degree to which it can impact on aspects of daily living.

Most members of the general population are aware of the SPF (sun protection factor) ratings for sunscreens, a laboratory-conditions rating which can be used to give a rough estimation of increasing protection the product affords against UVB. In the last 10 years an additional star rating (from 0 to 5) has been introduced by some manufacturers to give an indication, relative to the product's SPF, of the protection it affords against UVA. However individuals with EPP are more sensitive to longer wavelengths of UV into visible light, so an ideal sunscreen would require high reflectant properties against visible light in combination with high SPF and star rating.

Although a number of different therapeutic options have been reported, there are no large-scale trials assessing efficacy. Some treatments such as hospital phototherapy are not easily accessible, and others may be associated with side effects. The questionnaire sought to obtain the range of treatments that subjects had used or were currently using, and their views on overall usefulness. In addition the final open-ended question sought to obtain other therapeutic measures that individuals might have been tried.

There are currently no national guidelines on how often and by whom EPP patients should be followed up, although authors have made suggestions.¹³¹ A question was included to identify the current UK situation.

2.5.3 Previous medical history

xxix. Have you ever had any other medical conditions?

| | |
|------------------------|---------------------|
| <i>anaemia</i> | <i>Y/N details:</i> |
| <i>iron deficiency</i> | <i>Y/N details:</i> |
| <i>gallstones</i> | <i>Y/N details:</i> |
| <i>hepatitis</i> | <i>Y/N details:</i> |

| | |
|------------------------------|-----------------------|
| <i>other liver disease</i> | <i>Y / N details:</i> |
| <i>liver treatment</i> | <i>Y / N details:</i> |
| <i>arthritis</i> | <i>Y / N details:</i> |
| <i>haematological cancer</i> | <i>Y / N details</i> |
| <i>other</i> | _____ |

xxx. *Are you allergic to anything? Y / N If yes, what?*

xxxi. *Are you currently taking medicines and tablets? Y / N If yes, what?*

In common with a general medical history, participants were asked about other previous medical conditions in an open format. A number of specific conditions were then mentioned; these being conditions that were either study exclusion criteria, or conditions associated with EPP or liver dysfunction. A standard drug history was taken.

2.5.4 Family and social history

xxxii. *Are there any illnesses that run in your family? Y / N If yes, what?*

Family tree: parents/siblings/marital status/children

xxxiii. *Women only: Does your sensitivity change with your menstrual cycle? Y / N If yes, how?*

xxxiv. *If you had children, did you find any change in the EPP during your pregnancy? Y / N If yes, how?*

xxxv. *Place of birth: UK / northern Europe / Mediterranean Europe / other*

xxxvi. *What would you describe your ethnic origin as?*

caucasian / indo-asian / far east asian / afro-caribbean / other:

xxxvii. *Are you working? Y / N And if so, what is your job?*

xxxviii. *Was your choice of profession influenced by your skin? Y / N*

xxxix. *How much alcohol do you drink in an average week?*

Estimated units beer / wine / spirits / other

xl. *Have you ever noticed your skin more sensitive after drinking alcohol Y / N*

xli. *Do you smoke? Y / N And if yes, how much per day?*

xl.ii. *Vegetarian / Non-vegetarian?*

A family history and medical history was sought to investigate for associations. There are a number of reports of EPP symptoms improving during pregnancy, although the mechanism for this is unknown.^{8,115,165-167} Questions were asked to investigate this association, and to examine if this observation might be hormonally-mediated, and evident on a monthly cyclical basis.

Self-reported ethnicity was asked for: Although EPP appears to occur in all populations, there have been reports of increased incidence in certain ethnic groups (Professor Elder, personal communication), and it is possible that skin pigmentation provides additional protection against symptom onset in the UK following UV-exposure.

Whilst part of a general social history, a question on choice of profession was also included to assess if this was influenced by having EPP, and if any participants had occupations that resulted in them working outdoors. Alcohol and smoking were enquired about as part of a general medical history, but also because alcohol might be used as an analgesic during acute episodes, and for its potential to cause hepatotoxicity. Dietary preference was sought, as vegetarians, particularly strict vegans, might be more likely to have iron deficiency than omnivorous individuals.

2.6 Examination

Light-exposed skin was examined at the end of the history taking, particularly the face, neck, arms, hands and nails, but also any other affected skin volunteered by the subjects. Examination findings were grouped into acute (e.g. erythema, oedema, eczema, purpura, vesicobullous and crusted erosions) and chronic EPP signs (e.g. excoriations, thickened skin, hyperkeratosis, yellowing, excessively wrinkling, and scarring (varrioliform/pitted/stellate etc)), and other signs (e.g. evidence of liver dysfunction such as spider naevi or flap). The cutaneous signs were graded into a simple mild / moderate / severe.

2.7 Quality of life measurement

Skin conditions have been shown to exert profound effects on an individual's quality of life (QOL), through the disruption of family and social relationships, interference with sport, leisure and work, and adoption of specific clothing. Although there have been considerable advances in understanding the molecular genetics and treatment of porphyrias, little research has assessed the psychosocial consequences of these conditions. QOL was measured using previously validated dermatology-specific quality of life tools, the dermatology life quality index (DLQI – appendix vii)²⁹⁶ and the children's dermatology life quality index in a cartoon format (CDLQI – appendix viii)²⁹⁷. Each of these consists of ten questions, scored up to 3, giving a maximum possible score of 30: an increasing score indicates a more impaired QOL. They have both previously been validated using populations with dermatological conditions, and in normal individuals, for repeatability, internal consistency and sensitivity to change, and subsequently used in studies examining individuals, both adults and children, with a range of conditions including acne, atopic dermatitis, psoriasis, urticaria and vitiligo, and in a variety of languages.²⁹⁸

2.8 Blood samples, initial processing and transport

24 ml of blood was drawn from each patient into six tubes: two EDTA, one of which was light-protected, and four serum gel tubes, for the following analysis:

- | | |
|---------------------------|---|
| 1. EDTA | full blood count |
| 2. EDTA (light protected) | plasma porphyrin screen whole blood protoporphyrin screen whole blood protoporphyrin quantification DNA extraction and storage |
| 3. Serum sample | bilirubin aspartate transaminase alanine transferase alkaline phosphatase gamma glutamyl transferase albumin |
| 4. Serum sample | ferritin |

| | | |
|-----------------|-----------------------------|---------------------|
| | serum iron | |
| | total iron binding capacity | |
| | transferrin saturation | |
| 5. Serum sample | hepatitis serology | |
| 6. Serum sample | 25, hydroxy-vitamin D | serum calcium |
| | serum phosphate | parathyroid hormone |
| | stored for future analysis | |

Light-protected EDTA samples were placed in a thick brown envelope, which was then wrapped around the tube several times, secured with an elastic band, and kept in a darkened environment. Each serum sample was taken to the local biochemistry department, centrifuged at 3,000 r.p.m. for 8-10 minutes to allow the sample to separate, with the cellular component at the bottom of the tube below the gel, and the serum component in the area above the gel. The resulting serum was then stable to transport back to Cardiff. Depending on the location of visit, the samples and request forms were either sent to the porphyrin laboratory in Cardiff by post, or transported by car. Postal boxes used were Royal Mail first class postage pre-paid, diagnostic specimen containers (UN3373), which allowed transport of the six specimen tubes wrapped in a sealed plastic bag and wadding, in a water-tight inner compartment, with the request forms in an outer compartment. The reinforced outer plastic box was of a size suitable for postage through most post-boxes (figure 8).

All analyses were undertaken in the laboratories of the University Hospital of Wales, Cardiff. Automated processes were used for all the investigations listed above other than the porphyrin analysis and the DNA extraction and analysis.

Figure 8. The Royal Mail first class containers used for posting samples to the porphyria laboratories in Cardiff.



2.9 Biochemical analysis of samples

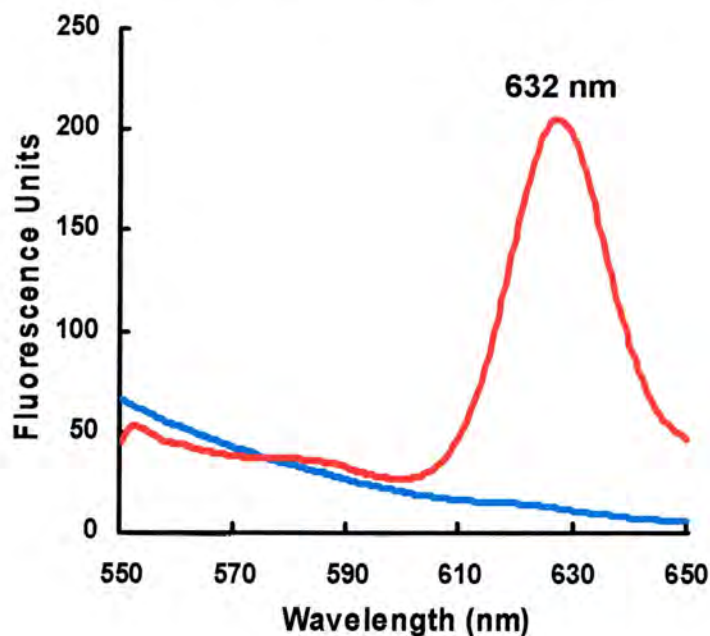
2.9.1 Routine automated biochemical analyses

Assessments of liver function were performed in the biochemical laboratories at the University Hospital of Wales, Cardiff, using the automated processes. The quantitative analysis of 25-hydroxyvitamin D (25-hydroxy cholecalciferol – 25-OH-D) was measured by radioimmunoassay following solvent extraction from stored patient's serum using a commercially available assay (^{125}I radioimmunoassay, DiaSorin, Stillwater, Minnesota, USA) in the Department of Medical Biochemistry and Immunology, University Hospital of Wales. 25-OH-D is the predominant circulating form of vitamin D, is more stable than 1,25-OH-D and is considered to be the most reliable index of vitamin D status. Serum 25-hydroxyvitamin D concentrations less than 10 and 20ng/ml (25 and 50nmol/L) were used to identify those vitamin D deficient (VDD) or insufficient (VDI).²⁹⁹

2.9.2 Plasma porphyrin screen

As porphyrins can absorb light energy and release this in the form of light emission in a different spectrum, a simple screen for porphyrins in samples can be undertaken by fluorescence emission detection. Plasma, diluted 10-fold in phosphate buffered saline is exposed to an excitation wavelength of 405nm light and screened for emission wavelengths between 550 and 650nm. Increased concentrations of coproporphyrin and uroporphyrin give a peak at around 615nm, protoporphyrin a peak at around 632nm, and protein-bound porphyrin, found only in variegate porphyria, a peak at around 626nm.

Figure 9. Plasma fluorescence emission of EPP sample compared to quality control sample (EPP patient sample in red, control sample blue).



2.9.2.1 Patient samples

5ml venous blood collected into heparin or potassium EDTA. An aliquot was centrifuged and the plasma removed. Samples were stored frozen at -20°C.

Sample processing was in the main undertaken by Mrs Jacqueline Woolf.

2.9.2.2 Equipment

1. Fluorimeter: Perkin-Elmer LS-50B luminescence spectrometer incorporating a R928 red sensitive photomultiplier (Hamamatsu 928).
2. Disposable soda glass cuvette tubes, 50 x 7.5 mm. Supplied by Kernicks Samco (code G010/24).

2.9.2.3 Materials and reagents

Phosphate buffered saline pH 7.3 (Oxoid BR14a)

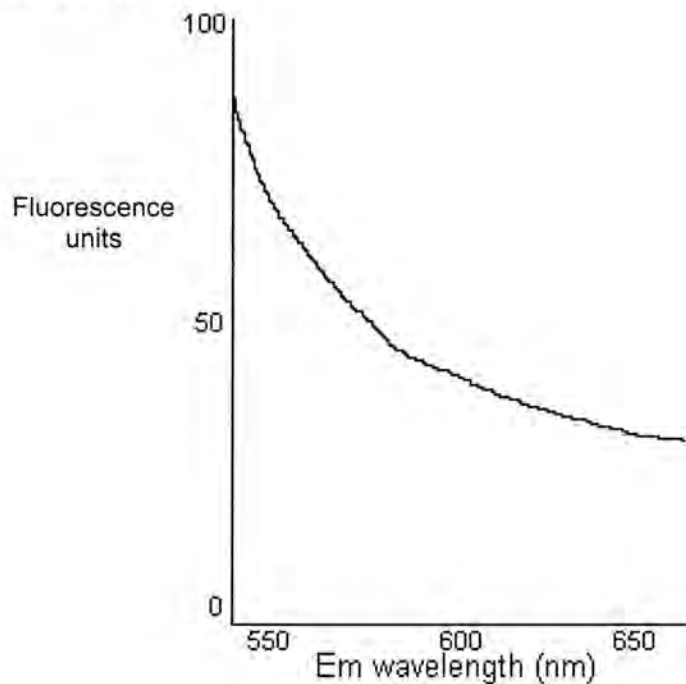
Dissolve one tablet in 100ml distilled water.

2.9.2.4 Method procedure

1. Thaw samples and QC (quality control sample).
2. Add 100ul plasma (QC and patient samples) to 900ul phosphate buffered saline in a soda glass cuvette. Mix by inversion.
3. Switch on fluorimeter, mertec disc drive, VDU and printer.
4. Click the 'FL Winlab' icon.
5. Call up 'LS50b setup' and change the excitation filter to 'clear (auto cut-off off)' and the emission monochromator to '515nm cut off'. Exit from the LS50b setup programme.
6. Click on 'pscan,mth' from the list of filenames: the 'setup parameters' screen will appear. At the 'result file' name box overwrite the day and month. Also select 'auto increment filename' and 'auto clear curves'.
7. The setup screen should be pre-set to scan emission between 550 and 650nm at an excitation wavelength of 405nm. The slit widths are both 10nm and the scan speed 240nm/min. Close the 'sample information' box.
8. At the 'spectrum 1' space overwrite the name of the sample or lab number.
9. Place the sample in the cuvette holder and close the lid.
10. Click on the traffic light icon. The light will turn red and the 'view scan' screen will appear when the scan begins. When it has finished, the light will turn green.

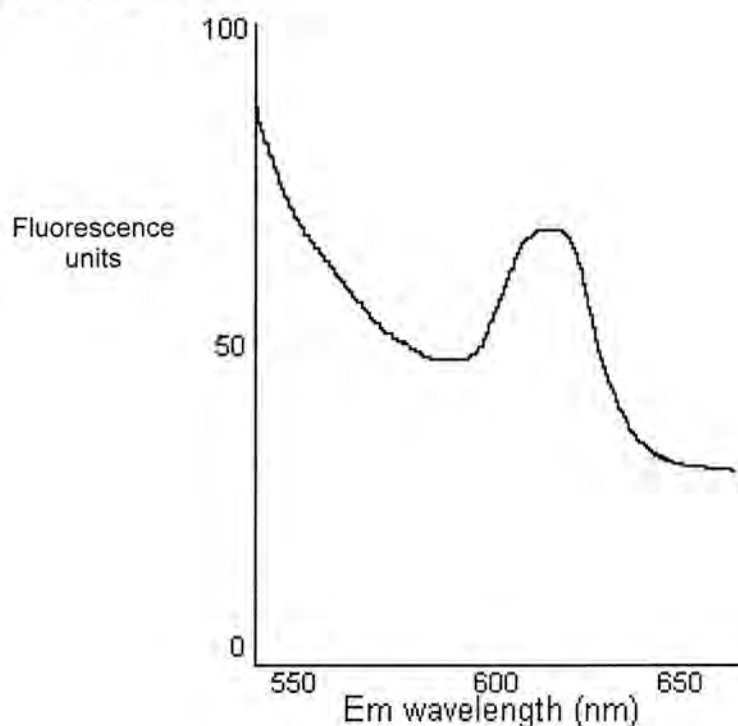
11. Turn off the fluorimeter and exit from the scan application.
12. To processing the scans, click on 'view' and from drop-down menu choose 'new graph window'. Click on 'file' and from the drop-down menu choose 'open'. Find the filename to be processed and double click on it. This will put the file into the new graph window. Adjust the ordinate by clicking on the second icon. Identify any peaks by clicking on the 7th icon and choosing 'peak' and 'OK'. If the trace appears negative close the graph window. Continue processing scans until a positive or borderline scan appears. Adjust the abscissa and identify the peaks as above but also click on 'file' and choose 'print'.
13. To calculating peak intensity, draw a tangent to the curve on the print out. Measure its height and calculate its intensity from the graph scale.

Negative scan:



If peak intensity is <5 FU (fluorescence units), this is reported as :
No porphyrin detected by fluorescence emission spectroscopy.

Positive scan:



If peak intensity is >4FU, this is reported as: Fluorescence emission spectrum shows a porphyrin peak at Xnm. If the intensity is >100FU, report as: Fluorescence emission spectrum shows a prominent porphyrin peak at Xnm

2.9.2.5 Clinical interpretation of results

Table 5. Clinical interpretation of fluorescent emission results

| Porphyria | Plasma fluorescence emission peak wavelength |
|-------------------------------------|--|
| Acute Intermittent Porphyria | ~615nm |
| Congenital Erythropoietic Porphyria | ~615nm |
| Porphyria Cutanea Tarda | ~615nm |
| Hereditary Coproporphyria | ~615nm |
| Variegate Porphyria | ~624-626nm |
| Erythropoietic Protoporphyria | ~632nm |

Porphyrin peaks at about 615nm may also be seen in chronic renal failure and cholestasis. A negative screen does not exclude porphyria.

2.9.3 Whole blood protoporphyrin screen

2.9.3.1 Principal of method

As in 2.9.2, this process uses the ability of protoporphyrin to absorb light energy in the Soret range and release this in the form of light emission in a different spectrum, to screen fluorimetrically for the presence and relative quantities of erythrocyte free and zinc protoporphyrin in whole blood samples. Zinc and free protoporphyrin have similar excitation maxima of 415 nm and 409 nm, but different emission maxima of 587 and 630 nm respectively. However, as analytical procedures in which acidic conditions are used the zinc protoporphyrin is removed, this method uses ethanol, a neutral extractant, to allow analysis for both.

The erythrocyte free protoporphyrin may be raised in EPP, and rarely in some sideroblastic anaemias and some homozygous porphyrias. The zinc protoporphyrin may be increased in lead poisoning, iron deficiency and some anaemias.

2.9.3.2 Patient samples

5 ml light-protected venous blood collected into potassium EDTA containers.

2.9.3.3 Equipment

1. Fluorimeter: Perkin-Elmer LS-50B luminescence spectrometer incorporating a R928 red sensitive photomultiplier (Hamamatsu 928).
2. Soda glass durham tubes, 7.5 mm, supplied by Kernicks Samco (code G010/24).

2.9.3.4 Reagents

1. Phosphate buffered saline pH 7.3
2. 95% ethanol

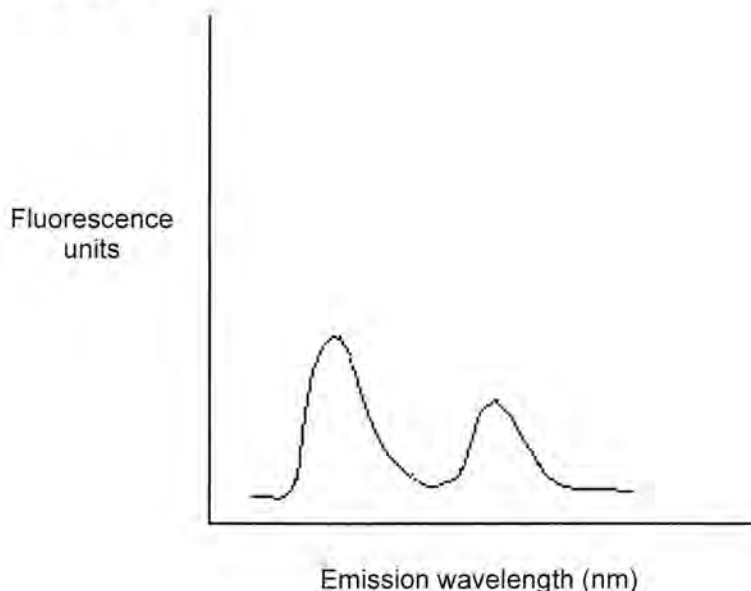
2.9.3.5 Method procedure

Add 50 µl of whole blood to 200 µl phosphate buffered saline in an eppendorf tube and mixed thoroughly by vortexing. Pipette 50 µl of this diluted blood into a fresh eppendorf tube containing 1 ml 95% ethanol, mixed for 15

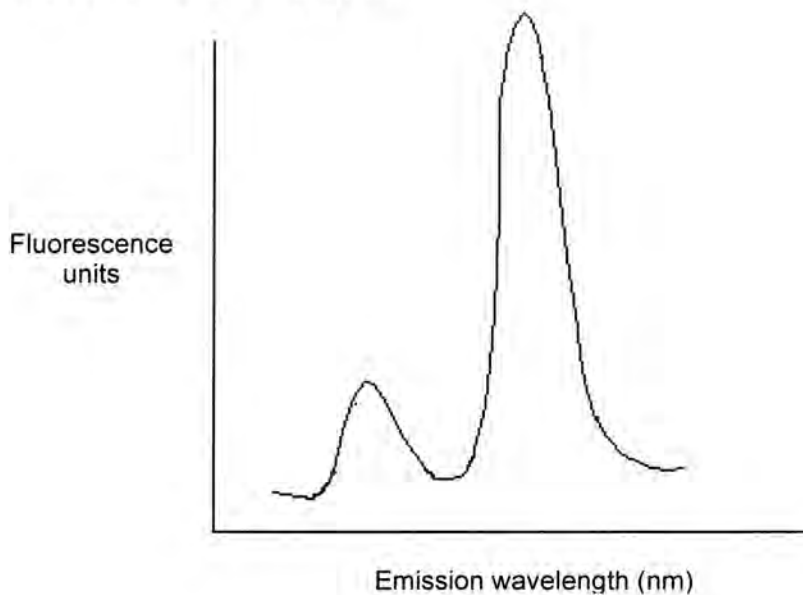
seconds then immediately centrifuge at 14,000 rpm for 2 minutes. Pipette supernatant into a disposable glass cuvette. Setup the fluorimeter to scan emission between 550 and 650 nm at an excitation wavelength of 415 nm. The slit widths are both 10nm and the scan speed 480 nm/minute. The sample is placed in the cuvette holder, the fluorimeter lid closed, and the traffic icon selected. During processing the green light turns red, and returns to green when the process is complete. The results are displayed on the fluorimeter VDU screen by selecting 'view', 'new graph window', 'file', and 'open' the filename to be processed. Double clicking the filename will place it into the new graph window. Clicking 'view' followed by 'horizontal cursor' allows the cursor to roughly estimate the FU response of zinc and free protoporphyrin peaks. A zinc protoporphyrin peak of <17 FU and a free protoporphyrin of <5 FU is reported as 'not increased (mainly zinc protoporphyrin)'. When a positive or borderline scan appears, roughly measure the peaks as about, but also click on 'file' and chose 'print'. Using the print out, measure the heights and intensity of the curves on the graph scale. A free protoporphyrin peak of >6 FU or zinc protoporphyrin of >17 FU is reported as raised and must be quantitated.

Interpretation of results:

Normal scan:

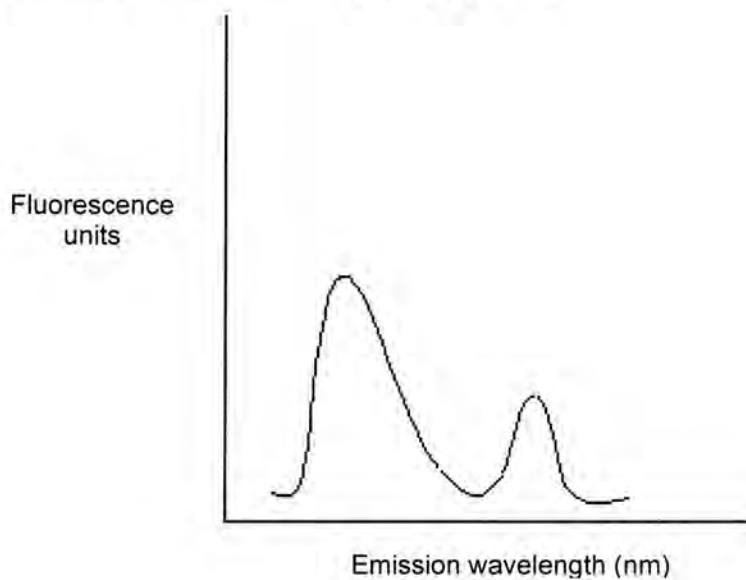


Raised free protoporphyrin:



Raised zinc protoporphyrin:

(Seen in X-linked dominant protoporphyria)



In erythropoietic protoporphyria, and rarely in some sideroblastic anaemias and some homozygous porphyrias, there is an increase in erythrocyte free protoporphyrin. In lead poisoning, iron deficiency and some anaemias, zinc protoporphyrin is increased.

2.9.4 Whole blood porphyrin quantitation

2.9.4.1 Principal of method

This method of quantifying porphyrins in whole blood uses comparison of the fluorescence of the sample against a standard molar coproporphyrin solution sample fluorescence. The samples are measured at the emission maxima of the porphyrins (in the case of coproporphyrin, the fluorimeter is calibrated using 595 and samples tested for protoporphyrin at 603nm respectively).

2.9.4.2 Patient samples

5 ml venous blood collected into potassium EDTA. The haematocrit is measured on a small sample of blood to enable the results to be expressed with reference to the volume of packed erythrocytes. The sample can then be frozen at -20°C and is stable for many months.

2.9.4.3 Equipment

1. A Perkin-Elmer LS-50B luminescence/Spectrophotometer incorporating a R928 red-sensitive photomultiplier. The proto factor is determined for the fluorimeter.
2. Cuvettes: Quartz glass tubes, 50 x 7.5mm supplied by Kernicks Samco. Code G010/24.

2.9.4.4 Materials and reagents

1. Phosphate buffered saline pH 7.3 (Oxoid BR14a)
Dissolve one tablet in 100ml distilled water.
2. Ethyl acetate 3:1 (BDH 10108 Analar)
3. Glacial acetic acid (BDH 10001 Analar)
Mix 6ml of ethylacetate with 2ml glacial acetic acid in fume cupboard.
4. HCL 1.5M (BDH 10307 Analar)
Dilute 129ml concentrated HCL to 871ml of distilled water.
5. HCL 5M (BDH 10307 Analar)
Dilute 429ml concentrated HCL to 571ml of distilled water.
6. Coproporphyrin I standard (Sigma COP-1-5)

2.9.4.5 Precautions and hazards

Ethyl acetate: glacial acetic acid mixture 3:1

- Area should be well ventilated
- Eppendorf tubes containing small volumes of ethyl acetate (μl) should be capped.

2.9.4.6 Method procedure

1. Equilibrate samples and coproporphyrin standard to room temperature, protected from light. Mix well before using. Fill in data sheet and turn on spectrophotometer to allow lamps to warm up.
2. Label Eppendorf tubes and add 60 μl phosphate buffered saline (PBS) to each.
3. Pipette 8 μl whole blood into PBS and vortex mix.
4. Add 400 μl ethylacetate/acetic acid while vortexing and continue to vortex mix for a further 15 seconds to break up clumps and vortex mix.
5. Centrifuge all tubes for 2 min. in Eppendorf centrifuge at 12,000 rpm.
6. Decant supernatant into clean Eppendorf tubes.
7. Add 800 μl 1.5M HCL and mix for 1 minute.
8. Centrifuge for 2 min. at 12,000 rpm. Leave to stand in the dark for at least 30 minutes.
9. Transfer approximately 1ml lower (aqueous) layer with a Pasteur pipette into labelled disposable glass cuvettes.
10. Measure absorbance of stock coproporphyrin standard against a bank of 1.5M HCL on the spectrophotometer by scanning between 350 and 450nm and using the 'trace' function to obtain the maximum absorbance. Record on a datasheet and attach a copy of the standard trace.
11. Prepare working copro standard in a soda glass cuvette. Add 10 μl of stock standard to 1ml 1.5M HCL and mix by inversion.
12. Switch on fluorimeter and set up the 'PSCAN' programme:
 - Excitation wavelength = 405nm
 - Emission wavelength = 550-650nm

- Ex an Em slit widths = 10nm

13. Scan the standard, QCs and samples and print out each one with the peak maxima.
14. Measure the peak heights and use the FU scale on the right hand side of each printout to work out the FU change.

2.9.4.7 Calculation of results

Erythrocyte porphyrin =

$$\frac{A_{400} \times 1}{0.489 \quad 101} \times 1000 \times \frac{FU(\text{sample})}{FU(\text{std})} \times \frac{0.94}{1000} \times \frac{1000}{8} \times \frac{1000}{1000} \times \frac{1.3}{Hct}$$

$$= A_{400} \times \frac{FU(\text{sample})}{FU(\text{std})} \times \frac{3.07 \mu\text{mol/RBC}}{Hct}$$

Components of equation:

1. Concentration of working standard

$$= \frac{A_{400}}{\mu\text{m}(0.489)} \times \frac{1}{101} (\text{Dil of stock std}) \times 1000 \text{ nmol/l}$$

2. $\frac{FU(\text{sample})}{FU(\text{standard})} = \frac{\text{fluorescence units of sample}}{\text{fluorescence units of standard}}$

3. $\frac{0.94}{1000} = \frac{\text{change in volume after addition of } 500 \mu\text{l organic phase to } 800 \mu\text{l of } 1.5 \text{ M HCL}}{1000}$

4. $1000 = \frac{\text{Converts } \mu\text{l to ml}}{\text{Volume in } \mu\text{l of blood assayed or } 160 \text{ volume of plasma assay}}$

5. Hct = Haematocrit

2.9.4.8 Reference range

Total erythrocyte porphyrin 0.4-1.7 $\mu\text{mol/L}$

2.9.4.9 Clinical interpretation

If the total porphyrin concentration is increased the proportion of zinc protoporphyrin to protoporphyrin is assessed (method: whole blood protoporphyrin screen). The presence of a major peak at 587nm (zinc

protoporphyrin) excludes protoporphyria. An increased zinc protoporphyrin can be caused by lead poisoning or iron deficiency, particularly if there is no evidence of protoporphyria. The coefficients of variation of the quantitative erythrocyte protoporphyrin assay are 9.8% at a level of 1.1 $\mu\text{mol/L}$ red blood cells and 11.1% at 39.6 $\mu\text{mol/L}$ red blood cells

2.10 Haematological analysis of samples

Full blood count, ferritin, serum iron, total iron binding capacity and transferrin saturation were determined in the Department of Haematology, University Hospital of Wales using automated processing. Serum iron (sFe),³⁰⁰ total iron binding capacity (TIBC),³⁰⁰ serum ferritin (sFn) (Elecsys 2010, Roche Diagnostics, IN, USA), soluble transferrin receptor-1 (sTfR) (R&D Systems, Abingdon, UK) and erythrocyte protoporphyrin³⁰¹ were determined as described. Other measurements were by standard automated methods. Data obtained previously for 611 male first time blood donors were used for comparisons.³⁰²

2.11 Virological analysis of samples

Samples were processed by the Public Health Laboratory Service, Cardiff for evidence of hepatitis B antibody and surface antigen, and hepatitis C. If either of the hepatitis B tests were positive, an IgG core antigen was also sought.

2.12 Mutational analysis of the *FECH* gene

Samples were processed by Dr Sharon D. Whatley in the Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff. Genomic DNA was extracted from whole blood using the QIAamp DNA purification kit (Qiagen, Crawley, UK). For identification of *FECH* mutations and SNPs, all exons with 20 - 300 bp flanking sequence and 1300 bp of the promoter region of the *FECH* gene were PCR-amplified and sequenced (primers and conditions in table 6). For sequencing, PCR-amplified double-stranded DNA was purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Cawley, UK) before being cycle sequenced using fluorescent ddNTPs (BigDye) and an ABI Prism 3100 Genetic Analyzer (PE Biosystems, Warrington, UK). The presence or absence of mutations

was confirmed by sequencing both strands. Partial or complete *FECH* gene deletions were excluded in apparent homozygotes by quantitative gene dosage analysis.³⁰³ Nucleotides are numbered from the cDNA sequence of human *FECH* (GenBank accession number D00726) with the A of the ATG initiation codon as '+1'.

PCR is the technique to amplify samples of DNA to facilitate analysis. It works on the basis of thermal cycling (heating and cooling) of the sample DNA with a DNA polymerase enzyme, sense and antisense primers, and mononucleotides. The sample DNA is denatured by heating to 95°C. At 55-69 °C the primers anneal to their target sequences. At 72 °C the polymerase extends the primers copying the sample DNA. The sample DNA and the copy are denatured at 95 °C and the cycle begins again. In each round of denaturation, not only is the original template replicated, but also all the copies made in previous rounds. Thus the amount of product is doubled in each round (20 rounds of PCR would amplify the target sequence one million fold). Primers are short fragments of DNA complimentary to the DNA template target region. The selectivity of PCR depends on having primers that will anneal only to the desired target and not to any other sequence in the whole genome. The necessary specificity is achieved by using short primers (usually 18-22 nucleotides) and taking the annealing temperature to the highest temperature at which the primer will still bind. If the temperature is too low the primers may be able to bind to mismatched targets, and if too high they will not hybridize at all. Where possible the choice of primers was according to the advice in the HotstarTaq PCR handbook (Qiagen):

1. Primers should be 18-30 bases in length.
2. G/C content should be between 40-60% of the total.
3. The two or three bases at the 3' ends should not be complementary to reduce primer-dimers formation.
4. Primers should end (3') in a C,G,CG or GC. If G/C bases are both the 5' and 3' ends they should be the same base.
5. Avoid runs of three or more C or G bases at the 3' end in GC rich sequences.
6. Avoid self-complementarity (> 4 bases) that may cause primer-primer binding or intra primer secondary structures (e.g. hairpin loops).

In our analyses, both DNA strands were sequenced to confirm the identified mutation.

Gene dosage is the number of copies of a gene present in a nucleus. Humans (being diploid) ordinarily have two copies of each gene, one on each chromosome. Abnormalities of dosage may occur due to deletion or duplication of a gene or part of a gene. PCR can be made quantitative (quantitative gene dosage analysis) by using a primer tagged with a fluorescent dye. PCR is carried out only during the linear phase of amplification and the amount of product measured using a fluorescence sequence analyser. The amount of product is compared with an internal standard and to a group of normal individuals to estimate the number of copies of the gene in the patient's sample.

Table 6. The primers required for each region of the *FECH* gene, their concentrations and PCR programmes:

| Region of gene | Primers | Primer sequence | pmol/μl | Programme |
|----------------|----------|--------------------------|---------|-----------------|
| Exon 1 | F37104 | GTGGTGGGGAGCGGGCTTCT | 65.1 | Hot 64°C + Q |
| | FECH g2 | GATCCTGGCCCTGGCGGC | 100 | |
| Exon 2 | F33401 | TTACCTGCCTGCAGAGAAATGC | 76.3 | Hot 65°C |
| | F34928 | CCTGTTGCAGGGAAGGATCC | 78.5 | |
| Exon 3 | F33402 | AAGTGTGACAAATCAACCGTTG | 74.5 | Hot 55°C |
| | FECH g6 | TTGAGAGACACACATGTCAATG | 100 | |
| Exon 4 | F33403 | AACATTTCTCAGTTGCTAAGCT | 48.1 | Hot 49°C |
| | FECH g8 | TTCATAACTACTTCGAAAGAACTA | 100 | |
| Exon 5 | F34929 | CTCTTGAGGCTCTTATGCC | 79.8 | Hot 65°C |
| | FECH g10 | ATTTTTGCCAGCACCGTATC | 100 | |
| Exon 6 | F37105 | CCACCGTGCCTGGCCGACATAC | 90.1 | Hot 66°C |
| | FECH g12 | ATGAGAAGCTGATTCACACTAGA | 100 | |
| Exon 7 | F33406 | TTTCCTCCATTTCTTCTTCC | 94.5 | Hot 46°C |
| | FECH g14 | GAAATCACCCAATCCTCTATCA | 100 | |
| Exon 8-9 | F34931 | GACCTTAGAGATGATTGACAG | 73.8 | Hot 55°C + DMSO |
| | FECH g18 | GATGGAAAAGGCAGATGG | 100 | |
| Exon 10-11 | F38652 | GAAGGGACATAAGAATTGAGG | 56 | Hot 59°C |
| | F38653 | CCATCAAGAGTCCAATCCTC | 83.8 | |
| Intron 3 | F69723 | GTGTTGTGTGTCCTGAATCTT | 57.3 | Hot 60°C |
| | F69724 | GAATGGTGCCAGCTTACTAAA | 70.5 | |

2.13 Prokaryotic expression of missense mutations

Prokaryotic organisms (mainly unicellular and bacteria) do not have nuclei and instead have a single loop of chromosomal DNA bound to protein in an area called the nucleoid. Thus, while eukaryote DNA is tightly bound in chromosomes within the DNA, the eukaryotic DNA loop is more accessible to retroviral insertion of study gene DNA, allowing exploration of its expression, and in the case of EPP, exploration of the effect of a variety of FECH mutations on the enzyme's activity.³⁰⁴

The effect of mutations on FECH activity was determined using the bacterial expression vector pHisTF20E.⁵⁶ Mutations were created using the Quickchange mutagenesis protocol (Stratagene) with 50ng of vector and 12 cycles of 30sec denaturation at 95°C, 30sec annealing at 55°C and 8min extension at 68°C. Primer sequences are available from the authors. After digestion with *DpnI* to eliminate the template vector DNA, the PCR products were used to transform chemically competent *E. Coli* JM109. Colonies were screened by automated fluorescent DNA sequencing.

For each mutation, a single bacterial colony was grown in 5ml of LB broth containing 100µg/ml carbenicillin for 6h. A 25µl aliquot was then used to inoculate 25ml LB (100µg/ml carbenicillin) and the culture grown at 37°C for 18h. Cells were harvested by centrifugation at 6000g for 15 min and resuspended in 1.5ml of 50mM Tris/HCl [pH 7.6] containing 20% glycerol and 1mM PMSF. Cells were disrupted by sonication on ice (3x 30sec) and centrifuged at 13500g for 2min. The supernatant, which contains the recombinant FECH, was stored at -70°C. FECH activity was determined as described by Gouya *et al.*¹⁷⁵ A blank without cell lysate was included and endogenous bacterial FECH activity was assayed using an empty vector control. Protein concentrations were determined using the BCA protein assay (Pierce).

3. Results

3.1 Results of case identification

The details of 401 UK patients with biochemically proven EPP were identified. Four of these individuals were under the age of five years, and were therefore excluded from the study. Three hundred and eighty nine replies were obtained from the patients' referring physicians. Of these, we were requested not to contact six patients, and were informed that 15 patients were deceased, one had emigrated to Australia, and that two additional patients had declined to take part (total = 24). Furthermore the physicians had either no records of the patients or no contact details in a further 17 cases. Three hundred and forty eight patients (or their consultants) were subsequently sent the study pack containing the invitation to participate, a reply sheet and a reply-paid envelope.

Replies were received from 223 following the initial mail shot, of which 215 indicated that they would like to take part (initial response rate 61%). One patient declined to take part, five letters were returned due to the patient no longer being resident at that address, and two replies stated that the patients to whom the letters had been addressed were dead. Follow-up letters were sent to the 122 non-responders (three patients were not sent a follow-up letter due to their inclusion towards the end of the trial recruitment period). Of this second posting, a further 39 patients replied to say they would like to take part, five declined to be included in the study, and there was no reply from the remaining 78. In addition, four further patients with EPP clinically but not known to the databases, were referred by other members of their family who had been invited to participate. Thus we obtained 258 individuals who wished to take part (final response rate 74%). Appointments were arranged for 254 of these study individuals (4 were not included due to study time constraints) and 223 subjects were seen by the study investigator. The remaining 32 either found the appointment time inconvenient or failed to attend.

3.2 Proforma results

3.2.1 Study participant characteristics

The study cohort contained 223 subjects (median age 34 years; range 5-87 years) with a history of acute photosensitivity. There were 114 females (median age 33.5 years, range 5-87 years) and 109 males (median age 35 years, range 5-77 years); the age distribution was similar in both sexes. All were white Caucasians apart from 2 Indo-Asians (originally from Pakistan and Iraq, both male and skin type IV).

3.2.2 Age at onset of photosensitivity

The median age at onset of symptom was 1.1 years (range birth to 12 years), usually at the time of first major exposure to sunlight (figure 10). However diagnosis was often delayed; the median age at diagnosis was 12 years (range 6 months to 86 years) with the diagnosis not being made in 38 subjects (19%) until after the age of 29 years (figure 11). The majority of subjects (81%; 180) were diagnosed by a dermatologist. Other diagnoses were made by general practitioners (6%; 13), the subject themselves (4%; 8), family members (4%; 8) (brother 1, sister 2, father 5), paediatricians (3%; 7), unknown (2%; 5), a biochemist (1) and a haematologist (1). Of the eight self-diagnoses, six recalled recognising the symptoms either after reading a magazine article (4), after researching in a medical library (1), or seeing a television programme (1) about EPP.

Figure 10. Age of symptom onset in EPP study cohort.

