MOLECULAR CHARACTERISATION OF ACUTE INTERMITTENT PORPHYRIA IN SOUTH AFRICA

BY

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Declaration

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

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To God in whom I trust, for all these doors didn't open by accident.

List of Abbreviations

ABA	acrylamide-bisacrylamide
AIP	acute intermittent porphyria
ALA	5-aminolaevulinic acid
ALAS	5-aminolaevulinic acid synthase
bp	base pair
CPG	coproporphyrinogen
CRIM	cross-reactive immunological material
dH_2O	distilled water
dNTPs	deoxynucleotide triphosphates
EBV	Epstein-Barr virus
EC	Enzyme Commission number
EDTA	ethylenediaminetetraacetic acid
HMBS	hydroxymethylbilane synthase
PBG	porphobilinogen
PBGD	porphobilinogen deaminase
PCR	polymerase chain reaction
siRNA	short interfering ribonucleic acid
TAE	Tris acetate-EDTA buffer
Tm	melting temperature
UPG	uroporphyrinogen
WT	wild type

PART A

Protocol

ORIGINAL PROTOCOL AS APPROVED BY THE DEPARTMENTAL RESEARCH COMMITTEE AND FACULTY OF HEALTH SCIENCES HUMAN RESEARCH ETHICS COMMITTEE

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Project Title

Molecular characterisation of acute intermittent porphyria in South Africa.

Short Description of the Project

Acute intermittent porphyria belongs to a group of inherited disorders of haem metabolism. The object of this project is to characterise the mutations in the hydroxymethylbilane synthase (*HMBS*) gene in a cohort of South African patients. The elucidation of these mutations will facilitate an understanding of the molecular basis of AIP in South Africa, and provide a platform for the screening of family members of affected patients. Identification of latent carriers would allow for education with respect to precipitants and how best to avoid them, so as to minimise the risk of provoking an acute attack.

Project Details

Aims

1. To understand the molecular basis of acute intermittent porphyria (AIP) in South Africa: specifically, this project aims to investigate the specific DNA sequence variations in the *HMBS* gene that exist in South African patients with AIP.
2. To determine the relative incidence of *HMBS* gene mutations in a South African cohort.
3. To provide a basis for genetic screening and counseling for AIP families in South Africa.

Objectives

1. Collect whole blood samples/culture stored Epstein-Barr virus (EBV) transformed lymphoblasts from AIP patients, and extract DNA.
2. Design primers to cover exons and surrounding introns of the *HMBS* gene using oligonucleotide design software.
3. Amplify, using polymerase chain reaction (PCR), all 15 exons (and surrounding introns) of the *HMBS* gene.
4. Directly sequence PCR products to identify any sequence variations (mutation/polymorphisms).

5. Compare identified mutations/polymorphisms with those published for AIP cohorts in other countries.
6. Based on results obtained above, establish restriction enzyme analyses where possible, to enable easy screening.
7. Screen family members and construct family pedigrees.
8. Provide appropriate genetic counseling.
9. Perform similar analyses, on an on-going basis, on newly diagnosed AIP patients that may present during the study period and thereafter.
10. Publish findings in an international peer-reviewed journal and as a MMed dissertation (Dr P. Fortgens).

Background

The porphyrias are a heterogeneous group of genetic disorders of haem biosynthesis. They have two characteristic clinical symptoms: the acute attack and/or photocutaneous sensitivity. There are eight enzymes in the pathway, and partial defects in each of these may result in a specific type of porphyria each with a characteristic build up of porphyrins with or without the precursors 5-aminolaevulinic acid (ALA) and porphobilinogen (PBG).[1, 2] There are two groups of porphyria viz. the acute and non-acute porphyrias. The distinction between these two groups appears to be the potential or non-potential for the precursors ALA and PBG to accumulate (only in the acute porphyrias will the precursors accumulate). Photosensitivity appears to be associated with the accumulation of the porphyrinogens or their oxidized forms, the porphyrins.

There are four acute porphyrias. These acute porphyrias are distinguished by the occurrence of potentially fatal acute neurovisceral attacks. One of these, acute intermittent porphyria (AIP), is an autosomal dominant disorder resulting from partially defective porphobilinogen deaminase (PBGD, also known as HMBS), the third enzyme in the haem biosynthetic pathway. In South Africa, AIP is found in all population groups, and is under diagnosed in the black population.[3] Worldwide, AIP is the most common of the neurological porphyrias with an estimated prevalence of 1-8 per 100 000. Indeed, even in South Africa where there is a well documented preponderance of another one of the acute

porphyrias, variegate porphyria, AIP acute attacks make up the majority of acute attack admissions in South African hospitals.[4]

While most people with AIP remain clinically and biochemically normal throughout their lives, potentially fatal acute neurological symptoms may be triggered (often by porphyrinogenic drugs, or in the case of females, hormonal fluctuations). Hence the importance of identifying individuals with the disease, and thus susceptible to the acute attack. The most sensitive and specific diagnosis in these individuals and their families is through molecular (mutational) analysis. Indeed, prior identification of individuals at risk is a good strategy in preventing acute attacks.[5] Furthermore, AIP frequently poses a diagnostic and therapeutic challenge to clinicians.

Worldwide, molecular heterogeneity of mutations causing AIP has been demonstrated. Over 300 mutations have been described in the *HMBS* gene, but only 8 of these mutations have been identified in the ubiquitous form of the enzyme alone.[6, 7] Mutations are equally distributed throughout the *HMBS* gene with no prevalent sites for mutation having been identified.[8]

In South Africa AIP remains poorly documented and only 5 mutations (Arg26His, Arg116Trp, Arg173Gln, c.771_772insThr and c.1002delGly) have been identified to date by workers abroad, in collaboration with earlier workers in our laboratory.[9, 10] Clearly the molecular characterization of other *HMBS* gene defects in South African individuals will be of interest to researchers in the field and of benefit to the patients and their relatives. The *HMBS* gene is located on chromosome11q23.3 and comprises 15 exons.[11] Two forms (erythroid specific and ubiquitous) of PBGD are generated by the use of separate promoters and alternative splicing of the two primary transcripts.[12] These forms differ at the amino-terminal ends where the ubiquitous form extends by another 17 amino acid residues.[13]

Methodology

Study design

Identification of disease causing mutations and polymorphisms will be undertaken in a cohort of South African AIP patients identified in our Lennox Eales (University of Cape Town) Porphyria Laboratories. DNA will be extracted, the exons of the *HMBS* gene amplified by PCR and the exact mutation identified by sequencing. Restriction analysis will be set up to identify the presence/absence of the mutation in other family members. Family pedigrees will be also be constructed. The methodology proposed above has been successfully utilized for mutation detection in the past both in our and other laboratories.