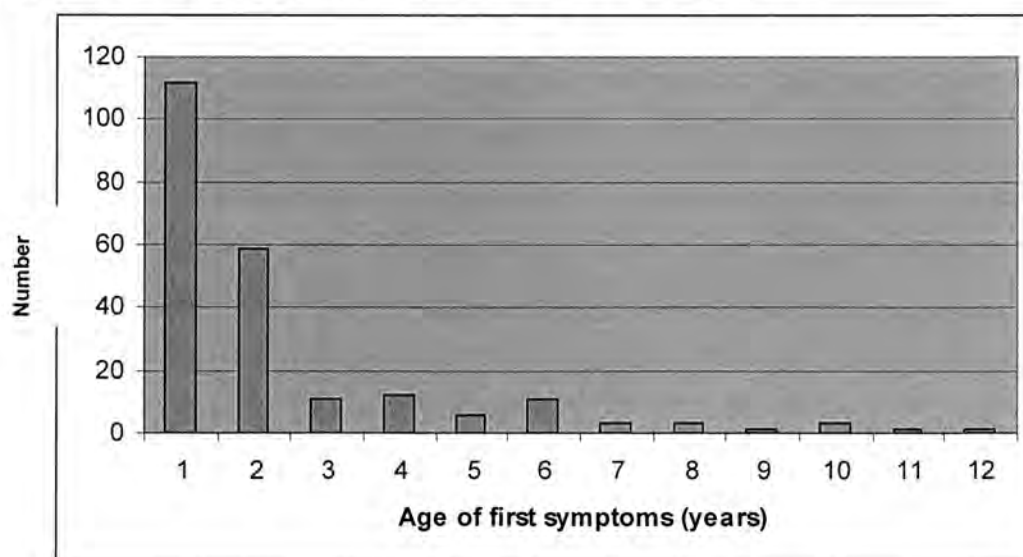
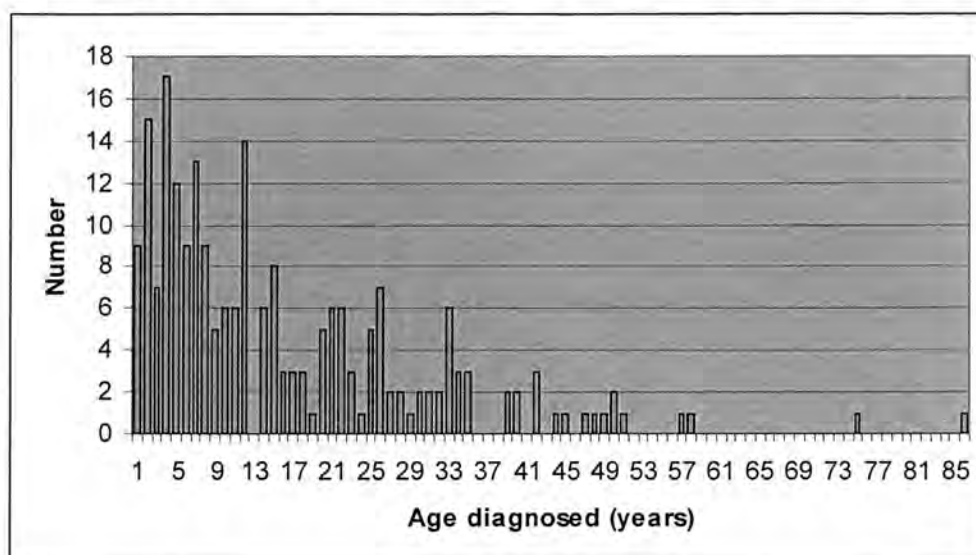


Figure 11. Age at which diagnosis of EPP was made in study cohort.



3.2.3 Symptoms of EPP

The cutaneous sensation following sunlight exposure was frequently said to be difficult to describe. The most frequently used terms included burning (85%), tingling (33%), prickling (4%) and stinging (3%). Descriptions of the sensation convey the severity: ‘like burn on a candle flame or bonfire’, ‘like being scalded and lots of red hot needles under the skin’, ‘like putting hands into a hot deep fat fryer, but under the skin, or getting into a chip pan at boiling point’, ‘having boiling water from a kettle or oil being poured all over the skin’, ‘like a thousand pins and needles’, ‘itching like stinging nettles’, ‘burning like blood boiling’, ‘burning like hot pins’ and ‘like iron burn all over’.

The median time for onset of symptoms following sunlight exposure was 20 minutes (lower quartile 10 minutes, upper quartile 60 minutes, range immediately to 12 hours/asymptomatic in the UK), and was significantly shorter in females than males (median 15 and 30 minutes, $P = 0.018$). The median time to resolution was three days (lower quartile 1, upper quartile 4 days, range immediately to 4 weeks).

Asked if they noticed any additional symptoms when they were affected (out of 220): 78% had problems sleeping (171), 30% irritability (66), 19% excessive temperature

sensitivity (41), 12% feeling down or depressed (26), 11% tired (24), 6% nausea (13), 5% malaise/generally unwell (10) and 3% headache (7). Fifteen percent of subjects (34) said they did not have any other symptoms apart from those of the skin. Only 25 of 199 subjects (12.5%) felt that their photosensitivity had changed with age; 14 subjects had improved and 11 subjects had become more sensitive. Other features of the cohort's EPP are summarised in table 7.

Table 7. Photosensitivity features of the EPP study cohort.

| Symptom | No. of subjects questioned | No. with symptom |
|---|----------------------------|------------------|
| Photosensitive through glass | 220 | 202 (92%) |
| Priming ¹ | 219 | 186 (85%) |
| Exacerbation by wind | 202 | 137 (68%) |
| Skin fragile/slow to heal | 115 | 75 (65%) |
| Improvement during summer ('hardening of skin') | 194 | 109 (56%) |
| Photosensitive during winter (November - February) ² | 165 | 73 (44%) |

¹Photosensitivity worse the day after sun exposure.

²Contributory factors: sunny weather (95%), snow (28%), wind (26%), water reflection (8%), seaside (5%), increased exposure to artificial light (1%).

The priming phenomenon was noticed by 85% (186/219), sensitivity to sunlight through glass by 92% (202/220), and exacerbation of the skin sensitivity by the wind (or in some cases stimulation of similar symptoms despite lack of sunlight) in 68% (137/202). Sixty five percent (75/115) felt their skin was more fragile or more difficult to heal when compared with others, 56% (109/194) that their skin sensitivity improved (hardened) over the summer. When asked if their skin had ever been sensitive over a UK winter (November – February) 44% said yes (73/165) and 56%

no (92/165). The circumstances that were most commonly associated with winter sensitivity were sunny weather (95%) with snow (28%) wind (26%) or water (8%; e.g. whilst sailing) as contributing factors. Other factors included being at the beach (5%), or the increased exposure in the winter to artificial light sources (1%). Five percent described occasions when they had been affected in overcast conditions, even whilst raining, in winter.

3.2.4 Menstruation & pregnancy

Twenty eight (47%) of 59 gravid female subjects reported an improvement in symptoms during pregnancy. Nine subjects spontaneously described this as “very significant” or that they became “asymptomatic”, with anecdotes of having a tan for the first time in their lives, or tolerating foreign sunny holidays. None reported deterioration, and several of the women who had not noticed a change commented that they had been pregnant mainly over winter or were too apprehensive to expose themselves to sunlight to test for a pregnancy-related change. Two women who had not noticed change were both seen in the study at an early stage of their first pregnancies and early in the year. Menses-related worsening of photosensitivity was reported by 8 (10%) of 81 menstruating females; 6 of these were gravid; 5 of whom had noticed an improvement in their sensitivity during pregnancy. No improvement during menses was reported. Three subjects mentioned their symptoms had improved since the menopause and none had noticed any post-menopausal worsening of photosensitivity.

3.2.5 Signs of EPP

Two hundred and eleven subjects (95%) said that the onset of symptoms was not associated with any immediate visible change to the skin. Immediate changes reported were reddening (7 subjects), swelling (3 subjects), purple discoloration and blanching (one subject each). Visible changes were generally related to the duration of exposure; minimal exposure tended not to result in visible changes, whilst prolonged exposure resulted in signs. Those most commonly reported included: swelling (179 subjects, 80%), reddening (45 subjects, 20%), blistering (37 subjects, 17%), crusting / eczema (32 subjects, 14%), petechiae and/or bruising (20 subjects,

9%) and fissuring (10 subjects, 4%). Thirteen of 103 subjects (13%) had noticed changes in their nails during acute episodes. Twenty two subjects (10%) reported no visible change at any time. Sixty one subjects estimated a median time for onset of swelling post-exposure of 6 hours (lower quartile 2, upper quartile 12 hours, range 15 minutes to the next morning).

3.2.6 Photoprotective measures and previous treatments

When asked if EPP interfered with their daily activities on sunny days, 92% (204) said “yes” and 8% said “no” (18). The main activities to avoid sunlight exposure were staying inside 65% (144), seeking shade 32% (71), and only going out in the evening or early morning 12% (26). Of the 18 subjects with no daily interference, some took care to avoid sports (1), foreign travel (2) or to limit time outside when attempting to hardening the skin (2).

Special clothes were worn by 87% (194) of subjects to protect themselves from sunlight, whilst 13% (28) wore no special clothes. These were most commonly as follows: 91% long sleeves (174/191) (two subjects sewed extra fabric to sleeves to make them longer), 78% trousers (149) (long skirts or dresses additionally 6), 75% hats (143) (where specified 29 baseball or cap, 14 brimmed, 5 foreign legion-style, 2 Arabic head-dresses), 50% gloves (95) (6 cotton, 4 leather, 1 each yachting and cycling) – for driving 18 and sport 7 (football, gardening and bowls). Other clothing included 3% UV-opaque bathing suits (6), 10% shoes and socks that covered feet (20), 4% sunglasses (8), 2% scarf (6), letting hair grow 3 and growing a beard 1, and 1 each of hooded tops, pillow case to drive, balaclava, face mask to play golf, plaster on nose.

When asked if they used a sunscreen, 31% (70) replied “no”, citing a number of reasons such as: tried most of them and none seem to work, sunscreens are too thick, cosmetically unacceptable, makes it feel like I’m frying, feels like they make my skin worse – stings and don’t like greasy sensation, feel they aggravate the skin by making it itchy, they are messy and expensive, don’t use them as always covered up with clothes. Of the 69% who used a sunscreen, the most frequent used was the

Tayside Pharmaceuticals' Dundee Reflectant Sunscreen, used by 25% of subjects, most commonly the beige colour, followed by conventional sunscreens with sun protection factor (SPF) 60 (23%), 50 (13%), 30 (13%), and 25 (11%). There was no obvious preference for any one brand of sunscreen manufacturer. Dundee sun cream was used regularly by 25% of the subjects, of whom 98% felt it was useful. Of the 25% who had previously used it, only 33% thought it to be helpful. Poor cosmesis was frequently mentioned in connection with this product. The range in the frequency of sunscreen application was between weekend or occasional use only, to 8 times a day (9% occasional use/use at weekends, 77% daily, and 14% more frequently than once a day). The circumstances in which the sunscreen was applied ranged from "only when abroad", to "all year" (6% abroad only, 61% in sunny weather, 24% all summer, 9% all year). Only 14% were aware of the UVA star rating of sunscreen.

Other commonly used treatments for EPP are shown in table 8. Seven percent of subjects currently used antihistamines, of which 50% thought them helpful, and 50% not. Forty five percent had previously used them, of whom 14% thought them helpful, and 86% not. Forty seven percent of subjects had never tried antihistamines.

Table 8. Treatments used for EPP.

| Treatment | % Currently Using | % Previously Used | % Never Used |
|-------------------|-------------------|-------------------|--------------|
| Antihistamines | 7 | 45 | 47 |
| Beta carotene | 28 | 56 | 24 |
| Dundee Cream | 25 | 25 | 50 |
| L-Acetyl Cysteine | 1 | 0.5 | 98 |
| UVB Phototherapy | 9 | 16 | 75 |
| Tanning Parlours | 2 | 15 | 83 |

Beta-carotene was used currently by 28% of whom 77% thought them to be helpful (median dose 180 mg, range 60-390 mg). Fifty six percent (124) had previously taken it, 24% of whom had felt it helped (median dose 240 mg, range 60-360 mg), and 16% had never tried it. Although not specifically asked, the main reason given

by those discontinuing it (other than efficacy) was an unacceptable orange discoloration of skin and body fluids (34), difficulty taking the tablets (5), retinal crystals from the canthoxanthine preparation (5) and media coverage of possible risks of malignancy (2).

L-acetyl cysteine was used by 3 subjects (1%), all of whom thought it helped. Two subjects had previously taken it, one thought it helpful and one did not (dose taken by all five was 500mg tablets twice daily).

Hospital phototherapy was used by 21 subjects (9%) at the time of the study, and all thought it helpful (due to geographical factors, one patient was using home phototherapy provided and monitored by a hospital department). Thirty five (16%) had previously used phototherapy (49% of whom thought it useful) and 166 (74%) had never had it. Commercially available sunbeds were currently used by 5 subjects (2%), all of whom found it helped. Thirty three (15%) had previously tried sunbeds, 53% of whom had found it useful, and 144 (83%) had never used them. Use of phototherapy was not statistically associated with use of commercial sunbeds (Spearman's correlation = 0.032; $n = 222$; $P = 0.639$).

The other measures most commonly used by subjects for symptom relief included immersion in cold water (51; 37%), wet cloths or flannels (47; 21%), use of a fan (12; 5%), putting affected areas on cold walls, metal or under pillows (11; 5%), drinking alcohol (10; 4%), emollients (8; 4%), ice packs (7; 3%), and emersion in hot water (7; 3%). Other treatments that small numbers found helpful included sleeping (8), analgesics (7), cold drinks cans kept in the fridge applied topically (7), fish oils (3), air-conditioning (3), antioxidants e.g. green tea and vegetable juices (3), sleeping tablets (2), blood transfusion (2), anaesthetic spray (1), acupuncture (1), oral iron supplementation (1), systemic steroids (1), cannabis (1) and make-up (1). Treatments that were said not to work included Chinese herbalist (1), zinc tablets (1), calamine lotion (3), topical steroid creams (1) and fish oils (1). Prescribed drugs which had been of no benefit included: a cystic fibrosis drug, mepacrine, activated charcoal and cholestyramine.

A large minority of the subjects (30%) were not seen regularly by any doctor for their EPP. None of these subjects had any monitoring of blood tests either for liver function or blood count. The majority of subjects 127 (57%) were under the care of a dermatologist, with either annual (54%), twice yearly (35%), three times yearly (4%) or four times yearly (7%) reviews. Eight subjects were under the care of a biochemist, seven were under a general practitioner, five were under a paediatrician and three were under a haematologist, all with annual reviews. Three subjects were under joint care by both a dermatologist and a gastroenterologist, and were seen twice yearly, and two subjects, one under the care of a hepatologist, and one under a gastroenterologist were seen weekly. Ninety-eight percent of those under the care of a dermatologist and all of those under the care of the biochemist, haematologists, gastroenterologists and hepatologists had blood checks at least annually. The six subjects, under the care of a general practitioner and paediatrician did not have regular blood tests.

3.2.7 Subjects' previous medical history

Asked about other illness and operations, 109 (49%) could not recall any other previous problems, and a further 60 (27%) had a single condition only. The most commonly reported conditions included: asthma 13, hayfever 12, hypothyroidism 11 (4.7% population), eczema 8, heart murmur 6, hiatus hernia 6, keratoderma 5, hypertension 4, ischaemic heart disease 4, peptic ulcer 4, osteoporosis 4, arrhythmias 4, appendectomy 3, irritable bowel 3, chronic fatigue 3, congenital cardiac anomalies 3, hyperthyroidism 3, peripheral vascular disease 2, liver failure 2, low platelets 2, ureteric anomalies 2, Perthe's disease 2, psoriasis 2, obstructive pulmonary disease 2, cardiac failure 2, stroke 2. Conditions which affected only one individual were: Osgood-Schlatter's syndrome, cataract, breast fibroma, colon carcinoma, rheumatoid arthritis, iritis, prostatic hypertrophy, prostatic carcinoma, leg ulcer, mechanical heart valve, rhinoplasty, onychomycosis, ovarian cyst, dyspraxia, pancreatitis, PLE, SLE, polymyalgia rheumatica, horse-shoe kidney, acne, acrodermatitis enteropathica, coeliac disease, vitiligo, Crohn's disease, thyroid cyst, leishmaniasis, pituitary gland tumour, ulcerative colitis, scleroderma, spastic

diplegia, testicular seminoma. Specific conditions were asked about: anaemia 18% previously, iron deficiency 22%, gallstones 9%, cholecystectomy 7%, hepatitis 1 patient, other liver problems 2 subjects (liver failure secondary to EPP), arthritis 7%, haematological malignancy 0%.

Ten percent of subjects gave a history of sensitivity to medication, most commonly penicillin and erythromycin. Fifty percent of subjects were taking medication at the time of the study, 51% of whom were taking only a single preparation. The medications taken most commonly were: beta-carotene 62, antihistamines 15, thyroxine 12, proton pump inhibitors 11, oral iron 10, paracetamol 9, NSAIDs 7, oral contraceptive 7, aspirin 7, cholesterol lowering agents 6, antidepressants 6, antidepressants 6, ACEI 5, beta blockers 5, fish oils 4, antimicrobials 4, opiates 4, H2 receptor antagonists 3, L-acetyl cysteine 3, warfarin 3, systemic steroids 3, calcium channel blockers 3, calcichew 3.

3.2.8 Family history

The 233 subjects came from 193 families. One hundred and twenty-five subjects (56%) had no family history of photosensitivity; 89 from 60 families had affected relatives (only 29 of whom were seen in the study) and a further 9 subjects were uncertain. In the 60 families with more than one affected individual, the disease was manifest in one or more siblings in 33 families, in seven of which a more distant relative was also affected. There were thirteen instances of parent to child transmission of photosensitivity, with transmission through three consecutive generations in two families. In 16 families only relatives more distant than first degree were affected.

Fifty four subjects from 24 families were related to another study subject. Forty one were siblings (20 families), and 3 parents with 4 affected children (3 families – one of these also included in the siblings families count). An affected great grandmother was also seen with one sibling pair, and the remaining 2 families were an uncle-niece, and a pair of first cousins. Thirty one (14%) of subjects had a family history

of another condition; the most commonly reported being ischaemic heart disease (13), diabetes (8) and hypothyroidism (3).

3.2.9 Social characteristics

Two hundred and twenty one were white Caucasian, the other two Indo-Asian (ethnically Pakistani and Iraqi), and all but four had been born in the United Kingdom (the exceptions being Canada, Germany, Iraq and Malawi). One hundred and twenty seven (56%) of the subjects were in employment, and a further 60 (27%) were either in school or were further education students. Half of those in employment felt their choice of profession had been significantly influenced by their skin.

Thirteen percent smoked tobacco (median 10 cigarettes/day, quartiles 4, 20, range 1-50), and 64% (143) of the study population, and 80% of those over the age of 16 drank alcohol regularly. The median number of units consumed was 7 per week (quartiles 1, 14.5, range 1-70), in the following formats: 55% wine, 50% beer, 26% spirits and 0.5% cider. Fourteen percent said they noticed their skin being more sensitive to the sun after drinking. All subjects apart from one ate meat regularly; the exception was a non-vegan vegetarian.

3.2.10 Skin examination

Only three subjects, all children, had acute changes at the time of examination; all three had erythema, mainly over the nose, cheeks, dorsae of hands and fingers, lower lips and upper helix of the ears (Figure 5). Thirty four (15%) subjects had no changes evident on any light-exposed skin (16 females, 18 males; median age 37 years, range 5-87 years). Scarring was the most commonly present chronic change, affecting mainly light-exposed skin of the face (149 subjects, 67%) and hands (138 subjects, 62%) (Figure 6, Table 9). The observer-perceived severity for mild and moderate face and hand signs was in roughly equal proportions (face 31% and 29% of subjects respectively, hands 30% and 27%). Only a minority of subjects had scarring that was assessed as severe (face 6%, hands 5%). Other chronic changes such as thickening of knuckle skin and lip rhagades were observed less frequently.

Table 9. Percentage of cohort with evident cutaneous changes on light-exposed skin

| | Face | Hand | Knuckle | Nail |
|----------|------|------|---------|------|
| none | 21 | 27 | 37 | 0 |
| mild | 37 | 35 | 34 | 0 |
| moderate | 35 | 32 | 25 | 1 |
| severe | 7 | 6 | 3 | 0 |

Compared to those with no thickening, the cohort with severe and moderate knuckle thickening were older (38 vs 34 years), had a higher proportion of male sex (63% vs 37%) and alcohol consumption (11.6 vs 7.6 units a week), shorter time to sensitivity following sunlight exposure (47 vs 52 minutes), but similar TEP (26.9 vs 25.3 $\mu\text{mol L}^{-1}$).

3.3 Quality of life analyses

Two hundred and twenty completed QOL questionnaires were obtained, comprising 176 DLQIs (age range 16-87, mean 39.6) and 44 CDLQIs (age range 5-16, mean 11.1). Scores were not significantly influenced by age or sex (table 10).

The mean total DLQI score was 13.95 (SD +/- 6.715, range 0-29, n = 176). It is possible to interpret the meaning of DLQI scores by applying simple validated descriptive score bands.³⁰⁵ Six adults scored 0 or 1 (3%) indicating no effect on their life over the preceding week and 15 scored 2-5 (9%), indicating a small effect: in total only 21 subjects (12%) scored 5 or less, indicating no or little effect on their QoL. Thirty two (18%) scored 6-10, indicating a moderate effect, 92 (52%) scored 11-20 indicating a very large effect and 31 (18%) scored 21-30 indicating an extremely large effect. Thus 123 of 176 adults (70%) scored over 10, indicating at least a very large effect on their QoL over the preceding week. The highest scoring DLQI questions were 4, 5 and 6, relating to clothing, social/leisure activities and sport. All six adults (4 female, 2 males; mean age 47.5 years, range 20-77 years) with a DLQI score of 0 or 1 had mild symptoms and were part of the group who felt

EPP did not interfere with daily activities (3.2.6); in three of these EPP was not diagnosed until over 43 years after the onset of symptoms.

The mean total CDLQI score was 13.02 (SD +/- 4.027, range 5-21) and the highest CDLQI questions were 5, 1 and 9, relating to social/leisure activities, skin symptoms and sleep. No child scored less than 5, and 80% (35) scored greater than 10.

Table 10. Total DLQI and CDLQI scores in EPP

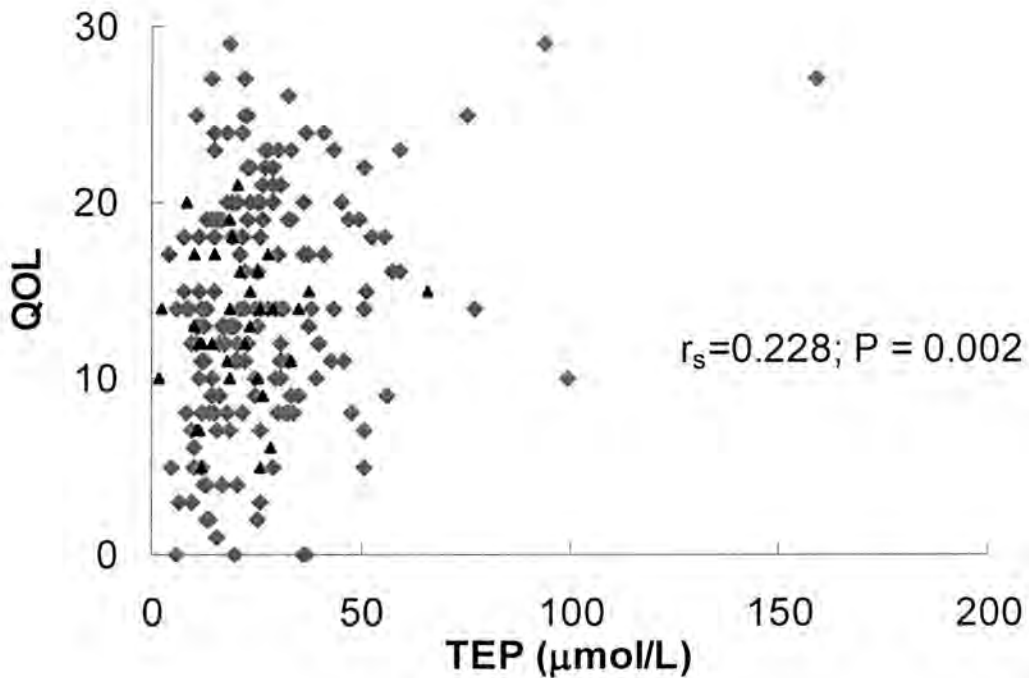
| Age range (years) | Sex | Number of subjects | Mean age (years) | QOL score (mean & range) |
|--------------------|-------|--------------------|------------------|--------------------------|
| 6-15 ¹ | F | 22 | 10.9 | 12.8 (5-20) |
| | M | 22 | 11.3 | 12.9 (6-21) |
| | F & M | 44 | 11.1 | 12.8 (5-21) |
| 16-35 ² | F | 40 | 27.3 | 14.4 (0-29) |
| | M | 37 | 26 | 13.9 (4-25) |
| | F & M | 77 | 26.7 | 14.1 (0-29) |
| 36-55 ² | F | 33 | 43.6 | 13.2 (0-25) |
| | M | 36 | 44 | 13.0 (1-29) |
| | F & M | 69 | 43.8 | 13.1 (0-29) |
| 56-87 ² | F | 17 | 62.8 | 16.1 (4-27) |
| | M | 13 | 62.3 | 15.3 (0-27) |
| | F & M | 30 | 62.6 | 15.76 (0-27) |
| 16-87 ² | F | 90 | 40 | 14.2 (0-29) |
| | M | 86 | 39 | 13.8 (0-29) |
| | F & M | 176 | 39.5 | 14.0 (0-29) |

¹CDQLI questionnaire; ²DQLI questionnaire

The relationships between three potential indicators of severity of disease and QoL were assessed. There was a positive correlation between TEP and adult DLQI scores ($r_s = 0.228$; $P = 0.002$; $n = 174$) but not between TEP and CDLQI scores ($r_s = 0.025$; $P = 0.888$; $n = 35$) (Figure 12). In contrast, there was a stronger correlation between times to onset of symptoms for CDLI scores ($r_s = -0.422$; $P = 0.004$; $n = 44$) than for adult DLQI scores ($r_s = -0.233$; $P = 0.002$; $n = 176$). There was no significant relationship between age at onset and either DLQI or CDQLI score.

Figure 12. Relationship between total erythrocyte protoporphyrin (TEP) and quality of life (QoL) scores for 220 subjects with EPP.

QoL: DLQI scores for 176 adults (grey diamonds) and CDQLI scores for 44 children (black triangles); TEP: total erythrocyte porphyrin; r_s = Spearman rank correlation.



3.4 Haematological analyses

3.4.1 Overall cohort results

Blood samples were obtained from 210 subjects for analysis. Overall, the mean haemoglobin was 12.54 g/dL, (range 8.6-17.5, quartiles 11.7, 13.37), with 34% (70) of subjects having levels below the normal range, in roughly equal proportions between males and females (34 of 107 (32%) females and 36 of 101 (36%) males). The RBC was normal in all females and abnormal in only two males who were both also anaemic. The haematocrit was abnormal in 3 females and 4 males (all of whom were anaemic). Overall 37% of subjects had a low MCV, and 47% a low MCH (abnormal MCV 77 (42 females, 35 males; 39 anaemic, 38 not anaemic), and 98

abnormal MCH (58 females and 40 males, 51 anaemic, 47 normal Hb, 19 anaemic, normal MCH). Low ferritin was seen in 42% subjects (1 male: 3.2 females), low serum iron in 35% (1 male: 1.8 females), and a raised TIBC in 17% (1 male: 1.4 females). Low ferritin ($P < 0.0005$), low serum iron ($P = 0.027$) and low transferrin saturation ($P = 0.019$) were all significantly associated with anaemia. A related-samples *t*-test showed significance between low haemoglobin and MCV beyond the .05 level: $t(207) = -211$; $P = 0.00$ (two-tailed); 95% confidence interval (-80.09, -78.61), and anaemia and MCH: $t(207) = -174.67$; $P = 0.00$ (two-tailed); 95% confidence interval (-25.56, -24.99).

3.4.2 Haematological analysis in dominant EPP

One hundred and ninety two subjects had one *FECH* mutation with one or two *FECH* IVS3-48C alleles and were classified as dominant EPP (dEPP);¹⁷⁵ 14 of these were excluded because they had diseases likely to affect iron metabolism, leaving 178 samples for more detailed analysis.

3.4.2.1 Red cell indices

By WHO criteria, 73 (41%; 95% CI: 34 – 48%) of our subjects with dEPP were anaemic. All had a mild microcytic, hypochromic anaemia; 48% of females and 33% of males being affected. The anaemic subjects did not form a separate subgroup. In both sexes, haemoglobin (Hb) (females: 11.9 ± 1.0 g/dL; males: 13.3 ± 1.0), mean cell volume (MCV) and mean corpuscular haemoglobin (MCH) were normally distributed with a shift in their means towards lower values (Table 11); the mean Hb for males being 1.2 g/L lower than in the general population (Fig 13a). This downward shift in Hb leads to some subjects falling within the definition of anaemia. Erythrocyte protoporphyrin concentrations (females: 21.9, 4.1 – 75.3 $\mu\text{mol/L}$; males: 25.5, 8.9 – 77.3 $\mu\text{mol/L}$) showed no correlation with Hb.

Table 11. Red cell indices in 178 subjects with dominant EPP

| | Female | | | Male | | |
|-----------------------------|------------------|---------------------|------------------------|------------------|---------------------|------------------------|
| | All (n = 98) | Anaemia (n = 47) | No anaemia (n = 51) | All (n = 80) | Anaemia (n = 26) | No anaemia (n = 54) |
| Hb (g/dL) | 11.9 ± 1.0 | 11.1 ± 0.6 | 12.7 ± 0.5*** | 13.3 ± 1.0 | 12.2 ± 0.5 | 13.8 ± 0.7*** |
| Hct | 0.361 ± 0.027 | 0.340 ± 0.018 | 0.380 ± 0.019*** | 0.398 ± 0.033 | 0.366 ± 0.019 | 0.414 ± 0.026*** |
| RBC (x10 ¹²) | 4.5 ± 0.3 | 4.4 ± 0.3 | 4.6 ± 0.3** | 4.9 ± 0.4 | 4.6 ± 0.3 | 5.0 ± 0.3*** |
| MCV (fL) | 80 ± 5 | 77 ± 5 | 82 ± 4*** | 82 ± 5 | 80 ± 6 | 83 ± 5** |
| MCH (pg) | 26.6 ± 2.5 | 25.5 ± 2.2 | 27.7 ± 2.2*** | 27.4 ± 1.6 | 26.7 1.9 | 27.7 ± 1.3** |

Figures are mean ± SD for 98 females aged 6 – 87 years, median 35 years; 80 males aged 8 – 87 years, median 36 years. Anaemia was defined as Hb less than 12.0 g/dL (females) or less than 13.0 g/dL (males) for adults and less than the lower limit of the age-related Hb range for children.³² For comparison of anaemia vs. no anaemia groups: ***P* < 0.01; *** *P* < 0.001.

3.4.2.2 Iron status

Both sexes showed evidence of iron depletion (Table 12). Differences in sFn and transferrin saturation (TS) between women and men suggested that more of the former had iron depletion due to iron loss in addition to abnormalities caused by EPP. Therefore, we restricted detailed analysis of iron indices to the 67 male subjects (Hb 13.5 ± 0.9 g/dL) aged over 15 years who had never received iron supplements.

The main abnormality was a marked shift in sFn towards lower values (Fig 13b) (Table 13); sFn correlated with Hb ($r_s = 0.415$; *P* < 0.001). Because protoporphyrin is hepatotoxic and accumulates in the liver in EPP, and liver cell damage may increase sFn, we assessed liver cell function by measuring liver enzymes. One or more of these was increased in 17 (25%) subjects; sFn correlated with γ -glutamyl transpeptidase ($r_s = 0.507$; *P* < 0.001) and alanine aminotransferase ($r_s = 0.392$; *P* < 0.001) but not with aspartate aminotransferase.

A second notable feature of iron depletion in dEPP was the finding that sFe (Table 12) and sTfR (18.6 ± 5.1 nmol/L) (Fig 13c) concentrations are normal. The normal sTfR was consistent with the degree of depletion of iron stores indicated by sFn and, together with the normal sFe, suggested that erythropoiesis is not limited by iron supply.³⁰⁶

Table 12. Iron status of 178 subjects with dominant EPP.

| Serum iron indices | Female subjects (n = 98) | | Male subjects (n = 80) | |
|----------------------------|--------------------------|-----------------------|------------------------|-----------------------|
| | All subjects | Iron insufficient (%) | All subjects | Iron insufficient (%) |
| sFe ($\mu\text{mol/L}$) | 11.9 (1 – 38.7) | - | 14.2 (1 – 29.5) | - |
| TIBC ($\mu\text{mol/L}$) | 65.4 ± 7.6 | - | 63.0 ± 6.7 | - |
| TS (%) | 19 (2 – 58) | 61 | 24 (2 – 49) | 15** |
| sFn ($\mu\text{g/L}$) | 13 (2 – 186) | 44 | 33 (8 – 246) | 27* |

Figures are means \pm SD or, for sFn, medians and ranges. Iron insufficient was defined as TS <16% and/or sFn < 15 $\mu\text{g/L}$. For comparisons of percentages of male and female subjects: * $P < 0.05$; ** $P < 0.001$.

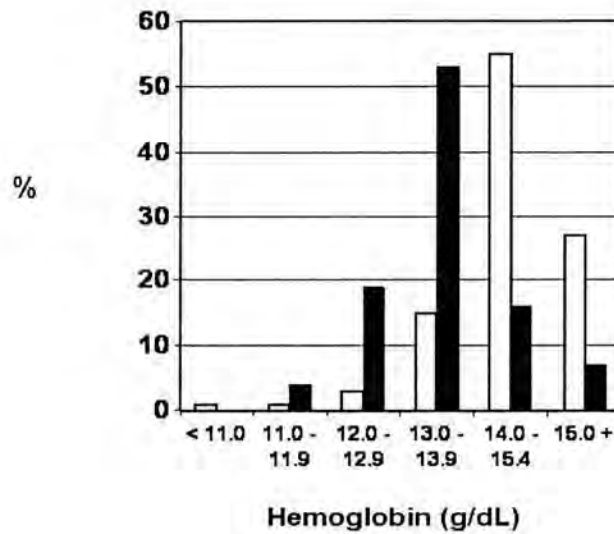
Table 13. Comparison of indicators of iron status in male subjects with dEPP and male first-time blood donors

| Serum iron indices | EPP subjects (n = 67) | First-time blood donors (n = 611) |
|----------------------------|----------------------------|-----------------------------------|
| sFe ($\mu\text{mol/L}$) | $15.1 \pm 6.6^{\text{ns}}$ | 16.7 ± 6.0 |
| TIBC ($\mu\text{mol/L}$) | $63.0 \pm 6.9^*$ | 54.5 ± 10.0 |
| TS (%) | $23.9 \pm 10.3^*$ | 31.1 ± 10.9 |
| sFn ($\mu\text{g/L}$) | 37 (10 – 119)* | 101 (35 – 220) |

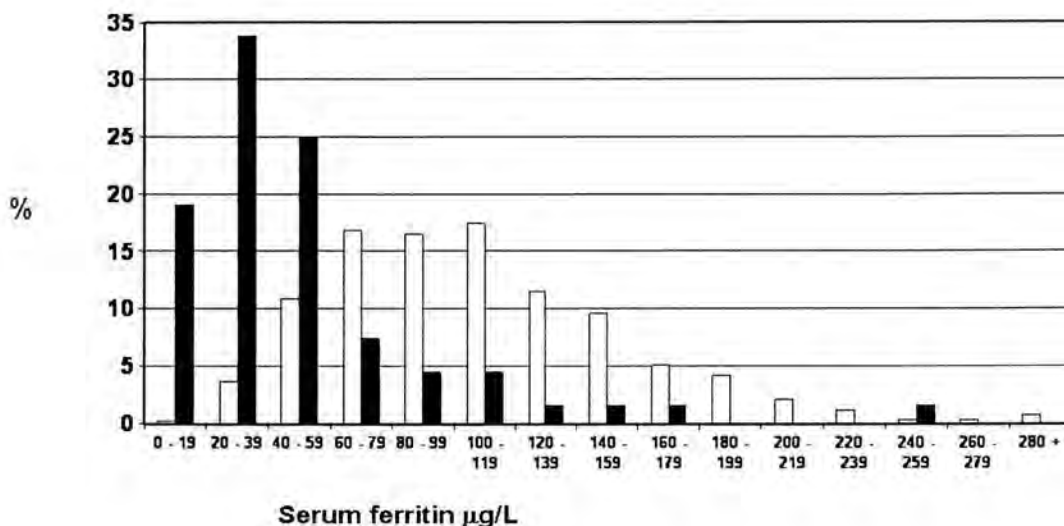
Figures are means \pm SD or, for sFn, medians and 95% ranges. EPP subjects are males aged 16 – 77 years who have never been prescribed iron supplements. Blood donors are male, first time donors aged 17 – 62 years from South Wales;³⁰¹ samples for analysis were obtained prior to first donation. * $P < 0.001$ compared with donors; ^{ns}not significant. Only TIBC showed any correlation with sFn ($r_s = -0.412$, $P < 0.001$).

Figure 13. Haemoglobin, serum ferritin and serum soluble transferrin receptor-1 concentrations in male subjects with dominant EPP.

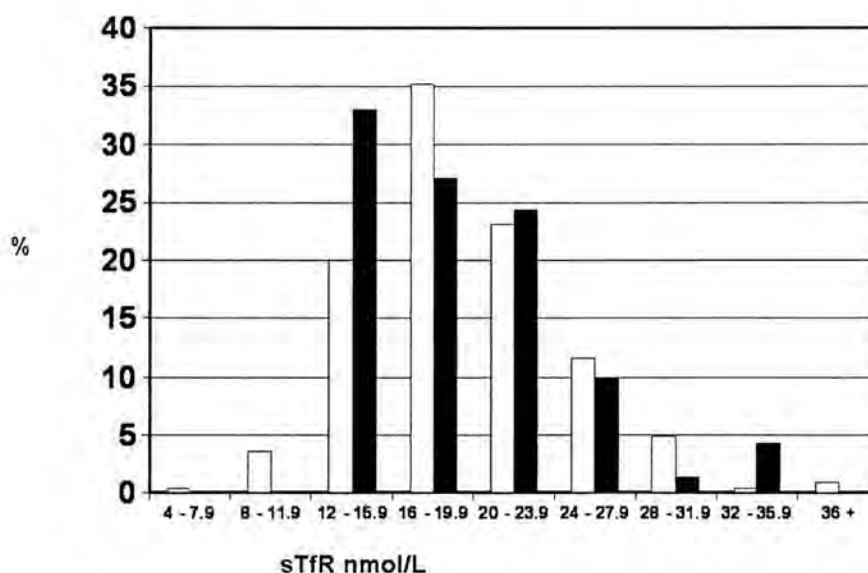
a) Haemoglobin concentrations in 66 male subjects with dEPP aged 16 or over (■) and in a sample of 5206 men aged 16 or over from the English population³⁰⁷ (□) .



b) Serum ferritin concentrations in 66 male subjects with dEPP aged 16 or over (■) and in 612 male first-time blood donors from south Wales aged 17 – 62 years³⁰² (□).



c) Serum soluble transferrin receptor-1 concentrations in 61 male subjects with dEPP aged 16 or over (18.6 ± 5.1 nmol/L) (■) and in 225 haematologically normal male and female subjects from the United States aged 17 – 97 years³⁰⁸ (□) assayed using the same method.



3.5 Biochemical assay of liver function in dEPP

Table 14. Liver dysfunction in reported EPP patient series.

| Paper Reference | Definition of abnormal liver function | Subject Number | Percentage (No.) with abnormal liver function | Virology | Comment |
|---------------------------|---------------------------------------|----------------|---|--------------|---|
| Holme | ↑ γGT, AST, ALT | 182 | 25 (45) | All negative | Current study |
| Frank ² | Not stated | 90 | 27 (24) | All negative | Retrospective analysis Possible selection bias |
| Doss ⁹ | Not stated | 55 | 35 (19) | Not reported | Retrospective analysis Possible selection bias |
| DeLeo ⁷ | ↑ AST Bi | 32 | 3 (1) | Not reported | Retrospective analysis |
| Schmidt ¹¹⁵ | ↑ AST Bi | 29 | 0 (0) | Not reported | Biochemistry not performed in all cases |
| Rademakers ¹³⁷ | ↑ AST, ALT | 11 | 36 (4) | Not reported | Retrospective analysis Small sample size |
| Mooyaart ¹⁶¹ | ↑ γGT, AST, ALT, LDH, Bi | 9 | 22 (2) | Not reported | Small sample size |
| Peterka ⁶ | ↑ Bi | 4 | 0 (0) | Not reported | Small sample size 3 other cases with no biochemistry |
| TOTAL | | 412 | 23 (95) | | |

From the overall study population of 223, 210 complete blood sample sets were obtained. Six result sets were withdrawn from the biochemical analysis for the following reasons: two subjects with malignancy (one colonic carcinoma, one testicular seminoma), one subject with liver failure, one recipient of a liver transplant, and two subjects with alcoholic liver disease. Of the remaining 204 sample sets, 182 were identified with mutation-positive dEPP (100 females and 82 males, median age 35 years, range 6-87). The precise mutations identified were: 24 missense (M), 10 of which were P334L missense mutations (MP), 75 null (N), and 83 splice (S), 44 of which were IVS3+2T>G splice mutations (SS).

Serum liver function tests were out-with normal limits in 25% of subjects (45/122) in keeping with results from previous EPP patient series (table 14). Total erythrocyte protoporphyrin (TEP) was higher in males (mean 29.6 $\mu\text{mol L}^{-1}$) than females (mean 23.6 $\mu\text{mol L}^{-1}$). Mean TEP was higher in this cohort with dEPP (mean 29.3 $\mu\text{mol L}^{-1}$) than those subjects with EPP inherited in an autosomal recessive pattern (rEPP; mean 10.8 $\mu\text{mol L}^{-1}$).³⁰⁹ Alcohol was drunk regularly by 132 subjects (73% (84% of the 157 aged 16 or over)). The median number of self-reported units consumed per week was 10 (quartiles 0, 14, range 0-70).

One hundred and seventy two subjects (95%) had no hepatitis B surface antigen and antibody, 8 had surface antibodies, but were negative for surface and core antigen, (suggesting hepatitis B vaccination - five were employed as health care professionals) and 2 were positive for both surface antibody and core antibody but negative for core antigen and e antigen (suggesting either latent or previous infection with no current replication). Neither of these two subjects had abnormal liver function results. None had evidence of infection with or exposure to the hepatitis C virus.

By creating a category of normal/abnormal AST/ALT, non-parametric analysis suggested associations with male gender (Mann-Whitney exact $P = 0.004$), TEP ($p=0.03$), haemoglobin ($p=0.049$), serum ferritin ($P = 0.002$) and GGT ($P < 0.0005$). Entry of these and borderline significant variables into a regression analysis revealed

significant associations with: male gender ($P = 0.002$), TEP ($P = 0.029$), haemoglobin ($P = 0.01$), haematocrit ($P = 0.017$), ferritin ($P = 0.03$), albumin ($P = 0.009$) and GGT ($P < 0.0005$). Adjusted $R^2 = 0.179$ and ANOVA significance $p < 0.0005$. There was no significant association between genotype and LFTS.

3.6 Assay of Protoporphyrin

Total erythrocyte porphyrin (TEP) was increased in the 211 subjects from whom blood was obtained (mean $26.1 \mu\text{mol L}^{-1}$, range $2.0 - 159.2 \mu\text{mol L}^{-1}$; normal subjects less than $1.7 \mu\text{mol L}^{-1}$). Erythrocyte porphyrin, mainly free protoporphyrin and plasma porphyrin (fluorescence emission maximum $626 - 634 \text{ nm}$), was increased in all subjects. TEP was significantly higher in males (mean $29.3 \mu\text{mol L}^{-1}$, range $2.0 - 159.2 \mu\text{mol L}^{-1}$) than females (mean $23.1 \mu\text{mol L}^{-1}$, range $2.3 - 75.3 \mu\text{mol L}^{-1}$) ($P = 0.003$). There was a slight increase with current age but this did not reach statistical significance in either sex ($P = 0.096$).

TEP results from analyses prior to this study were available for 113 study subjects (mean time from previous sample 5.9 years, range 0.5-12, mean TEP at time of study 26.41, mean previous TEP 27.59). There was no statistically significant difference between the two TEP groups (Wilcoxon test $P = 0.628$), suggesting no change in TEP with age.

3.7 Assay of 1,25 dihydroxy Vitamin D

Two hundred and ten samples were collected over a 7-month period between January and July, representing a period with minimal and maximal vitamin D levels in normal populations,³¹⁰ at latitudes ranging from 51°N to 57.5°N , and analysed for serum 25-hydroxyvitamin D. Nine subjects were withdrawn from the analysis due to non-white skin coloration (2 - both vitamin D insufficient (VDI)), systemic malignancy (2), renal failure (1), hepatic failure (2), post-orthotic liver transplant (1) and one patient taking ergocalciferol.

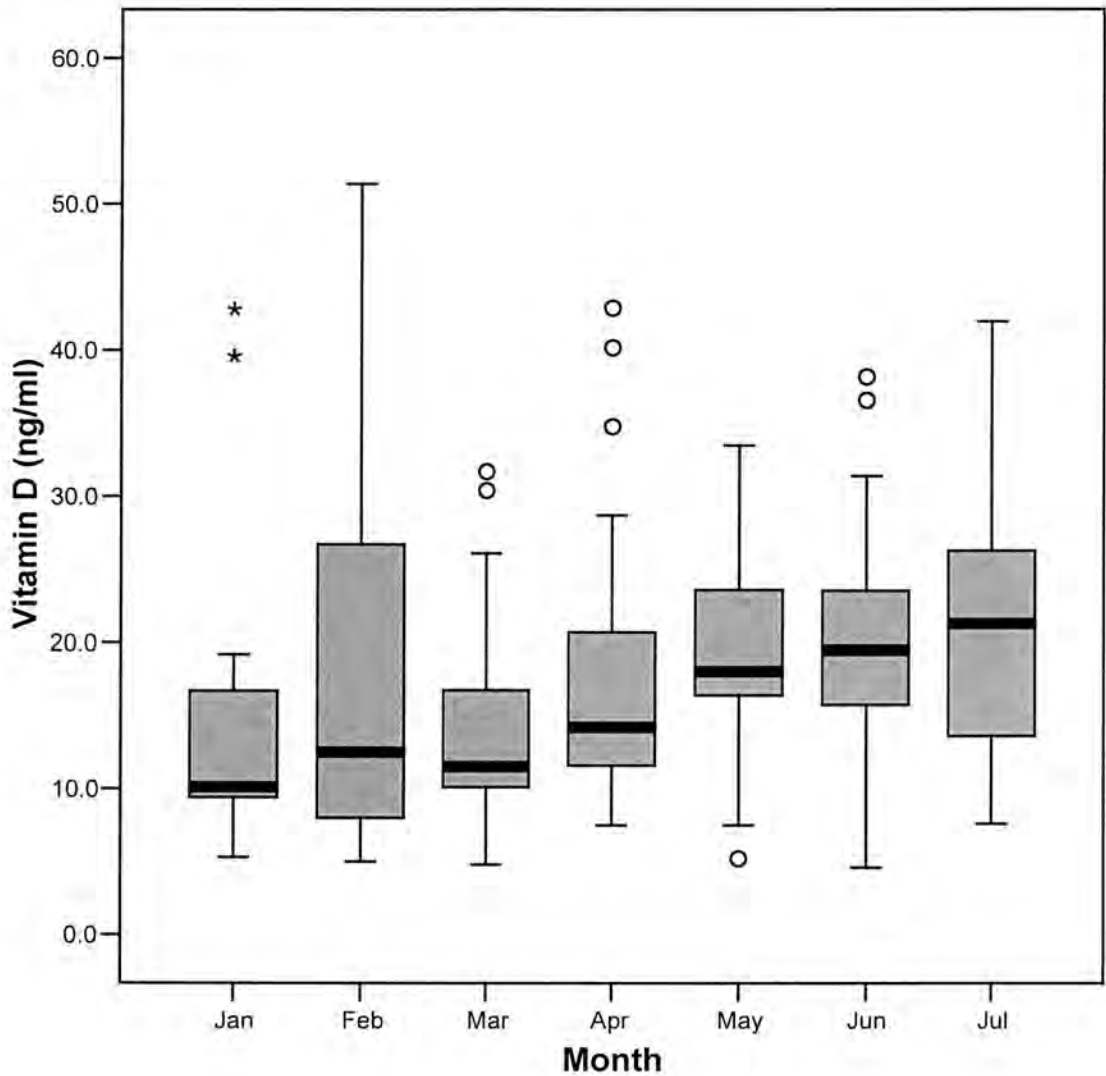
Eighty percent of the cohort regularly avoided sunlight, 87% wore long-sleeve occlusive clothing daily, 9% used a sun screen at least once daily all year and 68% used sunscreen once daily or more frequently in sunny weather. No subjects had ever had their vitamin D status checked by their physicians. Five subjects reported coexistent osteoporosis, but other than analgesics, were not taking any other treatments for this. Three subjects took fish liver oils daily as a health supplement, one of whom also took a calcium supplement. One further patient took calcium supplementation. Excepting a non-vegan vegetarian, all subjects were omnivores; a more detailed dietary history was not taken.

The mean serum hydroxyvitamin D was 18.32 ng/ml (range 4.9-51.4, quartiles 11.5, 23.5). One hundred and twenty six subjects (63%, 58 males, 68 females) were VDI of whom thirty four subjects were vitamin D deficient (VDD) (17%, 15 males, 19 females). Of three subjects taking dietary fish oil supplements, one was VDI. Twenty one subjects were receiving ultra-violet B (UVB) phototherapy to induce 'hardening' of their skin to sunlight sensitivity; 6 (29%) were VDI and none were VDD.

The mean monthly serum 25-hydroxyvitamin D rose over the study period January to July from 15.5 to 21.3 ng/ml (Figure 14). In the winter months of January and February 70% of subjects (19/27) were VDI and 44% (7/27) were VDD; In the summer months of June and July 45% (34/75) were VDI and 37% (28/75) VDD. There appeared to be a slightly smaller proportion of children aged 16 or under VDI or VDD compared to the overall population (3/34 - 11%, 18/92 - 20%).

One hundred and eighty one complete sample sets were available for analysis of calcium, phosphate and PTH biochemistry. Thirteen subjects (7%) were deficient in adjusted serum calcium (11 VDI, of whom 3 were VDD) and forty one (23%) had an elevated serum phosphate (21 VDI, of whom 3 were VDD). Hyperparathyroidism was seen in 12 subjects of whom 9 were VDI (2 VDD) and the remaining 3 had serum 25-hydroxyvitamin D at the lower end of the normal range of between 21 and 23 ng/ml.

Figure 14. Boxplot of serum 25-hydroxyvitamin D by month of sampling (deficiency <10 ng/ml, insufficiency < 20 ng/ml). The boxes contain results between the upper and lower quartiles and the dark bars within the boxes represent the median value.



Statistical analysis (Mann-Whitney test) suggested VDI was associated with total erythrocyte protoporphyrin (TEP) ($P = 0.009$) and inversely associated with the time in minutes to the onset of symptoms following sunlight exposure ($P = 0.008$). VDD was associated with the age of symptom onset ($P < 0.0005$), TEP ($P = 0.02$) and inversely with minutes to symptom onset ($P = 0.03$). There was no association with

calcium deficiency, raised phosphorus or elevated PTH, although elevated PTH approached significance with VDI ($P = 0.57$).

3.8 Autosomal recessive EPP

3.8.1 Palmar keratoderma is an uncommon feature of EPP.

From the study cohort, 7 rEPP subjects from 4 families had marked thickening of the palmar epidermis (palmar keratoderma). Subsequent to the close of the study, 2 further individuals with this uncommon phenotype were identified resulting in 5 males and 4 females, aged 8 to 63 years, from 6 families (Table 15), giving prevalences for palmar keratoderma in EPP of 3.3% for all subjects and 2.6% (95% confidence interval: 1.0 – 5.1%) for families.

In all subjects, keratoderma had been present since early childhood and in 7 subjects had developed before the diagnosis of EPP was made. In 3 subjects the development of mild palmar keratoderma was preceded during infancy by a scaly rash, mainly on the dorsum of the hands, initially thought to be eczema; a third patient had areas of hyperpigmentation and lichenified skin over her knees, neck and elbows which were present before the onset of photosensitivity and have persisted. Keratoderma was worse in summer and often resolved in winter. In one patient, occlusion of the skin with a plaster cast for 6 weeks following a wrist fracture led to almost complete resolution of the keratoderma. These features have not previously been reported for other palmoplantar keratodermas³¹¹ and were felt to represent a distinct rEPP-specific presentation.

The keratoderma ranged in severity from waxy keratoderma over the whole palm to mild keratoderma of the first interdigital web (Fig 15, Table 15). Palmar keratoderma was sharply demarcated at the wrist with, in most cases, minimal transgression onto the extensor surface, and without an erythematous border (Fig 15). Two subjects had mild onycholysis but otherwise nails were not affected. Sweating appeared unimpaired and there was no malodour. Two unrelated subjects also had mild plantar keratoderma (Table 15). Keratoderma was not particularly marked at sites of pressure or punctuate in pattern. No patient reported blistering,

hyperhydrosis, periodontitis or other teeth abnormalities, visual or auditory impairment.

In one patient, a very small punch biopsy of the hand had previously been obtained. The limited amount of epidermis present was slightly thickened and spongiotic and there were some prominent upper dermal vessels with a hint of PAS-positive hyaline change. There was not enough epidermis to determine whether the keratin layer was thickened.

The severity of photosensitivity varied within families but was similar to that in subjects with EPP without keratoderma (average time to symptom onset 24 minutes, quartiles 6, 45).³¹² All became photosensitive between the ages of 3 and 17 months. Symptoms started within 1 – 30 minutes after exposure to sunlight and quality of life, assessed by CDLQI or DLQI scores, was severely impaired in the 6 subjects in whom it was measured (mean DLQI 11, CDLQI 12).

Four subjects from 3 families had neurological abnormalities. Three subjects (P7, P8, P9) had slight or moderately severe cognitive and motor developmental delay, without regression since early childhood, and one of these suffered from fits until the age of 2 years. Older siblings in both families had neither EPP nor developmental delay. One patient (P4) presented in his early thirties with a spastic paraparesis which has progressed; extensive investigations to identify a cause were negative. Haemoglobin concentrations and biochemical tests of liver function were normal in all subjects, except for one female patient who had a haemoglobin concentration of 11.9g/dL.

Table 15. Recessive EPP with keratoderma: clinical features.

| Family | Patient | Sex | Age (yrs) | Onset age(yrs) | Family history | Keratoderma |
|-----------------|---------|-----|-----------|----------------|------------------|---|
| I | P1 | F | 8 | 1.4 | sib ¹ | Confluent waxy palmar keratoderma with fine peeling; sharp cut-off at wrists. |
| | P2 | M | 10 | 1.0 | sib ¹ | Keratoderma and peeling of first interdigital web and patchy keratosis and peeling over palmar surface of digit joints and pulps. |
| II ² | P3 | F | 58 | 0.5 | sib | Palmar keratoderma with sharp cut-off at wrist; transgression to involve first interdigital web; focal plantar keratoderma with fissuring over heels and MCT joints |
| | P4 | M | 52 | 0.4 | sib | Mild keratoderma of first interdigital web |
| III | P5 | M | 27 | 0.3 | - | Confluent palmar keratoderma with peeling; lateral aspects dry, white and cracked; slight onycholysis; keratoderma medial aspect of forefoot and great toes. |
| IV | P6 | M | 63 | <1 | - | Marked keratosis of first interdigital web and radial border of index fingers with fissuring at index finger joints |
| V | P7 | F | 17 | 0.25 | - | Waxy keratoderma over the whole palm; mild fine peeling; more obvious in summer, regresses in winter; sharp cut-off at wrist. Nail dystrophy. |
| VI | P8 | M | 13 | 0.25 | sib | Mild keratoderma of first interdigital web |
| | P9 | F | 12 | 2.0 | sib | Mild keratoderma of first interdigital web |

¹ Great grandmother has EPP without keratoderma (see text). ² Consanguineous parents.

Figure 15. Palmar keratoderma in EPP.





3.8.2 Erythrocyte protoporphyrin concentrations are lower in EPP with keratoderma than in EPP without keratoderma.

Erythrocyte porphyrin concentrations in the nine subjects with keratoderma were significantly lower (median 7.4 $\mu\text{mol/L}$, range 2.0-15.5 $\mu\text{mol/L}$; normal subjects, 0.4 – 1.7 $\mu\text{mol/L}$) than in 203 subjects without keratoderma (median 22.8 $\mu\text{mol/L}$, range 4.1-159.2 $\mu\text{mol/L}$) (Mann-Whitney test $P < 0.001$). When two members of the same family were affected, erythrocyte porphyrin concentrations were similar. The percentage of free erythrocyte protoporphyrin (41 – 91%) was increased in all subjects but in those with the lowest TEP was only approximately equal in amount to zinc-protoporphyrin. In contrast, the plasma protoporphyrin concentrations in the subjects with keratoderma (median 55.0 nmol/L , range 13.4 – 115.1 nmol/L ; normal subjects, less than 10.5 nmol/L) did not differ significantly from those in 11 randomly selected subjects with EPP without keratoderma (median 68.4, range 18.8 – 396.0) whose erythrocyte porphyrin concentrations ranged from 12.4 to 159.2 $\mu\text{mol/L}$ (median 30.1 $\mu\text{mol/L}$). Faecal total porphyrin excretion was substantially increased (median 1198 nmol/g dry weight; range 523 – 1940 nmol/g ; normal subjects, less than 200 nmol/g) in the seven subjects in whom it was measured, with protoporphyrin accounting for greater than 80% of the total.

3.8.3 EPP with palmar keratoderma is inherited in an autosomal recessive pattern

Three subjects had no family history of overt EPP or palmar keratoderma. In families II and VI, two siblings had EPP with keratoderma but no other relative had either condition. In family I, two of three siblings had EPP with keratoderma; the third had neither condition but their maternal great grandmother had a lifelong history of photosensitivity without keratoderma. Photosensitivity and keratoderma always occurred together when more than one member of the same generation had EPP.

Mutational analysis showed that all subjects with EPP and palmar keratoderma were either compound heterozygous or homozygous for *FECH* mutations and that only one patient (family III) had inherited the hypomorphic *FECH* IVS3-48C allele (Table 16). All but one of the mutations were missense; 4 of these (c.0302T>C; c.0854A>G; c.0898G>T; c.0502C>T) are novel. Mutational analyses of families II and VI have been reported.⁴ Both subjects in family I were compound heterozygotes for a missense mutation and a mutation (IVS3+2T>G) that is known to impair splicing of exon 3.³¹³ In their maternal grandmother, this mutation was *trans* to an *FECH* IVS3-48C allele; a genotype that has been identified in other patients with the typical dominant form of EPP (Whatley SD, unpublished information). Mutational analysis of 184 unrelated subjects without keratoderma identified 2 additional subjects with recessive EPP; one, a 14-year-old boy, had been reported previously with regard to his abnormal liver function.^{4,149} The other was homozygous for both a novel mutation (c.0502C>T; P168L) and the hypomorphic IVS3-48C allele.

Five of the eight missense mutations that we identified were expressed in a prokaryotic expression system and their effect on *FECH* activity determined (Table 17). These activities, together with those previously reported for the 3 other missense mutations,⁴ were used to calculate the *FECH* activities in our subjects (Table 17) on the assumption that they were similar to those expressed by these alleles in human tissues.

Table 16. EPP with palmar keratoderma: *FECH* genotypes with predicted *FECH* activities.

| Family | Allele | Mutation | Effect | <i>FECH</i> IVS3-48 | Predicted <i>FECH</i> activity (%) |
|-----------------|--------|-----------|---------------|---------------------|------------------------------------|
| I ¹ | 1 | c.0302T>C | L101P | T | 2.7 ² |
| | 2 | IVS3+2T>G | Splice defect | T | |
| II ¹ | 1 | c.0416A>T | Q139L | T | 18 |
| | 2 | c.0416A>T | Q139L | T | |
| III | 1 | c.0503C>T | P168S | T or C | 5.6 ³ |
| | 2 | c.0854A>G | Q285R | C or T | |
| IV | 1 | c.0820G>A | D274N | T | 5.9 |
| | 2 | c.0898G>T | V300L | T | |
| V | 1 | c.0707G>A | C236Y | T | 6.4 |
| | 2 | c.0820G>A | D274N | T | |
| VI ¹ | 1 | c.1137C>G | K379N | T | 25 |
| | 2 | c.0707G>A | C236Y | T | |

Previously unreported mutations are shown in bold; ¹both affected siblings had the same genotype; ²assuming no activity from the IVS3+2T>G allele; ³assuming IVS3-48C *cis* to P168S (see text).

Table 17. Prokaryotic expression of mutant and wild type *FECH* alleles

| <i>FECH</i> allele | <i>FECH</i> activity(nmol/h/mg) ¹ | Percent wild type activity |
|--------------------|--|----------------------------|
| Wild type | 5626 | 100 |
| L101P | 233 | 5.3 |
| P168S | 717 | 16 |
| D274N | 30 | 0.8 |
| Q285R | <10 | <1 |
| V300L | 489 | 11 |

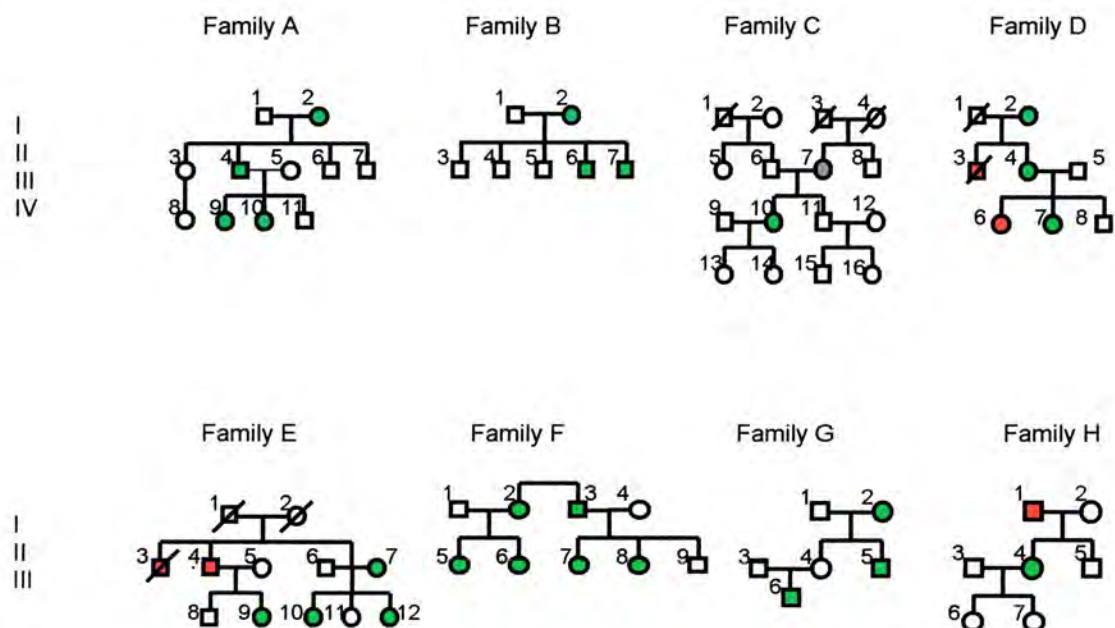
¹zinc-mesoporphyrin

Predicted *FECH* activity was calculated from data in Table 18 and Whatley et al, 2004.⁴

3.9 X-linked dominant protoporphyria (XLPP)

Mutational analysis fails to detect *FECH* mutations in about 7% of EPP families of which about 3% are homozygous for the wild type *FECH IVS3-48T* allele (rEPP).³¹⁴ We studied 8 families (Fig. 16) in which at least one individual had acute photosensitivity clinically indistinguishable from that of dEPP and who genotypically were not rEPP. Three families were identified from this study and the remainder through collaboration with colleagues in Paris, London and Cape Town.³¹⁵ They were of western European (4 families), Jewish, north African, Indo-Asian or Sudanese origin (one family each).

Figure 16. Pedigrees of families A – H with X-linked dominant protoporphyria.



● ■ = patient with EPP clinically. ● ■ = patient with EPP clinically and liver disease.

Clinical information was not obtainable for subjects C I, 3 and 4 or E I,1 and 2. All subjects with liver disease had symptoms attributable to liver disease which was confirmed at autopsy or by needle biopsy as being protoporphyric liver disease in all except patient H I,1 for whom a diagnosis has not been established. Patient E II,3 was previously described by Eales, L, Day, RS, Pimstone, NR. *Ann Clin Res* 1979; 10: 205-213. For families A – D, haplotyping showed no linkage of disease to *FECH* and was uninformative for family E. Haplotyping of

families A – C was consistent with a disease locus on the X chromosome; other families were not haplotyped.

We differentiated these subjects from others with mutation-negative EPP by showing that the percentage of erythrocyte PP that was present as its zinc chelate (ZnPP) (19-65%, median 44%) was markedly greater than in dEPP (4-13%, median 8%) (Fig. 17 and Table 20). Erythrocyte PP concentrations were also higher in our subjects, being increased 24-fold (range: 6 - 103-fold) compared with 14-fold (range: 4 - 44-fold) in 171 subjects with mutation-positive dEPP (Mann-Whitney test $P < 0.001$) (Fig. 17). Lymphocyte FECH activity was normal, indicating that PP accumulation was not caused by FECH deficiency resulting from a mutation of the ubiquitously expressed *FECH* gene (Fig. 18 and Table 20).

Figure 17. Spectrophotometric illustration of free erythrocyte protoporphyrin concentrations in mutation negative EPP subjects compared to dEPP and an unaffected population.

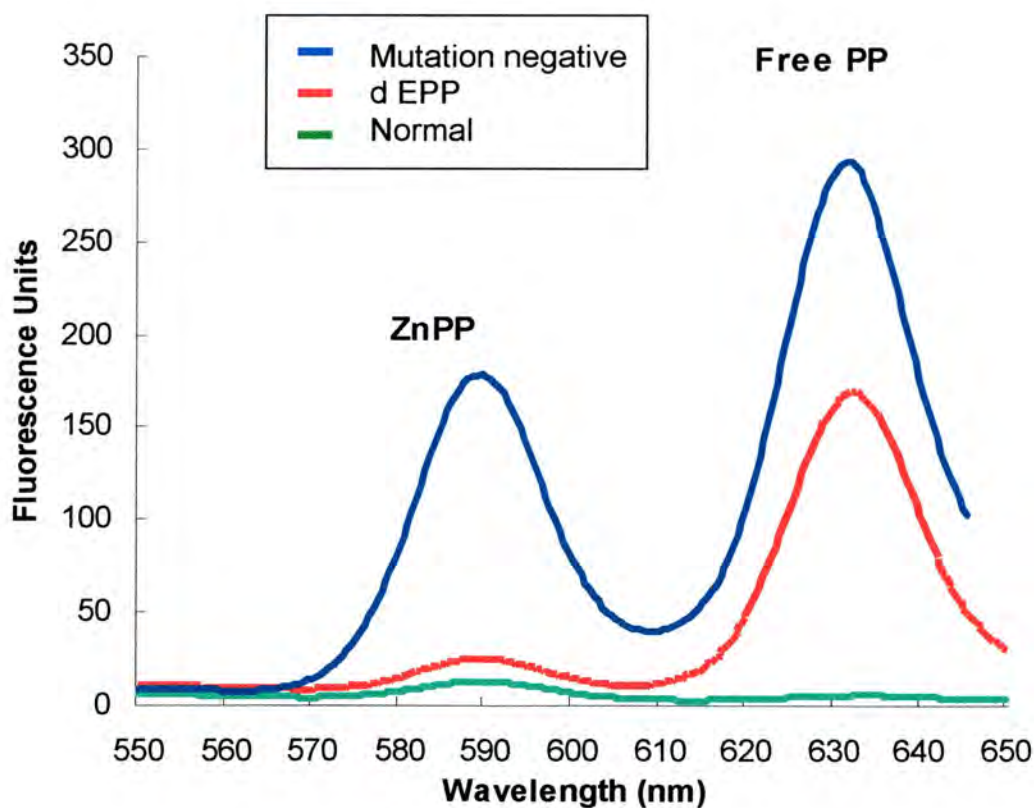
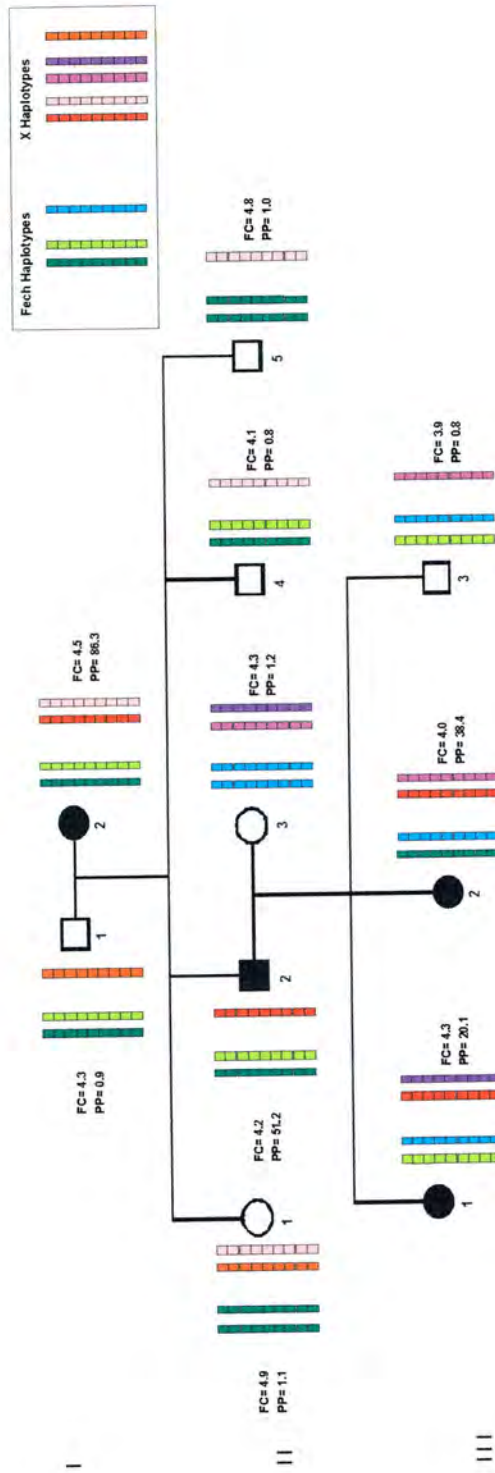


Table 18. Porphyrin and enzyme measurements for subjects with X-linked dominant protoporphyria

| Family/Patient | Sex | PP (µmol/L) | PP (fold increase) | ZnPP (% of PP) | FECH activity | ALAS2 mutation | |
|----------------|--------|-------------|--------------------|----------------|---------------|-----------------|---------|
| A | I,2 | 2 F | 86.3 | 45 | 30 | 4.5 | delAGTG |
| | II,4 | 4 M | 51.2 | 27 | 52 | 4.2 | |
| | III,9 | 9 F | 20.1 | 10.6 | 57 | 4.3 | |
| | III,10 | 10 F | 38.4 | 20.2 | 61 | 4.0 | |
| B | I,2. | 2 F | 52.4 | 27.6 | 57 | 3.8 | delAGTG |
| | II,6 | 6 M | 178.6 | 94 | 39 | 5.3 | |
| | II,7 | 7 M | 195.6 | 103 | 36 | 4.9 | |
| C | II,7 | 7 F | 56.7 | 29.8 | 40 | 4.1 | delAGTG |
| | III,10 | 10 F | 37.1 | 19.5 | 34 | 3.7 | |
| D | II,4 | 1 F | 6.0 ¹ | 24.0 | 27 | - | delAGTG |
| | III,6 | 2 F | 109 | 64.1 | 37 | 68 ² | |
| | III,7 | 3 F | 41.2 | 24.2 | 41 | - | |
| E | II, 4 | 1 F | 5.6 | 5.6 | 65 | - | delAGTG |
| | II,7 | 2 M | 11.1 | 11.1 | 45 | - | |
| | III,9 | 4 F | 9.3 | 9.3 | 61 | - | |
| | III,10 | 5 F | 8.1 | 8.1 | 65 | - | |
| | III,12 | 6 F | 27.3 | 27.3 | 47 | - | |
| F | I,3 | 1 M | 22.6 | 13.3 | 64 | - | delAT |
| | II,7 | 2 F | 61.7 | 36.3 | 46 | - | |
| | II,8 | 3 F | 40.8 | 24.0 | 42 | - | |
| G | I,2 | 1 F | 35.2 | 20.7 | - | - | delAT |
| | III,6 | 2 M | 22.5 | 13.2 | 58 | - | |
| H | I,1 | 1 M | 159 | 93.5 | 19 | - | delAGTG |

Reference ranges for erythrocyte PP are less than 1.9 µmol/L erythrocytes (families A-C), less than 1.0 µmol/L (family E) and 0.4-1.7 µmol/L (families D, F-H). ¹Free PP and ZnPP determined separately; reference ranges less than 0.2 µmol/L and less than 0.8 µmol/L respectively. Reference range for lymphocyte FECH activity: 5.0 ± 1.5 (mean ± 2SD) nmol zinc mesoporphyrin/h/mg protein;² indirect FECH assay, reference range: 42-92%, mean 68%, n = 22. There was no significant difference between erythrocyte PP in males and females (*P* = 0.37, Mann Whitney test).

Figure 18. Pedigree of family A.



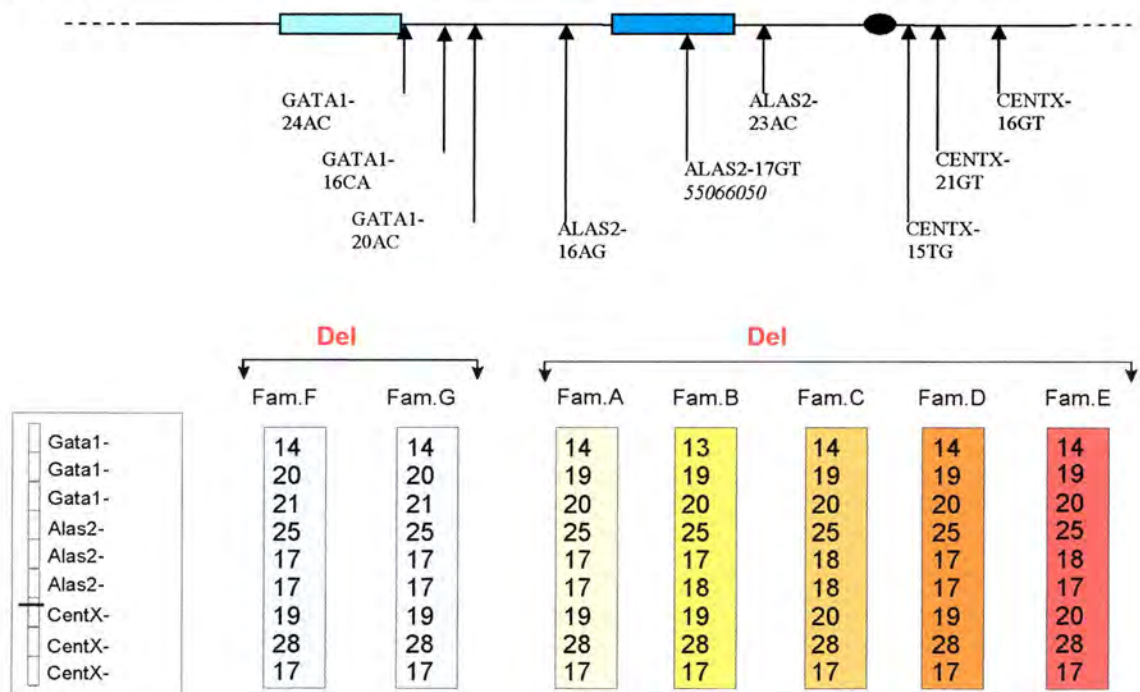
FC, lymphocyte FECH activity in nmol zinc-mesoporphyrin/h/mg protein;

PP, erythrocyte protoporphyrin in μmol/litre packed erythrocytes.

Involvement of *FECH* was further eliminated by showing that the disease did not segregate with *FECH* haplotypes (Fig. 18). Because abnormal expression of mRNA for mitoferrin has been implicated in the pathogenesis of a similar form of protoporphyria,³¹⁶ we sequenced *SLC25A37* in all 8 probands, but were unable to identify disease-specific mutations.

Parent-child transmission of overt disease is uncommon in dEPP.⁸ Our families were unusual in showing an apparent dominant pattern of inheritance with an absence of father-son transmission that suggested X-linkage (Figures 16 and 18). X-linkage was confirmed in 3 families using microsatellite markers for two candidate genes, *GATA1* and *ALAS2*, on the X chromosome (Fig. 19).

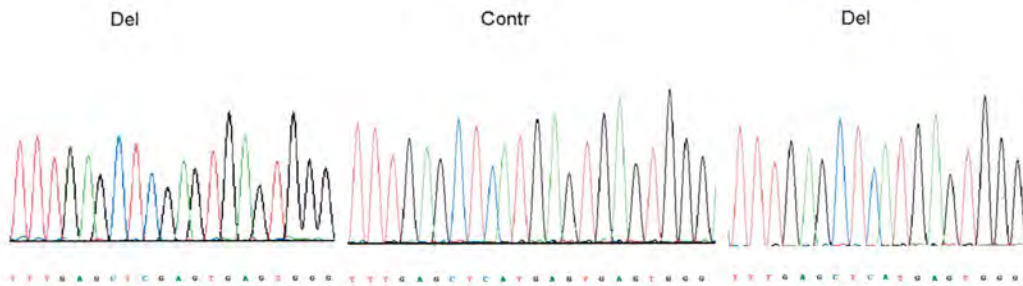
Figure 19. X chromosome microsatellite markers used for haplotyping and the X chromosome haplotypes associated with disease in families A – G.



Sequencing of genomic DNA excluded a mutation in *GATA1* but identified two different deletions in exon 11 of *ALAS2* (c.1706-1709delAGTG; p.Glu569GlyfsX24

in 6 families and c.1699-1700delAT; p.Met567GlufsX2 in 2 families) that predicted alterations of the 19 -20 C-terminal amino acids of ALAS2; either their replacement by a 23 residue sequence that extends the enzyme by 4 amino acids or their deletion (Fig. 20). These deletions segregated with photosensitivity (LOD score 8.13) and were absent from 23 unrelated FECH-mutation negative subjects, 106 unrelated dEPP subjects, and 100 normal subjects. The c.1706-1709delAGTG mutation occurred on 5 different haplotypes indicating that it has arisen on at least 5 separate occasions. This recurrent mutation involves a direct 4 base repeat in the coding sequence of *ALAS2*, such sequences being more prone to deletion (Fig. 20a). These data indicate that the deletions in *ALAS2* that we have identified are responsible for the disease in our families. They identify a previously unrecognised disorder, X-linked dominant protoporphyria (XLPP) that, in contrast to dEPP and other autosomal dominant porphyrias, has close to 100% penetrance.

Figure 20. a) Sequence analysis of genomic DNA from male subjects showing deletions in the *ALAS2* gene.



b) Alignment of *ALAS* C-terminal sequences; arrows indicate the effects of the deletions; the 26 conserved residues of *ALAS2* are boxed.



All previously described mutations in *ALAS2* have caused XLSA with frame shift (insertion or deletion of a number of nucleotides, not divisible by three, disrupting the triplet reading frame of the DNA) or other null *ALAS2* mutations (mutations which prevent the DNA being transcribed into RNA and / or translated into a functional protein product, which are embryonically lethal in males).³¹⁷ The mutations appear to be in the catalytic site of *ALAS2*, and reduce mitochondrial enzyme activity levels, resulting in dyserythropoiesis, with ring sideroblasts in the marrow, a microcytic hypochromic anaemia and a secondary (erythropoietic) haemochromatosis.³¹⁸ In contrast, in our families, both sexes were affected and subjects had neither anaemia (haemoglobin: 12.2 – 17.5g/dL) nor iron overload (Table 21). In one subject with iron deficiency caused by a gastric ulcer, treatment with iron led to a 3-fold decrease in TEP with little change in ZnPP concentrations; other subjects showed the mild disturbance of iron metabolism characteristic of dEPP,³¹⁹ consistent with the hypothesis that accumulation of protoporphyrin rather than FECH deficiency is responsible for this abnormality.¹²⁹ Five (16%) subjects had overt liver disease (Fig. 15), suggesting that XLPP, like autosomal recessive EPP,¹⁷⁵ carries a higher risk of liver disease than dEPP. Liver disease was commoner in males ($P = 0.008$, Fisher's exact test), and one obligate carrier was asymptomatic (Fig. 15, II, 4 in family G), but otherwise we found no evidence that X-inactivation led to milder disease in females; there was no significant difference between erythrocyte PP concentrations in males and females (Table 19).

The 26 C-terminal amino acids of *ALAS2* are highly conserved among species that have two *ALAS* genes (Fig. 19) but are not present in *ALAS* from *Rhodobacter capsulatus* (*ALAS_{Rc}*), the only *ALAS* for which the crystal structure has been reported.³²⁰ The conservation of this sequence and its absence from *ALAS1* suggest an important, but unknown, erythroid-specific function. It does not appear to be directly involved in catalysis and a missense mutation in this region (S568G) that produces XLSA probably acts by inducing a general conformational change.³²⁰

Table 19. Haematological and iron indices for subjects with X-linked dominant protoporphyria

| Family/Patient | Sex | PP ($\mu\text{mol/L}$) | Hb ($\mu\text{g/dL}$) | sFn ($\mu\text{g/L}$) | sTf sat (%) | sTfR |
|----------------|-----|-----------------------------|----------------------------|----------------------------|----------------|------|
| A I,2 | F | 45 | 14.1 | 65 | 23 | 2.24 |
| II,4 | M | 27 | 15.7 | 104 | 39 | 1.44 |
| III,9 | F | 10.6 | 13.8 | 21 | 33 | 1.32 |
| III,10 | F | 20.2 | 12.8 | 77 | 22 | 1.05 |
| B I,2 | F | 27.6 | 12.4 | 46 | 16 | 2.59 |
| II,6 | M | 94 | 14.3 | 24 | 7 | 2.47 |
| II,7 | M | 103 | 12.2 | 10 | 4 | 2.83 |
| C II,7 | F | 29.8 | 13.8 | 154 | 3 | 1.41 |
| III,10 | F | 19.5 | 13.0 | 19 | 12 | 1.24 |
| F I,3 | M | 22.6 | 17.5 | 28 | 24 | 18 |
| G III,6 | M | 22.5 | 12.4 | 12 | 16 | 17 |

Reference ranges: serum ferritin (sFn) 15 – 250 $\mu\text{g/L}$; serum transferrin saturation (sTf sat) 20 – 45 %; serum transferrin receptor (sTfR) 0.8 – 1.6 mg/L (families A-C); 8 – 30 nmol/L (families F and G).

4. Discussion

4.1 Case identification and prevalence of EPP

The study provides quantitative data about the frequency of clinical features (Tables 7 and 9). Differences, particularly in the frequencies of clinical features, between our data and that from previous descriptions of EPP^{7,115,166} are probably best explained by the larger number of patients in this study, the inclusion of a higher proportion of patients (32%) over the age of 40 years and possibly geographical location. Furthermore these results may be less susceptible to observer variation and participation bias, as all the subjects were seen by the same observer and selection bias was minimised by recruiting as high a percentage of known UK cases as possible.

We identified 389 living individuals within the UK with EPP and an additional 30 affected family members not included in the databases that we searched. This gives a minimum prevalence for EPP in the UK (population 59.8 million) of 1 in 143,000, close to the previously reported UK prevalence of 1:130,000³²¹ and higher than 1:200,000 reported in Sweden¹⁷⁷ but lower than the 1:79,000 and 1:75,000 for Northern Ireland and the Netherlands, where there is some evidence for founder effects.^{8,70} The true UK prevalence probably lies between these figures given the difficulty in obtaining a diagnosis reported by many of our patients, and our identification since the study ended of new cases that were symptomatic before the study period. Although it seems reasonable to assume that we studied just over half the total number of UK patients, some selection bias may have been introduced by using databases held by specialist referral centres and because only 76% of those invited volunteered to participate.

4.2 Clinical presentation of EPP

This study clearly defines for the first time the clinical features of EPP in a large cohort. The clinical onset is very much in early childhood, usually at the time of first significant sunlight exposure. All 223 patients in this study developed symptoms before they were 13 years old, although a few patients did not seek medical advice

until they were much older. This was an unexpected finding because other series have included patients whose disease became manifest only during their teens or later,^{7,115} and there are several reports of adult onset of EPP,¹¹⁰ distinct from those caused by somatic mutation of the *FECH* gene in haematopoietic cells.¹²² In contrast, the median age at diagnosis was 12 years and the diagnosis was not made in a third of our patients until they were 20 years of age or older. This has obvious implications for management and complications. Although laboratory methods for the diagnosis of EPP have improved over the past three decades, analysis of our data shows little change in the median time to diagnosis for each decade from 1965. Dermatologists diagnose most EPP individuals, with only a minority diagnosed by either GPs or Paediatricians, the healthcare professionals from whom most of these individuals will have first sought advice. While it is possible that GPs and Paediatricians refer individuals with such symptoms to Dermatologists to make the diagnosis, both the delay in diagnosis and participants' comments regarding the diagnostic process suggest that there is considerable ignorance of the condition in these professional groups. Thus there is a need for an increased awareness of EPP among GPs, Paediatricians, and also in the wider community, as 8% of our patients were self-diagnosed or diagnosed by a family member after seeing or reading information about porphyria.