A three year time frame in which to complete the study is realistic. The project will be performed in the Lennox Eales (University of Cape Town) Porphyria Laboratories which are internationally recognized for their contribution to research in the field of porphyria. They offer a good existing infrastructure for biochemistry and molecular studies. Together with clinicians in the Departments of Medicine at the University of Cape Town (Prof Spearman and Drs Sonderup and Hairwadzi) and the University of Kwa-Zulu Natal, these laboratories are dedicated to offering a comprehensive service to the porphyria community of South Africa: patients, medical practitioners and scientists alike. They are headed by Professor Meissner who is internationally recognised as a world authority in the field of porphyria. Dr Corrigall has many years of experience in the Liver Centre and porphyria laboratories, specializing in protein purification and molecular biology, and is also internationally recognised. These laboratories house the equipment required to carry out this study. In addition, use will be made of the sequencing facility at the University of Stellenbosch (Department of Genetics). All aspects of this research will conform to the requirements of the Declaration of Helsinki (World Medical Association, revised 2008).

Characteristics of the study population

Approximately 15 AIP patients from all racial groups, identified through our laboratories and the Liver Clinic at Groote Schuur Hospital/University of Cape Town will be studied. In addition, these probands will most likely give rise to approximately 60 family members for testing. It is not possible to give an exact number of participants as, as further AIP

families who are identified at the clinic over the 3 year period of the study, will be included. Likewise, it is impossible to estimate the precise size of patient families and how many of those will consent to testing.

Informed consent will be obtained from the parents of individuals under the age of 7 years. Minors over the age of 7 years will be asked to fill in the consent forms for minors. Adults with impaired decision making capacity and people in unequal relationships will not be included in the study.

Inclusion and exclusion criteria

AIP patients will be recruited and consent requested after a biochemical diagnosis of AIP has been made (see research procedure and data collection methods). Likewise, their family members will be recruited once the proband mutation has been identified. Subjects from all ethnic groups in South Africa will be included. The participants will be chosen after a discussion between the 3 primary investigators, and with consultation of participating medical consultants if necessary.

Recruitment and enrolment

The University of Cape Town Porphyria laboratories have a number of previously identified patients (12), and approximately 3 new patients per year are identified. We will be re-contacting patients with a previous diagnosis of AIP through the Liver Clinic, as well as investigating any new AIP patients who may be identified during the course of the study. The point of re-contacting patients will be to ask consent to use their blood samples (already obtained for routine porphyrin analyses in our labs) for further DNA analysis. In the case of newly identified patients, consent will be requested at the time of diagnosis. Once mutation(s) have been identified, blood samples from consenting family members will also be requested and obtained.

We have in storage at -180^{o}C a number of EBV transformed lymphoblast cultures from previously identified AIP patients. These may be cultured and DNA extracted, to provide suitable DNA for analysis, in which case drawing of further blood from these patients will

not be necessary. Any patient enrolled in the study will be asked to participate in the study by the Liver Clinic physicians or Dr Fortgens. They will be informed of the study, and given an information sheet and requested to read it, prior to giving consent. Subject to agreement to participate in the study, blood will be taken from them for study.

The following information will be collected and stored for each family: identifying and demographic data, full clinical and family history and, where possible and appropriate, further clinically relevant information including physical examination findings, biochemistry and haematology will be entered into a computerized database.

Research procedures and data collection methods

The diagnosis of AIP in probands is based on measurement of standard porphyrin biochemistry (raised uroporphyrin in the urine and raised precursors (ALA and PBG), a plasma porphyrin emission scan peak at 619nm together with a typical AIP clinical history (no photosensitive skin disease/potential and a history of acute neurovisceral attacks). We will further characterise patients with AIP in South Africa by DNA analysis to identify the disease-causing mutation present in each family. Dr Anne Corrigall has considerable experience in all the techniques required for this project apart from the sequencing. As mentioned previously this will be performed at the University of Stellenbosch. Dr Fortgens will be trained in all the techniques by Dr Corrigall.

Collection of blood samples from patients and cell culture

Following informed consent, blood samples will be obtained by venepuncture from known AIP patients, either at the Liver Clinic at Groote Schuur Hospital, or by arrangement with Dr Fortgens, or through a peripheral pathology laboratory if the subject is not based in Cape Town. Blood samples (2X ethylenediaminetetraacetic acid blood tubes, total 9ml) will be drawn by a qualified medical or nursing professional.

Control samples

We will make use of approximately 50 existing control DNA samples, drawn from a previous porphyria molecular study, stored within the Porphyria Laboratory. These were

drawn from blood donors and hospital staff, are anonymous and irretrievably dissociated from the identities of their donors. If additional control samples are necessary, these will be drawn with the consent of the donors, will be irretrievably anonymised and the donors informed of this.

DNA extraction

DNA will be extracted and isolated from cultured lymphoblasts (previously stored at -180^{o}C) or directly from whole blood (9ml) using the Promega DNA extraction kit.

Primer design

Primers will be designed using oligonucleotide designer software (Primer Designer) and checked for suitability by Oligoanalyzer, Blast and ePCR.