Clinical aspects of EPP which this study has confirmed include the previously reported priming phenomenon, nail changes in response to light exposure,¹¹⁵ acute symptoms usually in the absence of visible signs (swelling/oedema appear on average after 6 hours), and knuckle skin thickening. This latter sign appear more common in males than females and while this may relate to a direct sex effect, more probably reflects the increased light exposure of men's hands during differential occupational and leisure activities. The study also confirms the improvement in photosensitivity during pregnancy. Two novel findings related to this last observation were the identification that TEP was higher in males than females,⁷⁰ and the experience by some women of more severe symptoms around the time of their menses. These add to the evidence that sex hormones influence EPP, although the mechanism for the effect remains unclear. There are reports that TEP decreases^{166,167}

or remains unchanged during pregnancy.¹⁶⁵ The human ABCG2 multidrug transporter protein regulates intracellular protoporphyrin concentrations in erythroid and hepatic cells,³⁶ and its expression in human placental cells is increased by progesterone and 17 β -oestradiol,³⁷ an effect that might lead to removal of protoporphyrin from the maternal circulation during pregnancy. Other suggestions include a hormonal influence on *FECH* expression, the dilutional effects on the vasculature of pregnancy, enhanced biliary excretion during pregnancy or foetal detoxification of maternal PP.¹⁶⁸

Photo-induced hardening of the skin was reported in over half of participants. This is an important observation as it suggests that iatrogenic hardening using phototherapy (available in most Dermatology units) could be a helpful therapeutic modality if commenced in Spring, similar to treatments for polymorphic light eruption, to provide increase photo-tolerance over the summer months. Furthermore, such treatment would have an additional benefit of UV-induced endogenous vitamin D synthesis, as a further novel finding of this study was the identification that a large proportion of EPP individuals are vitamin D insufficient or deficient throughout the year, leaving individuals at risk of important clinical consequences. However three quarters of the study cohort had never been offered phototherapy, perhaps reflecting concerns of both patients and clinicians of using such treatment when the condition is precipitated by light radiation at a closely related wavelength.

Some of the study's findings suggested that several earlier reported features of the condition may not be correct. Subjectively there was no significant participant reported reduction in symptoms with increasing age and objectively, no reduction in DLQI scores with age. In contradistinction, there may be a trend to increasing sensitivity with age due to the association between TEP and increasing age. Participants' comments on this issue were generally that with increasing age they were better able to judge situations and avoid activities at times when symptoms might be induced.

Although there is a perceived increased in hypothyroidism in our EPP study population (4.7% versus 3.3% in a normal British population (Tayside)),³²² a chi square test failed to detect a significant difference ($\chi^2 = 1.19$; $df = 1$; $P = 0.274$).

In contrast, gallstones have been reported to be more common in individuals with EPP than might be expected;^{7,323} in our population 9% had gallstones and 6% previous cholecystectomy (1% men and 5% women). It is recognised that gallstones are often asymptomatic and may only be discovered incidentally, and that the incidence of gallstones and cholecystectomy rises with age. The mean age of our study population was 34 years, and the mean age of the Scottish population in 2000 was 38.76 (General Register Office for Scotland) and in England in 2001 was 38.6 (2001 census, Office for National Statistics), suggesting that normal British populations have a similar mean age to our study population. Previous studies of large normal British populations in Bristol³²⁴ and Teesside³²⁵ reveal cholecystectomy rates of 1.67 – 1.3% for men and 3.5 – 4.1% for women respectively. While there appeared to be no significant differences in cholecystectomy rates between the Bristol and Teesside populations ($\chi^2 = 0.06$; $df = 1$; $P = 0.803$), there appeared to be a significance difference in rates between the EPP population and the Bristol ($\chi^2 = 7.72$; $df = 1$; $P = 0.005$) and Teesside populations ($\chi^2 = 7.57$; $df = 1$; $P = 0.006$). Closer analysis showed no difference in rates in males with EPP versus those resident in Bristol ($\chi^2 = 0.64$; $df = 1$; $P = 0.424$) or Teesside ($\chi^2 = 1.52$; $df = 1$; $P = 0.218$), but there appeared to be an increased rate in females with EPP over those in Bristol ($\chi^2 = 9.55$; $df = 1$; $P = 0.002$) or Teesside ($\chi^2 = 7.53$; $df = 1$; $P = 0.006$). The explanation behind this observation is not apparent. Due to the small numbers of men with EPP (3) who had undergone cholecystectomy, statistical analysis of this group may not be accurate. The increased rate in women with EPP may be a true reflection of an increase of symptomatic gallstone disease in the condition, or a reflection of the preconception that individuals with EPP are more likely to have gallstones leading to over-investigation and over-treatment.

Management of study individuals' EPP was often not optimal, possibly reflecting a relative lack of knowledge amongst physicians, perhaps resulting from the dearth of

robust studies. Although all individuals knew about sunscreen use to limit photosensitivity, it was striking that the majority of those currently using sunscreen were using propriety products which would be unlikely to protect effectively against those visible wavelengths precipitating acute symptoms. Furthermore, nearly a third did not use any sunscreen. Regular follow-up of EPP individuals would provide an opportunity for physicians to educate them, suggest treatment options (including phototherapy discussed above), and undertake regular blood investigations such as liver function tests (LFT), serum 25 hydroxy vitamin D and possibly for FBC and iron status (sFn and sTfR) to assess for possible complications. However 30% of the study cohort was under no regular review of any medical practitioner. Although there are no published follow-up guidelines for EPP either within the UK or internationally, it would seem sensible for individuals to have at least an annual review with a medical practitioner who has knowledge of the condition. An example of an important area for education is the consumption of alcohol. Not only was alcohol intake associated with abnormal LFTS, but 4% of the cohort used excess alcohol intake as an analgesic approach during symptomatic episodes. While it may be argued that there is no current evidence linking alcohol or abnormal LFTS and the development of liver failure in EPP, it would seem prudent to limit intake of additional hepatotoxins in the presence of TEP, an endogenous hepatotoxin.

Forty percent of participants recounted a family history of the condition, most usually in siblings, but also parent-child inheritance and in more distant relations. In only 2 families was transmission recorded in an apparent dominant pattern, between at least 3 consecutive generations. Both these families were subsequently discovered not to have classical EPP, but instead the novel XLPP, suggesting a good family history is a useful discriminating feature between the two conditions.

4.3 Quality of life in EPP

The substantial impact of EPP on the lifestyle of most patients is indicated by their QoL scores (Table 10) which were unexpectedly high in comparison with those for other skin diseases generally regarded as more severe, such as severe eczema and epidermolysis bullosa (Table 20).²⁹⁸ In contrast, relatively asymptomatic but

visually disfiguring conditions scored much lower.²⁹⁸ There are no published studies of QoL in other cutaneous porphyrias, although the impact of non-cutaneous symptoms in acute porphyrias can be marked.³²⁶ The high scores, with EPP having a very large effect on QoL in 70% of adults, are partly explained by high scoring in the social and leisure categories. This may reflect the lifestyle restrictions imposed by the need to avoid exposure to sunlight, whereas in most other skin disorders symptom-related questions score highest.^{327,328} Children, who may be less aware of the social implications of their illness, tended to score higher on questions about symptoms and sleeping, reflecting their reduced control over sunlight exposure and the pain experienced when avoidance is not possible. Thus the main effect of EPP on QoL comes from the morbidity produced by acute and prolonged pain following sunlight exposure together with the social consequences of the measures needed to avoid this. This very significant impact of having EPP on an individual's QoL has probably not previously been fully appreciated by clinicians.

The previously reported trend for higher DLQI scores in younger adults³²⁸ was not seen in EPP (Table 10). Indeed, scores did not improve with age, again in keeping with our observation above of no subjective change with age and a trend for higher TEP levels to be associated with increasing age, refuting the earlier suggestion of the condition improving with age (which may have been more a reflection of better ability to avoid sunlight in adulthood).¹¹⁵

Table 20. Comparison of previous DLQI and CDLQI study mean scores

| Condition | Adults | Children |
|------------------------------------|--|---------------------------|
| EPP (current study) | 14.0 | 12.8 |
| Acne | | 5.7 ³²⁹ |
| Atopic eczema | 4.14 ³³⁰ -16.2 ³³¹ | 7.7-12.7 ^{21,24} |
| Epidermolysis bullosa simplex | 10.7 ²⁵ | 15 ²⁵ |
| Epidermolysis bullosa - dystrophic | 7.5 ³³² | 11.5 ³³² |
| Erythrokeratoderma variabilis | 5.3 ²⁶ | 2.75 ²⁶ |
| Psoriasis | 4.5 ³³⁰ -13.9 ³³¹ | 5.4 ³²⁹ |
| Melanocytic naevi | | 2.3 ³²⁹ |
| Vitiligo | | 5.6 ²⁷ |
| Unaffected population | | 0.38 ³²⁹ |

4.4 Haematological analyses

Part of the previously reported wide variation in incidence of anaemia can be explained by the use of different definitions.^{7,70,106,115,124,127} Using WHO references, 41% of the dEPP subjects were anaemic, all with a microcytic, hypochromic pattern. In both sexes haemoglobin, mean cell volume (MCV), and mean corpuscular haemoglobin (MCH) were normally distributed with a shift in their means towards lower values. This is similar to a downward shift in haemoglobin (Hb) previously noted in Dutch EPP patients,⁸ which leads to some patients falling within the definition of anaemia. FECH activity in dEPP is about 35% of normal.¹⁷⁵ Our data show that this decrease is sufficient to produce in all patients a mild defect of erythropoiesis that impairs haemoglobinisation. Defective erythropoiesis persists throughout life and our findings and previous reports^{126,333} suggest that it may not be corrected by oral iron unless there is evidence of co-existing iron loss.

The study showed a marked shift in serum ferritin (sFn) towards lower values, correlating with Hb. Since sFn correlates with mobilizable iron stores,³³⁴ the downward shift in sFn by approximately two-thirds (Figure 13b; Table 12) suggests that iron stores in dEPP are decreased to a similar extent or a little more if the effect of liver dysfunction is taken into account. Turnbull *et al*¹²⁶ found that storage iron, determined by venesection, was less than 250mg in 3 patients; otherwise, quantitative measurements of tissue iron have not been reported in EPP. However in contrast to iron deficiency due to iron loss, sustainable iron is present in erythroblasts.¹²⁷ In homozygous *FECH*^{mlPas} mice, total body iron is normal but iron is redistributed from peripheral tissues to an enlarged haematopoietic spleen.¹²⁹ Although these mice have liver disease, lower FECH activity and more severe anaemia,¹²⁹ it seems unlikely that FECH deficiency limits erythropoiesis and disturbs iron metabolism by different mechanisms in the two species. The anomalous observation in EPP of accumulation of iron in erythroblasts¹²⁷ suggests that there may also be redistribution of iron stores towards the site of erythropoiesis in EPP. Thus in both species, FECH deficiency appears to provoke a response that leads to

accumulation of PP but prevents accumulation of the other, more toxic substrate, iron.

The analysis also showed that serum ferritin (sFe) and soluble transferrin receptor-1 (sTfR) concentrations were normal. The normal sTfR in our patients is consistent with the degree of depletion of iron stores indicated by sFn and, together with the normal sFe, suggesting that erythropoiesis is not limited by iron supply.³⁰⁶ This indicates that the reduction in iron stores has not led to iron deficient erythropoiesis. Furthermore, the rate of erythropoiesis is not increased as this would also increase sTfR levels. These findings suggest FECH deficiency in dEPP leads to the establishment of a steady state in which iron absorption and supply is diminished but matches the requirement for reduced erythropoiesis.

The mechanism of these changes in iron metabolism has not been established. Iron metabolism is also altered in griseofulvin-induced protoporphyria.³³⁵ Because serum transferrin is increased in *FECH^{mlPas}* BALB/c mice and correlates with erythrocyte protoporphyrin concentration, it has been suggested that PP may act as a signal to increase hepatic transferrin synthesis when iron supply to erythroid cells is insufficient and thus modulate iron metabolism.¹²⁹ We found only a slight increase in TIBC (Table 12) and no correlation with TEP. Alternatively, FECH deficiency within enterocytes might affect duodenal iron transport by altering enterocyte mitochondrial iron status.³³⁶ Finally, measurement of sTfR, in addition to sFn, may help to distinguish those patients in whom the anaemia of EPP is exacerbated by iron loss and who might benefit from iron replacement.²⁷³

4.5 Iron metabolism in EPP

We have shown anomalous haematopoiesis in EPP presenting as a microcytic anaemia with apparent features of iron deficiency, a pattern which has previously been reported.⁷ As discussed above, haematopoiesis does not appear to be limited by iron supply.

The role of iron supplementation in EPP is controversial and the literature limited, consisting mainly of small series and case reports (Table 4). Oral iron and transfusion of whole blood or haematin have been reported to normalise liver function and erythrocyte protoporphyrin levels.²⁶⁹⁻²⁷² However others report symptomatic and biochemical deterioration of EPP following oral iron supplementation^{159,273} or whole blood transfusion,²⁷⁴ with improvement following the discontinuation of treatment. Theories for the beneficial effects of iron supplementation in EPP include reduced enterohepatic circulation due to chelation with protoporphyrin in the intestine, increased non-enzymatic intracellular chelation with protoporphyrin, or facilitated chelation by residual active ferrochelatase.²⁶⁹ Conversely deterioration following supplementation may result from stimulation of haematopoiesis and increased production of protoporphyrin, or interaction with other products such as the oral contraceptive, which predispose to cholestasis.²⁷³ Looking at the entire EPP study population (not just the dEPP in whom the detailed haematological analysis above was undertaken), ten subjects were taking oral iron supplementation at the time of the study. Other than the expected lower Hb, MCV and MCH, TIBC and sTfR were normal in all but 3, suggesting that supplementation for the other 7 was unnecessary.

One study subject reported symptoms significantly improved whilst taking oral iron (appendix VI (2)),³³³ which is only the second report of this phenomenon in EPP. The first reported case was administered oral cholestyramine in addition to oral iron, and the improved symptoms were associated with a reduction in erythrocyte and hepatic protoporphyrin.²⁶⁸ In contrast, protoporphyrin levels in our patient remained constant throughout and following the course of iron therapy. Although a placebo effect cannot be discounted in our case, a significant improvement did not occur with a course of β -carotene. The demonstration of our patient's stable protoporphyrin levels throughout the period of treatment with iron, suggests that there may be an alternative, as yet unknown, mechanism for iron in improving the symptoms of EPP.

4.6 Liver function in dominant EPP

Liver dysfunction in EPP patients was recognised shortly after the condition's initial description in the 1960s.⁴³⁻⁴⁵ Despite advances in the understanding of the condition's biochemistry, and genetic inheritance,^{5,247} there remains a lack of prospective clinical data from studies containing large numbers of affected individuals (table 3). Previous studies are retrospective analyses, often from tertiary centres specialising in the treatment of liver disease, and report subjects in common, many of whom are from large family pedigrees.^{2,6,7,9,115,137,161} Limitations of ascertainment bias and small population size have restricted accurate investigation of fundamental clinical issues around EPP such as risk factors for, and incidence of, liver dysfunction.

While liver dysfunction in EPP is common and probably represents a range of clinical involvement, liver failure is infrequent but usually rapidly fatal. The use of transaminases to indicate the presence or otherwise of dysfunction can be regarded at best as an accurate proxy. Although one study has suggested EPP individuals with normal liver function had normal liver architecture, while those with liver dysfunction had protoporphyrin accumulation and liver damage,¹³⁷ others have reported the presence of fibrosis even in those with normal transaminases.¹⁶¹ Despite this reservation, it was felt that liver biopsy would not be practicable for the large geographically disparate cohort in this study, and therefore biochemical markers were used. The incidence of 25% of subjects with liver dysfunction in this study sits within the range reported from previous studies (table 3), suggesting the approach to be reasonable.

The study demonstrated male sex, current age, age at symptom onset, time to onset of symptoms after sunlight exposure, haemoglobin concentration, and elevated TEP are significantly associated with LFTS, concurring with some previously suggested risk factors of sex,¹³² alcohol consumption,^{9,153} autosomal recessive inheritance^{147,149} and TEP.¹⁵⁴ The demonstration of a link between the biologically active aetiological chemical (TEP) in EPP and the resulting symptoms seems intuitive, yet has only previously been recognised in fulminant hepatic failure.^{115,247} Despite this, and a

demonstrated association between genotype and TEP, and TEP with LFTS, it is perplexing that we did not identify a direct association between genotype and LFTS. It seems reasonable to assume that the TEP level is a major factor in LFTS, and its serum concentration is under a degree of genetic influence, but perhaps the TEP and its effect on LFTS are both considerably influenced by multiple other factors, making a direct genotype-LFTS association weak. The complex association between self-reported alcohol consumption and liver dysfunction might be consequent to a degree of under-reporting of intake by individuals. Common sense dictates that patients with EPP should restrict alcohol consumption to modest levels and physicians caring for patients with EPP should be alert to other causes of liver dysfunction. Overall these findings provide a rationale for regular monitoring of haemoglobin, sFn, sTfR, TEP and liver function to detect evidence of hepatic dysfunction in EPP, and to look for trends that might suggest potential for hepatic failure.

Examination of the TEP results from those subjects who had a previous estimation prior to the study, confirmed previous observations that the level of protoporphyrin remains relatively constant for any one individual.¹¹⁵ One year following the end of the study, one subject developed hepatic failure and had undergone orthotic liver transplantation. At the time of the study, he had mild anaemia, elevated ALT and γ GT, and a TEP markedly higher than when measured 6 months previously. As with the incidence of liver dysfunction, the prevalence of liver failure in EPP is not known, and systematic long-term studies will be required both to answer this, and to confirm that risk factors for dysfunction are also predictive for liver failure.

4.7 Vitamin D analyses

We identified a high prevalence of vitamin D deficiency (VDD) and vitamin D insufficiency (VDI) in a large cohort of patients with EPP, whose main risk factors were latitude of residence and their photodermatosis. In keeping with previous findings, we demonstrated an increase in median 25-hydroxyvitamin D between winter and summer.^{221,222,229,310} However, a sizable proportion were VDI even in summer, implicating the photodermatosis and sun-avoidance measures as the most

plausible explanation, and supporting the demonstrated association with sensitivity and TEP.

Although previously recognised in cases reports, studies of photosensitive populations with xeroderma pigmentosum (XP) and Smith-Lemli-Opitz syndrome (SLOS), and normal populations using sunscreens have not shown similar levels of vitamin D insufficiency.^{221,222,228,229,310} Explanations include low study sensitivities due to smaller patient numbers, residency in sunnier environments, lack of skin discomfort in XP (an efficient prompt for rigorous sunlight avoidance in EPP), or the presence in SLOS of abnormally high concentrations of the vitamin D precursor, 7-dehydrocholesterol.

Since completion of this study, controversy over the correct values of the 25-hydroxyvitamin D normal range,²¹⁶ has led to our laboratory now using 30ng/mL as the lower end of the normal range, increasing the proportion of VDI in our cohort to 91%. Thus, a sizeable proportion of the EPP study cohort are VDI and at risk of important clinical outcomes. Treatment with oral calcium and vitamin D increases bone mass and reduces the risk of fractures,^{337,338} so clinicians advising patients with EPP about sunlight avoidance should consider monitoring both serum 25-hydroxyvitamin D and PTH, and giving supplementation throughout the year. As the cutaneous synthesis of vitamin D is initiated by UVB-mediated photolysis of 7-dehydrocholesterol, use of UVB phototherapy to reduce symptoms may have additional therapeutic benefit.

4.8 Autosomal recessive EPP

The combination of palmar keratoderma, a relatively low TEP concentration and autosomal recessive inheritance that we describe here in 9 patients from 6 families constitutes a hitherto unrecognized subtype of EPP. Two of the 13 patients from 12 families with rEPP described previously^{4,65,116,147,175,186} are also included in this report (P4, P8). Thus, of the 20 symptomatic patients from 16 families with rEPP now reported, 9 from 6 families (45% of patients; 38% of families) have palmar keratoderma. To date we have not seen a patient with keratoderma who has not had

rEPP but more patients need to be studied before the reliability of palmar keratoderma as a clinical indicator of rEPP can fully be assessed.

Keratoderma was not reported as a clinical feature of either dominant or recessive EPP^{7,65,70,115,147,175,186} prior to this study; possibly because it is uncommon and, unless large numbers of patients are investigated, the association of EPP with keratoderma may be regarded as chance, as was initially the case for two of our families. However, it seems unlikely that keratoderma has been overlooked in all previously reported patients with rEPP. Where clinical descriptions have been provided,^{116,147,186} it has not been noted and three other patients with recessive disease that we have identified (this report and Whatley *et al*, 2004)⁴ did not have keratoderma. There does not appear to be a clear association between keratoderma and the knuckle skin thickening frequently reported in EPP – the finding of thickening was present in four males and one female from the keratoderma cohort, and absent in one male and three females.

Our findings are unlikely to be explained by an association of two separate disorders. First, the keratoderma of EPP differs clinically from other syndromes that include palmar keratoderma.³¹¹ The hyperkeratosis fluctuates, being worse in summer, tends to be relatively mild on the soles, and may almost disappear with complete exclusion of light; all of which suggest a role for UV exposure in its aetiology. This is supported by the observation that those with mild involvement have signs only in the first interdigital web. None of our patients described blistering, suggesting a non-epidermolytic pattern; none had hyperhydrosis, the keratosis was not particularly at sites of pressure, and was not punctuate in pattern. Second, we observed hyperkeratosis only in rEPP and no family showed independent inheritance of the two conditions. Third, the allelic heterogeneity of our patients makes close linkage with a previously undescribed recessive form of keratoderma very unlikely.

Our patients with keratoderma also differed in other respects from other patients with recessive or dominant EPP. TEP concentrations were lower and, in family I, concentrations were increased by such a small amount (TEP: 2.0 and 2.3 $\mu\text{mol/L}$)

that the diagnosis would not have been made without plasma and faecal porphyrin measurements. In spite of the relatively low erythrocyte concentrations, plasma porphyrin concentrations were the same as in EPP without keratoderma, photosensitivity was severe and faecal PP concentrations were substantially increased. These features suggest that the low TEP concentration is more likely due to a redistribution of the equilibrium between erythrocytes and plasma than to less formation of protoporphyrin by erythroid cells in the bone marrow than in EPP without keratoderma.

There may also be clinical differences apart from keratoderma. Neurological abnormalities were present in four of our patients but, apart from a reversible polyneuropathy after transplantation for protoporphyric liver failure,²⁷⁹ have not previously been described as associated with any form of EPP. Developmental delay has been reported in the recessive or 'homozygous' forms of the dominant acute porphyrias, usually in association with other neurological and skeletal abnormalities,³³⁹ which were not seen in our patients. Furthermore, 5 of the 12 (42%) reported patients with rEPP, in which keratoderma was apparently not present, had liver disease.^{4,116,147,175} In contrast, none of our nine patients had liver dysfunction. This raises the possibility that patients with keratoderma may be at lower risk of liver disease than other patients with rEPP. Liver disease is accompanied by markedly increased TEP although it is not clear whether these are present before liver function deteriorates and their role as a risk factor for liver disease is uncertain.^{130,132} The relatively low TEP and apparently effective biliary elimination of PP shown by our patients may indicate the presence of a special pattern of porphyrin metabolism that does not lead to excessive hepatic accumulation of PP. However, only prolonged follow-up can determine whether a lower risk of liver disease is another component of the syndrome that we describe.

The unusual phenotype that we describe here is inherited in a recessive pattern but it is not clear why it is restricted to rEPP or present in only about 40% of such families. Although *FECH* mutations on both alleles appear to be essential for its expression, it seems unlikely that the phenotype is determined solely by the nature of the mutations

at the *FECH* locus. There appear to be no features of the genotypes shown in table 16 that clearly distinguish them from those of other patients with rEPP.^{4,175} Most mutations are missense; most are on *FECH* IVS3-48T alleles and the nine different mutations are not clustered together in the *FECH* molecule.³⁴⁰ In four families, one of the mutations has been identified previously either in dEPP (IVS3+2T>G),³¹³ or in rEPP without keratoderma (P168S, D274N) with the D274N mutation being present in two of our families and in two of those reported by Gouya *et al*, 2006.¹⁷⁵ Another mutation (P168S) was present in two of our patients; a homozygote without keratoderma where it occurred on a hypomorphic allele and a compound heterozygote with keratoderma (Table 17), the only one of our patients with a hypomorphic allele, presumably again containing this mutation. The four other mutations have only been reported in EPP with keratoderma. *FECH* genotype is the main determinant of *FECH* activity in EPP.¹⁷⁵ In general, *FECH* activities in rEPP are lower than in dEPP though the presence on one or both alleles of a missense mutation that has only a small effect on *FECH* activity may lead to activities within the range for dEPP.¹⁷⁵ Prokaryotic expression of the missense mutations predicted *FECH* activities for our patients with keratoderma of 1 – 25 % (mean 10%) normal (Tables 16 and 17),⁴ close to the range of 4 – 29% reported for rEPP.¹⁷⁵ Thus it seems unlikely that the unusual phenotype shown by our patients is directly determined either by *FECH* genotype or expression.

4.9 X-linked Dominant Protoporphyrin

Prokaryotic expression studies showed that both deletions markedly increase ALAS2 activity. These findings of gain-of-function strongly suggest that PP accumulates in XLPP because the rate of ALA formation is increased to such an extent that insertion of Fe²⁺ into PP by *FECH* becomes rate limiting for haem synthesis. The resulting accumulation of PP presents as an EPP clinical phenotype. The significantly higher levels of TEP seen in XLPP compared to dEPP may explain the apparent increased incidence of liver dysfunction in these individuals. Gain of function mutations have not previously been identified in genes of the haem biosynthetic pathway³⁴¹ but, as in our families, characteristically cause dominant disorders.

Urinary excretion of haem precursors is normal in XLPP indicating that most of the ALA produced by erythroid cells is metabolised to PP. Some is used for haemoglobin synthesis but the fate of the rest is uncertain. Since FECH activity in erythroid cells exceeds that required for haemoglobin synthesis,³¹⁷ some may be converted to free haem and exported from the cytoplasm.³⁴² However the accumulation of ZnPP in XLPP, indicating utilisation by FECH of its alternative metal substrate, suggests that formation of excess haem is prevented by lack of available iron. The phenotype of iron deficiency in XLPP (Table 19) closely resembles that of the *Ireb2*^{-/-} mice in which deletion of iron-regulatory protein 2 (IRP2) leads to over-expression of ALAS2, erythroblast iron deficiency and microcytic anaemia.³⁴³ In one patient (Table 19 BII,7), iron repletion decreased PP accumulation and corrected the anaemia but, in contrast to iron deficiency without XLPP, did not decrease ZnPP, possibly because its synthesis becomes limited by intra-mitochondrial Zn²⁺ availability when PP synthesis is increased.³⁴³ Thus, our findings suggest that the regulatory system that enables efficient utilisation of iron for haem synthesis during erythroid differentiation³⁴⁴ allows matching of erythroblast iron uptake to intra-mitochondrial haem synthesis to be maintained in the presence of excess PP. The mechanism is unknown but may involve regulation of transferrin receptor-1 expression in erythroblasts through haem-mediated degradation of IRP2.³⁴⁵

The 26 C-terminal amino acids of ALAS2 are highly conserved and have diverged from ALAS1 (figure 19 b) which suggests that this sequence may have an important, but unknown, erythroid-specific function. During erythropoiesis, tight co-ordination of substrate supply to FECH normally prevents accumulation of toxic amounts of PP. Co-ordination is largely achieved through iron-dependant post-transcriptional regulation of the synthesis of ALAS2.³¹⁷ It seems probable that this system fails in XLPP because the mutations that we have described stabilise the ALAS2 against degradation, permitting excessive erythropoiesis in the absence of a matched supply of iron. The marked increase in activity, the absence of C-terminus of the ALAS2 from ALAS_{RC}, (5-amino levulinate synthase from *Rhodobacter capsulatus*, the only ALAS for which the crystal structure has been reported)³⁴⁶ and evidence that it is not

directly involved in ALAS2 catalysis,^{346,347} favour this hypothesis over any intrinsic increase in specific activity. The discovery of gain-of-function mutations of ALAS2 provides new information about the regulation of substrate supply for haem synthesis during erythroid differentiation and identifies a potential tool for increasing erythroid haem synthesis in experimental systems.

4.10 Conclusion

This work represents a study of the largest cohort of individuals with EPP reported to date. It has clarified the presentation of the condition both in subjective and objective clinical terms, and also in biochemical and haematological investigations. Knowledge of the condition appears limited in general practice and among paediatricians which can result in considerable delay in diagnosis, and suggests a stratagem for focussed education of these groups. The previously unrecognised significant impact of the condition in terms of quality of life has been documented. Many previously described features of the condition, often only reported from case reports or limited series, have been confirmed, while others have been refuted or not supported. The apparent microcytic anaemia has been identified to be due to a downward shift in haematopoiesis of the whole EPP population, and erythropoiesis does not appear to be limited by iron supply. Abnormal liver function has been linked to a number of factors, including for the first time the concentration of erythrocyte protoporphyrin. The design of the study was such that risks for developing liver failure were not directly sought and future longitudinal projects will be required to investigate this. A clinically important deficiency of vitamin D was identified for the first time to affect a considerable proportion of the population even in summer, and presents an area in which supplementation should be considered. Important differences in the clinical diagnosis and management of individual EPP patients have been highlighted. The identification of haematological and biochemical changes seen within the population, allows a stratagem for annual review and monitoring of EPP patients. Two further novel findings have been the identification of an apparent sub-type of recessive EPP associated with palmar keratoderma, which may convey a lower risk of liver dysfunction, and a new entity, X-linked dominant protoporphyria. This results from gain-of-function mutations to

5-aminolevulinate synthase, the initial rate-limiting enzyme in the haem biosynthetic pathway, which clinically presents as EPP, but has important haematological and biochemical differences.

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Appendix 1. Abbreviations

| | |
|--------------------|--|
| AIP | acute intermittant porphyria |
| ALA | 5-amino levulinate |
| ALAD | 5-amino levulinate dehydrogenase |
| ALADP | 5-amino levulinate dehydrogenase porphyria |
| ALAS | 5-amino levulinate synthase |
| ALAS _{Rc} | 5-amino levulinate synthase from <i>Rhodobacter capsulatus</i> |
| Alk | |
| Phos | serum alkaline phosphatase |
| bp | base pairs |
| cDNA | complemenatary DNA |
| CEP | congenital eythropoietic porphyria |
| CP | coproporphyria |
| dEPP | dominant erythropoietic protoporphyria |
| EPP | erythropoietic protoporphyria |
| FBC | full blood count |
| FECH | ferrochelataase |
| GGT | serum gamma glutamyl transferase |
| GPs | general practitioners |
| Hb | haemoglobin |
| HCP | hereditary coproporphyria |
| Hct | haematocrit |
| IgG | immunoglobulin G |
| IRE | iron responsive element |
| IRP2 | iron-regulatory protein 2 |
| ISCP | isocoproporphyrin |
| LFT | liver function tests |
| LFTSt | liver function test status |
| MCH | mean corpuscular haemoglobin |
| MCV | mean cell volume |
| mRNA | messenger ribonucleic acid |
| MSH | melanocyte stimulating hormone |
| PAS | periodic acid schiff |
| PBG | porphobilinogen |
| PBGD | porphobilinogen deaminase |
| PCT | porphyria cutanea tarda |
| PP | protoporphyrin |
| rEPP | recessive erythropoietic protoporphyria |
| rs | Spearman rank correlation |
| sFe | serum iron |

| | |
|------------------|----------------------------------|
| sFn | serum ferritin |
| SLOS | Smith-Lemli-Opitz Syndrome |
| SPF | sun protection factor |
| sTfR | soluble transferrin receptor-1 |
| TEP | total erythrocyte protoporphyrin |
| TIBC | total iron binding capacity |
| TS | transferrin saturation |
| UK | United Kingdom |
| UPF | UV protection factor |
| URO | uroporphyrin |
| UV | ultra-violet |
| UVB | ultra-violet B |
| VDD | vitamin D deficient |
| VDI | vitamin D insufficient |
| VP | variagate porphyria |
| WHO | world health organisation |
| χ^2 | chi squared |
| XP | xeroderma pigmentosum |
| Zn ²⁺ | zinc |
| ZnPP | zinc protoporphyrin |

Appendix II: Grants

Royal College of Physicians, Lewis Thomas Gibbon Jenkins of Britton Ferry Memorial Trust. “The influence of molecular and other factors on the development of liver dysfunction in erythropoietic protoporphyria.” (September 2003 for 12 months): £65,000

Gwent Healthcare NHS Trust, Small Grant Award. Laptop computer, software, printer and digital camera. (October 2003): £2,645

British Skin Foundation Grant No. S020. Is vitamin D deficiency a significant problem in severe photodermatoses? An investigation of 200 individuals with erythropoietic protoporphyria. (July 2004 for 12 months): £3,440

University of Wales College of Medicine, William Morgan Thomas Bequest Fund WMT/03/20. Travelling Scholarship. (November 2003): £125

British Porphyria Association (October 2004): £2,000

Professor AV Anstey Dermatology Research Fund – travelling costs.

Appendix III: Study Proforma

Date _____

Patient's initials _____

D.O.B. _____

Sex M / F

Age in years _____

1. How old were you when you first experience problems with the EPP? _____
2. What were these initial symptoms?
crying / screaming / burning / itching / tingling / other _____
3. How old were you when the diagnosis was made? _____
4. Who made diagnosis? GP / dermatologist / paediatrician / gastroenterologist /
other doctor / other person _____
5. What symptoms do you have now after sun exposure?
nil / crying / screaming / burning / itching / tingling / other _____
6. How long does it take for the symptoms to start after sunlight exposure? _____
7. How long does it take for the symptoms to settle? _____
8. Does your skin have any visible changes immediately after sunlight exposure?
nil / redness / swelling / eczema / bruising / blistering / crusting / other _____
9. Are there any visible changes later on?
nil / redness/ swelling/ eczema/ bruising/ blistering/ crusting/ other _____
10. Apart from the skin symptoms, do you feel unwell in any other way after sunlight exposure?
nil / fever / generally unwell / can't sleep / feel down / irritable / other _____
11. On the second day of sun exposure, are the symptoms the *same*, *better* or *worse*?
12. Is your skin sensitive to sunlight through window glass Y / N
13. Is your skin more sensitive on windy days? Y / N
14. Do your nails ever get affected by the sunlight? Y / N
15. Do you think your skin is more fragile / difficult to heal than others? Y / N
16. Do you think your skin sensitivity *improves* over the summer or *stays about the same*?
17. Do you think there has been any change in sensitivity as you've got older? Y / N
If yes, *improved* / *deteriorated*, and any thoughts why? _____
18. Is your skin ever sensitive over winter (November – February)? Y / N
If yes, how often / circumstances etc _____
19. Does your sunlight sensitivity interfere with your daily activities in summer? Y / N
20. Do you regularly try to avoid sunlight? Y / N
What kind of things do you do? stay inside / seek shade/ go out in evening / at night/ _____

21. Do you regularly wear special clothes to go out in sunlight? Y / N
 hat / high collar / long sleeves / trousers / gloves / other _____
22. Do you use sunscreen? Y / N (if no go to 22)
 22)
23. What type and sun protection factor (SpF)? _____
24. How often? daily / at weekends / less often _____
 all year / in any sunny weather / over summer only / abroad only / other _____
25. Are you aware what the star ratings for sunprotection mean? Y / N _____
26. Have you tried any of the following, and were they helpful?
- | | | |
|-----------------------|--------------------------------|-----------------------|
| antihistamines | currently / previously / never | helpful / not helpful |
| β-carotene | currently / previously / never | helpful / not helpful |
| Dundee sunscreen | currently / previously / never | helpful / not helpful |
| cysteine | currently / previously / never | helpful / not helpful |
| hospital phototherapy | currently / previously / never | helpful / not helpful |
| sunbeds | currently / previously / never | helpful / not helpful |
| fake tan | currently / previously / never | helpful / not helpful |
| other treatments | _____ | _____ |
27. Who is involved in the care of your skin problem?
 No-one / GP / dermatologist / paediatrician / gastroenterologist / other _____
28. How often does each of these people see you? _____

Previous Medical History

29. Have you ever had any other medical conditions?
- _____

| | | |
|-----------------------|-------|----------------|
| anaemia | Y / N | details: _____ |
| iron deficiency | Y / N | details: _____ |
| gallstones | Y / N | details: _____ |
| hepatitis | Y / N | details: _____ |
| other liver disease | Y / N | details: _____ |
| liver treatment | Y / N | details: _____ |
| arthritis | Y / N | details: _____ |
| haematological cancer | Y / N | details: _____ |

30. Are you allergic to anything? Y / N
 If yes, what _____
31. Are you currently taking medicines and tablets? Y / N
 If yes, what _____

Family and Social History

32. Are there any illnesses that run in your family? Y / N

If yes, what _____

33. Family tree: _____ parents/siblings/marital status/children

Women only:

34. Does your sensitivity change with your menstrual cycle? Y / N

If yes, how? _____

35. If you had children, did you find any change in the EPP during your pregnancy? Y / N

significantly deteriorated / got worse / no change / improved / significantly better

36. Place of birth: UK / northern Europe / Mediterranean Europe / other _____

37. What would you describe your ethnic origin as?

caucasian / indo-asian / far east asian / afro-caribbean / other: _____

38. Are you working? Y / N

And if so, what is your job? _____

Was your choice of profession influenced by your skin? Y / N

39. How much alcohol do you drink in an average week? Estimated units _____

beer / wine / spirits / other _____

40. Have you ever noticed your skin more sensitive after drinking alcohol Y / N

41. Do you smoke? Y / N

And if yes, how much per day? amount _____

42. Vegetarian / Non-vegetarian? V / C

Examination

Acute changes – erythema (RED), oedema (O), eczema (X), purpura (P), vesicobullous (B), crusted erosions (E)

Chronic changes – excoriation (EXCOR) / thickened (TH) / hyperkeratosis (HK) / yellow (Y) / excessively wrinkled (WR) / scarring (varrioliform/pitted/other) (SCAR V, P)

Evidence jaundice, hepatomegally, chronic liver dx – spider naevi, flap

Degree of involvement: + = mild, ++ = moderate, +++ = severe

| SITE | ACUTE CHANGES | CHRONIC CHANGES | OTHER FINDINGS |
|-------|---------------|-----------------|----------------|
| FACE | | | |
| NECK | | | |
| ARMS | | | |
| HANDS | | | |
| NAILS | | | |
| LEGS | | | |
| FEET | | | |

Appendix IV: The Dermatology Life Quality Index

Patient Initials:

DLQI SCORE:

Date:

The aim of this questionnaire is to measure how much your skin problem has affected your life **OVER A SUNNY WEEK IN SUMMER**. Please tick one box for each question.

- | | | | |
|-----|--|--|--|
| 1. | Over a sunny week, how itchy , sore , painful or stinging would your skin be? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 2. | Over a sunny week, how embarrassed or self conscious would you have been because of your skin? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 3. | Over a sunny week, how much would your skin interfere with you going shopping or looking after your home or garden ? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 4. | Over a sunny week, how much would your skin influenced the clothes you wear? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 5. | Over a sunny week, how much would your skin affect any social or leisure activities? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 6. | Over a sunny week, how much would your skin made it difficult for you to do any sport ? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 7. | Over a sunny week, how much would your skin been a problem at work or studying ? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 8. | Over a sunny week, how much would your skin create problems with your partner or any of your close friends or relatives ? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 9. | Over a sunny week, how much would your skin caused any sexual difficulties ? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |
| 10. | Over a sunny week, how much of a problem would the treatment for your skin been, for example by making your home messy, or by taking up time? | Very much A lot A little Not at all | <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> |

Trouble with Skin

The aim of the questionnaire is to measure how much your skin problem has affected you OVER THE LAST WEEK. Please ✓ one box for each question.

OVER THE LAST WEEK



How itchy, 'scratchy', sore or painful has your skin been?

Very much

 Quite a lot

 A little

 Not at all



How upset or embarrassed, self conscious or sad have you been because of your skin?

Very much

 Quite a lot

 A little

 Not at all



How much has your skin affected your friendships?



How much have you changed or worn different or special clothes/shoes because of your skin?

Very much

 Quite a lot

 A little

 Not at all



How much has your skin trouble affected going out, playing or doing hobbies?



How much have you avoided swimming or other sports because of your skin trouble?



OVER THE LAST WEEK



Either

If school time: How much did your skin affect your **school work**?

- Very much
- Quite a lot
- A little
- Not at all



Or

If holiday time: How has your skin problem interfered with your **holiday plans**?



How much trouble have you had because of your skin with other people **calling you names, teasing, bullying, asking questions or avoiding you**?

- Very much
- Quite a lot
- A little
- Not at all



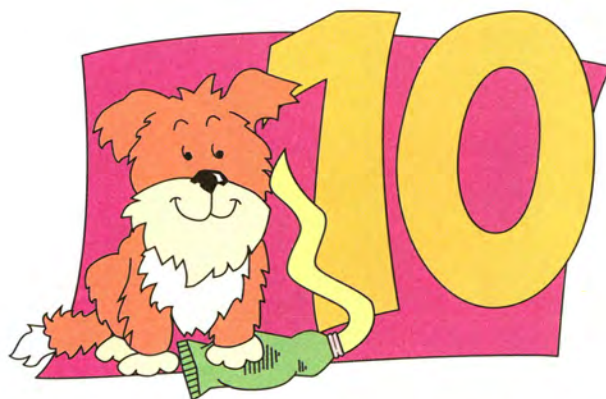
How much has your **sleep** been affected by your skin problem ?

Hospital No.:
 Name :
 Age:
 Address:

 Diagnosis:
 Date:
CDLQI SCORE:

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- Very much
- Quite a lot
- A little
- Not at all



How much of a problem has the **treatment** for your skin been ?

Please check that you have answered EVERY question. Thank you.