PCR amplification

Primers will be designed to cover all 15 exons of the *HMBS* gene and their surrounding introns. PCR amplification will be carried out in a total volume of 50µL containing 10mM Tris, 1.5mM magnesium chloride, 0.2units GoTaq flexi polymerase (Promega), 50µM of each deoxynucleotide, 0.5µM of each primer and 100ng of genomic DNA. The cycles will consist of an initial denaturation step at 95^{o}C for 1 min, 35 cycles of denaturation at 95^{o}C for 30s, annealing at relevant temperatures for 30s, and extension at 72^{o}C for 30sec, and then final extension for 7min at 72^{o}C followed by cooling at 4^{o}C for 6min. This is performed in a Robocycler Thermal cycler. If necessary the amplification conditions will be altered to accommodate the primers.

Direct sequencing

PCR products, after verifying with polyacrylamide electrophoresis for specificity, will be purified using the GFX Amersham purification kit and sent to the University of Stellenbosch Central Sequencer for capillary zone electrophoresis-based sequence analysis.

Once a mutation has been identified restriction analysis will be set up, if possible, to enable easy screening of other family members and control individuals. Approximately fifty controls, representative of the ethnicity of the patients, will be selected and investigated for the presence of sequence variations/mutations identified during the course of the study.

Data safety and monitoring

Security and confidentiality will be maintained, and no person other than the principal researchers will have access to the information entered into the computerized database and laboratory workbooks. No adverse events are expected.

Data analysis

Sequencing will be performed at the sequencing facility at the University of Stellenbosch, which has an excellent track record for good quality and reliable sequencing. Sequencing will be performed in both a forward and reverse direction (two different PCR amplicons) to confirm the exact mutation. After identification of the disease causing mutation and any polymorphisms in each family, data will be closely examined and assessed for any potential founder effect. Clinical, biochemical and molecular data will be evaluated and correlated after examination of the clinical and biochemical database. Pedigrees will be constructed in the families.

Description of Risks and Benefits

The participants in this study are not subjected to risk. Benefits to the patients will be a definitive and 100% specific and sensitive diagnosis of AIP, identification of latent cases (i.e. gene carriers who may unknowingly be at risk of the acute attack) and hence advice can be given on how to avoid precipitating an acute attack.

Informed Consent Process

Previous consent

Existing EBV transformed lymphoblasts were collected for porphyria diagnosis and characterisation thereof, approximately 15-20 years ago. In each case, verbal consent was

given for lymphoblasts to be transformed and stored indefinitely for future DNA analysis assuming such analysis became possible. Collection of such blood samples fell within the definition of basic standard of care since both biochemical and DNA blood analysis are regarded as integral to the diagnosis of AIP.

Prospective consent

We acknowledge that current guidelines for consent require a more formalized process. Any further subjects we identify for possible inclusion in our study, their families, and family members of already identified AIP patients, will provide full informed consent in writing, using consent forms (Part D). Informed consent will be ongoing throughout the study. Any minor who does not wish to give participate in the study will not be pressurized in any way to do so. Consent will be obtained by Dr Philip Fortgens, Prof Spearman, Dr Sonderup or Dr Hairwadzi. Consent forms will be translated into Afrikaans and Xhosa as required.

Comprehension of information

All potential participants will have a one-on-one discussion with a medical doctor involved in the study. They will be given an information sheet which they will be required to read prior to signing the consent form. If a subject appears not to understand the medical doctor will explain further.

Withholding information

No information will be withheld from participants in the study.

Consent and assent forms

See attached forms (Part D). Different forms will be used for adults and minors (age 7-17 years). Once the forms have been approved by the ethics committee, they will be translated into Afrikaans and Xhosa.

Privacy and Confidentiality

Strict regulations for patient confidentiality will be adhered to. Subject information will be recorded in a database for sole use by the principal researchers involved in the study. Patients names will be encoded and not appear on sample tubes used during the study.

Reimbursement for Participation

The participants in the study will not receive any financial reimbursement for participation, but travel costs will be covered as necessary.

Emergency Care and Insurance for Research-Related Injuries

No research related injuries are expected in this study.

What Happens at the End of the Study

Patients and family members will be informed of the findings of the study (providing they wish to know the outcome). Appropriate clinical management will be discussed with patients and genetic counseling will be offered.

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Envisaged Outputs/Outcomes

This study will allow for the molecular characterisation of AIP in a South African cohort of patients, the screening of family members and the counseling of individuals found to have mutations. It is envisaged that this work be presented at the International Congress of Porphyrins and Porphyrias 2013 (Luzern, Switzerland) and at the South African Laboratory Medicine Congress 2013 in Cape Town. Both of these objectives have subsequently been met. The study will further provide material for Dr Fortgens' MMed dissertation and will also provide an opportunity to publish the results in a peer-reviewed journal.

Institutional Approval

This proposal has been approved by the University of Cape Town Research Ethics Committee.

Funding

Funding is provided by the Medical Research Council, self-initiated research grant awarded to PN Meissner, the NRF Incentive award for rated researchers (PN Meissner), The University of Cape Town Research Committee and the National Health Laboratory Service Research Trust.

Amendments to Original Protocol

1. EBV-transformed lymphoblasts from AIP patients were not used as stored DNA was found to be of suitable quality for further study.

2. The number of AIP patients and their family members was initially over-estimated due to difficulties in subsequently tracing them. Access to families, particularly in remote locations was also challenging, hence tracing of relatives and the generation of pedigrees was not part of this study.

3. During the study the Robocycler Thermal cycler was replaced by the Labnet Mutigene Optimax necessitating the re-optimisation of some PCR reactions.

4. PCR products were visualized using both polyacrylamide and agarose gel electrophoresis systems.

5. PBGD enzyme activity was measured in a patient with clinical and biochemical parameters consistent with AIP, but where mutation analysis showed no abnormality.

6. Aim 2 of the study (to determine the relative incidence of *HMBS* gene mutations in a South African cohort) revealed itself to be beyond the scope of the study for MMed purposes.

PART B

Literature Review: Acute Intermittent Porphyria

INTRODUCTION

The porphyrias are a group of largely inherited metabolic diseases, each characterised by impairment of a specific enzymatic step in haem biosynthesis. This results in the accumulation of haem precursors and the manifestation of characteristic clinical features (1). This literature review serves firstly to introduce the porphyrias as a group, then focus on acute intermittent porphyria (AIP; OMIM 176000), the mutations associated with this disease, and finally to discuss its importance in the South African context.

There are eight enzymes involved in haem biosynthesis (Figure 1), and a defect at any one of these steps can result in a characteristic accumulation of porphyrins with or without the haem precursors 5-aminolaevulinic acid (ALA) and porphobilinogen (PBG). The word "porphyria" is derived from the Greek word *porphuros* meaning purple, which alludes to the typical discolouration of urine in patients with porphyria. It is often cited that Hippocrates (460-375 BC) was the first to recognise porphyria, but the first cases of the appearance of human urinary porphyrin pigments in modern scientific literature was reported in 1874 (2, 3). Thereafter, in 1889, a Dutch physician Stokvis, described a woman with dark-red urine who died after taking the hypnotic Sulfonal (4). This is commonly thought to be the first description of the acute attack of porphyria.

HAEM BIOSYNTHESIS

While haem synthesis is necessary for all mammalian cells that obtain energy from mitochondrial fuel oxidation, the major producers are the liver for the synthesis of haemoproteins (mitochondrial or microsomal cytochromes, peroxidase, catalase, tryptophan pyrrolase enzymes, amongst many) and erythropoietic cells chiefly for the production of haemoglobin (5). The first enzyme in the biosynthetic pathway, 5-aminolaevulinic acid synthase (ALAS; Enzyme Commission (EC) number 2.3.1.37), is coded by two tissue-specific genes (6). The ubiquitously expressed ALAS1 (on chromosome 3) is regarded as a house-keeping gene for the synthesis of

haemoproteins, while ALAS2 (on chromosome X) is expressed only in erythroid cells for haemoglobin production. Approximately 80% of haem synthesis occurs in the bone marrow and about 15% in the liver. Indeed, regulation of the pathway is tissue and cell specific, subject to modulation by a variety of developmental and environmental pressures through diverse transcriptional factors and mechanisms (7). In general, however, as is typical for many biosynthetic pathways, synthesis of the first committed intermediate, in this case the generation of ALA by ALAS, is considered rate-limiting. ALAS2 transcription is induced during active haemoglobin synthesis, a process co-ordinated by transcription factors according to the state of cell maturation (8). Unlike ALAS2, the ubiquitously expressed ALAS1 transcript is subject to negative feedback regulation by intra-cellular haem (9), and can further be induced by a number of other factors, including starvation, drugs and hormones. Indeed, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is induced under conditions of energy depletion and, in turn, is able to up-regulate mitochondrial biogenesis, gluconeogenic enzymes and the transcription of ALAS1 (10). The latter probably occurs to increase the synthesis of mitochondrial respiratory haem proteins, thereby allowing more efficient energy utilisation.

Haem biosynthesis (Figure 1) commences in the mitochondrion with two simple compounds, glycine and succinyl CoA. The first reaction, catalyzed by ALAS, using pyridoxal phosphate (vitamin B6) as co-factor, results in the formation of ALA. The second reaction occurs in the cytosol during which 2 molecules of ALA condense to form a monopyrrole, PBG, eliminating two molecules of water. This is catalyzed by the enzyme ALA dehydratase (EC 4.2.1.24) and requires zinc as co-factor. Next, PBG deaminase (PBGD; EC 2.5.1.61), also known as hydroxymethylbilane synthase, catalyzes the condensation of 4 molecules of PBG with the release of 4 molecules of ammonia to form hydroxymethylbilane, a linear tetrapyrrole. During the fourth reaction, uroporphyrinogen (UPG) III synthase (EC 4.2.1.75) is responsible for closing the tetrapyrrole ring and catalyzing isomerisation to UPG III, containing 8 carboxyl groups on the side chains. Non-enzymatic cyclization to the I isomer can occur. Sequential decarboxylation by UPG decarboxylase (EC 4.1.1.37) through 7, 6 and 5 carboxyl groups occur to generate the 4 carboxyl coproporphyrinogen (CPG) III. The remainder of the pathway, including the final haem producing reaction, occurs within the mitochondrion.

The sixth reaction consists of the oxidative decarboxylation of CPG III to form the dicarboxylic protoporphyrinogen IX via the enzyme CPG oxidase (EC 1.3.3.3). The penultimate step involves the enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX by protoporphyrinogen oxidase (EC 1.3.3.4). Ferrochelatase (EC 4.99.1.1) catalyzes the final reaction which uses ferrous iron and protoporphyrin to produce haem.

The fundamental defect in all porphyrias is the overproduction of haem intermediates resulting in the clinical sequelae of the disease. Porphyrias may be classified as either erythropoietic or hepatic, based on the organ from which precursors arise (Figure 1). From a clinical perspective, however, it is more useful to divide porphyrias into those causing acute attacks, those resulting in cutaneous lesions, and the rare autosomal recessive porphyrias. The remainder of this review will focus on AIP, including clinical aspects of presentation and diagnosis, and will also address the molecular pathology of this disease.