Erythropoietic protoporphyria in the U.K.: clinical features and effect on quality of life

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Summary

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clinical features, erythropoietic protoporphyria, photosensitivity, porphyria, quality of life

Conflicts of interest

A.Y.F. is joint copyright owner of the DLQI and CDLQI.

Background Erythropoietic protoporphyria (EPP) is a rare inherited photodermatosis that causes lifelong painful photosensitivity. Neither its full clinical spectrum nor its impact on quality of life (QoL) has been investigated in a large cohort of patients.

Objectives To document the clinical features of EPP and its impact on QoL in a high proportion of all patients with EPP resident in the U.K.

Methods Patients with EPP were identified from U.K. clinical databases and assessed by the same clinical investigator over a 7-month period using a standardized proforma and validated adult (Dermatology Life Quality Index, DLQI) and children's (Children's Dermatology Life Quality Index, CDLQI) QoL questionnaires.

Results Three hundred and eighty-nine living patients with EPP were identified, of whom 223 [114 females, 109 males; median age 34 years (range: 5–87), from 193 families] were investigated. Total erythrocyte porphyrin (TEP) was higher in males (median: $25.3 \mu\text{mol L}^{-1}$) than females (median: $19.3 \mu\text{mol L}^{-1}$). The median ages at onset and diagnosis were 1 and 12 years, respectively. Median times for onset of symptoms after sun exposure, onset of signs (oedema, erythema) and resolution of symptoms were 20 min, 6 h and 3 days, respectively. Most patients reported absence of protection by glass (92%), priming (85%), exacerbation by wind (68%), no family history of photosensitivity (56%), no symptoms during winter (56%) and had chronic skin lesions (79%). Symptoms changed little with age but improved during pregnancy in 47% of gravid women. Most patients used protective clothing and a sunscreen; 28% were taking β -carotene and a further 56% had taken it; 29% were not under regular medical care. Two patients (1%) had liver failure and 8% reported gallstone disease. QoL was markedly impaired, with scores similar to those in severe dermatological disease (mean DLQI score 14.0, $n = 176$; mean CDLQI score 12.8, $n = 44$), indicating a large effect on patients' lives. DLQI scores correlated weakly with TEP ($r_s = 0.228$; $P = 0.002$) and time to onset of symptoms ($r_s = -0.233$; $P = 0.002$) but not with age at onset.

Conclusions EPP is a persistent, severely painful, socially disabling disease with a marked impact on QoL. Its diagnosis is often overlooked. None of TEP, age at onset nor time to onset of symptoms is a useful predictor of impaired QoL in individual patients.

Erythropoietic protoporphyria (EPP, MIM 177000) is a rare inherited photodermatosis with systemic complications that has only been clearly recognized since 1961.¹ Partial deficiency of ferrochelatase (FECH), the terminal enzyme of haem biosynthesis, causes excessive formation, mainly in the bone marrow, of protoporphyrin IX which accumulates in erythrocytes, plasma, liver and skin. Protoporphyrin in the skin,

through its photodynamic action, causes lifelong acute photosensitivity of sun-exposed areas,^{2,3} while its accumulation in the liver leads to potentially fatal liver failure in 1–4% of patients.^{2,4,5} In most families, increased protoporphyrin formation sufficient to cause photosensitivity occurs only in individuals who have inherited a low expression FECH gene polymorphism, present in 13% of the U.K. population,⁶ trans

to an FECH mutation that abolishes or markedly decreases FECH activity.⁷

The clinical features of EPP, which are distinct from those of other cutaneous porphyrias, have been described in various case reports and small series, none containing > 32 patients,^{2,8-10} while larger studies have focused on particular aspects of the disease, such as genetics¹¹ or liver disease.⁴ Information from such sources may be biased by selection and may not reflect the full clinical spectrum. In addition, no study has addressed the psychosocial consequences of EPP despite the apparent severity of symptoms and substantial changes to lifestyle necessary to avoid episodes of acute photosensitivity. Here we report findings from a cross-sectional study of a large cohort of patients with EPP which was designed to record clinical features, methods of sun avoidance and treatment, and impact on quality of life (QoL).

Patients and methods

Patients

The study was conducted in accord with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments. Prior approval was obtained from the North West Multicentre Research Ethics Committee and 84 local research ethics committees. All patients or their parents gave informed consent. Three hundred and eighty-nine living subjects, resident in all parts of the U.K., previously diagnosed as having EPP, were identified from databases held in five referral centres for either porphyrias or photodermatoses, located in Cardiff, Dundee, Leeds, London and Manchester. The referring physician for each subject was identified from the records and sent details of the study asking for his/her cooperation. Exclusion criteria were: children under the age of 5 years, haematological malignancy or hepatic malignancy. Permission was obtained to contact 352 patients of whom 258 (73%) replied to say they wished to take part. Of these, 223 (86%) subjects were seen in 30-min consultations at their local National Health Service outpatient facility by a single clinician (S.A.H.) over a 7-month period. The remaining individuals either found their appointment times inconvenient or failed to attend. History and clinical features were recorded using a standardized proforma that avoided ambiguous or leading questions. The questions covered four main areas: photosensitivity, photoprotective measures and treatments, general medical history, and family, social and economic background. Any additional comments about EPP made during the interview were recorded. All light-exposed skin was examined, particularly that of the face, neck, arms, hands and nails, and also any other affected skin volunteered by the subjects. Examination findings were grouped into acute (e.g. erythema, oedema, eczema, purpura, vesicobullous and crusted erosions), chronic (e.g. excoriations, thickened skin, hyperkeratosis, yellowing, excessive wrinkling, scarring), and other signs.

Quality of life indices

QoL was assessed using dermatology-specific tools: the Dermatology Life Quality Index (DLQI)¹² or, for patients aged < 16 years, the cartoon version of the Children's Dermatology Life Quality Index (CDLQI)¹³ (both of which may be viewed at <http://www.dermatology.org.uk>). Both contain 10 questions, each being scored up to 3 to give a maximum possible score of 30, indicating the worst QoL. Both have been validated in normal populations and those with dermatological conditions for repeatability, internal consistency and sensitivity to change.

Biochemical analyses

Erythrocyte and plasma porphyrins were determined using standard methods.¹⁴

Statistical analyses

Data were analysed using SPSS software (SPSS, Chicago, IL, U.S.A.). The Mann-Whitney test was used to test the significance of differences between quantitative variables. Spearman rank correlation (r_s) was used to characterize and test the significance of the relationships between all possible pairs of the four variables: total erythrocyte porphyrin (TEP), age at onset, time to symptoms and QoL. Confidence intervals (CIs) were calculated by the conventional inverse hyperbolic tangent method.

Results

Patients

The study cohort contained 223 patients (median age: 34 years, range: 5-87) with a history of acute photosensitivity. There were 114 females (median age: 33.5 years, range: 5-87) and 109 males (median age: 35 years, range: 7-77); the age distribution was similar in both sexes. All were white skinned apart from two Indo-Asians (originally from Pakistan and Iraq, both skin phototype IV). The 223 patients came from 193 families: 125 patients (56%) had no family history of photosensitivity; 89 patients from 60 families had affected relatives (only 29 of whom were seen in the study) and a further nine patients were uncertain. Only four patients were excluded according to the exclusion criteria, all due to age < 5 years.

Porphyrin analysis

TEP was increased in all the 211 patients from whom blood was obtained (median: 25.3 $\mu\text{mol L}^{-1}$, range: 2.0-159.2; normal subjects: < 1.7). Erythrocyte porphyrin was mainly free protoporphyrin, and plasma fluorescent scanning indicated that plasma protoporphyrin was increased in all subjects (emission maxima: 626-634 nm). TEP was significantly

higher in males (median: 25.3 $\mu\text{mol L}^{-1}$, range: 2.0–159.2) than females (median: 19.3 $\mu\text{mol L}^{-1}$, range: 2.3–75.3; $P = 0.003$), and there was a slight increase with current age but this did not reach statistical significance in either sex ($r_s = 0.115$; $P = 0.096$).

Photosensitivity

Age at onset

The median age at onset of symptoms was 1.0 years (range: birth–12 years) (Fig. 1a), usually at the time of first major exposure to sunlight, and was inversely correlated with TEP ($r_s = -0.241$, 95% CI: -0.364 to -0.110 ; $P = 0.001$). However, diagnosis was often delayed: the median age at diagnosis was 12.0 years (range: 6 months–86 years), with the diagnosis not being made in 75 patients (34%) until the age of 20 years or more (Fig. 1b).

Symptoms

Patients often reported that the cutaneous sensation following sunlight exposure was difficult to describe. The most frequently used terms included burning (85%), tingling (33%), prickling (4%) and stinging (3%). The following descriptions

of the sensation convey the severity: 'like a burn on a candle flame or bonfire', 'red hot needles under the skin', 'having boiling water or oil poured on the skin', 'like blood boiling and being burnt from the inside out'. The median time for onset of symptoms following exposure to sunlight was 20 min (lower quartile: 10 min, upper quartile: 60 min, range: immediately to 12 h or asymptomatic in the U.K.), was significantly shorter in females (median: 15 min) than in males (median: 30 min; $P = 0.018$) and showed a weak correlation with age at onset ($r_s = 0.214$, 95% CI: 0.084 – 0.336 ; $P = 0.004$) but not with TEP ($r_s = -0.134$; $P = 0.054$). The median time to resolution was 3 days (lower quartile: 1 day, upper quartile: 4 days, range: immediately to 4 weeks).

Two hundred and eleven (95%) patients said that onset of symptoms was not associated with any immediate visible change to the skin. Immediate changes reported were reddening (seven patients), swelling (three patients), purple discoloration and blanching (one patient each). Visible changes were generally related to the duration of exposure: minimal exposure tended not to result in visible changes, while prolonged exposure resulted in signs. Those most commonly reported included swelling (179 patients, 80%), reddening (45 patients, 20%), blistering (37 patients, 17%), crusting/eczema (32 patients, 14%), petechiae and/or bruising (20 patients, 9%) and fissuring (10 patients, 5%). Thirteen (13%) of 103 patients had noticed changes in their nails during acute episodes. Sixty-one subjects estimated a median time for onset of swelling postexposure of 6 h (lower quartile: 2 h, upper quartile: 12 h, range: 15 min to the next morning). Twenty-two subjects (10%) reported no visible change at any time. Only 25 (13%) of 199 patients felt that their photosensitivity had changed with age: 14 had improved and 11 had become more sensitive. Other features of the photosensitivity of EPP are summarized in Table 1.

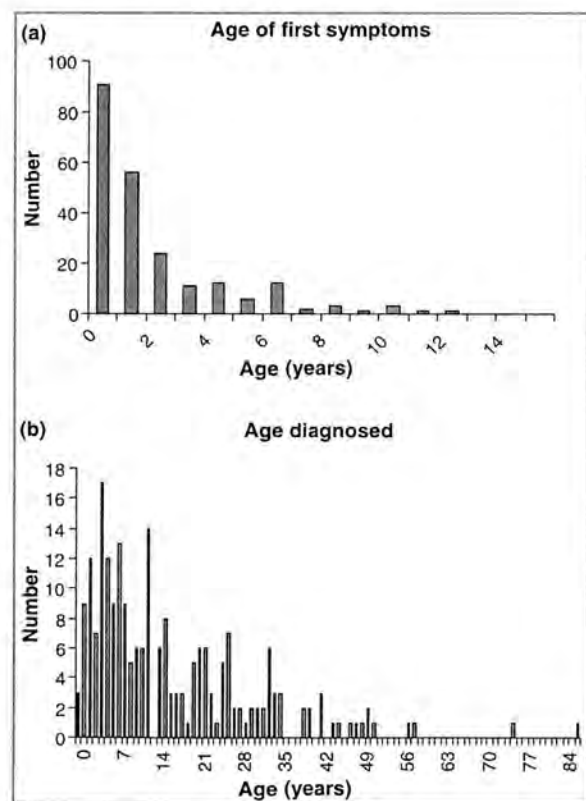


Fig 1. Ages at onset of symptoms (a) and at diagnosis (b) for 223 patients with erythropoietic protoporphyria.

Table 1 Photosensitivity in erythropoietic protoporphyria

| Symptom | No. of patients responding to question | No. of patients with symptom (%; 95% CI) |
|---|--|--|
| Priming ^a | 219 | 186 (85; 80–89) |
| Photosensitive through glass | 220 | 202 (92; 87–95) |
| Exacerbation by wind | 202 | 137 (68; 61–74) |
| Improvement during summer ('hardening of skin') | 194 | 109 (56; 49–63) |
| Skin fragile/slow to heal | 115 | 75 (65; 56–73) |
| Photosensitive during winter (November–February) ^b | 165 | 73 (44; 37–52) |

CI, confidence interval. ^aPhotosensitivity worse the day after sun exposure.^bContributory factors: sunny weather (95%), snow (28%), wind (26%), water reflection (8%), seaside (5%), increased exposure to artificial light (1%).

Endocrine factors

Twenty-eight (47%) of 59 gravid female patients had noticed an improvement in symptoms during pregnancy. Nine patients spontaneously described this as very significant or that they became asymptomatic. Several of the women who had not noticed any change commented that they had been pregnant mainly over winter or too scared to expose themselves to sunlight; two others were seen early during their first pregnancies and early in the year. Menses-related worsening of photosensitivity was reported by eight (10%) of 81 menstruating females; six of these were gravid and five had noticed improvement during pregnancy. Three women mentioned that their symptoms had improved after their menopause. None had noticed any worsening of photosensitivity during pregnancy or postmenopause, or improvement during menses.

Other symptoms

Noncutaneous symptoms associated with photosensitive episodes were reported by 189 patients and included problems with sleeping (171 patients, 77%), irritability (66 patients, 30%), excessive temperature sensitivity (41 patients, 18%), feeling down or depressed (26 patients, 12%), tired (24 patients, 11%), nauseated (13 patients, 6%) or generally unwell (10 patients, 5%), and headache (seven patients, 3%).

Photoprotective measures and treatments

Sixty-seven subjects (29%) were not seen regularly by any doctor for their EPP. The rest were under the care of a dermatologist (58%), other hospital specialist (10%) or general practitioner (3%) and reviewed at least once (59%) or twice (29%) a year; all but six subjects in this group had blood tests at least annually.

Photoprotection

Two hundred and four patients (92%) said that EPP interfered with their daily activities on sunny days. The main sunlight avoidance activities were staying inside (144 patients, 65%), seeking shade (71 patients, 32%), and only going out in the evening or early morning (26 patients, 12%). One hundred

and ninety-four patients (87%) wore special clothes to protect themselves from sunlight: long sleeves (174 patients, 90%), trousers or long skirts (155 patients, 80%), hats (143 patients, 74%) and gloves (95 patients, 49%).

Sunscreen use

Seventy patients (31%) never used a sunscreen, citing reasons such as poor efficacy, unacceptable cosmetics on skin and clothing, and expense. Among the 152 patients (68%) who used a sunscreen, 40 patients used the Tayside Pharmaceuticals' Reflectant Sunscreen (Appendix 1; usually the beige colour); others used sun protection factor 60 (35 patients), 50 (20 patients), 30 (20 patients) or 25 (16 patients) sunscreens from a variety of manufacturers. The frequency of application ranged from occasional (9%) through once daily (77%) to more than once a day (14%), with a maximum of eight times a day. Sunscreen was applied only in sunny weather (61%), all summer (24%), all year (9%) or only when abroad (6%). Only 30 of 221 patients (14%) were aware of the U.K. UVA star rating of sunscreens.

Physician-suggested therapies

Table 2 shows the number of patients who had used or were currently using physician-suggested therapies, and their opinions of efficacy. β -Carotene was prescribed for 187 patients but two-thirds of these had stopped it. Reasons given were cosmetically unacceptable orange discoloration of skin and body fluids (71%), retinal crystals associated with the withdrawn canthaxanthine preparation (15%), problems swallowing large capsules (7%), gastrointestinal side-effects (4%) and media coverage of potential carcinogenic effects (2%).

Other treatments

Measures used to relieve the pain of acute photosensitivity included immersion in cold water (52 patients, 23%), wet cloths or flannels (47 patients, 21%), putting affected areas on cold walls, metal cans from the refrigerator or under pillows (16 patients, 7%), electric fans (12 patients, 5%), drinking alcohol (10 patients, 5%) and, for 15 patients (7%), emollients, ice packs or immersion in hot water.

Table 2 Subjects currently using or previously using commonly suggested therapies for erythropoietic protoporphyria

| Treatment | No. (%) cohort currently using | No. (%) think useful | No. (%) cohort previously used | No. (%) think useful | No. (%) cohort never used |
|-------------------|--------------------------------|----------------------|--------------------------------|----------------------|---------------------------|
| Antihistamines | 16 (7) | 8 (50) | 101 (45) | 14 (14) | 105 (47) |
| β -Carotene | 62 (28) ^a | 48 (77) | 124 (56) ^b | 30 (24) | 36 (16) |
| Tayside sunscreen | 40 (20) | 38 (95) | 41 (20) | 13 (32) | 142 (64) |
| L-Acetylcysteine | 3 (1) ^c | 3 (100) | 2 (0.5) | 1 (50) | 218 (98) |
| Phototherapy | 21 (9) | 21 (100) | 35 (16) | 17 (49) | 167 (75) |
| Sun parlours | 5 (2) | 5 (100) | 34 (15) | 18 (53) | 183 (82) |

^aMedian dose 6 \times 30 mg daily (range: 2–13 \times 30 mg); ^bmedian dose 8 \times 30 mg daily (range: 2–12 \times 30 mg); ^c500 mg twice daily.

Medical history

Two patients (1%) had protoporphyric liver failure: a 43-year-old man, who was under assessment for liver transplantation, and a 35-year-old man who had received a liver transplant 1 year previously. Eighteen patients (8%; 15 women and three men) had gallstones, of whom 11 women and three men (mean age: 51 years, range: 28–66) had had a cholecystectomy. One hundred and fourteen patients (51%) reported an additional illness, none of which appeared to be associated with EPP.

Family, social and economic background

One hundred and twenty-seven subjects (57%) were in employment, and a further 60 (27%) were either school or further education students. Sixty (47%) of the 127 patients in employment felt their choice of profession had been significantly influenced by their skin condition. One hundred and forty-three subjects (81% of those over the age of 16 years) drank alcohol regularly. The median number of units consumed was seven units per week (range: 1–70). Twenty patients (14%) noticed that their skin became more photosensitive for up to 2 days after drinking. Thirty-two patients (14%) smoked tobacco (median: 10 cigarettes per day, range: 1–50).

Cutaneous examination

Only three patients, all children, had acute changes at the time of examination: all had erythema, mainly over the nose, cheeks, dorsa of hands and fingers, lower lips and upper helix of the ears. Thirty-four (15%) patients had no changes evident on any light-exposed skin (16 females, 18 males; median age: 37 years, range: 5–87). Scarring was the most commonly pre-

sent chronic change, affecting mainly light-exposed skin of the face (149 patients, 67%) and hands (138 patients, 62%; Table 3). The observer-perceived severity for mild and moderate face and hand signs was in roughly equal proportions (face, 31% and 29% of subjects, respectively; hands, 30% and 27%). Only a minority of subjects had scarring that was assessed as severe (face, 6%; hands, 5%). Other chronic changes such as thickening of knuckle skin were observed less frequently (Table 3).

Quality of life

Dermatology Life Quality Index scores

Two hundred and twenty patients completed QoL questionnaires: 176 the adult DLQI and 44 the CDQLI. Their scores are summarized in Table 4. Scores were not significantly influenced by age or sex. The median total DLQI score was 14 (range: 0–29, $n = 176$). It is possible to interpret the meaning of DLQI scores by applying simple validated descriptive score bands.¹⁵ Six adults (3%) scored 0 or 1, indicating no effect on their life over the preceding week and 15 (9%) scored 2–5, indicating a small effect: in total only 21 subjects (12%) scored 5 or less, indicating no or little effect on their QoL. Thirty-two (18%) scored 6–10, indicating a moderate effect, 92 (52%) scored 11–20, indicating a very large effect, and 31 (18%) scored 21–30, indicating an extremely large effect. Thus 123 of 176 adults (70%) scored over 10, indicating at least a very large effect on their QoL over the preceding week. The highest-scoring DLQI questions were 4, 5 and 6, relating to clothing, social/leisure activities and sport. All six adults (four women and two men; mean age:

Table 4 Total Dermatology Life Quality Index (DLQI) and Children's Dermatology Life Quality Index (CDLQI) scores in erythropoietic protoporphyria

| Age range (years) | Sex | Number of patients | Mean age (years) | Mean quality of life score (range) |
|--------------------|---------|--------------------|------------------|------------------------------------|
| 6–15 ^a | F | 22 | 10.9 | 12.8 (5–20) |
| | M | 22 | 11.3 | 12.9 (6–21) |
| 16–35 ^b | F and M | 44 | 11.1 | 12.8 (5–21) |
| | F | 40 | 27.3 | 14.4 (0–29) |
| 36–55 ^b | M | 37 | 26.0 | 13.9 (4–25) |
| | F and M | 77 | 26.7 | 14.1 (0–29) |
| | F | 33 | 43.6 | 13.2 (0–25) |
| 56–87 ^b | M | 36 | 44.0 | 13.0 (1–29) |
| | F and M | 69 | 43.8 | 13.1 (0–29) |
| | F | 17 | 62.8 | 16.1 (4–27) |
| 16–87 ^b | M | 13 | 62.3 | 15.3 (0–27) |
| | F and M | 30 | 62.6 | 15.8 (0–27) |
| | F | 90 | 40.0 | 14.2 (0–29) |
| 16–87 ^b | M | 86 | 39.0 | 13.8 (0–29) |
| | F and M | 176 | 39.5 | 14.0 (0–29) |

^aCDLQI questionnaire; ^bDLQI questionnaire.

Table 3 Chronic changes on light-exposed skin of 223 subjects

| Sign | No. patients (%) |
|----------------------------|------------------|
| Scarring (total) | 187 (84) |
| Facial scarring | |
| Total | 149 (67) |
| Depressed pits/varioliform | 89 (40) |
| Macular | 115 (52) |
| Dorsal hand scarring | |
| Total | 138 (62) |
| Macular | 136 (61) |
| Thickened knuckle skin | 78 (35) |
| Premature facial wrinkling | 20 (9) |
| Rhagades and lip furrows | 18 (8) |
| Palmar keratoderma | 6 (3) |
| Pebbled facial skin | 3 (1) |
| Pebbled hand skin | 3 (1) |
| Thickened waxy hand skin | 3 (1) |
| Thickened waxy facial skin | 2 (1) |
| Nail changes | 1 (0.4) |

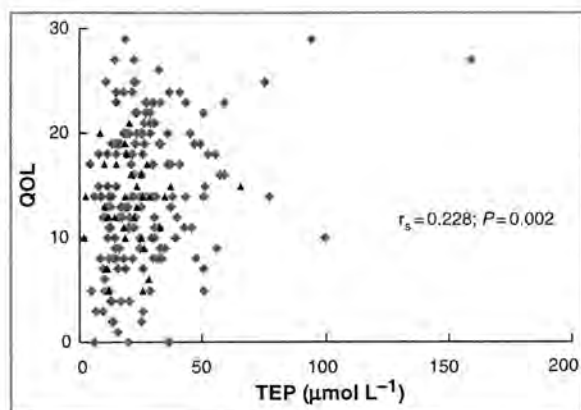


Fig 2. Relationship between total erythrocyte porphyrin (TEP) and quality of life (QoL) scores for 220 patients with erythropoietic protoporphyria. QoL: Dermatology Life Quality Index scores for 176 adults (grey diamonds) and Children's Dermatology Life Quality Index scores for 44 children (black triangles); r_s = Spearman rank correlation.

47.5 years, range: 20–77) with a DLQI score of 0 or 1 had minor symptoms which were not affecting their QoL; in three of these EPP was not diagnosed until over 43 years after the onset of symptoms.

The median total CDLQI score was 13 (range: 5–21, $n = 44$); no child scored < 5, and 35 (80%) scored > 10. The highest-scoring CDLQI questions were 5, 1 and 9, relating to social/leisure activities, skin symptoms and sleep.

Indices of severity of disease

The relationships between three potential indicators of severity of disease and QoL were assessed. There was a positive correlation between TEP and adult DLQI scores ($r_s = 0.228$; $P = 0.002$; $n = 174$) but not between TEP and CDLQI scores ($r_s = 0.025$; $P = 0.888$; $n = 35$; Fig. 2). In contrast, there was a stronger inverse correlation with time to onset of symptoms for CDLQI scores ($r_s = -0.422$; $P = 0.004$; $n = 44$) than for adult DLQI scores ($r_s = -0.233$; $P = 0.002$; $n = 176$). There was no significant relationship between age at onset and either DLQI or CDLQI score.

Discussion

In addition to confirming previous descriptions of the clinical features of EPP,^{2,8–11,16,17} our study provides quantitative data about the frequency of these features (Tables 1 and 3) in a much larger group than has been reported previously. It also documents the measures taken to avoid acute photosensitivity. Most patients depended mainly on physical protection from direct sunlight, usually supplemented by the use of sunscreens. Our study showed that patient choice of sunscreen was varied and often uninformed, highlighting the need for both patient information and expert advice. Subsequent to the completion of this study a new patient information sheet for EPP was

prepared by us and is now available online (<http://www.bad.org.uk/public/leaflets/erythropoietic.asp>). The Tayside Pharmaceuticals' Reflectant Sunscreen is specially formulated to protect the skin against visible light as well as ultraviolet radiation, and details of this product are included in Appendix 1.¹⁸ The clinical effectiveness of the most widely used drug treatment for EPP, β -carotene, is doubtful.^{2,10,19} Our study confirms that two-thirds of those for whom β -carotene was prescribed were no longer taking it, which suggests that its benefits, if any, may be marginal (Table 2). Despite the high rate of discontinuation for most forms of therapy, for those remaining on treatments other than antihistamines there appears to be a high rate of perceived efficacy. While a placebo effect cannot be discounted, it is possible that these treatments are indeed useful for selected patients, in which case affected individuals could be offered a sequential trial of treatment options to assess for potential benefits.

We identified 389 living individuals with EPP within the U.K. and an additional 30 affected family members not included in the databases that we searched. This gives a minimum prevalence for EPP in the U.K. (population 59.8 million) of 1 in 143 000, close to the previously reported U.K. prevalence of 1 : 130 000²⁰ but lower than the 1 : 79 000 and 1 : 75 000 for Northern Ireland² and the Netherlands¹⁰ where there is some evidence for founder effects.^{2,11} The true U.K. prevalence probably lies between these figures, given the difficulty in obtaining a diagnosis reported by many of our patients and our identification since the study ended of new patients who were symptomatic before the study period.

Differences, particularly in the frequencies of clinical features, between our data and those from previous descriptions of EPP^{8–10,16,17} are probably explained mainly by the larger number of patients and the inclusion of a higher proportion of patients (32%) over the age of 40 years, but may also reflect geographical differences. There may also be less observer variation and participation bias in this study. All our patients were seen by the same observer and we attempted to minimize selection bias by recruiting as high a percentage of known U.K. patients as possible. However, only 223 of an identified 389 (57%) living U.K. patients with EPP were seen in the study. There is therefore the possibility of bias either through more mildly affected patients seeing little point in participating, or perhaps more severely affected individuals disillusioned with medical therapy declining to take part. Furthermore, identification of individuals using databases held by specialist referral centres and the difficulty experienced in obtaining a diagnosis might have led to fewer mildly affected individuals being included.

The substantial impact of EPP on the lifestyle of most patients is indicated by their QoL scores (Table 4) which were unexpectedly high by comparison with those for other skin diseases generally regarded as more severe, with levels of pain comparable with severe eczema²¹ and epidermolysis bullosa²² (Table 5^{21–27}). In contrast, relatively asymptomatic but visually disfiguring conditions score lower overall.²⁸ There are no published studies of QoL in any other cutaneous porphyria,

Table 5 Comparison of previous Dermatology Life Quality Index and Children's Dermatology Life Quality Index study mean scores

| Condition | Adults | Children |
|---|--|---------------------------------------|
| Erythropoietic protoporphyria (current study) | 14.0 | 12.8 |
| Acne | | 5.7 ²³ |
| Atopic eczema | 4.14 ²⁴ –16.2 ²¹ | 7.7 ²³ –12.7 ²⁵ |
| Dystrophic epidermolysis bullosa | 7.5 ²² | 11.5 ²² |
| Epidermolysis bullosa simplex | 10.7 ²² | 15 ²² |
| Erythrokeratoderma variabilis | 5.3 ²⁶ | 2.75 ²⁶ |
| Psoriasis | 4.5 ²⁴ –13.9 ²¹ | 5.4 ²³ |
| Melanocytic naevi | | 2.3 ²³ |
| Vitiligo | | 5.6 ²⁷ |
| Unaffected population | | 0.38 ²³ |

although the impact of acute, noncutaneous symptoms in the acute porphyrias can be extensive.²⁹ The high scores in EPP are partly explained by high scoring in the social and leisure categories, which may reflect the lifestyle restrictions imposed by the need to avoid exposure to sunlight, whereas in most other skin disorders symptom-related questions score highest.^{30,31} Children, who may be less aware of the social implications of their illness, tended to score higher on questions about symptoms and sleeping, reflecting their reduced control over sunlight exposure and the pain experienced when avoidance is not possible. Thus the main effect of EPP on QoL comes from the morbidity produced by acute and prolonged pain following sunlight exposure together with the social consequences of the measures needed to avoid this. The previously reported trend for higher DLQI scores in younger individuals and adults³¹ was not seen in EPP (Table 4). Like others,^{9,10} we were unable to confirm that symptoms improve with age.⁸

Photosensitivity increases with TEP concentration in EPP patients with liver failure³² and it has been suggested that EPP severity correlates directly with protoporphyrin concentration.³³ We found correlations between TEP and quantifiable indicators of severity such as QoL score and age at onset of symptoms but not time to onset of symptoms. However, these correlations were weak and CIs too wide for TEP to be a useful predictor of severity for individual patients (Fig. 2). It is possible that plasma porphyrin concentrations, which were not measured in this study, may provide a better correlation with severity of disease than TEP.

In addition to confirming the improvement in symptoms during pregnancy,^{8,11,17,34,35} we found that TEP was higher in males than females, a difference that has not been noted previously,² and that some women experience more severe symptoms around the time of their menses. These observations add to the evidence that sex hormones influence EPP. The mechanism for this effect remains unclear. There are reports that TEP decreases^{17,34} or remains unchanged during pregnancy.³⁵ The human ABCG2 multidrug transporter protein regulates intracellular protoporphyrin concentrations in erythroid and hepatic cells.³⁶ Its expression in human placental cells

is increased by progesterone and 17 β -oestradiol,³⁷ an effect that might lead to removal of protoporphyrin from the maternal circulation during pregnancy.

All 223 patients in this study developed symptoms before they were 13 years old, although a few patients did not seek medical advice until they were much older. This was an unexpected finding, because other series have included patients whose disease became manifest only during their teens or later^{8,9} and there are several reports of adult onset of EPP,³⁸ distinct from those caused by somatic mutation of the FECH gene in haematopoietic cells.³⁹ In contrast, the median age at diagnosis was 12 years and the diagnosis was not made in a third of our patients until they were 20 years of age or older. Although laboratory methods for the diagnosis of EPP have improved over the past three decades, analysis of our data shows little change in the median time to diagnosis for each decade from 1965. Such a finding highlights the need to increase awareness of EPP, particularly among general practitioners, the primary point of medical contact, and paediatricians to whom the majority of patients will have been referred following the onset of their symptoms. Increased awareness in the wider community through the written and broadcast media also has a role to play; 8% of our patients were self-diagnosed or diagnosed by a family member after seeing such information.

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Appendix 1. Formulation of Tayside Pharmaceuticals' Reflectant Sunscreen

Tayside Pharmaceuticals' Reflectant Sunscreen Creams are available in three colours: coral pink, beige and coffee. The active ingredients in the sunscreen are titanium dioxide and zinc oxide in a base containing colours and preservatives. The creams do not contain lanolin and are fragrance free. They contain the following ingredients:

Zinc oxide, titanium dioxide, distilled water, light liquid paraffin BP, 1,3-butylene glycol, cetyl dimeticone copolyol-EM90, glyceryl isostearate/sorbitan isostearate, silicon fluid 200/100 CS, sodium chloride BP, almond oil BP, butylated hydroxytoluene, methyl hydroxybenzoate BP, bronoprol BP.

The pigment in the 'coral pink' product is red iron oxide, in the 'beige' product is yellow and black iron oxide, and in the 'coffee' product is red, yellow and black iron oxide.

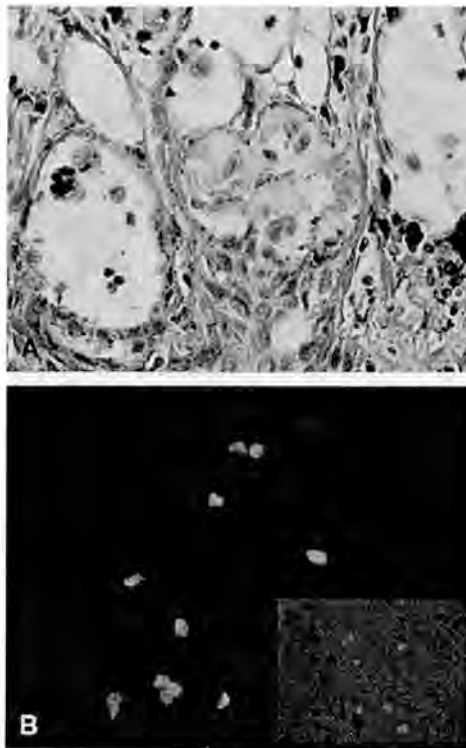


Fig 2. **A**, Large pale-staining cells with vacuolated cytoplasm and small basophilic nuclei. (Hematoxylin-eosin stain, original magnification: $\times 400$.) **B**, TdT-mediated dUTP-biotin nick end labeling—positive balloon cells containing melanin in papillary dermis. Positive cells were viewed under halogen and fluorescence lamp (*inset*).

Our data based on TdT-mediated dUTP-biotin nick end labeling analysis suggest that apoptosis is involved in the development of balloon cell nevus. Interestingly, TdT labeling was not observed in nuclei of ballooned cells without melanin. This finding suggests either the limitation of TdT-mediated dUTP-biotin nick end labeling assay to detect different phases of apoptosis, which was suggested by Zhao et al⁷ in hepatocytes, or that cells exhibiting amelanotic ballooning change may not have undergone a pathway related to apoptosis. Melanogenesis may be related to the apoptosis.^{8,9} Expression of apoptosis regulatory molecules (ie, Bcl down-regulation and Bax up-regulation) are closely involved in melanogenesis.⁸ In addition, it was reported that mature melanocytes may tend to undergo apoptosis after all-*trans* retinoic acid treatment.⁹ Further study is needed for more precise understanding of the ballooning changes in association with apoptotic activity.

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Symptomatic response of erythropoietic protoporphyria to iron supplementation

To the Editor: Erythropoietic protoporphyria (EPP) is an inherited disorder of heme biosynthesis characterized by a relative deficiency of the mitochondrial enzyme ferrochelatase, which catalyses the incorporation of ferrous iron into protoporphyrin IX to form heme. The defect causes increased free protoporphyrin IX, producing acute cutaneous photosensitivity and scarring beginning in childhood and, rarely, hepatic fibrosis and failure. The anomalous hematopoiesis often presents as a microcytic anemia with features of iron deficiency. Despite these findings, the role of iron supplementation in EPP remains unclear. Following oral supplementation, both exacerbation and improvement of laboratory indices and symptoms have been reported.¹⁻⁵ We report a case of apparent mild iron deficiency in EPP in which the hematologic response to oral supplementation was accompanied by an increased tolerance to

Table I. Laboratory values of patient 1 before and after iron supplementation

| Parameter (reference range) | Before iron supplementation | After iron supplementation |
|--|-----------------------------|----------------------------|
| Erythrocyte total porphyrin (0.4-1.7 $\mu\text{mol/L}$) | 18.0 | 20.0 |
| Hemoglobin (13.5-16.5 g/dL) | 12.7 | 12.9 |
| Mean corpuscular volume (80-98 fl) | 83.5 | 85.1 |
| Mean corpuscular hemoglobin (27-34 pg) | 27.6 | 28.0 |
| Serum ferritin (15-300 $\mu\text{g/L}$) | 13 | 42 |
| Serum iron (8.8-32.4 $\mu\text{mol/L}$) | 16.6 | 40.2 |
| Total iron binding capacity (44.8-80.6 $\mu\text{mol/L}$) | 83.2 | 63.1 |
| Transferrin saturation (16-50%) | 20.0 | 63.7 |

sunlight, the second such report in the literature, and uniquely without an apparent change in protoporphyrin concentrations.

A white male had experienced burning paresthesia since his early childhood after about 3 hours of sunlight exposure. The sensation generally took 3 days to completely resolve. He was diagnosed biochemically and genotypically with EPP at 10 years of age. At a routine outpatient review at the age of 17, his blood count revealed a mild anemia associated with an apparent iron deficiency (Table I).

Tests for fecal occult blood were negative. He was prescribed oral ferrous sulphate 200 mg twice daily for 16 weeks, before reducing to 200 mg once daily. After 6 months, the ferritin- and iron-binding capacities were within the normal range, although his hemoglobin had not returned to normal (Table I). The patient reported significant improvement in sunlight tolerance (up to 8 hours symptom-free exposure and resolution within 24 hours). Over this period, there was no significant change in either his protoporphyrin levels or biochemical tests of liver function.

Hematologic investigations of EPP patients often suggest an iron-deficient picture, although the mechanism remains uncertain because no studies have examined the etiology. It seems unlikely that individuals are losing iron; the mechanism probably involves either failure to absorb dietary iron adequately or failure to utilize it. The role of iron supplementation in EPP is controversial and the literature limited (Table II). Oral iron and transfusion of whole blood or hematin have been reported to normalize liver function and erythrocyte protoporphyrin levels.¹ However, others report symptomatic and biochemical deterioration of EPP following oral supplementation^{2,3} or whole blood transfusion.⁴ Theories for beneficial effects include reduced enterohepatic circulation caused by gastrointestinal chelation, nonenzymatic intracellular protoporphyrin chelation, or facilitated chelation by residual active ferrochelatase.¹ Conversely, deterioration following supplementation may result from stimulation of hematopoiesis and production of protoporphyrin or interaction with other products predisposing to cholestasis.²

Table II. Summary of previous EPP literature reporting effect of oral iron supplementation

| Year of paper | Number of subjects | Hematologic investigations | Treatment | Results of oral iron therapy | Reference |
|---------------|--------------------|--|------------------------------|--|----------------|
| 2007 | 1 | Low Hb, serum ferritin raised TIBC | Oral iron | Normalization of ferritin and TIBC, improved Hb, no change LFT or EPP-reduced photosensitivity | Current report |
| 1990 | 1 | Low Hb, MCV, serum ferritin | Oral iron | Increased photosensitivity raised LFT | 2 |
| 1988 | 4 | Case 1, iron deficiency anemia; case 2, anemia; case 3, iron deficiency anemia; case 4, iron deficiency anemia | Oral iron | Case 1, increased photosensitivity; case 2, increased photosensitivity; case 3, increased photosensitivity raised EPP; case 4, increased photosensitivity raised EPP | 3 |
| 1986 | 1 | Low Hb, MCV, iron, serum ferritin raised TIBC, LFT | Oral iron | Reduced EPP improved LFT; photosensitivity effect not reported | 1 |
| 1970 | 1 | Intrahepatic porphyristasis; iron deficiency anemia | Oral iron and cholestyramine | Reduced photosensitivity; reduced PP deposition on liver rebiopsy | 5 |

EPP, Erythrocyte protoporphyrin; Hb, hemoglobin; LFT, liver function tests; MCV, mean corpuscular volume; TIBC, total iron binding capacity.

Our patient's experience of significantly improved symptoms while taking oral iron supplements is the second report of this phenomenon in EPP. The other reported patient was administered oral cholestyramine and the improved symptoms were associated with reduced erythrocyte and hepatic protoporphyrin.⁵ In contrast, our patient's protoporphyrin levels remained constant throughout and beyond iron supplementation. Although a placebo effect cannot be discounted in the current case, a similar improvement did not occur with beta carotene. This demonstration of our patient's stable protoporphyrin levels throughout the period of treatment with iron suggests that there may be an alternative, as yet unknown mechanism for iron in improving the symptoms of EPP.

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Squamous cell carcinoma over tattoos

To the Editor: Tattooing has been associated with a variety of complications, including inflammatory



Fig 1. Ulcerated nodular lesion on right hand



Fig 2. Well-differentiated squamous cell carcinoma, with pigment granules at periphery of tumor (case 2). (Hematoxylin-eosin stain; original magnification: $\times 20$.)

reactions, the transmission of infections, and neoplasms. We report two cases of squamous cell carcinoma (SCC) and a keratoacanthoma appearing over tattoos.

Case 1 involved a 35-year-old man who presented with an ulcerated nodular lesion. It had appeared 4 months earlier and was located over a tattoo on the right arm that had been applied 10 years previously with black ink (Fig 1). Complete removal of the lesion was carried out. The histopathologic study revealed islets of epithelial cells exhibiting marked atypia and aberrant keratinization. Some zones showed basal membrane rupture and spread of the keratinized cells toward the dermis. Well-differentiated SCC was diagnosed.

Case 2 involved a 30-year-old woman who presented with a recent ulcerated and indurated nodular lesion over a tattoo that had been applied 10 months earlier, overlying a black pigment zone. The lesion was completely removed, and the histologic study revealed an epidermis with irregular acanthosis and endophytic prolongations composed of cells with an abundant and eosinophilic cytoplasm that invaded the dermis. Nuclear atypia, individual keratinization, and keratin pearls were identified. The lesion margins were of a pseudoepitheliomatous

Gene Dosage Analysis Identifies Large Deletions of the *FECH* Gene in 10% of Families with Erythropoietic Protoporphyrria

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Erythropoietic protoporphyria (EPP) is an inherited cutaneous porphyria characterized by partial deficiency of ferrochelatase (*FECH*), accumulation of protoporphyrin IX in erythrocytes, skin, and liver, and acute photosensitivity. Genetic counseling in EPP requires identification of *FECH* mutations, but current sequencing-based procedures fail to detect mutations in about one in six families. We have used gene dosage analysis by quantitative PCR to identify large deletions of the *FECH* gene in 19 (58%) of 33 unrelated UK patients with EPP in whom mutations could not be detected by sequencing. Seven deletions were identified, six of which were previously unreported. Breakpoints were identified for six deletions (c.1-7887-IVS1+2425insTTCA; c.1-9629-IVS1+2437; IVS2-1987-IVS4+352del; c.768-IVS7+244del; IVS7+2784-IVS9+108del; IVS6+2350-TGA+95del). Five breakpoints were in intronic repeat sequences (AluSc, AluSq, AluSx, L1MC4). The remaining deletion (Del Ex3-4) is likely to be a large insertion-deletion. Combining quantitative PCR with routine sequencing increased the sensitivity of mutation detection in 189 unrelated UK patients with EPP from 83% (95% CI: 76-87%) to 93% (CI: 88-96%) ($P=0.003$). Our findings show that large deletions of the *FECH* gene are an important cause of EPP. Gene dosage analysis should be incorporated into routine procedures for mutation detection in EPP.