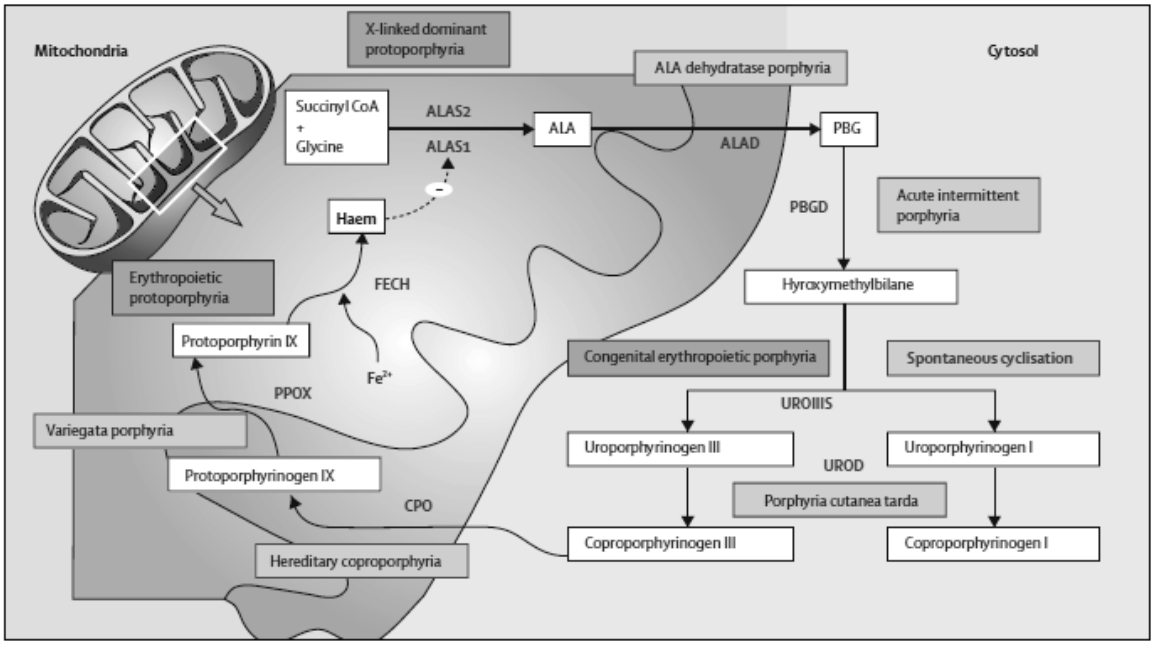

Figure 1: Haem biosynthetic pathway and the porphyrias

Light grey boxes: hepatic porphyrias; dark grey boxes: erythropoietic porphyrias; white boxes: intermediates.

ALAD: ALA dehydratase; PBGD: PBG deaminase; UROIIIS: uroporphyrinogen III synthase; UROD: uroporphyrinogen decarboxylase; CPO: coproporphyrinogen oxidase; PPOX: protoporphyrinogen oxidase; FECH: ferrochelatase (reproduced from (11)).

ACUTE INTERMITTENT PORPHYRIA

AIP is the most common and severe of the inherited acute porphyrias world-wide, and is classified as a hepatic porphyria due to the overproduction of PBG (and ALA and other porphyrin intermediates) in the liver. The disease belongs to a group of autosomal dominantly inherited acute porphyrias, which include variegate porphyria and hereditary coproporphyria but these, unlike AIP, are also characterised by cutaneous involvement. An extremely rare, fourth acute porphyria also exists and is termed ALAD deficiency porphyria. Like AIP it only manifests with acute porphyria symptoms. AIP results from a partial deficiency of the third enzyme in the haem biosynthetic pathway, PBGD. Markedly elevated ALA and PBG occur during an acute attack, and often mildly elevated PBG during remission. Urinary porphyrins are also frequently raised.

The European Porphyria Network recently collected information over a 3 year period regarding the number of newly diagnosed patients with an inherited porphyria. The study received information from 335 patients in 11 different European countries, and showed that the disease occurs in many population groups, with an incidence of symptomatic AIP of 0.13 per year per million population. In northern Sweden, due to a founder effect, the incidence is as high as 0.51 per year per million (12). The prevalence of 5.4 per million (12) is significantly lower than the 50 per million stated in the United States of America (Anderson 2001). AIP has also been reported in patients of African and Afro-Caribbean extraction (13).

Acute porphyrias manifest more commonly in women than men, and rarely occur before puberty or after menopause (14). Most patients who develop the phenotype have only a few attacks and then remain symptom free for the rest of their lives. Indeed, about 80-90% of patients who inherit the defective autosomal dominant AIP gene remain clinically latent for life (15), reflecting the poor penetrance of this disease. Severe, recurrent acute attacks of porphyria occur in a minority of patients and, in women, may be related to the menstrual cycle, particularly during the luteal phase when progesterone is elevated (16).

Porphobilinogen Deaminase Protein

PBGD is a cytosolic protein which catalyses the sequential condensation of four molecules of PBG in a linear head-to-tail fashion, with a covalent enzyme-intermediate at each step. This yields the tetrapyrolle intermediate, hydroxymethylbilane, which is converted to uroporphyrinogen III by uroporphyrinogen III synthase. In the absence of this enzyme, hydroxymethylbilane is able to undergo spontaneous cyclisation to the uroporphyrinogen I isomer. PBGD has an interesting mechanism in which activity is dependent upon a dipyromethane co-factor which is bound covalently to a cysteine residue, and serves as an attachment site for subsequent PBG molecules (17, 18). The co-factor is generated from PBG itself, but does not undergo catalytic turnover. The human structure of PBGD (Protein Data Bank code: 3EQ1 and 3ECR) reveals a monomeric enzyme composed of three domains namely: I (N-terminal), II (central), and III (C-terminal). The active site is located in a cleft between domains I and II and contains the dipyrromethane cofactor (19, 20).

Hydroxymethylbilane synthase Gene

The human *HMBS* gene consists of 15 exons spanning over 10kb of genomic DNA, and is located at chromosome 11q23.3. Two tissue specific mRNA isoforms arising from this single gene have been characterised; the two transcripts arise from two separate promoters via alternative splicing of exons 1 and 2 (Figure 2). The mRNA of the housekeeping (tissue non-specific) isoform contains exons 1 and 3 to 15, which code for an enzyme of 361 amino acids (Mr 42 000), whereas the erythroid isoform is encoded by exons 2 to 15 and thus lacks the first 17 amino acids of the NH_2-terminus (Mr 40 000). Eleven of these amino acids are encoded for by exon 1, and 6 by a short sequence which precedes the start codon of the erythroid isoform. The upstream promoter is active in all tissues while the second promoter, located 3kb downstream, is active only in erythroid cells (1, 5, 21). Two erythroid *trans*-acting factors, GATA-1 and NF-E2, recognise sequences in the downstream *HMBS* promoter, suggesting a role for these transcription factors during erythroid development (22).

Prior to the advent of DNA technology, AIP mutations were divided between two sub-types dependent upon the relative amounts of enzyme activity versus immunologically reactive protein in erythrocytes. A mutation was classified as cross-reactive immunological material (CRIM)-negative when the amount of immunoreactive protein corresponded to the amount of enzymatically active protein (1, 23, 24). CRIM-negative mutations were further sub-divided into two groups according to PBGD activity. In type I mutations, both enzyme activity and protein content are reduced by approximately 50%, as a result of the mutation rendering the mRNA or its protein product unstable. This group (Type I) accounts for about 85% of all mutations, and results from nonsense and missense mutations, small deletions and insertions, splicing defects or polypeptide truncation. Type II mutations arise from single base substitutions at the intron/exon boundary of exon 1, resulting in a splicing defect in the non-specific form of PBGD. Erythroid-specific PBGD is unaffected, as the mutation is upstream from the gene transcription initiation site. The level of erythrocyte PBGD activity is, therefore, normal while activity in non-erythroid tissue (e.g. liver) is half normal. Fewer then 5% of patients with AIP reveal this type of mutation. Type III mutations are CRIM-positive, and produce an approximately 50% decrease in enzyme activity in the presence of normal levels of structurally stable but catalytically inactive enzyme. These mutations occur mostly in exon 12, resulting in a catalytically impaired but

stable mutant due to abnormal splicing (25), or in exon 10 which encodes a well conserved region that is thought to be essential for catalytic activity (18).

To date 390 mutations in the *HMBS* gene have been reported (Human Gene Mutation database; http://hgmd.org; last accessesed January 2014), with most being private and described in only one or a few families. There are, however, several 'founder effect' populations based on the high prevalence of specific mutations in several geographical areas including Sweden, Canada, the Netherlands and Argentina (5, 12). To date ten patients in South Africa have had a diagnosis of AIP confirmed at the gene level, of whom four carry the p.Arg116Trp mutation (26-28), described as occurring commonly in the Netherlands due to a founder effect (29). The ancestry of three of these patients suggests mixed Malay, European and Khoi genetic makeup. The European component may well be Dutch, thereby explaining the presence of this mutation in these apparently unrelated patients. There is also a group of extremely rare recessive mutations of the *HMBS* gene, which manifests in early childhood. The clinical presentation is different from that seen in dominant AIP, with features including mental and developmental retardation, cataracts, ataxia and convulsions (30).

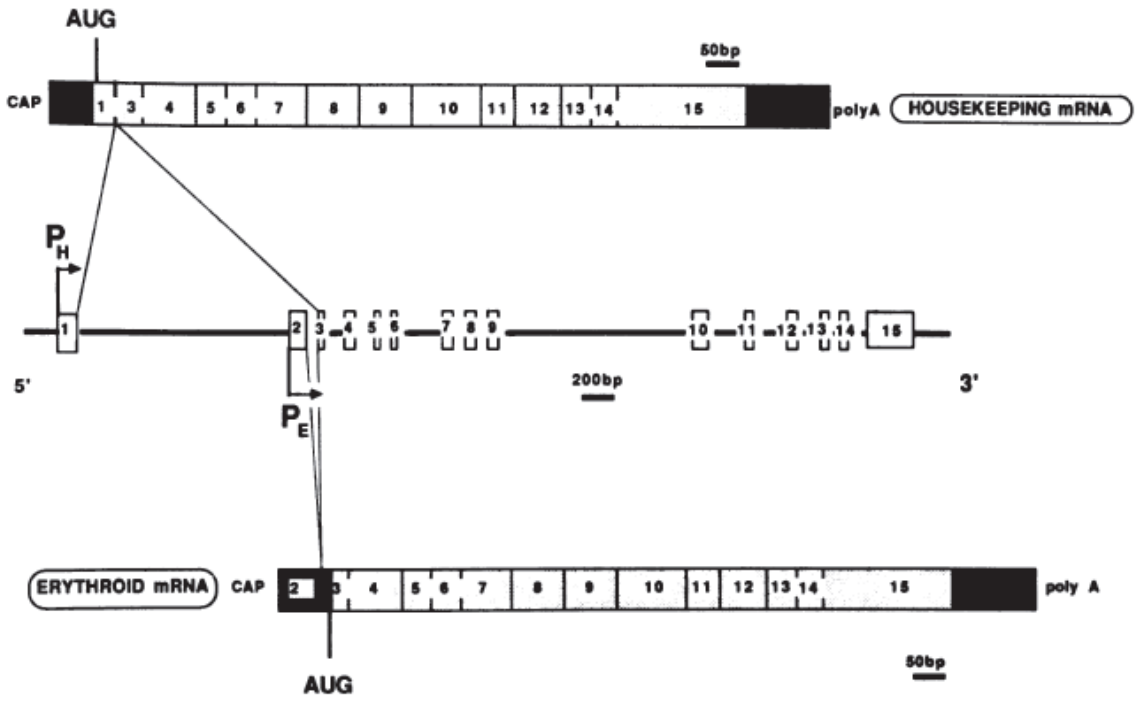

Figure 2: Structure of the human *HMBS* gene and the tissue non-specific and erythroid-specific transcripts

P_H: housekeeping promoter; P_E: erythroid promoter. The exons and introns are drawn to scale (reproduced from (21)).