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INTRODUCTION

Erythropoietic protoporphyria (EPP) (OMIM 177,000) is an inherited cutaneous porphyria caused by partial deficiency of ferrochelatase (*FECH*) (EC 4.99.1.1), the final enzyme in the heme biosynthetic pathway, which catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme. Deficiency of *FECH* leads to accumulation of protoporphyrin IX in erythrocytes, plasma, skin, and liver. Accumulation of protoporphyrin in the skin produces lifelong acute photosensitivity while its deposition in the liver leads to hepatobiliary disease in some patients (Todd, 1994; Meerman, 2000).

The inheritance of EPP is complex. In most families, clinical expression requires inheritance of a hypomorphic *FECH* IVS3-48C *trans* to a disabling *FECH* mutation that is inherited in an autosomal dominant fashion (Gouya *et al.*,

2006) but rare families with autosomal recessive EPP have been reported (Lamoril *et al.*, 1991; Sarkany *et al.*, 1994; Poh-Fitzpatrick *et al.*, 2002; Whatley *et al.*, 2004; Gouya *et al.*, 2006). The frequency of the hypomorphic *FECH* allele varies between populations (Gouya *et al.*, 2006), being present in about 12% of white Europeans (Gouya *et al.*, 2002, 2006; Whatley *et al.*, 2004) but in 68% of Japanese (Saruwatari *et al.*, 2006). Molecular analysis to identify the IVS3-48C allele and disease-specific mutations in the *FECH* gene is important for accurate genetic counseling of families with EPP (Gouya *et al.*, 2002).

Over 120 mutations in the *FECH* gene, which contains 11 exons spread over 45 kb, have been reported in EPP, most of which are restricted to one or a few families (Gouya *et al.*, 2006; Saruwatari *et al.*, 2006; Aurizi *et al.*, 2007). Apart from one complete gene deletion (Magness *et al.*, 1994) and a partial deletion (Wood *et al.*, 2006), all have been detected using methods that depend only on sequencing of exons and their flanking regions, including up to 1.3 kb of 5' non-coding sequence, to identify mutations. This approach fails to identify an *FECH* mutation in about one in six families (Rüfenacht *et al.*, 1998; Whatley *et al.*, 2004; Gouya *et al.*, 2006). Here we show that gene dosage analysis by quantitative PCR detects gross deletions of one or more *FECH* exons in 19 of 33 (58%) of such "mutation-negative" families and that deletions of this type are present in 10% of UK families with EPP.

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Abbreviations: EPP, erythropoietic protoporphyria; *FECH*, ferrochelatase; WT, wild type

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RESULTS

Direct sequencing of all exons, 30–250 bp of intronic flanking regions, and 1,000 kb of 5' untranslated region (UTR) identified an FECH mutation on one or both alleles in 156 (83%) of our 189 unrelated patients, 149 of whom had a mutation on only one allele. Genotyping of the 33 mutation-negative patients showed that 28 (85%) patients carried an FECH IVS3-48C allele, a significantly lower frequency than in the 149 patients who were heterozygous for an FECH mutation, all but one of whom had at least one IVS3-48C allele (χ^2 13.3; $P < 0.005$).

Gene dosage analysis by quantitative PCR of genomic DNA from the 33 mutation-negative patients identified deletions encompassing one or more exons in 19 (58%) patients, 18 of whom were either FECH IVS3-48C/T heterozygotes or had the IVS3-48C allele *trans* to a deletion (Table 1). Seven different deletions were detected, one of which has been described previously (Wood et al., 2006) (Table 1). Exon 1 was deleted in five patients; genotyping with three highly informative microsatellite markers, two flanking the FECH gene and one in intron 4, identified two haplotypes that differed only at the 5' flanking site. Two different deletions were identified: three patients had a 10,379 bp deletion with a small 4 bp insertion and two patients had a 12,133 bp deletion (Table 1). Two patients had lost exons 3 and 4; one has previously been described (Wood et al., 2006), the other had a deletion for which breakpoints were not identified (Table 1). In two patients, a small deletion involving part of exon 7 and 244 bp of 3' flanking sequence removed the region in which one of the primers for amplification of this exon is usually based. The presence of a stop codon 68 codons 3' to this deletion suggests that any mRNA produced from this allele will be truncated and unstable. Exons 8 and 9 were deleted in eight patients; genotyping with three microsatellite markers showed that this mutation was present in at least two different FECH haplotypes. The largest deletion removed exons 7, 8, 9, 10, and 11 and was present in two patients.

Breakpoints for six deletions were defined by sequencing (Table 2). The 10.4 kb exon 1 deletion contained a four-base insert with a sequence that was repeated in the 5' flanking region (Table 2) and 30 bp 3' to the insertion. For four of the five other deletions, the breakpoints shared the same 3, 4, or

5 bp sequences at the deletion junctions (Table 2). The 3' breakpoints of both exon 1 deletion and the exon 7 deletion and the 5' breakpoint of the exon 8–9 deletion were all sited in Alu repeat regions while the 5' breakpoint of the exon 7–11 deletion was in an L1MC4 repeat sequence (Table 2). These features have been reported for large deletions in other genes and are believed to facilitate homologous recombination (Woods-Samuels et al., 1991; Laccone et al., 2004).

We were unable to identify the breakpoint for one deletion. This patient was heterozygous for a deletion that included exons 3 and 4 and surrounding sequence from IVS2-88 to IVS4+2046 and thus differed from the exon 3–4 deletion with defined breakpoints recently described in two Italian families (Di Pierro et al., 2007). PCR amplification using primers flanking the deleted region gave a 7 kb band consistent with the size of the deletion as identified by gene dosage analysis. No smaller product was identified using amplification conditions that are capable of detecting fragments as small as 100 bp. Restriction enzyme (*NdeI*) digestion of the 7 kb amplicon gave a normal pattern consistent with the amplicon coming from the normal allele alone. These findings exclude the presence of a 7 kb insertion-deletion but not of an insertion-deletion too large to amplify.

DISCUSSION

Most patients with EPP are heterozygous for disabling mutations in the FECH gene (Gouya et al., 2006). Intragenic deletions involving entire exons and their flanking regions, including primer binding sites, are not identified in heterozygotes by methods for mutation detection that rely on direct sequencing of amplified genomic DNA because sequencing does not distinguish between amplification of one or both alleles. Quantitative analysis of amplified genomic DNA is required for this purpose. Here, we have used gene dosage analysis to show that large deletions of the FECH gene, likely to abolish all FECH activity, were present in 19 (58%) of 33 patients with EPP in whom FECH mutations were not identified by sequencing.

Such deletions are an important cause of human disease and represent 5.6% of all mutations in the human gene mutation database (www.hgmd.cf.ac.uk). There is evidence that they result from various molecular interactions that are

Table 1. Deletions in the FECH gene in patients with EPP

| Number of patients | Deleted exons | Deletion | Size of deletion (bp) | FECH IVS3-48 genotypes |
|--------------------|------------------|------------------------------|-----------------------|------------------------|
| 3 | Exon 1 | c.1-7887 to IVS1+2425insTTCA | 10,379 | C/T |
| 2 | Exon 1 | c.1-9629 to IVS1+2437 | 12,133 | C/T |
| 1 ¹ | Exons 3, 4 | IVS2-1987 to IVS4+352 | 4,425 | C/- |
| 1 | Exons 3, 4 | See text | >7,000 | C/- |
| 2 | Exon 7 (partial) | c.768 to IVS7+244 | 280 | C/T, T/T |
| 8 | Exons 8, 9 | IVS7+2784 to IVS9+108 | 2,196 | C/T |
| 2 | Exons 7–11 | IVS6+2350 to TGA+95 | 9,889 | C/T |

¹Wood et al. (2006).

Table 2. Deletion breakpoints in the FECH gene

| Deleted exon(s) | Sequence | Breakpoints | Repeat sequence |
|-------------------|------------|-------------------------------|-----------------|
| 1 (10.4 kb) | Junctional | gttcacgcacaTTCaggctcactgca | — AluSc |
| | 5'WT | gtgcattcaggttcacgcaca | |
| | 3'WT | ggctcactgcaacctccgct | |
| 1 (12.1 kb) | Junctional | tttgctgttgTGCCCGgctgtttgt | — AluSq |
| | 5'WT | tacacagagtttgctgttgTGCCCG | |
| | 3'WT | TGCCCGgctgtttgtaggcctcta | |
| 3, 4 ¹ | Junctional | 5' ctttagtttcGAGctgctgctat 3' | — — |
| | 5'WT | atcatagatcttttagtttcGAG | |
| | 3'WT | GAGctgctgctattattgccttt | |
| 7 | Junctional | 5' agagaagcgagGTGtaccaggct 3' | — AluSx |
| | 5'WT | ccacttgagaagagaagcgagGTC | |
| | 3'WT | GTGtaccaggctggagtgcaat | |
| 8, 9 | Junctional | 5' ctattttctcTTTGcatgtacgg 3' | AluSx — |
| | 5'WT | ctaaaattactattttctcTTTG | |
| | 3'WT | TTTGcatgtacgggtcctcattc | |
| 7, 8, 9, 10, 11 | Junctional | 5' tgggaccacag cactctcctt 3' | L1MC4 — |
| | 5'WT | tgctgagtagctgggaccaca | |
| | 3'WT | cactctccttgatatata | |

Wild-type (WT) sequences 5' and 3' to the breakpoints are shown. Junction points, except for the flush junction indicated by ||, and homologous sequences present at both the 5' and 3' breakpoints are in capitals. The inserted sequence is indicated by italic capitals.
¹Previously reported (Wood *et al.*, 2006).

dependent on the features of the surrounding DNA sequence (Abeyasinghe *et al.*, 2006). Repetitive elements, which were present in all but one of the deletions for which we defined breakpoints, are known to facilitate the formation of secondary structures such as hairpin loops and cruciforms (non-B DNA conformations) that have been shown to coincide with the breakpoints of gross deletions (Chuzhanova *et al.*, 2003; Bacolla *et al.*, 2004).

The 33 patients came from a cohort of 189 apparently unrelated patients identified by a cross-sectional study of EPP in the UK (Holme *et al.*, 2006). FECH mutations were identified by sequencing in 156 (83%) of these 189 patients. Here we show that a further 19 (10%) patients had large intragenic deletions. Thus sequencing combined with gene dosage analysis identifies mutations in 175 of UK patients with EPP, increasing the sensitivity of mutation detection to 93% (95% confidence interval: 88–96%) (χ^2 8.7; $P=0.003$), which is higher than those reported previously (Rüfenacht *et al.*, 1998; Whatley *et al.*, 2004; Gouya *et al.*, 2006).

Only one of the 19 patients with deletions was not heterozygous for the hypomorphic FECH IVS3-48C allele (Table 1). This patient had the smallest deletion that we identified as involving exon 7 and its 3' flanking region (Tables 1 and 2). However, it is unlikely that the mutant allele gives rise to a stable truncated protein (see Results) capable of exerting a dominant-negative effect on FECH activity, as reported for an exon 10 deletion in the mouse (Magness *et al.*, 2002). At present, it is not clear why rare patients with the dominant form of EPP who have a mutation *trans* to a normal FECH IVS3-48T allele develop photosensitivity (Wiman *et al.*, 2003).

Sequencing combined with gene dosage analysis failed to identify a FECH mutation in 14 (7%) of UK patients with EPP. Ten of these patients were heterozygous for the hypomorphic FECH IVS3-48C allele, a proportion that is significantly higher than in the UK population (χ^2 26.0, $P<0.001$). This suggests that most, if not all, of these 10 patients may be heterozygous for deleterious FECH mutations that lie in regions of the gene that determine expression but are outside the regions that we sequenced. The other four patients all had the genotype FECH IVS3-48T/T, a proportion that is much higher than that in other groups of EPP patients (Risheq *et al.*, 2003; Gouya *et al.*, 2006) except for those with mutations on both FECH alleles (Whatley *et al.*, 2004; Gouya *et al.*, 2006). It seems unlikely that these patients are homozygous or compound heterozygotes for FECH mutations that were not detected by our methods since all patients with recessive EPP yet reported have had at least one intra-exonic mutation (Whatley *et al.*, 2004; Gouya *et al.*, 2006). Alternatively, at least some of these patients may have mutations in genes other than FECH. Deletion of the Irf2 gene has recently been shown to produce protoporphyria in mice (Cooperman *et al.*, 2006), and the possibility that EPP may also be caused by mutation of genes that regulate the supply of substrates to FECH remains to be excluded.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 189 apparently unrelated patients with EPP as part of a cross-sectional study of EPP in the

UK (Holme *et al.*, 2006). We investigated 33 of these patients (32 white British, one of Iraqi descent) in whom an *FECH* mutation could not be identified by direct sequencing of all exons, 30–250 bp of intronic flanking regions, and 1,000 kb of 5' UTR. Total erythrocyte porphyrin was increased in all 33 patients (mean 25.6 $\mu\text{mol/l}$, range 6.2–65.7 $\mu\text{mol/l}$; reference range 0.4–1.7 $\mu\text{mol/l}$) and was mainly free protoporphyrin.

The study was conducted in accordance with the Declaration of Helsinki Principles for medical research involving human subjects and its subsequent amendments. Prior approval was obtained from the North West Multicentre Research Ethics Committee and 84 local research ethics committees. All patients or their parents gave informed consent.

Preparation of DNA

Genomic DNA was extracted from whole blood using the QIAamp DNA purification kit (Qiagen, Crawley, UK) for sequencing and the Flexigene kit (Qiagen) for dosage studies.

Gene dosage analysis

A multiplex PCR was designed to simultaneously amplify exons 2–9 and 11 of the *FECH* gene, along with exon 9 of the *HMBS* gene and exon 5 of the *PPOX* gene as internal controls. Exons 1 and 10 did not amplify consistently within the multiplex, so they were analyzed separately with exon 1 of the *UROS* gene and exon 4 of the *HMBS* gene as internal controls. A fluorescent (5' FAM) label was incorporated into one of each primer pair. Details of the primers used for gene dosage analysis are available from the authors. Amplification was carried out in 20 μl volumes containing 5.39 μl DNA, 3.45 μl primer mix (0.5–1.5 pmol (final concentration) of each primer), and 10 μl 2 \times Qiagen multiplex PCR master mix (containing HotStarTaq DNA polymerase, Qiagen multiplex PCR buffer (6 mM MgCl_2 , pH 8.7), dNTP mix). An initial denaturation of 15 minutes at 95°C was followed by 20 cycles of denaturation for 30 seconds at 94°C, annealing for 1 minute 30 seconds at 64°C, and extension for 1 minute 30 seconds at 72°C, with a final extension for 10 minutes at 72°C. The PCR products (2 μl) were added to a mixture containing formamide (10 μl) and GS500 standard (0.3 μl) (PE Applied Biosystems, Cheshire, UK) and denatured at 95°C for 3 minutes. The samples were run on an ABI Prism 310 analyser at 60°C using POP4 polymer (PE Applied Biosystems).

To determine the gene dosage for each exon, the peak areas were compared with each other and against controls (Yau *et al.*, 1996). A locus with a double copy will give a theoretical value of 1.0 whereas a locus with a deletion will give a value of 0.5 and 2.0 depending on whether the deleted locus is the numerator or the denominator. To exclude the presence of polymorphisms at the primer sites, all deletions were confirmed using at least one other set of primers to eliminate false positive results due to mis-priming. Primer sequences are available from the authors.

Identification of breakpoints

To identify deletion breakpoints, pairs of primers were designed for quantitative PCR-amplification of intronic regions around the deleted exon; these primer sequences are available from the authors. These amplicons were multiplexed with internal controls and analyzed for dosage. The results indicated whether each amplicon was within the deleted region or not. Pairs of primers were

redesigned at increasing or decreasing distance from the deletion, depending on these results, partially localizing the deletion. Where possible, the forward primer from a diallelic region 5' of the deletion was used with the antisense primer from a diallelic region 3' of the deletion to amplify a PCR product straddling the deletion. This amplicon was sequenced and the breakpoints were identified. Amplification across breakpoints was carried out using Elongase enzyme mix (Invitrogen, Paisley, UK) according to the manufacturer's instructions, using a final concentration of 1.8 mM magnesium with an elongation time between 5 and 20 minutes.

Haplotype analysis

Genotyping with two microsatellite markers flanking the *FECH* gene (315.0 kb 5' to the start codon, 267.6 kb 3' to the stop codon) and with one microsatellite marker in intron 4 was carried out as described by Parker (2006).

Sequencing of genomic DNA

PCR-amplified double-stranded DNA was purified from agarose gels using the QIAquick gel extraction kit (Qiagen) before being cycle sequenced using fluorescent ddNTPs (BigDye) and an ABI Prism 3100 Genetic Analyzer (PE Biosystems, Warrington, UK). Nucleotides are numbered from the cDNA sequence of human *FECH* (GenBank accession number D00726) with the A of the ATG initiation codon as "+1".

Other methods

Erythrocyte porphyrins were analyzed as described (Deacon and Elder, 2001).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Brief report

Erythropoiesis and iron metabolism in dominant erythropoietic protoporphyria

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Erythropoietic protoporphyria (EPP) results from deficiency of ferrochelatase (FECH). Accumulation of protoporphyrin IX causes life-long acute photosensitivity. Microcytic anemia occurs in 20% to 60% of patients. We investigated 178 patients with dominant EPP confirmed by molecular analysis. Erythropoiesis was impaired in all patients; all had a downward shift in hemoglobin (Hb), and the mean decreased

in males by 12 g/L (1.2 g/dL). By World Health Organization criteria, 48% of women and 33% of men were anemic. Iron stores, assessed by serum ferritin (sFn), were decreased by two-thirds, but normal serum soluble transferrin receptor-1 and iron concentrations suggested that erythropoiesis was not limited by iron supply. FECH deficiency in EPP appears to lead to a steady state in

which decreased erythropoiesis is matched by reduced iron absorption and supply. This response may in part be mediated by protoporphyrin, but we found no correlation between erythrocyte protoporphyrin and Hb, sFn, total iron-binding capacity, or transferrin saturation. (Blood. 2007;110:4108-4110)

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Introduction

Erythropoietic protoporphyria (EPP, MIM 177000) is an inherited disorder caused by partial deficiency of ferrochelatase (FECH; EC 4.99.1.1) that catalyzes the chelation of ferrous iron by protoporphyrin IX. FECH deficiency leads to accumulation of protoporphyrin in normoblasts, erythrocytes, plasma, skin, and liver, causing lifelong acute photosensitivity and, in approximately 2% of patients, severe liver disease.^{1,2}

Microcytic anemia occurs in 20% to 60% of patients.³⁻⁶ In contrast to other inherited disorders of erythroid heme biosynthesis,⁷ the anemia is not dyserythropoietic, there is no iron overload, and there is evidence for iron deficiency^{5,6,8,9} without iron loss.⁹ A mouse model of EPP, the homozygous *Fech^{mlpas}* mutant, develops a similar microcytic anemia.^{10,11} Although it is probable that the anemia of EPP reflects limitation of heme formation by FECH deficiency, its incidence, mechanism, and relationship to disordered iron metabolism remain unclear.

Hematologic and biochemical measurements

All analyses were carried out in the same laboratory. Serum iron (sFe),¹² total iron-binding capacity (TIBC),¹⁴ serum ferritin (sFn, Elecsys 2010; Roche Diagnostics, Indianapolis, IN), soluble transferrin receptor-1 (sTfR; R&D Systems, Abingdon, United Kingdom), and erythrocyte protoporphyrin¹⁵ were determined as described. Other measurements were by standard automated methods. Data obtained previously for 611 male first time blood donors were used for comparisons.¹⁶

Statistical methods

Results were expressed as mean plus or minus a standard deviation (SD) for normally distributed data and median and range for data (sFn, protoporphyrin) with a log-normal distribution. Differences between quantitative variables were assessed by the Mann-Whitney test. Spearman rank correlation (r_s) was used to test the significance of relationships between pairs of variables and the chi-square test for differences between proportions.

Patients and methods

Patients and control subjects

Blood samples were obtained from 210 patients with EPP during a cross-sectional study of EPP in the United Kingdom.¹² One hundred ninety-two patients had one *FECH* mutation with 1 or 2 *FECH* IVS3-48C alleles and were classified as dominant EPP (dEPP)¹³; 14 of these were excluded because they had diseases likely to affect iron metabolism. Ethical approval was obtained from the North West Multicentre Research Ethics Committee and 84 local research ethics committees. Informed consent was obtained in accordance with the Declaration of Helsinki from all patients or their parents.

Results and discussion

Red cell indices

By World Health Organization criteria, 73 (41%; 95% confidence interval: 34%–48%) of our patients with dEPP were anemic. All had a mild microcytic, hypochromic anemia; 48% of females and 33% of males were affected. The anemic patients did not form a separate subgroup. In both sexes, hemoglobin (Hb; females: 119 ± 10 g/L [11.9 ± 1.0 g/dL]; males: 133 ± 10 g/L [13.3 ± 1.0 g/dL]), mean cell volume (MCV), and mean corpuscular hemoglobin (MCH) were normally distributed with a shift in their means toward lower values (Table S1, available on the *Blood* website; see the

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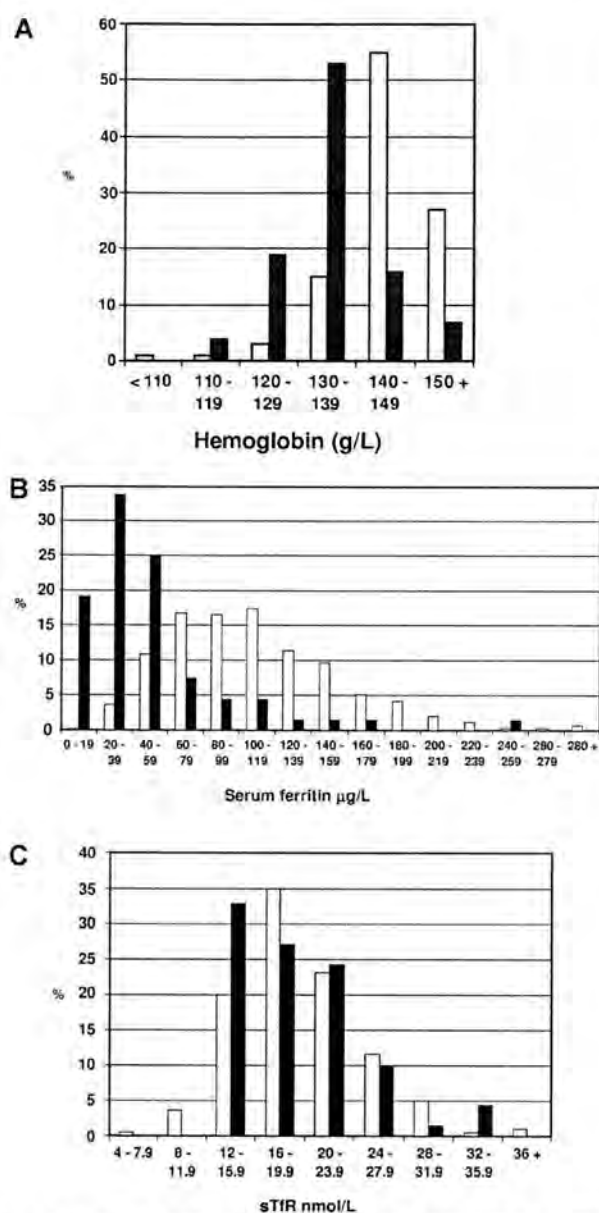


Figure 1. Hemoglobin, serum ferritin, and serum soluble transferrin receptor-1 concentrations in male patients with dominant EPP. (A) Hemoglobin concentrations in 66 male patients with dEPP aged 16 years or older (■) and in a sample of 5206 men aged 16 years or older from the English population¹⁷ (□). (B) Serum ferritin concentrations in 66 male patients with dEPP aged 16 years or older (■) and in 612 male first-time blood donors from south Wales aged 17 to 62 years¹⁵ (□). (C) Serum soluble transferrin receptor-1 concentrations in 61 male patients with dEPP aged 16 years or older (18.6 ± 5.1 nM) (■) and in 225 hematologically healthy male and female subjects from the United States aged 17 to 97 years¹⁸ (□) assayed using the same method.

Supplemental Materials link at the top of the online article); the mean Hb for males was 12 g/L (1.2 g/L) lower than in the general population (Figure 1A). A similar shift has been noted in Dutch EPP patients.¹⁹ This downward shift in Hb leads to some patients falling within the definition of anemia; part of the wide variation in reported incidences can be explained by use of different definitions.^{2-6,9} Erythrocyte protoporphyrin concentrations (females: $21.9 \mu\text{M}$ [range, 4.1-75.3 μM]; males: $25.5 \mu\text{M}$ [range, 8.9-77.3 μM]) showed no correlation with Hb.

Table 1. Comparison of indicators of iron status in male patients with dEPP and male first-time blood donors

| Serum iron indices | EPP patients, n = 67 | First-time blood donors, n = 611 |
|----------------------|--------------------------|----------------------------------|
| sFe, μM | $15.1 \pm 6.6^*$ | 16.7 ± 6.0 |
| TIBC, μM | $63.0 \pm 6.9^\dagger$ | 54.5 ± 10.0 |
| TS, % | $23.9 \pm 10.3^\dagger$ | 31.1 ± 10.9 |
| sFn, $\mu\text{g/L}$ | 37 (10-119) [†] | 101 (35-220) |

Values are means plus or minus SD, except for sFn, which are medians and 95% ranges. EPP patients are males aged 16 to 77 years who have never been prescribed iron supplements. Blood donors are male first-time donors aged 17 to 62 years from South Wales¹⁵; samples for analysis were obtained prior to first donation. Only TIBC showed any correlation with sFn ($r_s = -0.412$, $P < .001$).

*P value is not significant.

[†] $P < .001$ compared with donors.

FECH activity in dEPP is approximately 35% of normal.¹³ Our data show that this decrease is sufficient to produce in all patients a mild defect of erythropoiesis that impairs hemoglobinization. Defective erythropoiesis persists throughout life and our findings (Table S1 legend) and previous reports^{8,20} suggest that it may not be corrected by oral iron unless there is evidence of coexisting iron loss.

Iron status

Both sexes showed evidence of iron depletion (Table S2). Differences in sFn and transferrin saturation (TS) between women and men suggested that more of the former had iron depletion due to iron loss in addition to abnormalities caused by EPP. Therefore, we restricted detailed analysis of iron indices to the 67 male patients (Hb, 135 ± 9 g/L [13.5 ± 0.9 g/dL]) older than 15 years who had never received iron supplements.

The main abnormality was a marked shift in sFn toward lower values (Figure 1B; Table 1); sFn correlated with Hb ($r_s = 0.415$; $P < .001$). Because protoporphyrin is hepatotoxic and accumulates in the liver in EPP, and liver cell damage may increase sFn, we assessed liver cell function by measuring liver enzymes. One or more of these was increased in 17 (25%) patients; sFn correlated with γ -glutamyl transpeptidase ($r_s = 0.507$; $P < .001$) and alanine aminotransferase ($r_s = 0.392$; $P < .001$) but not with aspartate aminotransferase. Since sFn correlates with mobilizable iron stores,²¹ the downward shift in sFn by approximately two-thirds (Figure 1B; Table 1) suggests that iron stores in dEPP are decreased to a similar extent or a little more if the effect of liver dysfunction is taken into account. Turnbull et al⁸ found that storage iron, determined by venesection, was less than 250 mg in 3 patients; otherwise, quantitative measurements of tissue iron have not been reported in EPP. However, in contrast to iron deficiency due to iron loss, stainable iron is present in erythroblasts.⁹

In homozygous *Fech*^{mlPns} mice, total body iron is normal but iron is redistributed from peripheral tissues to an enlarged hematopoietic spleen.¹¹ Although these mice have liver disease, lower FECH activity and more severe anemia,¹¹ it seems unlikely that FECH deficiency limits erythropoiesis and disturbs iron metabolism by different mechanisms in the 2 species. The anomalous observation in EPP of accumulation of iron in erythroblasts⁹ suggests that there may also be redistribution of iron stores toward the site of erythropoiesis in EPP. Thus, in both species, FECH deficiency appears to provoke a response that leads to accumulation of protoporphyrin IX but prevents accumulation of the other, more toxic, substrate iron.

A second notable feature of iron depletion in dEPP is our finding that sFe (Table 1) and sTfR (18.6 ± 5.1 nM; Figure 1C) concentrations are normal. The normal sTfR in our patients is consistent with the degree of depletion of iron stores indicated by sFn and, together with the normal sFe, suggests that erythropoiesis is not limited by iron supply.²² This indicates that the reduction in iron stores has not led to iron-deficient erythropoiesis. Furthermore, the rate of erythropoiesis is not increased as this would also increase sTfR levels. These findings suggest FECH deficiency in dEPP leads to the establishment of a steady state in which iron absorption and supply is diminished but matches the requirement for reduced erythropoiesis.

The mechanism of these changes in iron metabolism has not been established. Iron metabolism is also altered in griseofulvin-induced protoporphyria.²³ Because serum transferrin is increased in *Fech^{mlPus}* BALB/c mice and correlates with erythrocyte protoporphyrin concentration, it has been suggested that protoporphyrin may act as a signal to increase hepatic transferrin synthesis when iron supply to erythroid cells is insufficient and thus modulate iron metabolism.¹¹ We found only a slight increase in TIBC (Table 1) and no correlation with erythrocyte protoporphyrin. Alternatively, FECH deficiency within enterocytes might affect duodenal iron transport by altering enterocyte mitochondrial iron status.²⁴

Finally, measurement of sTfR, in addition to sFn, may help to distinguish those patients in whom the anemia of EPP is exacerbated by iron loss and who might benefit from iron replacement.²⁵

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Authorship

Contribution: S.A.H. collected the clinical data and patient samples; M.W. and M.N.B. supervised laboratory analyses; A.V.A. supervised patient contact and clinical aspects; G.H.E. and M.W. wrote the paper; and all authors participated in the design of the research and checked the final version of the paper.

Conflict of interest disclosure: The authors declare no competing financial interests.

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Serum 25-hydroxyvitamin D in erythropoietic protoporphyria

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Summary

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Key words

photodermatosis, photoprotection, porphyria, protoporphyria, sunscreen, vitamin D

Conflicts of interest

None declared.

Background Vitamin D, produced by the action of sunlight on skin, is an important hormone for calcium homeostasis and has been implicated as tumour-protective agent. Some previous studies of photosensitive patients who actively avoid sunlight have failed to show convincing evidence of vitamin D insufficiency.

Objectives The aim of this study was to characterize the vitamin D status of a large cohort of patients with erythropoietic protoporphyria (EPP).

Methods U.K. patients with EPP were recruited prospectively and seen locally by a single study investigator. A blood sample was taken for vitamin D assay. All blood analyses were performed in the same laboratory.

Results A cohort of 201 patients with known EPP was seen over a 7 month period between January and July. Thirty-four patients (17%) were deficient in vitamin D and 126 (63%) had insufficient vitamin D. Both insufficiency and deficiency were significantly associated with the total erythrocyte protoporphyrin concentration and inversely with the time in minutes to the onset of symptoms following sunlight exposure.

Conclusions This is the first report of significant levels of vitamin D deficiency and insufficiency in a large cohort of patients with a photodermatosis. Such individuals are at risk of associated adverse events. In future, clinicians should consider monitoring 25-hydroxyvitamin D levels and instigating oral supplementation or dietary advice if appropriate.

Vitamin D is an essential fat-soluble hormone required for bone integrity and calcium homeostasis.¹ It may also protect against the development of other conditions such as diabetes mellitus, hypertension, tuberculosis and some malignancies.²⁻⁴ Approximately 90% of requisite vitamin D is formed within the skin as a result of sunlight photolysis of 7-dehydrocholesterol by ultraviolet (UV) B radiation, before a temperature-dependent isomerization to cholecalciferol.⁵ Previous studies of photosensitive patients with xeroderma pigmentosum (XP) and Smith-Lemli-Opitz syndrome (SLOS) who actively avoid sunlight have failed to show convincing evidence of vitamin D insufficiency.^{6,7}

Erythropoietic protoporphyria (EPP, MIM 177000) is a rare photodermatosis with systemic complications which results from an inherited partial deficiency of ferrochelatase, the terminal enzyme of haem biosynthesis. Excessive formation of its substrate, protoporphyrin IX, results in protoporphyriaemia and accumulation in erythrocytes, plasma, skin and liver, prior to excretion in the bile.⁸ Protoporphyrin can absorb light energy, damaging surrounding tissues through the generation

of free radicals and clinically manifesting as painful photosensitivity within minutes of skin exposure to sunlight. The discomfort may last for several hours, cutaneous tolerance to sunlight may be reduced for several days afterwards and some individuals experience the symptoms even on cloudy days and in winter.⁹ Management of EPP is based mainly on minimizing the acute adverse effects of sunlight by use of broad-spectrum sunscreens, occlusive clothing and behavioural measures to avoid direct sunlight.

Patients and methods

During a prospective study of U.K. patients with EPP,⁹ we sought to characterize their vitamin D status. A cohort of 210 outpatients was seen over a 7-month period between January and July, representing a period with minimal and maximal vitamin D levels in normal populations,¹⁰ at latitudes ranging from 51°N to 57.5°N. Each provided a blood sample for vitamin D assay and all analyses were performed in the same laboratory using a commercial radioimmunoassay (Diasorin Ltd,

Wokingham, U.K.). Serum 25-hydroxyvitamin D concentrations of < 10 and < 20 ng mL⁻¹ (25 and 50 nmol L⁻¹) were used to identify those who were vitamin D deficient (VDD) or insufficient (VDI).¹¹ Nine patients were withdrawn from the analysis due to nonwhite skin coloration ($n = 2$: both VDI), systemic malignancy ($n = 2$), renal failure ($n = 1$), hepatic failure ($n = 2$), post-orthotic liver transplant ($n = 1$) and a patient taking ergocalciferol ($n = 1$). Statistical analysis was performed using the Mann-Whitney test.

Eighty per cent of the cohort regularly avoided sunlight, 87% wore long-sleeved occlusive clothing daily, 9% used a sunscreen at least once daily all year and 68% used sunscreen once daily or more frequently in sunny weather. No patients had ever had their vitamin D status checked by their physicians. Five patients reported coexistent osteoporosis, but other than analgesics, were not taking any other treatments for this. Three patients took fish liver oils daily as a health supplement, one of whom also took a calcium supplement. One further patient took calcium supplementation. Excepting a nonvegan vegetarian, all patients were omnivores; a more detailed dietary history was not taken.

Results

The mean serum hydroxyvitamin D was 18.32 ng mL⁻¹ (range 4.9–51.4, quartiles 11.5, 23.5). One hundred and twenty-six patients (63%; 58 males, 68 females) were VDI, of whom 34 were VDD (17%; 15 males, 19 females). Of three patients taking dietary fish oil supplements, one was VDI. Of the twenty-one patients receiving UVB phototherapy to induce 'hardening' of their skin to sunlight sensitivity, only six (29%) were VDI and none was VDD.

The mean monthly serum 25-hydroxyvitamin D rose over the study period January to July from 15.5 to 21.3 ng mL⁻¹ (Fig. 1). In the winter months of January and February 70% of patients (19 of 27) were VDI and 44% (seven of 27) were VDD; in the summer months of June and July 45% (34 of 75) were VDI and 37% (28 of 75) VDD. There appeared to be a slightly smaller proportion of children aged 16 years or under who were VDI or VDD compared with the overall population [three of 34 (11%) vs. 18 of 92 (20%)]. One hundred and eighty-one complete sample sets were available for analysis of calcium, phosphate and parathyroid hormone (PTH) biochemistry. Thirteen patients (7%) were deficient in adjusted serum calcium (11 VDI, of whom three were VDD) and 41 (23%) had an elevated serum phosphate (21 VDI, of whom three were VDD). Hyperparathyroidism was seen in 12 patients, of whom nine were VDI (two VDD) and the remaining three had serum 25-hydroxyvitamin D at the lower end of the normal range, at between 21 and 23 ng mL⁻¹.

Statistical analysis suggested that being VDI was associated with total erythrocyte protoporphyrin (TEP) ($P = 0.009$) and inversely associated with the time in minutes to the onset of symptoms following sunlight exposure ($P = 0.008$). Being VDD was associated with the age at symptom onset ($P < 0.0005$), TEP ($P = 0.02$) and inversely with minutes to

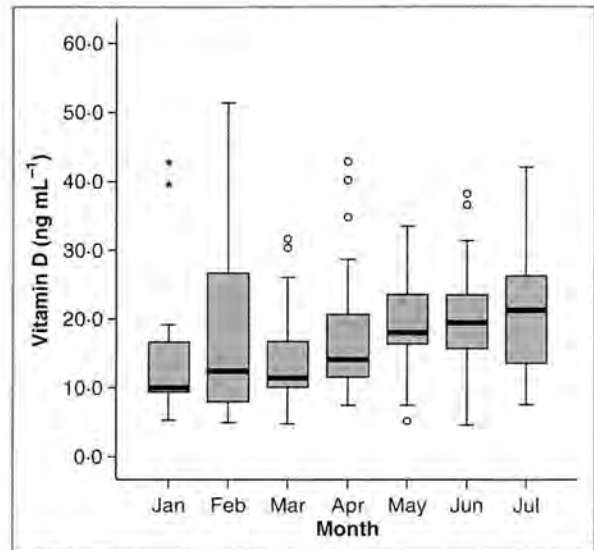


Fig 1. Boxplot of serum 25-hydroxyvitamin D by month of sampling (deficiency < 10 ng mL⁻¹, insufficiency < 20 ng mL⁻¹). The boxes contain results between the upper and lower quartiles and the dark bars within the boxes represent the median value. The whiskers represent smallest and largest values which are not outliers, while the circles are outliers (more than 1.5 box lengths above or below the box) and the stars extreme outliers (more than 3 box lengths above the box).

symptom onset ($P = 0.03$). There was no association with calcium deficiency, raised phosphorus or elevated PTH, although elevated PTHII approached significance with VDI status ($P = 0.57$).

Discussion

We have shown a high prevalence of VDD and VDI status in a large cohort of patients with EPP, whose main risk factors were latitude of residence and their photodermatosis. In keeping with previous findings, we demonstrated an increase in median 25-hydroxyvitamin D between winter and summer.^{7,10,12,13} However, a sizable proportion was VDI even in summer, implicating the photodermatosis and sun-avoidance measures as the most plausible explanation, and supporting the demonstrated association with sensitivity and TEP.

Although previously recognized in cases reports, studies of photosensitive populations with XP and SLOS, and normal populations using sunscreens, have not shown similar levels of vitamin D insufficiency.^{6,7,12,13} Explanations include low study sensitivities due to smaller patient numbers, residency in sunnier environments, lack of skin discomfort in XP (an efficient prompt for rigorous sunlight avoidance in EPP), or the presence in SLOS of abnormally high concentrations of the vitamin D precursor, 7-dehydrocholesterol.

Since completion of this study, controversy over the correct values of the 25-hydroxyvitamin D normal range⁵ has led to

our laboratory now using 30 ng mL⁻¹ as the lower end of the normal range, increasing the proportion of VDI patients in our cohort to 91%. The findings of this study suggest that a sizeable proportion of an EPP cohort is VDI and at risk of important clinical outcomes. Treatment with oral calcium and vitamin D increases bone mass and reduces the risk of fractures,^{14,15} so clinicians advising patients with EPP about sunlight avoidance should consider monitoring both serum 25-hydroxyvitamin D and PTH, and giving supplementation throughout the year. As the cutaneous synthesis of vitamin D is initiated by UVB-mediated photolysis of 7-dehydrocholesterol, use of UVB phototherapy to reduce symptoms appears to have additional therapeutic benefit.

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Skin Cancer, Sunlight and Vitamin D Deficiency

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Sir,

The BMJ editorial by Professor Michael Holick on Vitamin D deficiency highlights a dilemma for clinicians and public health physicians when advising patients and the public on sun exposure. [1] Too much sun is the main risk factor for skin cancer; on the other hand, too little sun may lead to vitamin D deficiency. Holick has reported previously that white populations may become vitamin D deficient in winter and that only minimal exposure to ultraviolet (UV) radiation (5-10 minutes sun exposure three times a week of hands, arms and face) is required during spring, summer and autumn to reverse this. [2] The message from the articles by Holick [1] and Sievenpiper [3] highlighting the consequences of vitamin D deficiency should not confuse the health promotion message emphasising the importance of UV protection to prevent skin cancer. Non-melanoma skin cancer is now the UK's commonest malignancy with a steeply rising incidence, and malignant melanoma is the commonest cancer in the 15-34 year group. [4]

In his editorial figure, Holick suggests sunscreen use to be a cause of vitamin D deficiency. This is misleading as there is no convincing evidence to support this claim in normal populations not actively avoiding sunlight, although sunscreen use does appear to cause a modest reduction in 25-hydroxyvitamin D levels. [5,6] In cases where sunscreen use has been implicated in deficiency, other risk factors such as ethnic skin pigmentation, institutional residency, age or occlusive clothing, have also been present. Two recent publications have reported an additional risk factor for vitamin D deficiency to be the presence of severe photosensitivity such as occurs in erythropoietic protoporphyria or cutaneous lupus erythematosus. [7,8] It is individuals with risk factors for vitamin D deficiency, such as the Asian women described by Sievenpiper, who should be targeted for education or assessment, not the white population in whom the risk of skin cancer is higher.

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C-Terminal Deletions in the *ALAS2* Gene Lead to Gain of Function and Cause X-linked Dominant Protoporphyrin without Anemia or Iron Overload

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All reported mutations in *ALAS2*, which encodes the rate-regulating enzyme of erythroid heme biosynthesis, cause X-linked sideroblastic anemia. We describe eight families with *ALAS2* deletions, either c.1706-1709 delAGTG (p.E569GfsX24) or c.1699-1700 delAT (p.M567EfsX2), resulting in frameshifts that lead to replacement or deletion of the 19–20 C-terminal residues of the enzyme. Prokaryotic expression studies show that both mutations markedly increase *ALAS2* activity. These gain-of-function mutations cause a previously unrecognized form of porphyria, X-linked dominant protoporphyrin, characterized biochemically by a high proportion of zinc-protoporphyrin in erythrocytes, in which a mismatch between protoporphyrin production and the heme requirement of differentiating erythroid cells leads to overproduction of protoporphyrin in amounts sufficient to cause photosensitivity and liver disease.

Each of the seven inherited porphyrias results from a partial deficiency of an enzyme of heme biosynthesis. Mutations that cause porphyria have been identified in all the genes of the heme biosynthetic pathway except *ALAS1* and *ALAS2*, which encode the ubiquitously expressed (*ALAS1*) and erythroid-specific (*ALAS2*) isoforms of mitochondrial 5-aminolevulinic synthase (*ALAS*) (EC 2.3.1.37), the initial, rate-regulating enzyme of the pathway.¹ *ALAS2* is essential for hemoglobin formation by erythroid cells, and *ALAS1* cannot replace this function. No mutations have been identified in *ALAS1*, but pathogenic mutations in the 14.4 kb, 11-exon-containing *ALAS2* cause X-linked hereditary sideroblastic anemia (XLSA [MIM 301300]) with iron overload.²

Erythropoietic protoporphyria (EPP [MIM 177000]) is an inherited disorder caused by partial mitochondrial deficiency of ferrochelatase (*FECH*) (EC 4.99.1.1), the terminal enzyme of heme biosynthesis. Accumulation of protoporphyrin IX in erythrocytes and other tissues leads to lifelong photosensitivity and, in about 2% of patients, severe liver disease.³ Most patients have autosomal-dominant EPP (dEPP), in which clinical expression normally requires co-inheritance of an *FECH* mutation that abolishes or markedly reduces *FECH* activity *trans* to a hypomorphic *FECH IVS3-48C* allele carried by about 11% of western Europeans.⁴ About 4% of families have autosomal-recessive EPP.⁴ However, mutational analysis fails to detect *FECH*

mutations in about 7% of EPP families, of which about 3% are homozygous for the wild-type *FECH IVS3-48T* allele,⁵ suggesting possible involvement of another locus.

Within this subgroup of families with mutation-negative EPP, we studied eight families in which at least one individual had acute photosensitivity clinically indistinguishable from that of dEPP. These families were identified through referral to specialist porphyria centers or during surveys of EPP in the UK⁶ or South Africa⁷ and were of western European (four families), Jewish, north African, Indo-Asian, or Sudanese (one family each) ancestry. Our study was conducted in accord with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments. All patients or their parents gave informed consent to investigation. Prior ethical approval was obtained for patient surveys.^{6,7}

Genomic DNA was extracted from whole blood. For sequencing of *ALAS2*, *FECH*, and *SLC25A37* (MIM 610387) (GenBank accession numbers: human *ALAS2* [NM000032.3], *FECH* [NM000140], and *SLC25A37* [NM0166112] cDNAs and *ALAS2* [NT011630] and *SLC25A37* [NT023666] genes), all exons and their flanking sequences were amplified by polymerase chain reaction (PCR) (primers and conditions are available from the authors). PCR-amplified double-stranded DNA was purified from agarose gels with the QIAquick gel extraction kit

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Table 1. Erythrocyte Porphyrins, Hematological Measurements, and Serum Iron Indices in Patients with X-Linked Dominant Protoporphyrinuria and Their Unaffected Relatives

| | XLDPP | | Unaffected Relatives | |
|--------------------------------------|-------------------------------|---------------------|-----------------------------|---------------------|
| | Male | Female | Male | Female |
| Total protoporphyrin (fold increase) | 27, 11–103 (7) | 24, 6–64 (16) | <i>Less than 1.0</i> | |
| Zinc protoporphyrin (% total) | 45, 19–58 (7) | 46, 30–65 (15) | <i>Less than 80</i> | |
| Hemoglobin (g/dl) | 14.3, 12.2–17.5 (5) | 13.0, 12.4–14.1 (8) | 14.2, 13.1–14.7 (8) | 12.5, 11.9–14.2 (5) |
| MCV (fl) | <i>87, 78–92 (13)</i> | | <i>88, 84–96 (13)</i> | |
| Ferritin ($\mu\text{g/liter}$) | 24, 10–104 (5)* | 56, 21–154 (6) | 102, 41–231 (8) | 51, 33–91 (5) |
| Transferrin (g/liter) | <i>2.87, 1.93–3.81 (9)</i> | | <i>2.27, 1.59–2.84 (13)</i> | |
| Transferrin saturation (%) | <i>16, 3–39 (11)*</i> | | <i>26, 22–35 (13)</i> | |
| Transferrin receptor-1 (mg/liter) | <i>1.44, 1.24–2.59 (10)**</i> | | <i>1.16, 0.84–1.66 (13)</i> | |

Measurements are medians and ranges. * $p = 0.02$ versus unaffected male relatives; ** $p = 0.04$ versus unaffected relatives; differences between other groups are not significant. Total porphyrin is expressed as n-fold increase (times upper limit of normal); for 17 patients with XLDPP in whom total erythrocyte porphyrin was measured by the same method,⁶ the median concentration was 51.2 $\mu\text{mol/liter}$, range 20.1–195.6 $\mu\text{mol/liter}$ (reference range: less than 1.7 $\mu\text{mol/liter}$). Values in italics include male and female subjects.

(QIAGEN, Crawley, UK) before being cycle sequenced with fluorescent ddNTPs (BigDye) and an ABI Prism 3130XL Genetic Analyzer (PE Biosystems, Warrington, UK). We confirmed the presence or absence of mutations by sequencing both strands. Genotyping with *FECH* intragenic single-nucleotide polymorphisms (SNPs),⁴ and microsatellite markers for *FECH*⁷ and *ALAS2* was performed on the ABI PRISM 3100 automated sequencer. The *ALAS2* microsatellite markers (16AG at position 54992283, 17GT at position 55066050, and 23AC at position 55535957) were identified at the UCSC genome bioinformatics site (Santa Cruz, CA; see Web Resources). Results were analyzed with the ABI PRISM GeneMapper software version 3.0. Erythrocyte porphyrins were measured as previously described.^{7,8} The percentage of zinc protoporphyrin was calculated from fluorescence emission spectra of ethanol⁸ or acetone⁹ extracts of erythrocyte haemolysates. *FECH* activity was measured as described⁴ or indirectly from the amount of protoporphyrin formed from 5-aminolevulinic acid in the presence and absence of Fe^{2+} .¹⁰ Differences between quantitative variables were assessed with the Mann-Whitney test, and those between proportions were assessed with Fisher's exact test.

We differentiated patients in these eight families from others with *FECH* mutation-negative EPP by showing that the percentage of erythrocyte protoporphyrin present as its zinc chelate (19%–65%, median 44%) was markedly greater than in patients with dEPP (4%–13%, median 8%). Erythrocyte protoporphyrin concentrations were also higher in our patients, in whom they were increased 24-fold (range: 6- to 103-fold) (Table 1) compared with 14-fold (range: 4- to 44-fold) in 171 patients with dEPP ($p < 0.001$). In one patient with iron deficiency, erythrocyte protoporphyrin increased markedly (101-fold) but then decreased as iron stores were replenished (Figure 1). Lymphocyte *FECH* activity, measured in ten patients, ranged from 74%–106% (median 85%) of the mean normal value, indicating that protoporphyrin accumulation

was not caused by *FECH* deficiency resulting from a mutation of the ubiquitously expressed *FECH* gene. We further eliminated involvement of *FECH* by using intragenic SNPs⁴ or microsatellite markers⁷ to show that protoporphyrin accumulation did not segregate with *FECH* haplotypes in two families; other families were uninformative or not tested. Because abnormal expression of mRNA for mitoferrin has been implicated in the pathogenesis of a similar form of protoporphyrinuria (Shaw et al., Blood 108, ASH Annual Meeting Abstracts, 6a), we sequenced all exons of *SLC25A37* and their flanking sequences in all eight probands but were unable to identify any disease-specific mutation.

Parent-child transmission of overt disease is uncommon in EPP.¹¹ Our families were unusual in showing an apparent dominant pattern of inheritance with an absence of father-son transmission, which suggested X-linkage (Figure 2). We therefore investigated two candidate genes that are located on the X chromosome and are involved in heme formation, *GATA1* (MIM 305371) (data not shown) and *ALAS2*. Protoporphyrin accumulation segregated with an X chromosome haplotype defined by microsatellite markers around *ALAS2* in three families (data not shown). Sequencing of genomic DNA identified two different deletions (c.1706–1709 delAGTG in six families; c.1699–1700 delAT in two families) in *ALAS2* exon 11, an exon that is present in all *ALAS2* transcripts.¹² The frame-shifts produced by these deletions lead to predicted alterations of the 19–20 C-terminal amino acids of *ALAS2* (Figures 3A and 3B); either deletion (delAT) or replacement by a 23 residue sequence (delAGTG) that extends the enzyme by 4 amino acids and alters the predicted secondary structure (Supplemental Data; Figure 1). These mutations segregated with photosensitivity (LOD score 7.8) and were absent from 129 unrelated EPP patients (106 dEPP; 23 *FECH*-mutation-negative EPP) and 100 normal chromosomes. The delAGTG mutation occurred on five

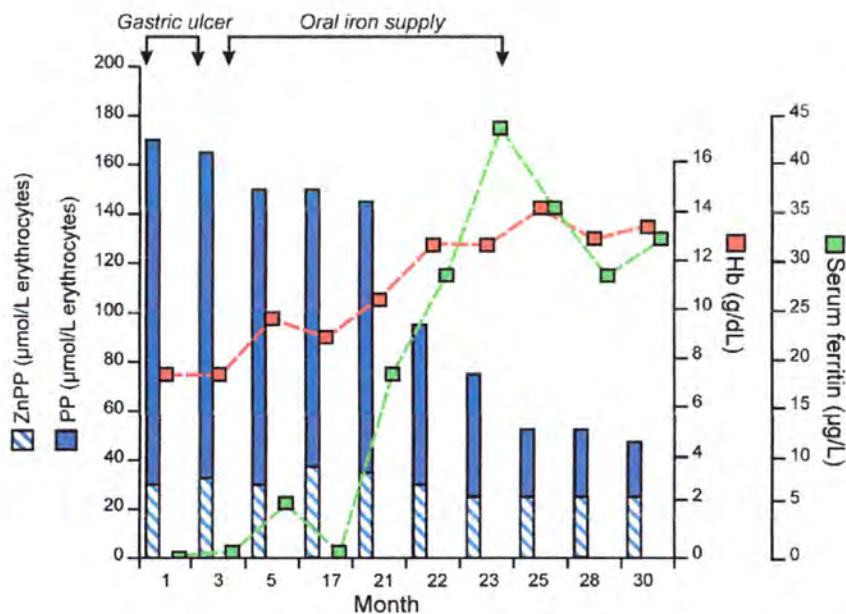


Figure 1. Treating Iron Deficiency Decreases Erythrocyte Protoporphyrin Concentrations

Iron deficiency caused by bleeding from a gastric ulcer was treated with omeprazole and oral iron.

null *ALAS2* mutations causing XLSA have been embryonically lethal in males.² In our families, both sexes were affected, and patients had neither anemia nor iron overload (Table 1). Instead, there was some evidence of diminished iron stores, particularly in males (Table 1). Similar abnormalities have been observed in dEPP¹⁴ and might result from accumulation of protoporphyrin rather than FECH deficiency.¹⁵ Five (17%) patients had overt liver disease, suggesting that

different haplotypes, indicating that it has arisen on at least five separate occasions, whereas the two delAT families, both from southwest England, had the same background haplotype and may come from a single extended family (data not shown). These findings show that these deletions in *ALAS2* cause a previously unrecognized X-linked dominant protoporphyria (XLDPP) that, in contrast to dEPP and other autosomal-dominant porphyrias,¹ has close to 100% penetrance (Figure 2).

All previously described mutations in *ALAS2* have caused XLSA (Human Gene Mutation Database), including a missense mutation (S568G) in the region lost or replaced in our patients.¹³ Previously reported frameshift or other

XLPP, like autosomal recessive EPP^{3,4}, carries a higher risk of liver disease than dEPP. Liver disease was more common in males ($p = 0.008$), and one obligate carrier was asymptomatic (Figure 2; family G, II4), but otherwise we found no evidence that X inactivation led to milder disease in females. Erythrocyte protoporphyrin concentrations in photosensitive patients were not significantly different between the sexes (Table 1). These data show that disruption of the C-terminal region of *ALAS2* leads to the production of protoporphyrin in excess of the amount required for hemoglobinization and in quantities sufficient to cause photosensitivity and liver damage, in spite of normal FECH activity; this is a situation unique in human disease.

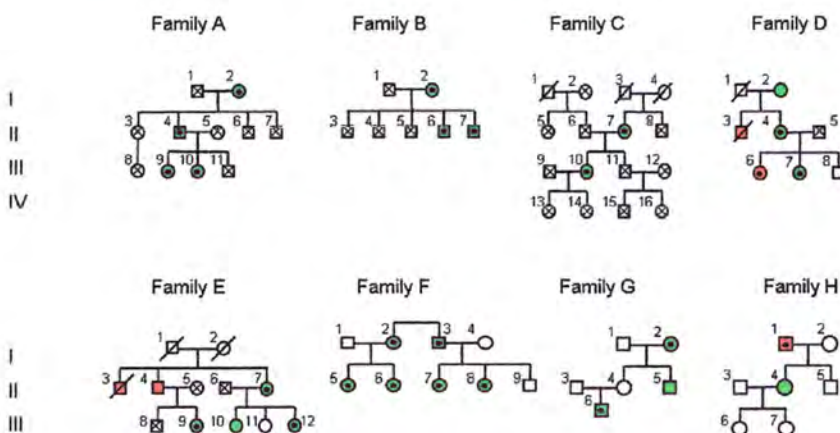


Figure 2. Pedigrees of Eight Families with FECH-Mutation-Negative Protoporphyrin

Green circles and squares represent patients with photosensitivity. Red circles and squares represent patients with photosensitivity and clinically overt liver disease. Protoporphyrin liver disease was confirmed at autopsy or by needle biopsy in all these patients except patient I1 (family H), for whom a diagnosis has not been established. Patient II3 (family E) has been reported previously.²³ Clinical information was not obtainable for patients C I, 3 and 4 or E I, 1 and 2.

Black dots within circles or squares indicate individuals in whom either the delAGTG (families A–E, H) or delAT (families F and G) *ALAS2* mutations were identified.

Crosses within circles or squares indicate individuals in whom sequencing excluded the presence of an *ALAS2* mutation. The absence of a black dot or cross indicates an individual from whom a DNA sample was not available for analysis. The LOD score for linkage between photosensitivity and the *ALAS2* mutation was calculated for families A–E.

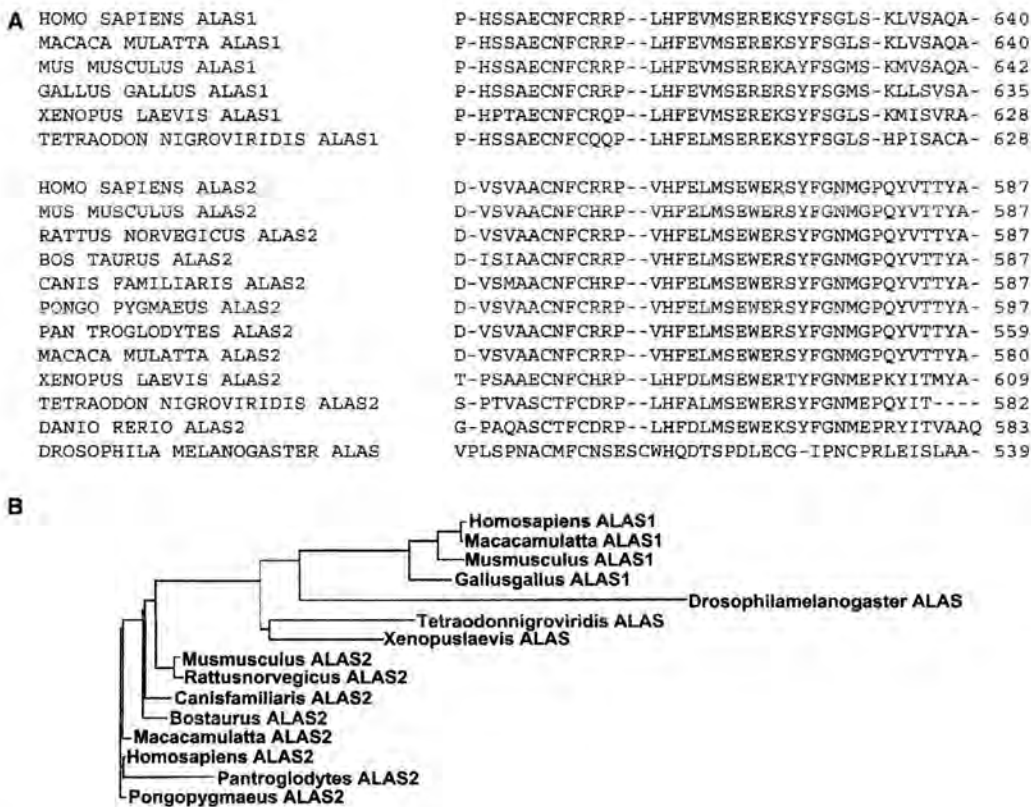


Figure 4. Comparison of C-terminal Sequences of ALAS enzymes

(A) Alignment of ALAS C-terminal sequences.

(B) Phylogenetic tree showing relationships between ALAS genes constructed with ClustalW.

its alternative metal substrate, suggests that formation of excess heme can be prevented by lack of available iron. The phenotype of iron deficiency in XLDPP (Figure 1) closely resembles that of *Ireb2*^{-/-} mice, in which deletion of iron-regulatory protein 2 (IRP2) leads to overexpression of ALAS2, erythroblast iron deficiency, and microcytic anemia.¹⁸ In the patient whose clinical course is shown in Figure 1, iron repletion decreased protoporphyrin accumulation and corrected the anemia. However, zinc protoporphyrin did not decrease, as it does in uncomplicated iron deficiency.¹⁹ This might indicate that the synthesis of zinc protoporphyrin becomes limited by intra-mitochondrial availability of Zn²⁺ when the protoporphyrin pool is greatly expanded.¹⁸ These findings are consistent with the hypothesis that the regulatory system that enables efficient utilization of iron for heme synthesis during erythroid differentiation²⁰ allows matching of erythroblast iron uptake to intra-mitochondrial heme synthesis to be maintained even when excess protoporphyrin is present. The mechanism by which this is achieved is unknown but might involve regulation of transferrin receptor-1 expression in erythroblasts through heme-mediated degradation of IRP2.²¹

The 26 C-terminal amino acids of ALAS2 are highly conserved and have diverged from ALAS1 (Figure 4), which suggests that this sequence might have an important, but unknown, erythroid-specific function. During erythropoi-

esis, tight coordination of substrate supply to FECH normally prevents accumulation of toxic amounts of protoporphyrin. Coordination is largely achieved through iron-dependent post-transcriptional regulation of synthesis of ALAS2.² This system fails in XLDPP because the mutations that we have described greatly increase ALAS2 activity. A possible mechanism might be stabilization against degradation or an intrinsic increase in specific activity. This C-terminal sequence is not present in ALAS_{RC}, the only ALAS for which the crystal structure has been reported.²² Sequence similarities between ALAS_{RC} and human ALAS2²² suggest that it is not directly involved in pyridoxal 5-phosphate-dependent catalysis. Our findings indicate that it modulates enzyme activity, but the mechanism of this effect remains to be determined. The discovery of gain of function mutations in ALAS2 identifies a previously undefined type of human porphyria, provides new information about the regulation of substrate supply for heme synthesis during erythroid differentiation, and identifies a potential tool for increasing erythroid heme synthesis in experimental systems.

Supplemental Data

Supplemental Data include one figure, available online at <http://www.ajhg.org/>.

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Web Resources

URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for EPP, XLSA)

Human Gene Mutation Database (HGMD), <http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html/>

For ALAS2 microsatellite markers, <http://www.genome.ucsc.edu/>
Protein sequence comparisons, ClustalW, <http://www.ebi.ac.uk/clustalW/>

Network protein sequence analysis, http://pbil.univ-lyon1.fr/pf_bioinfo/rubrique9.html

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Seasonal Palmar Keratoderma in Erythropoietic Protoporphyrinemia Indicates Autosomal Recessive Inheritance

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Erythropoietic protoporphyria (EPP) is an inherited disorder that results from partial deficiency of ferrochelatase (FECH). It is characterized clinically by acute photosensitivity and, in 2% of patients, liver disease. Inheritance is usually autosomal dominant with low penetrance but is recessive in about 4% of families. A cross-sectional study of 223 patients with EPP in the United Kingdom identified six individuals with palmar keratoderma. We now show that these and three additional patients, from six families, have an inherited subtype of EPP which is characterized by seasonal palmar keratoderma, relatively low erythrocyte protoporphyrin concentrations, and recessive inheritance. No patient had evidence of liver dysfunction; four patients had neurological abnormalities. Patients were hetero- or homoallelic for nine different *FECH* mutations; four of which were previously unreported. Prokaryotic expression predicted that FECH activities were 2.7–25% (mean 10.6%) of normal. Neither mutation type nor FECH activity provided an explanation for the unusual phenotype. Our findings show that palmar keratoderma is a clinical indicator of recessive EPP; identify a phenotype that occurs in 38% of reported families with recessive EPP that to our knowledge is previously unreported, and suggest that patients with this phenotype may carry a lower risk of liver disease than other patients with recessive EPP.

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INTRODUCTION

Erythropoietic protoporphyria (EPP) is an inherited disease characterized by life-long acute photosensitivity with minimal cutaneous signs and occasional liver dysfunction (Todd, 1994; Cox, 2003). It results from partial deficiency of the enzyme ferrochelatase (FECH) which leads to accumulation of the photosensitizing pigment, protoporphyrin, in erythrocytes, plasma, skin, and liver. Reduction of FECH activity to below about 35% of normal is required for protoporphyrin to

accumulate sufficiently to cause photosensitivity (Nordmann and Deybach, 1990). In most families, this decrease is brought about by coinheritance of a hypomorphic *FECH* IVS3-48C allele *trans* to a *FECH* mutation that markedly decreases or abolishes FECH activity (Gouya *et al.*, 2002; Gouya *et al.*, 2006). The hypomorphic allele is present in 13% of the United Kingdom population (Whatley *et al.*, 2004). In these families, EPP is inherited as a dominant disorder with low clinical penetrance (Gouya *et al.*, 2002, 2006). More rarely, EPP may be transmitted as an autosomal recessive trait with photosensitivity resulting from the presence of *FECH* mutations on both alleles (Gouya *et al.*, 2006). To date, only 12 families with autosomal recessive EPP have been reported (Lamoril *et al.*, 1991; Sarkany *et al.*, 1994a; Poh-Fitzpatrick *et al.*, 2002; Whatley *et al.*, 2004; Gouya *et al.*, 2006; Herrero *et al.*, 2007). Recessive EPP carries a higher risk of liver disease than the dominant form (Sarkany *et al.*, 1994a; Whatley *et al.*, 2004; Gouya *et al.*, 2006; Herrero *et al.*, 2007).

Chronic skin lesions in EPP are the result of repeated episodes of acute photosensitivity. They are restricted to sun-exposed areas and rarely amount to more than minor pitting and scarring of the face with some waxy thickening of the skin, particularly over the joints on the backs of the hands (Schmidt *et al.*, 1974; DeLeo *et al.*, 1976; Todd, 1994; Holme *et al.*, 2006). In a cross-sectional study of 223 patients with

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Abbreviations: EPP, erythropoietic protoporphyria; FECH, ferrochelatase

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EPP (Holme et al., 2006), six patients were found to have palmar keratoderma; a chronic skin condition that had not previously been recognized as a feature of EPP. Here we show that these and three additional patients have a previously unreported inherited subtype of EPP characterized by palmar keratoderma, relatively low erythrocyte protoporphyrin concentrations and autosomal recessive inheritance.

RESULTS

Palmar keratoderma is an uncommon feature of EPP

We identified nine patients with EPP and palmar keratoderma; five males and four females, aged 8–63 years, from six families (Table 1). Six of these, from five families, were identified during a cross-sectional study of the clinical features of 223 patients with EPP in the UK, of whom 193 were unrelated, (Holme et al., 2006), giving prevalences for palmar keratoderma in EPP of 2.7% for all patients and 2.6% (95% confidence interval: 1.0–5.1%) for families.

In all patients, hyperkeratosis had been present since early childhood and in seven patients had developed before the diagnosis of EPP was made. In three patients the development of mild palmar keratoderma was preceded during infancy by a scaly rash, mainly on the dorsum of the hands, initially thought to be eczema; a third patient has areas of hyperpigmentation and lichenified skin over her knees, neck, and elbows which were present before the onset of photosensitivity and have persisted. Hyperkeratosis was worse in summer and often resolved in winter. In one patient, occlusion of the skin with a plaster cast for 6 weeks following a wrist fracture led to almost complete resolution of the keratoderma.

The hyperkeratosis ranged in severity from waxy keratoderma over the whole palm to mild hyperkeratosis of the first interdigital web (Figure 1; Table 1). Palmar keratoderma was sharply demarcated at the wrist with, in most cases, minimal transgression onto the extensor surface, and without an erythematous border (Figure 1). Two patients had mild onycholysis but otherwise nails were not affected. Sweating appeared unimpaired. All patients had thickened skin over their knuckle joints, a sign which is present in 35% of 223 UK patients with EPP (Holme et al., 2006). This thickening was mild or moderate in severity. No patient with dominant EPP and thickened knuckle skin, even those with severe thickening and scarring, showed any evidence of changes on the palmar surface. Two unrelated patients also had mild plantar hyperkeratosis (Table 1). Hyperkeratosis was not particularly marked at sites of pressure or punctate in pattern. No patient reported blistering or hyperhidrosis. Only a very small punch biopsy from the hand of one patient (patient 2) was examined histopathologically. The limited amount of epidermis present was slightly thickened and spongiotic and there were some prominent upper dermal vessels with a hint of periodic acid Schiff (PAS)-positive hyaline change. There was not enough epidermis to determine whether the keratin layer was thickened.

The severity of photosensitivity varied within families but was similar to that in patients with EPP without keratoderma (Holme et al., 2006). All became photosensitive between the ages of 3 and 17 months. Symptoms started within 1–30 minutes after exposure to sunlight and quality of life, assessed by childhood dermatology quality of life index or dermatology quality of life index scores (Holme et al., 2006), was severely impaired in the six patients in which it was measured (mean scores 12, range 5–18).

Table 1. EPP with keratoderma: clinical features

| Family | Patient | Sex | Age (years) | Onset age (years) | Family history | RBC porphyrin ($\mu\text{mol l}^{-1}$) | Keratoderma |
|-----------------|---------|-----|-------------|-------------------|------------------|--|--|
| I | P1 | F | 8 | 1.4 | sib ¹ | 2.3 | Confluent waxy palmar hyperkeratosis with fine peeling; sharp cutoff at wrists. |
| | P2 | M | 10 | 1.0 | sib ¹ | 2.0 | Hyperkeratosis and peeling of first interdigital web and patchy keratosis and peeling over palmar surface of digit joints and pulps. |
| II ² | P3 | F | 58 | 0.5 | sib | 7.4 | Palmar hyperkeratosis with sharp cutoff at wrist; transgression to involve first interdigital web; focal plantar hyperkeratosis with fissuring over heels and MCT joints |
| | P4 | M | 52 | 0.4 | sib | 6.2 | Mild hyperkeratosis of first interdigital web. |
| III | P5 | M | 27 | 0.3 | — | 15.5 | Confluent palmar hyperkeratosis with peeling; lateral aspects dry, white, and cracked; slight onycholysis; hyperkeratosis on medial aspect of forefoot and great toes. |
| IV | P6 | M | 63 | <1 | — | 7.9 | Marked keratosis of first interdigital web and radial border of index fingers with fissuring at index finger joints. |
| V | P7 | F | 17 | 0.25 | — | 6.8 | Waxy keratoderma over the whole palm; mild fine peeling; more obvious in summer, regresses in winter; sharp cutoff at wrist. Nail dystrophy. |
| VI | P8 | M | 13 | 0.25 | sib | 10.5 | Mild hyperkeratosis of first interdigital web. |
| | P9 | F | 12 | 2.0 | sib | 11.2 | Mild hyperkeratosis of first interdigital web |

RBC, red blood cell

¹Great grandmother has EPP without keratoderma (see text).

²Consanguineous parents.

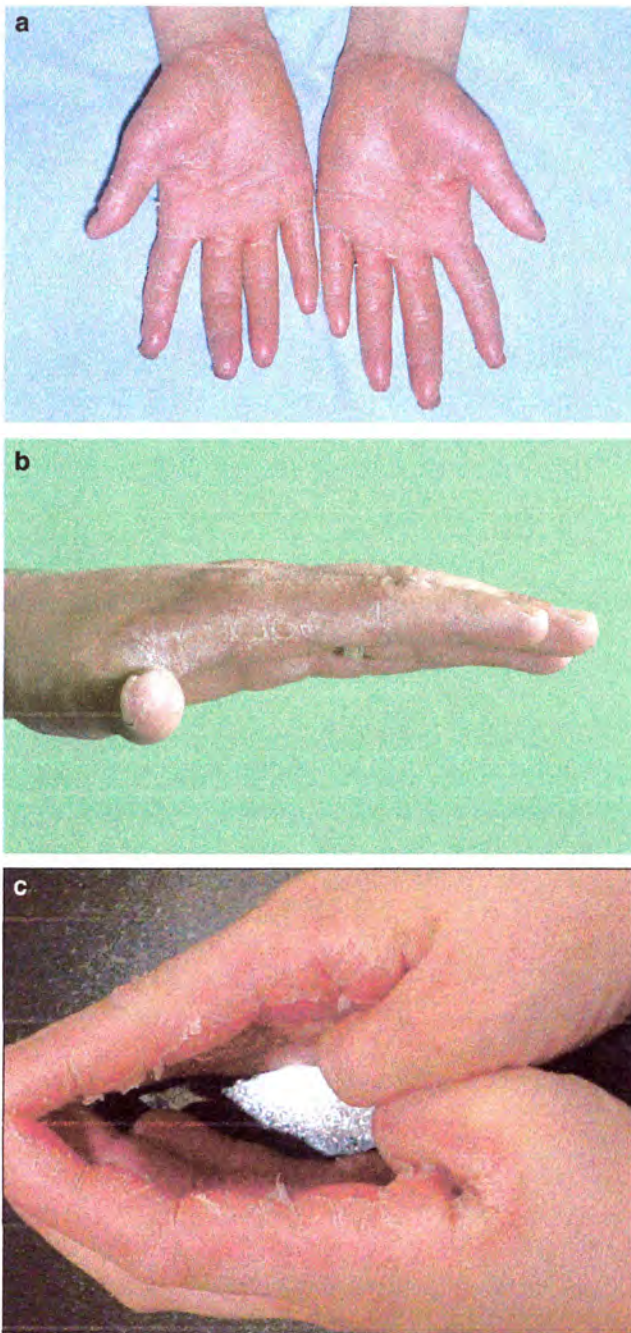


Figure 1. Palmar keratoderma in EPP. For clinical details see Table 1; (a) patient 7, (b) patient 6, (c) patient 2.

Four patients from three families had neurological abnormalities. Three patients (P7, P8, P9) had slight or moderately severe cognitive and motor developmental delay, without regression since early childhood, and one of these suffered from fits until the age of 2 years. Older siblings in both families had neither EPP nor developmental delay. One patient (P4) presented in his early 30s with a spastic paraparesis that has progressed; extensive investigations to identify a cause were negative. Hemoglobin concentrations

and biochemical tests of liver function were normal in all patients, except for one female patient who had a hemoglobin concentration of $11.9 \text{ g (100 ml)}^{-1}$.

Erythrocyte protoporphyrin concentrations are lower in EPP with keratoderma than in EPP without keratoderma

Erythrocyte porphyrin concentrations in the 9 patients with keratoderma (median $7.4 \mu\text{mol l}^{-1}$, range $2.0\text{--}15.5 \mu\text{mol l}^{-1}$; normal subjects, $0.4\text{--}1.7 \mu\text{mol l}^{-1}$) were significantly lower than in 203 patients without keratoderma (median $22.8 \mu\text{mol l}^{-1}$, range $4.1\text{--}159.2 \mu\text{mol l}^{-1}$) ($P < 0.001$). When two members of the same family were affected, erythrocyte porphyrin concentrations were similar. The percentage of free erythrocyte protoporphyrin (41–91%) was increased in all patients but in those with the lowest total erythrocyte porphyrin was only approximately equal in amount to zinc-protoporphyrin. In contrast, the plasma protoporphyrin concentrations in the patients with keratoderma (median 55.0 nmol l^{-1} , range $13.4\text{--}115.1 \text{ nmol l}^{-1}$; normal subjects, less than 10.5 nmol l^{-1}) did not differ significantly from those in 11 randomly selected patients with EPP without keratoderma (median 68.4, range 18.8–396.0) whose erythrocyte porphyrin concentrations ranged from 12.4 to $159.2 \mu\text{mol l}^{-1}$ (median $30.1 \mu\text{mol l}^{-1}$). Fecal total porphyrin excretion was substantially increased (median $1,198 \text{ nmol g}^{-1}$ dry weight; range $523\text{--}1,940 \text{ nmol g}^{-1}$; normal subjects, less than 200 nmol g^{-1}) in the seven patients in whom it was measured, with protoporphyrin accounting for greater than 80% of the total.

EPP with palmar keratoderma is inherited in an autosomal recessive pattern

Three patients had no family history of overt EPP or palmar keratoderma. In families II and VI, two siblings had EPP with keratoderma but no other relative had either condition. In family I, two of three siblings had EPP with keratoderma; the third had neither condition but their maternal great grandmother had a lifelong history of photosensitivity without keratoderma. Photosensitivity and keratoderma always occurred together when more than one member of the same generation had EPP and overall keratoderma was not reported in any family member who did not have EPP.

Mutational analysis showed that all patients with EPP and palmar keratoderma were either compound heterozygous or homozygous for *FECH* mutations and that only one patient (family III) had inherited the hypomorphic *FECH* IVS3-48C allele (Table 2). All but one of the mutations were missense; four of these (c.0302T>C; c.0854A>G; c.0898G>T; c.0502C>T) are previously unreported. Mutational analyses of families II and VI have been reported (Whatley *et al.*, 2004). Both patients in family I were compound heterozygotes for a missense mutation and a mutation (IVS3 + 2T>G) that is known to impair splicing of exon 3 (Sarkany *et al.*, 1994b). In their maternal grandmother, this mutation was *trans* to an *FECH* IVS3-48C allele; a genotype that has been identified in other patients with the typical dominant form of EPP (Whatley SD, unpublished information). Mutational analysis of 184 unrelated patients without

Table 2. EPP with palmar keratoderma: FECH genotypes with predicted FECH activities.

| Family | Allele | Mutation | Effect | FECH IVS3-48 | Predicted FECH activity (%) |
|-----------------|--------|-----------|---------------|--------------|-----------------------------|
| I ¹ | 1 | c.0302T>C | L101P | T | 2.7 ² |
| | 2 | IVS3+2T>G | Splice defect | T | |
| II ¹ | 1 | c.0416A>T | Q139L | T | 18 |
| | 2 | c.0416A>T | Q139L | T | |
| III | 1 | c.0503C>T | P168S | T or C | 5.6 ³ |
| | 2 | c.0854A>G | Q285R | C or T | |
| IV | 1 | c.0820G>A | D274N | T | 5.9 |
| | 2 | c.0898G>T | V300L | T | |
| V | 1 | c.0707G>A | C236Y | T | 6.4 |
| | 2 | c.0820G>A | D274N | T | |
| VI ¹ | 1 | c.1137C>G | K379N | T | 25 |
| | 2 | c.0707G>A | C236Y | T | |

FECH, ferrochelatase.

Previously unreported mutations are shown in bold.

¹Both affected siblings had the same genotype.

²Assuming no activity from the IVS3+2T>G allele.

³Assuming IVS3-48C cis to P168S (see text). Predicted FECH activity was calculated from data in Table 3 and Whatley et al. (2004).

Table 3. Prokaryotic expression of mutant and wild type FECH alleles

| FECH allele | FECH activity (nmol h ⁻¹ mg ⁻¹) ¹ | Percent wild-type activity |
|-------------|---|----------------------------|
| Wild type | 5626 | 100 |
| L101P | 233 | 5.3 |
| P168S | 717 | 16 |
| D274N | 30 | 0.8 |
| Q285R | <10 | <1 |
| V300L | 489 | 11 |

FECH, ferrochelatase.

¹Zinc-mesoporphyrin.

keratoderma identified 2 additional patients with recessive EPP; one of whom has been reported previously (Whatley et al., 2004). The other was homozygous for both a previously unreported mutation (c.0502C>T; P168L) and the hypomorphic IVS3-48C allele.

Five of the eight missense mutations that we identified were expressed in a prokaryotic expression system and their effect on FECH activity determined (Table 3). These activities, together with those previously reported for the three other missense mutations (Whatley et al., 2004), were used to calculate the FECH activities in our patients (Table 2) on the assumption that they were similar to those expressed by these alleles in human tissues.

DISCUSSION

The combination of palmar keratoderma, a relatively low erythrocyte protoporphyrin concentration and autosomal recessive inheritance that we describe here in nine patients

from six families constitutes a hitherto unrecognized subtype of EPP. Of the 13, 2 patients from 12 families with recessive EPP described previously (Lamoril et al., 1991; Sarkany et al., 1994a; Poh-Fitzpatrick et al., 2002; Whatley et al., 2004; Gouya et al., 2006; Herrero et al., 2007) are also included in this report (P4, P8). Thus, of the 20 symptomatic patients from 16 families with recessive EPP now reported, 9 from 6 families (45% of patients; 38% of families) have palmar keratoderma. To date we have not seen a patient with keratoderma who has not had recessive EPP but more patients need to be studied before the reliability of palmar keratoderma as a clinical indicator of recessive EPP can fully be assessed.

Keratoderma was not reported as a clinical feature of either dominant or recessive EPP (Schmidt et al., 1974; DeLeo et al., 1976; Lamoril et al., 1991; Todd, 1994; Sarkany et al., 1994a; Poh-Fitzpatrick et al., 2002; Gouya et al., 2006) before our study of 223 UK patients with this disorder (Holme et al., 2006); possibly because it is uncommon and, unless large numbers of patients are investigated, the association of EPP with keratoderma may be regarded as chance, as was initially the case for two of our families. However, it seems unlikely that keratoderma has been overlooked in all previously reported patients with recessive EPP. Where clinical descriptions have been provided (Lamoril et al., 1991; Sarkany et al., 1994a; Herrero et al., 2007), it has not been noted and three of the patients with recessive disease that we have identified (this report; Whatley et al., 2004) did not have keratoderma.

Our findings are unlikely to be explained by an association of two separate disorders. First, the keratoderma of EPP differs clinically from other syndromes that include palmar keratoderma (Itin and Fistarol, 2005). The hyperkeratosis fluctuates, being worse in summer, tends to be relatively mild on the

soles, and may almost disappear with complete exclusion of light; all of which suggest a role for UV exposure in its etiology. This is supported by the observation that those with mild involvement have signs only in the first interdigital web. None of our patients described blistering, suggesting a nonepidermolytic pattern; none had hyperhydrosis, the keratosis was not particularly at sites of pressure, and was not punctuate in pattern. Second, we observed hyperkeratosis only in recessive EPP and no family showed independent inheritance of the two conditions. Third, the allelic heterogeneity of our patients makes close linkage with a previously undescribed recessive form of keratoderma very unlikely and fourthly the incidence of inherited keratoderma in normal populations has been estimated at between 0.05 (Culati *et al.*, 1997) and 0.55% (Gamborg Nielsen *et al.*, 1994) whereas it was present in 2.7% of our EPP population.

Our patients with keratoderma also differed in other respects from other patients with autosomal recessive or dominant EPP. Erythrocyte protoporphyrin concentrations were lower and, in family 1, concentrations were increased by such a small amount (total erythrocyte porphyrin: 2.0 and 2.3 $\mu\text{mol l}^{-1}$) that the diagnosis would not have been made without plasma and fecal porphyrin measurements. In spite of the relatively low erythrocyte concentrations, plasma porphyrin concentrations were the same as in EPP without keratoderma, photosensitivity was severe and fecal protoporphyrin concentrations were substantially increased. These features suggest that our patients may be producing protoporphyrin in amounts that are similar to those in EPP without keratoderma but the explanation for their low erythrocyte protoporphyrin concentrations is unknown.

There may also be clinical differences apart from keratoderma. Neurological abnormalities were present in four of our patients but, apart from a reversible polyneuropathy after transplantation for protoporphyrin liver failure (McGuire *et al.*, 2005), have not previously been described as associated with any form of EPP. Developmental delay has been reported in the recessive or "homozygous" forms of the dominant acute porphyrias, usually in association with other neurological and skeletal abnormalities (Elder, 1997), which were not seen in our patients. Also, 5 of the 12 (42%) reported patients with autosomal recessive EPP, in which keratoderma was apparently not present, had liver disease (Sarkany *et al.*, 1994a; Whatley *et al.*, 2004; Gouya *et al.*, 2006; Herrero *et al.*, 2007). In contrast, none of our nine patients had liver dysfunction. This raises the possibility that patients with keratoderma may be at lower risk of liver disease than other patients with recessive EPP. Liver disease is accompanied by markedly increased erythrocyte protoporphyrin concentrations though it is not clear whether these are present before liver function deteriorates and their role as a risk factor for liver disease is uncertain (Bloomer, 1988; Meerman, 2000). The relatively low erythrocyte porphyrin concentrations and apparently effective biliary elimination of protoporphyrin shown by our patients may indicate the presence of a special pattern of porphyrin metabolism that does not lead to excessive hepatic accumulation of

protoporphyrin. However, only prolonged follow-up can determine whether a lower risk of liver disease is another component of the syndrome that we describe.