Clinical Aspects of AIP

The clinical manifestations of an acute porphyric attack all result from extensive involvement of the peripheral, autonomic or central nervous systems. Such neurological involvement may be due to axonal degeneration rather than demyelination (1). An attack often begins with a prodromic phase, which may include behavioural changes such as restlessness, anxiety and insomnia (31). The cardinal feature of the attack is abdominal pain which is severe and generalised, shows no signs of peritonism, and may be accompanied by other gastrointestinal symptoms such as nausea, vomiting and constipation. Pain may also be experienced in the chest, back or thighs. It is continuous and not cramping or colicky, and is usually severe enough to warrant opioid analgesia. Signs of increased sympathetic activity such as tachycardia, sweating and hypertension may be present. Urine may be dark in colour (orange or red) due to the

presence of porphobilin, an auto-oxidised product of PBG, and due to non-enzymatic cyclisation of PBG to form uroporphyrin I (5).

Pain in the extremities is often described as muscular, and may reflect early peripheral neuropathy. Not all patients with an acute attack will develop peripheral neuropathy, but it often occurs as a consequence of ingestion of harmful drugs (see below for further discussion on "Precipitating factors"). Although almost any type of neuropathy can occur, motor neuropathies predominate and generally begin in proximal muscles, affecting arms more frequently than legs. Weakness can be symmetrical, asymmetrical, or localised, and nerve involvement may be progressive, giving rise to quadriplegia, cranial nerve (mostly seventh and tenth) palsies, respiratory and bulbar paralysis and death. Seizures may also occur, thereby producing a therapeutic conundrum as many anti-epileptic drugs themselves exacerbate acute porphyria. Seizures may occur as a result of the porphyria itself or hyponatraemia. Central nervous system involvement is uncommon, although cerebellar and pyramidal signs, transitory blindness and alterations in the level of consciousness do occur (1, 5, 11). There has been a widely held belief that porphyrias are associated with chronic psychotic illnesses such as schizophrenia and bipolar mood disorder. There is, however, no evidence to support this contention (32), although disturbances such as anxiety, depression, acute paranoia and hallucinations do occur (33). Posterior reversible encephalopathy syndrome is a well-described feature of acute porphyria (33) and may occur due to abnormal cerebral autoregulation or cerebral endothelial dysfunction.

Hyponatraemia is a common electrolyte abnormality during an acute attack, and may be severe. The syndrome of inappropriate anti-diuretic hormone secretion is often suggested as the aetiology, but salt depletion from vomiting with poor intake and salt-losing nephropathy are further possible mechanisms (1, 16). Other organ systems may also be affected, causing chronic abnormalities of liver function and increased risk of hepatocellular carcinoma (34). Interestingly, Swedish patients with AIP have a higher risk of hepatocellular carcinoma than other AIP populations, an observation which may, in part, be explained by the high penetrance W128X mutation (35). Whether this finding is generalisable to all AIP patients, and whether global screening of AIP patients will improve outcomes, is unclear (36). Long-standing clinically expressed AIP may further result in sustained hypertension and impaired renal function

(37), while endocrine abnormalities such as glucose intolerance (38) have also been described. The course of an acute attack is variable, with some patients experiencing partial or complete neurological recovery, while others may be left with long-term motor or sensory sequelae. Patients, mostly female, who suffer frequent attacks tend to become increasingly immobilised by motor neuropathy. The outlook for patients suffering from acute attacks has, however, improved significantly over the recent past, with the majority of patients with AIP or variegate porphyria reporting that they lead normal lives (1). Despite the apparent increased understanding and awareness of acute porphyria, the differential diagnosis of particularly undifferentiated neurological presentations can be difficult. Delayed diagnosis is problematic as it can lead to the use of contra-indicated drugs, and delay specific therapy that potentially increases the risk of irreversible neurological damage.

It is clear, therefore, that pre-symptomatic diagnosis of AIP would be of great advantage to individuals shown to carry a proven disease-causing mutation. Such studies are essential in the management of families, and are best performed using DNA analysis (15). Appropriate counselling can be given as to how to minimise risk of suffering an acute attack and, equally importantly, unaffected family members can be reassured as to their non-porphyric status.

Pathophysiology of the Acute Attack

It is interesting to speculate on the possible mechanisms underlying the neuropathology in acute porphyria, and several hypotheses have been proposed. The two leading hypotheses are haem/haemoprotein deficiency in nerve cells and the neurotoxicity of ALA, PBG and/or other porphyrin metabolites (39). If the pathogenesis of AIP was caused by haem deficiency, then it would be expected to cause early neurological features, reminiscent of those seen in mitochondrial disorders associated with encephalopathies. Indeed, the white matter abnormalities seen in patients with AIP (the homozygous recessive variant) differ from those reported in mitochondrial disorders, and muscle mitochondrial respiratory chain enzymes from these patients are normal. Based on magnetic resonance imaging studies, it has been proposed that ALA-mediated neurotoxicity appears to be the most likely mechanism causing the pathological features of AIP (39). Brain white matter appears to be selectively affected in AIP, and it has been proposed that the homology of ALA with glutamate and aspartate (excitatory

neurotransmitters) could affect regions of the brain susceptible to excitotoxicity. The foetal brain may be protected from the toxic effects of haem precursors due to their post-placental clearance from circulation by the maternal renal system (39). Peripheral neurotoxicity induced by ALA has been demonstrated to cause de-myelination, most likely as a result of the oxidative properties of ALA (40). Interference by ALA with the action of another neurotransmitter, γ-aminobutyric acid, on the basis of structural similarity, has also been suggested and may inhibit presynaptic GABA release or act postsynaptically as a partial GABA receptor agonist/antagonist (41). Interestingly, administration of ALA to a healthy volunteer did not precipitate acute symptoms (5).