The unusual phenotype that we describe here is inherited in a recessive pattern but it is not clear why it is restricted to this type of EPP or present in only about 40% of such families. Although *FECH* mutations on both alleles appear to be essential for its expression, it seems unlikely that the phenotype is determined solely by the nature of the mutations at the *FECH* locus. There appear to be no features of the genotypes shown in Table 2 that clearly distinguish them from those of other patients with recessive EPP (Whatley *et al.*, 2004; Gouya *et al.*, 2006). Most mutations are missense; most are on *FECH* IVS3-48T alleles and the nine different mutations are not clustered together in the *FECH* molecule (Wu *et al.*, 2001). In four families, one of the mutations has been identified previously either in dominant EPP (IVS3 + 2T > G) (Sarkany *et al.*, 1994b) or in recessive EPP without keratoderma (P168S, D274N) with the D274N mutation being present in two of our families and in two of those reported by Gouya *et al.* (2006). Another mutation (P168S) was present in two of our patients; a homozygote without keratoderma where it occurred on a hypomorphic allele and a compound heterozygote with keratoderma (Table 2), the only one of our patients with a hypomorphic allele, presumably again containing this mutation. The four other mutations have only been reported in EPP with keratoderma. *FECH* genotype is the main determinant of *FECH* activity in EPP (Gouya *et al.*, 2006). In general, *FECH* activities in recessive EPP are lower than in dominant EPP though the presence on one or both alleles of a missense mutation that has only a small effect on *FECH* activity may lead to activities within the range for dominant EPP (Gouya *et al.*, 2006). Prokaryotic expression of the missense mutations predicted *FECH* activities for our patients with keratoderma of 1–25% (mean 10%) normal (Table 2 and Table 3; Whatley *et al.*, 2004), close to the range of 4–29% reported for autosomal recessive EPP (Gouya *et al.*, 2006). Thus it seems unlikely that the unusual phenotype shown by our patients is directly determined either by *FECH* genotype or expression.

MATERIALS AND METHODS

Patients

The study was conducted in accordance with the Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments. Prior approval was obtained from the North West Multicentre Research Ethics Committee and 84 local research ethics committees. All patients or their parents gave informed consent, including consent for publication of photographs. Six of the patients with palmar keratoderma were identified during a cross-sectional study of EPP in the United Kingdom during which the clinical features of 223 patients from 193 families were noted by the same investigator (SAH) (Holme *et al.*, 2006). Two additional patients were referred to SAH; one other was a sibling of a patient included in the cross-sectional study. Blood samples were obtained from all patients with keratoderma and from 203 EPP patients who did not have keratoderma (Holme *et al.*,

2006). Quality of life was assessed using dermatology-specific questionnaires (Holme et al., 2006).

DNA analysis

Genomic DNA was extracted from whole blood using the QIAamp DNA purification kit (Qiagen, Crawley, UK). For identification of FECH mutations and SNPs, all exons with 20–300 bp flanking sequence and 1,300 bp of the promoter region of the FECH gene were PCR amplified (primers and conditions are available from the authors) and sequenced. For sequencing, PCR-amplified double-stranded DNA was purified from agarose gels using the QIAquick gel extraction kit (Qiagen) before being cycle sequenced using fluorescent ddNTPs (BigDye) and an ABI Prism 3100 Genetic Analyzer (PE Biosystems, Warrington, UK). The presence or absence of mutations was confirmed by sequencing both strands. Partial or complete FECH gene deletions were excluded in apparent homozygotes by quantitative gene dosage analysis (Wood et al., 2006). Nucleotides are numbered from the cDNA sequence of human FECH (GenBank accession number D00726) with the A of the ATG initiation codon as “+1”.

Prokaryotic expression of missense mutations

The effect of mutations on FECH activity was determined using the bacterial expression vector pHisTF20E (Burden et al., 1999). Mutations were created using the Quickchange mutagenesis protocol (Stratagene, Stockport, Cheshire, UK) with 50 ng of vector and 12 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 55 °C and 8 minutes extension at 68 °C. Primer sequences are available from the authors. After digestion with DpnI to eliminate the template vector DNA, the PCR products were used to transform chemically competent *E. coli* JM109. Colonies were screened by automated fluorescent DNA sequencing.

For each mutation, a single bacterial colony was grown in 5 ml of Luria-Bertani broth containing 100 µg ml⁻¹ carbenicillin for 6 hours. A 25 µl aliquot was then used to inoculate 25 ml Luria-Bertani (100 µg ml⁻¹ carbenicillin) and the culture grown at 37 °C for 18 hours. Cells were harvested by centrifugation at 6,000 g for 15 minutes and resuspended in 1.5 ml of 50 mM Tris/HCl (pH 7.6) containing 20% glycerol and 1 mM phenylmethylsulphonyl fluoride. Cells were disrupted by sonication on ice (3 × 30 seconds) and centrifuged at 13,500 g for 2 minutes. The supernatant, which contains the recombinant FECH, was stored at -70 °C. FECH activity was determined as described by Gouya et al. (2006). A blank without cell lysate was included and endogenous bacterial FECH activity was assayed using an empty vector control. Protein concentrations were determined using the BCA protein assay (Pierce, Cramlington, Northumberland, UK).

Other methods

Porphyryns in erythrocyte, plasma, and feces were determined by standard methods (Deacon and Elder, 2001). Percentages of protoporphyrin and its zinc chelate were determined by fluorescence emission spectroscopy of ethanol extracts of whole blood, using protoporphyrin and zinc-protoporphyrin as standards.

Statistical methods

Data were analyzed using Microsoft Excel and Minitab v 13. Measurements were expressed as medians and ranges. The

significance of differences between quantitative variables was assessed by the Mann-Whitney test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Key words: generalized pustular psoriasis, systemic glucocorticosteroids, treatment

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A homozygous mutation in the ferrochelatase gene underlies erythropoietic protoporphyria associated with palmar keratoderma – reply

DOI: 10.1111/j.1365-2133.2009.09407.x

SIR, We were interested to read the recent report by Méndez *et al.*,¹ which describes a single patient with the unusual combination of erythropoietic protoporphyria (EPP) and palmar keratoderma. They reported homozygous inheritance of a ferrochelatase (FECH) gene mutation and stated erroneously that this is 'the first incidence of homozygous inheritance of a novel missense mutation in the FECH gene underlying the uncommon phenotype of EPP associated with palmar keratoderma'.

Our group undertook a cross-sectional study of 223 U.K. individuals with EPP in 2004, and from this cohort we identified six individuals with similar coincident seasonal palmar keratoderma.² In a subsequent publication we reported these, and three additional British patients identified with the same phenotype.³ In addition to palmar keratoderma, all nine individuals from six families exhibited autosomal recessive inheritance, being hetero- or homo-allelic for nine different FECH mutations, four of which were novel. Prokaryotic expression predicted that FECH activities were 2.7–25% (mean 10.6%) of normal. Neither mutation type nor FECH activity provided an explanation for the unusual phenotype. In addition, we demonstrated that these patients had low erythrocyte protoporphyrin concentrations when compared with the larger EPP cohort, in combination with plasma porphyrin concentrations at levels usually seen in dominant EPP. Furthermore, none of these nine patients had evidence of liver dysfunction while four had neurological abnormalities. Prior to our publication only 12 families with autosomal recessive EPP had been reported;^{4–9} based on these individuals it had been suggested that recessive EPP carried a higher risk of liver disease than the dominant form. Our findings challenged this dogma by confirming that this recessively inherited clinical variant of EPP appears to carry a decreased risk of liver disease compared with recessive EPP without this phenotype. Thus, palmar keratoderma is

both a clinical indicator of recessive EPP, and appears to be associated with a decreased risk of liver disease. This is in marked contrast to the recently described X linked dominant protoporphyria which has the clinical phenotype of severe EPP, yet is caused by a mutation in a different haem biosynthetic gene and is associated with an increased risk of liver disease.¹⁰

The report by Méndez *et al.* is a useful addition to the EPP literature. However, their case should be interpreted in the context of what is already known (and published) about this interesting clinical phenotype of EPP. This vignette illustrates the continuing importance of phenotype/genotype studies in addressing clinically relevant issues.

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Key words: autosomal recessive EPP, liver disease

Conflicts of interest: None declared.

Successful treatment of Hailey–Hailey disease with topical 5-fluorouracil

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SIR, Hailey–Hailey disease (HHD) is a rare, autosomal dominantly inherited genodermatosis, also known as familial benign chronic pemphigus, first discovered by the Hailey brothers in 1939.¹ Current therapeutic alternatives are based on topical steroids or surgical excision without high efficacy. We report an alternative therapeutic approach with topical 5-fluorouracil (5-FU), used until now for pre-epitheliomatous keratosis, with encouraging results.

A 43-year-old white man, with a sedentary profession, presented with typical lesions of HHD that had evolved over a period of 23 years. The patient complained of chronic pruritic erythematous and fissured plaques complicated by four or five acute painful exacerbations per year. He was significantly bothered by the discomfort, which interfered with his daily physical and professional activities and his sex life, especially during the relapses. There was no family history of HHD.

Examination revealed moist erythematous papulovesicular patches bilaterally measuring 3–4 cm in diameter, scaling, and crusting in the axillae as well as in the deep folds of the groin (Fig. 1a). Skin biopsy showed broad, nearly full-thickness areas of numerous acantholytic cells overlying suprabasilar clefts and underlying chronic inflammation. Direct and indirect immunofluorescence was negative. Topical corticosteroid application was unsuccessful, as were other treatments including topical and oral antibiotics.

We administered 5-FU cream at a concentration of 5% three times weekly for 3 months followed by once weekly for 3 months with monthly clinical follow-up and 3-monthly full blood count. An almost complete remission was obtained after 3 months (Fig. 1b), and this clinical improvement was maintained during treatment. No local or systemic side-effects have been observed to date. He has been followed up for 1 year. No relapses occurred during the first 6 months after the end of treatment. A minor relapse with pruritic lesions was treated with the same cream and remission was obtained in few days. Quality of life was significantly improved.

HHD is a rare intraepidermal blistering disorder, which manifests as recurrent erosions on the axillae and groin. It is caused by mutations in the ATP2C1 gene encoding the secretory pathway calcium ATPase 1 (SPCA1).^{2–4} This protein resides in the Golgi apparatus, and functions as an intracellular

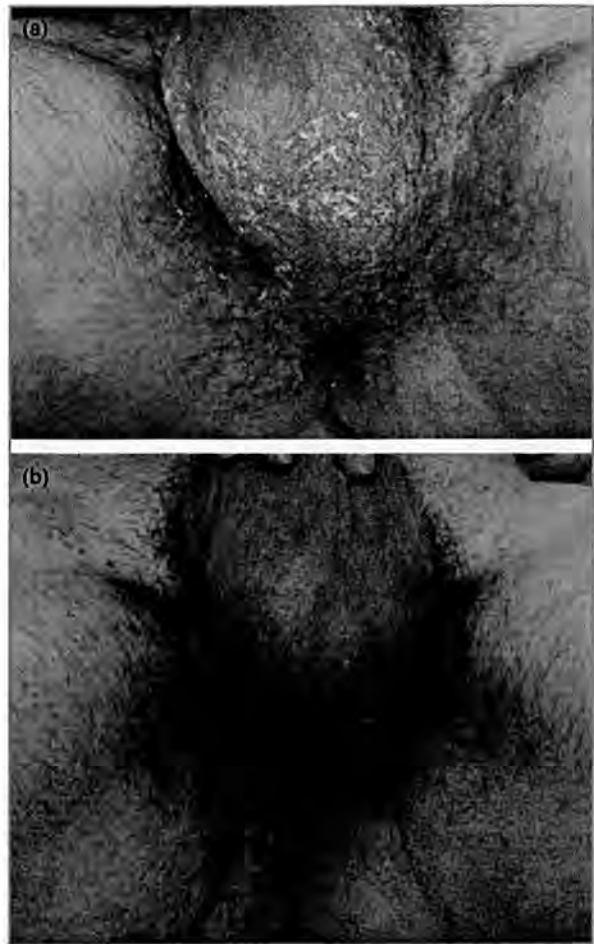


Fig 1. (a) Inflammatory moist erythematous papular patches in the deep folds of the groin. (b) Clinical improvement after 3 months of treatment with topical 5-fluorouracil.

$\text{Ca}^{2+}/\text{Mn}^{2+}$ pump.⁵ These findings suggest that intracellular Ca^{2+} stores play an important role in regulation of epidermal cell–cell adhesion and differentiation.⁴

Topical 5-FU is an analogue of nucleic acid uracil resulting in inhibition of DNA synthesis. It has been extensively used to treat actinic keratoses in photodamaged skin using twice-daily applications for 3–6 weeks.⁶ To the best of our knowledge this is the first therapeutic trial of topical 5-FU in HHD.

The efficiency of topical low-dose 5-FU was demonstrated in Darier disease (DD) which shares a similar pathogenesis with HHD.^{7–9} DD was shown to be caused by mutations in the ATP2A2 gene on 12q24.1, which encodes the sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 2 (SERCA2).⁵

It has been hypothesized that 5-FU restores normal intracytoplasmic calcium concentrations.⁹ Given the similar pathogenesis of HHD and DD and the improvement of our patient by 5-FU, we consider that this hypothesis is likely. Another hypothesis was based on the inhibition of hyperproliferation and abnormal cytokeratin production in DD,⁷ but there is no dyskeratosis or keratinocyte proliferation in HHD.

Molecular epidemiology of erythropoietic protoporphyria in the U.K.

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Summary

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Background Erythropoietic protoporphyria (EPP) is a cutaneous porphyria caused by mutations in the ferrochelatase (FECH) or, less frequently, the delta-aminolaevulinate synthase 2 (ALAS2) gene. Predictive genetic counselling requires accurate molecular diagnosis and knowledge of patterns of inheritance.

Objectives To investigate the molecular epidemiology of EPP in the U.K.

Methods DNA samples from 191 unrelated patients resident in the U.K. were analysed for mutations in the FECH and ALAS2 genes and for the FECH IVS3-48 dimorphism.

Results Mutations were identified in 179 (94%) patients. Most (169; 94%) had a FECH mutation on one allele and were classified as having pseudodominant EPP (psdEPP); seven (4%) patients had FECH mutations on both alleles (autosomal recessive EPP) and three (2%) patients had ALAS2 mutations (X-linked dominant protoporphyria). The FECH IVS3-48C allele was strongly associated with psdEPP and with the absence of mutations at the FECH or ALAS2 loci. Fifty-six FECH mutations were identified, 19 being previously unreported. Missense mutations were predominant in autosomal recessive EPP (82%) but not in psdEPP (32%). One mutation (c.314 + 2T>G) was present in 41 (24%) of EPP families, most of whom appeared to be descended from a common ancestor resident in the north of England.

Conclusions These data define the prevalence and molecular epidemiology of each type of EPP in the U.K.

Erythropoietic protoporphyria (EPP) is a cutaneous porphyria that affects at least one in 140 000 of the population of the U.K.¹⁻³ Increased concentrations of protoporphyrin IX in blood and other tissues lead to life-long acute photosensitivity and, in about 2% of patients, severe liver disease. Three patterns of inheritance have been identified. In most families, accumulation of protoporphyrin results from partial deficiency of ferrochelatase (FECH) (OMIM 177000).^{1,2} In the majority of these, photosensitivity is inherited in a pseudodominant fashion (psdEPP) and is normally manifest only in individuals in whom a deleterious FECH mutation, that markedly decreases or abolishes FECH activity, is inherited *trans* to a hypomorphic allele (FECH IVS3-48C, c.315-48C)³ that is present in 13% of the U.K. population.⁵ About 4% of families have autosomal recessive EPP (arEPP) in which a FECH mutation is present on both alleles.^{5,6} In a few families, overproduction of protoporphyrin is caused by gain-in-function mutations in the ALAS2 gene that encodes the initial enzyme of erythroid haem biosynthesis, 5-aminolaevulinate synthase.⁷ This variant form of

EPP has been named X-linked dominant protoporphyria (XLDPP; OMIM 300752).⁷

Although 110 FECH mutations have now been reported in EPP families,⁸ there are only a few descriptions of the molecular epidemiology of EPP in defined populations.^{6,9,10} Here we describe the molecular characterization of 191 apparently unrelated patients investigated in a cross-sectional study of EPP in the U.K.³

Patients and methods

Patients

Samples for DNA analysis were obtained from 223 patients during a cross-sectional study of EPP in the U.K.;³ 191 of these samples were from patients who were unrelated. Clinical details of these patients have been reported.^{3,11} Prior approval was obtained from the North West Multicentre Research Ethics Committee and 84 local research ethics

committees. All patients or their parents gave informed consent.

Molecular analyses

Genomic DNA was extracted from whole blood using the QIAamp DNA purification kit (Qiagen, Crawley, U.K.) or, for gene dosage analysis, the Flexigene kit (Qiagen). The FECH gene was screened for mutations and genotyped for the IVS3-48C/T dimorphism by heteroduplex analysis using a WAVE™ denaturing high-performance liquid chromatography (dHPLC) instrument (Transgenomic, Omaha, NE, U.S.A.) with absorbance detection at 260 nm. Following polymerase chain reaction (PCR) amplification of individual exons and their flanking regions (30–300 bp) reaction mixtures were cooled by 1 °C s⁻¹ to 4 °C. PCR products (5 µL) were injected on to a reverse-phase HPLC column at a temperature determined theoretically using the Navigator software (Transgenomic) and optimized empirically using DNA containing known mutations whenever possible. Primer sequences and temperatures are available from the authors on request. The dimorphism at IVS3-48C/T separated into two homoduplex peaks in addition to heteroduplex peaks enabling identification of the genotype. Exons and flanking regions showing shifts in the dHPLC trace that indicated possible mutations were further analysed by sequencing. Exon 1 was sequenced in all patients. When no shift was detected all exons including 30–250 bp of flanking intronic sequence and 250 bp of the promoter region were sequenced.¹² When no FECH mutation was detected by sequencing, gene dosage analysis to identify large deletions and characterization of deletion breakpoints was carried out.¹² FECH haplotypes were determined by genotyping with two microsatellite markers (chr18:53101375–53101421, chr18:53719877–53719917) flanking the FECH gene and with one intragenic marker (chr18:53388333–53388377) as described.⁹ The ALAS2 gene was sequenced as previously described.⁷

Nucleotides are numbered from the cDNA sequence of human FECH (NCBI reference sequence NM_000140.3) and human ALAS2 (NM_000032.4) with the A of the ATG initiation codon as '+1'. Functional effects of missense mutations were predicted using AGVGD (<http://agvgd.iarc.fr>), SIFT

(<http://blocks.fhcrc.org>) and Polyphen (<http://genetics.bwh.harvard.edu/pph/>) programs.

In addition, erythrocyte porphyrins were analysed as described.¹³

Statistical methods

Data were analysed using Microsoft Excel and Minitab v 13. Measurements were expressed as medians and ranges. The significance of differences between quantitative variables was assessed by the Mann–Whitney test.

Results

Mutations were identified in 179 (94%; 95% confidence interval, CI 89–96%) of 191 unrelated patients. Of these, 169 patients had a FECH mutation on one allele and were classified as having psdEPP (Table 1). Seven patients had FECH mutations on both alleles (arEPP) and three patients had ALAS2 mutations (XLDPP) (Table 1). The arEPP and XLDPP families have been reported previously.^{5,7,11}

The hypomorphic FECH IVS3-48C allele was strongly associated with psdEPP, being absent from only two patients (Table 1) ($P < 0.001$), but its frequency was not increased in the patients with arEPP or XLDPP in comparison with the general U.K. population (Table 1). There was also a strong association with this allele in the group of 12 patients in whom no mutations were detected ($P < 0.001$), only two of whom had the genotype IVS3-48T/T (Table 1). One of these patients had the biochemical features of XLDPP (erythrocyte protoporphyrin 45.6 µmol L⁻¹; 33% zinc protoporphyrin) but no family history of photosensitivity. Erythrocyte protoporphyrin concentrations (median 18.2 µmol L⁻¹, range 11.2–32.5) in the other 11 patients in whom no mutation was detected did not differ from those in psdEPP (Table 2) ($P = 0.15$) and no distinctive clinical features were observed. The proportions of the IVS3-48C/C or IVS3-48T/T genotypes in this group of patients did not differ significantly from those in psdEPP ($P = 0.27$ and 0.17, respectively).

Among patients in whom FECH mutations were identified, erythrocyte porphyrin concentrations were lower in the patients with missense mutations than in other patients

Table 1 Frequency of different types of protoporphyria in the U.K.

| Type of protoporphyria | No. of unrelated patients (% of total; 95% CI) | FECH IVS3-48 genotype (no. of patients) | | |
|--------------------------------------|---|---|-----|-----|
| | | C/C | C/T | T/T |
| FECH mutation on one allele (psdEPP) | 169 (88.5; 83.2–92.3) | 3 | 164 | 2 |
| FECH mutation on two alleles (arEPP) | 7 (3.7; 1.8–7.4) ^a | 1 | 1 | 5 |
| ALAS2 mutation (XLDPP) | 3 (1.6; 0.5–4.5) ^a | 0 | 0 | 3 |
| No FECH or ALAS2 mutation | 12 (6.3; 3.6–10.7) | 1 | 9 | 2 |

^aarEPP vs. XLDPP $P = 0.17$. The difference between the prevalences of arEPP and XLDPP is not statistically significant.

CI, confidence interval; psdEPP, pseudodominant erythropoietic protoporphyria; arEPP, autosomal recessive erythropoietic protoporphyria; XLDPP, X-linked dominant protoporphyria.

Table 2 Erythrocyte protoporphyrin concentrations in erythropoietic protoporphyria

| | No of unrelated patients | Erythrocyte protoporphyrin ^a (μmol L ⁻¹), median (range) |
|---------------------------------------|--------------------------|---|
| psdEPP | 169 | 23.7 (4.1–77.3) |
| Pro334Leu | 10 | 11.1 (4.1–47.2) |
| Missense mutations ^b | 26 | 13.9 (4.1–47.2) |
| Splicing mutations | 73 | 22.6 (7.9–99.5) |
| Frameshift, nonsense, gross deletions | 70 | 24.8 (6.2–94.5) |

^aIncludes zinc protoporphyrin. ^bIncludes Pro334Leu. psdEPP, pseudodominant erythropoietic protoporphyria.

(*P* = 0.0002). This difference was largely due to the presence of 10 patients (24%) with the mutation Pro334Leu among those with missense mutations. This mutation may be associated with a mild form of EPP.¹⁴ Erythrocyte porphyrin concentrations in patients with mutations predicting defective RNA splicing did not differ from those in patients with null mutations (frameshift, nonsense or gross deletion) (*P* = 0.30).

Fifty-six different mutations were identified in the FECH gene, of which 19 have not previously been reported (Fig. 1). In psdEPP, 25% of mutations were small deletions or insertions, 16% were splice site mutations, 32% were missense mutations, 14% were nonsense mutations and 13% were large deletions that were detected only by gene dosage analysis.¹²

In contrast, nine (82%) of the 11 different mutations in arEPP were missense, none of which was found in psdEPP (Fig. 1).

Eight mutations were found in five or more families and together accounted for the mutations in 58% (98/169) of psdEPP families (Fig. 1). One mutation (c.314 + 2T>G) was particularly common, being present in 41 (24%) families, including one family with arEPP. Genotyping with microsatellite markers within and flanking the FECH gene in one family identified a haplotype that segregated with this mutation. The microsatellite polymorphisms comprising this haplotype were present in 37 of the apparently unrelated patients with this mutation, suggesting that they were descended from a common ancestor. Mapping the places of residence of these 37 patients suggested that their common ancestor may have lived in south Yorkshire or Lancashire (Fig. 2).

Discussion

Our 191 apparently unrelated patients were recruited to a cross-sectional study of EPP in the U.K. using a procedure that minimized selection bias.³ Thus the prevalence for each type of EPP shown in Table 1 is likely to reflect that among all U.K. patients. A similar prevalence for arEPP has been reported for France.⁶ Although we identified fewer families with XLDPP than arEPP, their prevalences were not significantly different.

Our data show that, as in other countries,^{6,9,15–20} several different FECH mutations are found in EPP in the U.K. However, allelic heterogeneity is less than for the autosomal dominant acute porphyrias,²¹ largely due to the presence of a single large extended family with the mutation c.314 + 2T>G. Similarly high frequencies of a single mutation

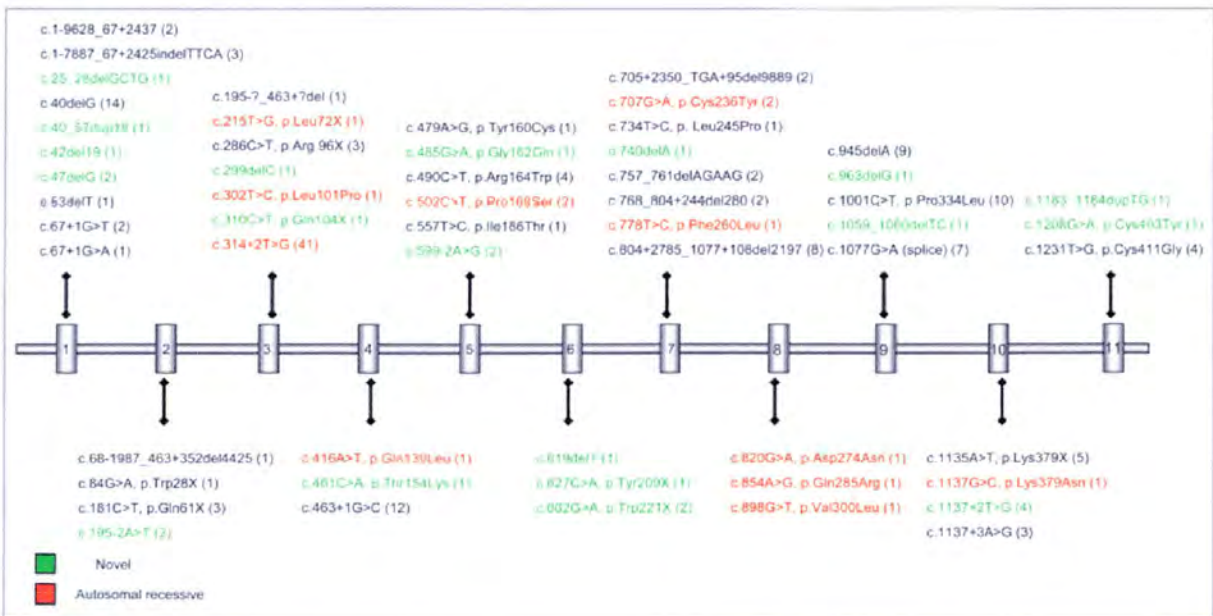


Fig 1. FECH mutations in U.K. patients. Previously unreported mutations are shown in green and mutations found only in autosomal recessive families in red. Numbers in parentheses indicate the number of unrelated patients with that mutation.

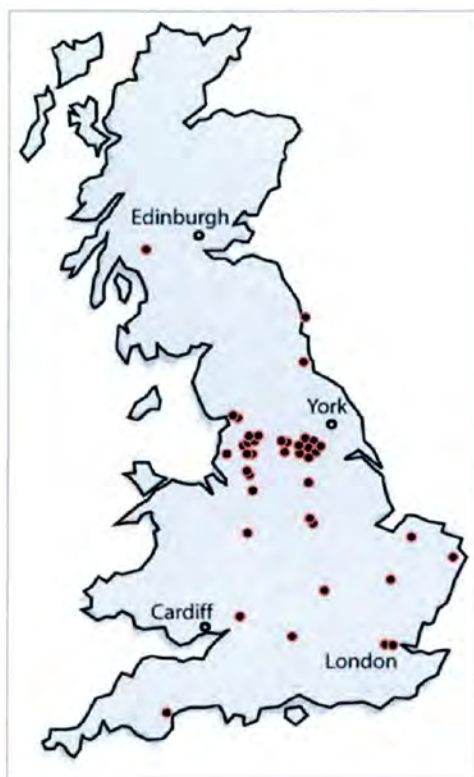


Fig 2. Geographical distribution of families with FECH c.314 + 2T>G mutation.

among apparently unrelated families have been reported for EPP in Northern Ireland,²² in South Africans of European descent⁹ and in Switzerland.¹⁰

The percentages of different types of mutation showed a marked difference between arEPP and psdEPP, with missense mutations predominating in arEPP. No missense mutation was found in both arEPP and psdEPP. This difference probably reflects the need for at least one allele in arEPP to carry a mutation that preserves sufficient residual FECH activity to maintain normal development; such 'mild' missense mutations may be less likely to produce psdEPP when *trans* to a hypomorphic allele. Reports of mutation patterns from other countries have not distinguished between the two forms of EPP or included patients with large deletions but otherwise show no marked differences from the overall pattern in U.K. patients.⁶

Nineteen (34%) of the mutations that we identified have not been reported previously. Most were small deletions or insertions leading to frameshifts, splice site mutations predicted to cause aberrant RNA splicing or nonsense mutations introducing premature stop codons (Fig. 1) and therefore predicted to have a marked effect on FECH activity. Three were missense mutations. Of these, p.Cys403Tyr altered one of the four ligating cysteine residues in the iron-sulphur cluster that is essential for human FECH activity²³ while the other two (p.Thr154Lys, p.Gly162Gln) were highly conserved, indicating likely interference with protein function.

All but two of our patients in whom a single FECH mutation was identified had the genotypes IVS3-48C/T or IVS3-48C/C (Table 1). This strong association is consistent with the notion that clinical expression of psdEPP requires the hypomorphic IVS3-48C allele to be *trans* to a deleterious mutation,⁶ although we were unable to demonstrate this directly because we did not determine phase. The two patients without these genotypes both had mutations (c.314+2T>G; c.768_804+244del280) that are known to cause psdEPP in conjunction with a hypomorphic allele. They had lower erythrocyte protoporphyrin concentrations (8.9, 6.2 $\mu\text{mol L}^{-1}$) than most patients with psdEPP (4.7% had < 9 $\mu\text{mol L}^{-1}$) and milder disease with one patient (c.314+2T>G) not presenting until the age of 10 years and neither being recognized to have EPP until their fourth decades. Others have also reported rare patients with psdEPP and the genotype IVS3-48T/T.^{6,16} In one such patient, delayed appearance of symptoms was apparently provoked by partial hepatectomy for multiple adenomas.⁶ It is not clear why these rare patients with a single FECH mutation *trans* to an IVS3-48T allele develop photosensitivity; an unidentified hypomorphic allele or acquired factors, separately or together, may be required. However, the finding that 1.2% (95% CI 0.3–4.2%) of patients with psdEPP have not inherited an IVS3-48C allele does have consequences for predictive counselling as it shows that a small risk of having an affected child remains even when the unaffected partner has the genotype IVS3-48T/T.

No FECH or ALAS2 mutation was identified in 12 patients (Table 1). One patient with the IVS3-48T/T genotype had the biochemical features of XLDPP and may have an unidentified mutation in ALAS2 or a gene that regulates ALAS2. In the other patients, the distribution of FECH IVS3-48 genotypes was not significantly different from psdEPP (Table 1). FECH activity was not measured in these patients because nucleated cells were not available for analysis but the strong association with the hypomorphic allele suggests that it may be decreased. Thus, FECH mutations in regions of the gene not analysed by our procedures (approximately 40 kb of noncoding sequence) seem the most likely explanation for EPP in the majority of these patients. One of our mutation-negative patients had the genotype IVS3-48C/C that decreases FECH activity by 30–40%.^{4,8} However, our patient, unlike a recently reported patient with this genotype who had mild photosensitivity, a small increase in protoporphyrin concentration and normal plasma porphyrin,⁸ had typical EPP (erythrocyte porphyrin 18.2 $\mu\text{mol L}^{-1}$).

Finally, what is the role of molecular analysis in the management of EPP? Predictive genetic counselling requires prior identification of the pattern of inheritance in individual families. Our findings identify the extent of genetic heterogeneity of EPP in the U.K. and add to the evidence that mutation identification, in addition to FECH IVS3-48C/T genotyping, is required to support accurate genetic counselling. However, the usefulness of mutation identification as part of the initial diagnostic protocol in all patients with EPP requires further evaluation.

What's already known about this topic?

- Erythropoietic protoporphyria (EPP) is a cutaneous porphyria that affects at least one in 140 000 of the population of the U.K.
- Patients have life-long acute photosensitivity with about 2% developing severe liver disease.
- EPP is caused by mutations in the ferrochelatase (FECH) or, less frequently, the delta-aminolaevulinate synthase 2 (ALAS2) gene.

What does this study add?

- This study of EPP is the largest to date and defines the prevalence and molecular epidemiology of EPP in the U.K.
- Most patients were found to have a FECH gene mutation associated with a low expression allele and were classified as having pseudodominant EPP. The autosomal recessive form of EPP was identified in 4% of patients while 2% were found to have the recently identified X-linked dominant protoporphyria.
- One mutation was found to be present in 24% of families with pseudodominant EPP.

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ERYTHROPOIETIC PROTOPORPHYRIA

What are the aims of this leaflet?

This leaflet has been written to help you understand more about erythropoietic protoporphyria (EPP). It tells you what it is, what causes it, what can be done about it and where you can find out more about it.

What is erythropoietic protoporphyria?

The word 'erythropoietic' means associated with red blood cells ('erythro-') and their formation ('-poietic'). The porphyrias are a group of uncommon diseases caused by something going wrong with the production of chemicals known as porphyrins. These chemicals are the building blocks of haem, which, when combined with a protein (globin), forms haemoglobin, the material in red blood cells that carries oxygen round the body. In the case of EPP, there is a build up of one of these porphyrins (protoporphyrin) in the blood, especially in the red blood cells. This leads to a sensitivity to sunlight.

What causes EPP?

An enzyme is a protein that helps to convert one chemical substance into another. In EPP, there is a shortage of one particular enzyme (ferrochelatase), which normally helps to convert protoporphyrin into haem by adding iron to it. As a result of this enzyme deficiency, protoporphyrin levels build up in the blood. As blood passes through the skin, the protoporphyrin absorbs the energy from sunlight and this sets off a chemical reaction that can damage surrounding tissues. The nerve endings in the skin interpret this

as itching or burning pain, and if the blood vessels are affected, they can leak fluid, causing swelling.

The light that protoporphyrin absorbs is different from that which causes ordinary sunburn. Usually sunburn is caused by the shorter wavelengths of ultraviolet light (UVB), but in EPP the skin is more sensitive to visible light and to longer ultraviolet wavelengths (UVA).

Is EPP hereditary?

Yes, but there is not always a family history of the condition. Everyone has two genes for ferrochelatase in each cell in their body (one coming from their mother and one from their father). In most families, EPP occurs when an affected individual inherits a gene for a severely underactive ferrochelatase enzyme from one parent, and a less severely affected gene from the other parent. The less severely affected gene is quite common, being present in about 10% of the general population, but it never causes EPP by itself. The genetics is quite complex and advice from your local genetics service may be useful.

What are the symptoms of EPP?

Typically EPP starts with abnormal sensitivity to sunlight. Exposure to sunlight causes tingling, itching or burning, which may be associated with redness and swelling. These symptoms usually occur within a few minutes of skin exposure to sunlight, and often they take hours or days to resolve. During this time the skin may feel more sensitive than usual to sunlight and extremes of temperature. The light producing these changes need not be direct – light reflected off water and sand, or passing through window glass, including car windscreens, can also cause the symptoms.

EPP usually starts in childhood, and affects males and females equally. Infants may cry or scream after being taken out into the sunlight and older children may complain of burning, try to wave their hands in the air, or put them into cold water to try to relieve the pain. A very small number of people

who have had with EPP for many years may develop liver damage. Fortunately this is rare.

What does EPP look like?

Despite severe discomfort, there may be nothing abnormal to see on the skin. Sometimes there can be swelling of the skin, initially like a nettle rash. With time, some people develop thickening of the skin over their knuckles, and small scars on sun-exposed skin such as that on the cheeks, nose, and backs of the hands. However these skin changes show wide variation between different individuals.

How is EPP diagnosed?

The diagnosis is usually suspected from the story, and can be confirmed by a blood test. This measures the amount of protoporphyrin in the blood (serum protoporphyrin) and in the red blood cells (erythrocyte free protoporphyrin). Some doctors will also ask for a stool sample to measure the level of protoporphyrin in the faeces. No urine tests are relevant to this condition except to exclude other types of porphyria.

Although it is unlikely that you will develop liver problems as a complication of EPP, your doctor may monitor the way your liver is working by yearly blood tests. If there is any evidence of a deterioration in liver function, there are certain interventions that may help to halt or reverse this.

As EPP affects the production of haemoglobin, it is not uncommon for people with EPP to be slightly anaemic. Your doctor will probably also measure your blood count to make sure that you are not becoming too anaemic.

Can EPP be cured?

At present there is no cure for EPP.

How can EPP be treated?

The aim of most treatments is to give your skin extra protection from sunlight, so that you tolerate sunlight better. Advice about clothing and sunscreens is given later in this leaflet.

Medical treatments for EPP include the use of:

- **Beta-carotene.** This is derived from the chemical that makes carrots orange. Some people with EPP find that taking beta-carotene capsules is helpful. The capsules are available on prescription (supplier details are given at the end of the sheet), are taken by mouth, and usually give the skin a slightly orange colour. This medicine is considered to be safe, but may occasionally cause a slight tummy upset. Large studies showed some health gains e.g. fewer strokes, but there may also be an increased risk of lung cancer in smokers.
- **Antihistamines.** These tablets or syrups may help the few people for whom the nettle rash response of the skin is a major problem.
- **Phototherapy.** Narrow-band UVB therapy is a type of ultraviolet light treatment used in dermatology departments. It involves careful exposure to artificial ultraviolet light, usually three times a week for about five weeks in the spring, to allow the skin to thicken slightly and develop a tan. This acts as a natural sun block and may improve tolerance to sun exposure over the summer.
- Treatments still being developed include the possible use of L-acetyl cysteine, MSH (Melanotan), and dihydroxyacetone paint.

What can I do?

It is sensible to avoid unnecessary exposure to sunlight. Other helpful measures include the wearing of protective clothing and the use of sunscreens:

- **Clothing** – simple measures include the wearing of clothes made from tightly woven dark-coloured cloth, long sleeves, a hat (ideally brimmed or Foreign Legion-style); shoes rather than sandals, and gloves, particularly for driving. Some manufacturers provide information about the degree of protection provided by clothing using the UV protection factor (UPF) rating which measures protection from clothing against UVB and UVA. While it does not automatically follow that this equates to visible-light protection, in practice this is likely to be the case.
- **Sunscreens** - as EPP is characterised by sensitivity mainly to visible light, conventional sunscreens that are formulated to protect against ultraviolet (particularly UVB) are usually not effective. Reflectant sunscreens that are based on titanium dioxide or zinc oxide will be more effective as they cover both UVA, UVB, and visible light to a degree. In the UK, the SPF (sun protection factor) number tells you how effective the sunscreen is for UVB, and the star rating (usually found on the back of the bottle, with a maximum 4 stars) gives a measure of the UVA protection.

A tinted reflectant sunscreen is available on prescription from Dundee Pharmaceuticals in three colours: coral pink, beige, and coffee. These can be mixed to obtain a good colour match with your skin (Dundee Pharmaceuticals, Ninewells Hospital, Dundee, DD1 9SY, telephone: 01382 632052).

- **Reactions to other medications.** Unlike other types of porphyria, EPP does **not** cause porphyria 'crises' as an effect of certain medicines and anaesthetics. Some doctors and pharmacists confuse EPP with these other porphyrias and may tell you to avoid certain medicines. In general you can take whatever medicines your health requires.

Where can I get more information about EPP?

As EPP is so uncommon, many general practitioners will have had little experience of dealing with it. However, dermatologists see most people with EPP, but if they too have little personal experience of the problem, they may refer you to a colleague with particular expertise, for investigations and discussion. If you are concerned about the likelihood of passing the condition onto your children, you may be referred to a geneticist for information about this complex area. There are a number of other sources of information, most of which are on the Internet. Most give details about all forms of porphyria, although a few specialise just in EPP.

1. Organisations specialising in EPP.

Netherlands EPP Foundation – click the Union Jack flag to translate

www.epp.info

EPPREF

www.brighamandwomens.org/eppref

National Centre for Biotechnology Information - OMIM

www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=177000

Pubmed – search for medical journal articles

www.ncbi.nlm.nih.gov/entrez/query.fcgi

EPP information, links and results from recent research

<http://Alzuko.tripod.com/epp.htm>

2. Organisations dealing with all forms of porphyria

British Porphyria Association

14 Mullison Rise, Gravesend, Kent, DA1Z 4QJ Tel: 01474 369 231

www.porphyrria.org.uk

European Porphyria Initiative

www.porphyrria-europe.com

Canadian Porphyria Foundation CPF

Box 1206, CA-Neepawa, R0J 1H0 Phone/Fax: Country code, then 204 476 2800

www.cpf-inc.ca

American Porphyria Foundation

P.O.Box 22712 US-Houston, TX 77227 USA

University of Cape Town Porphyria Service, South Africa

<http://web.uct.ac.za/depts/porphyria/>

University of Queensland, Australia

www.uq.edu.au/porphyria/

CLIMB – children living with inherited metabolic diseases

176, Nantwich Road, Crewe. CW2 6BG Telephone: 0870 7700 326

www.climb.org.uk

3. Other information:

Beta-Carotene - sources and dosage:

Beta-carotene is available on prescription, and the dosage required may be up to 200mg per day. In the UK, the only licensed preparation is a 3mg capsule. Higher strength preparations are available via a company called IDIS, which imports them from abroad. As these preparations do not have a product licence in the UK, IDIS requires written confirmation from your consultant of the reason why these higher strength capsules are required. This is to satisfy the Medicines Control Agency that there is a need to import the product even though a licensed product is available in the UK. The contact details for IDIS can be found on their website: www.idisonline.com.

Window film to cut out UV / to cover operating theatre lights:

Bonwyke Window Films Ltd., 41-43 Redlands Lane, Fareham, Hampshire.
PO14 1HL Telephone: 01329 289621

Madico (Madico CLS200XR) 45 Industrial Parkway, Woburn, MA 01888,
USA. Telephone: 001 800 225 1926. Email: info@madico.com

(Whilst every effort has been made to ensure that the information given in this leaflet is accurate, not every treatment will be suitable or effective for every person. Your own doctor will be able to advise in greater detail.)

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