The most convincing evidence for the role of haem precursors in acute attacks is the report of a 19-year-old female, with heterozygous AIP, who underwent an allogeneic liver transplant after 37 hospital admissions over 29 months. Her ALA and PBG concentrations normalised within 24 hours and she had not had any further attacks 1.5 years after transplant (42). The gene defect was corrected in the liver hence the normalisation of precursor levels, but this is clearly not the case in other tissues, including the nervous system. One would expect that if haem deficiency was an important component of the pathophysiology of the acute attack, the patient would not have such a dramatic resolution of her symptoms. A functional haem deficiency could still be considered, however, based on the elevated plasma levels and brain delivery of tryptophan in the rat, as a consequence of reduced activity of hepatic tryptophan pyrrolase, a haem-dependent enzyme (43). Furthermore, gene targeting has produced a mouse model of PBGD deficiency and these animals develop a chronic peripheral neuropathy at normal or only slightly elevated levels of ALA (44), thus supporting the hypothesis that haem deficiency in murine nervous tissues is also involved in the disease pathogenesis. This issue may, however, have finally been put to rest by the results of recent domino liver transplantations (45). Patients with hepatoma, who were transplanted with liver from AIP patients, developed acute attacks including symptoms of peripheral neuropathy, associated with elevated ALA and PBG excretion. It is clear that the only source of neurotoxin is the liver, establishing that haem deficiency in neuronal tissue plays little role in the pathogenesis of acute attacks in porphyria.

Precipitating Factors

The majority of asymptomatic heterozygotes with a documented *HMBS* gene defect will remain clinically latent for the duration of their lives, although they may exhibit baseline PBG concentrations up to 50-fold higher than the upper reference limit (46). A relatively small proportion of individuals with latent or previously clinically expressed AIP, may manifest an acute AIP attack after exposure to precipitants which may be endogenous, or exogenous environmental factors. It is generally held that most precipitating factors are related to an increase in liver ALAS activity, the rate-limiting enzyme in haem biosynthesis, presumably as a consequence of increased hepatic haem demand. However, increased ALAS activity, in the presence of an approximately 50% decrease in PBGD activity, converts the latter enzyme into the rate-limiting step, causing the accumulation of ALA and PBG and precipitating an acute attack. Under normal conditions therefore, the demand on the haem biosynthetic pathway is below the threshold at which the rate of PBGD activity becomes rate-limiting.

A variety of precipitants may induce an acute attack, and the leading endogenous cause is endocrine. Clinically expressed AIP is rare before puberty and after menopause, and is more common in women, especially during the pre-menstrual period. Naturally occurring sex steroid metabolites induce *de novo* synthesis of hepatic ALAS and increase hepatic cytochromes P-450, while synthetic steroids are also known to induce porphyria (1, 5). Interestingly, and perhaps paradoxically, women with AIP fare well during pregnancy despite the large elevations in sex steroid concentrations (47), however first time mothers with active acute porphyria appear to have elevated risk of perinatal loss (48). An underestimated precipitating factor is reduced caloric intake. Hepatic haem oxygenase is induced by fasting (49), which results in decreased hepatic haem concentrations thereby de-repressing ALAS1. Also, as discussed previously, a low glucose concentration activates PGC-1α which increases ALAS1 expression (10), thereby increasing the flux through the haem biosynthetic pathway. These mechanisms probably account for fasting-induced acute attacks in AIP.

One of the primary culprits for precipitating an acute attack is drugs and, indeed, many patients do well after a diagnosis of AIP has been made if they avoid harmful drugs. It is well known, however, that induction of ALAS, the rate controlling enzyme of the haem biosynthetic pathway,

is crucial to drug porphyrogenicity. The defective gene in the haem biosynthetic pathway becomes rate-limiting, resulting in the accumulation of precursor metabolites. Indeed, drugs are able to induce or suicidally inactivate hepatic cytochrome P-450, an event which is central to this process (50). Many drug safety lists, categorising drugs as safe or unsafe in porphyria, have been published including those lists found on our University of Cape Town website (http://web.uct.ac.za/depts/porphyria), and perhaps more fully, and certainly the most up to date, those lists on the Norwegian (NAPOS) component of the European Porphyria Network website (http://www.drugs-porphyria.org).

In addition, a number of other factors such as smoking (51), infections, surgery, inter-current illness and alcohol excess are known to contribute to an exacerbation of AIP (1, 5). An intriguing finding is noted in a small study in northern Sweden, where a group of 16 patients with AIP subsequently developed type 2 diabetes mellitus. Of this group 8 had clinically manifest AIP but remained free from acute attacks after the onset of diabetes (52). The mechanism of this seemingly protective effect of diabetes is unclear.

Diagnosis

The signs and symptoms of an acute attack are very non-specific, and a high index of suspicion is required, particularly in the context of patients from a non-porphyric background or in children. The abdominal pain of the acute attack can mimic many other often more common conditions, and the yield of screening for acute porphyria should be expected to be low.

An acute porphyric attack is associated with the characteristic increases in serum ALA and PBG, which are excreted into urine. The first-line investigation for a patient suspected of suffering from an acute attack is, therefore, examining the urine for these precursors (1, 5, 11). Both compounds are excreted in large amounts during acute attacks with a mean PBG excretion of 50-fold above the reference interval (53). Although not essential to measure ALA, it can be useful to detect autosomal recessive ALA dehydratase porphyria, lead poisoning or tyrosinaemia type 1, all of which are associated with depressed ALA dehydratase activity. About 66% of latent AIP carriers show normal urinary PBG excretion while the remainder exhibit slightly increased excretion (53). Patients with clinically expressed AIP excrete variably increased PBG and ALA

between attacks but approximately 10% of such patients remain undetected by urinary PBG analysis (53). Uroporphyrin and coproporphyrin are usually only moderately elevated in urine during an acute attack, while stool porphyrins are normal or only slightly increased. Variegate porphyria and hereditary coproporphyria, the other two porphyrias associated with the acute attack, are associated with much greater increases of stool porphyrins, and may also manifest photocutaneous symptoms. Variegate porphyria can often be identified by the presence of a peak at 626nm on plasma fluorescence emission spectroscopy (54), although this peak may shift to a slightly shorter wavelength (e.g. 620nm) during the acute attack (UCT Porphyria lab experience). Serial measurements of urinary PBG are a useful reflection of disease activity, and are used to evaluate the biochemical response to therapy. These measurements are particularly useful in patients who present late, where clinical improvement, which is often used to gauge response to therapy, may be limited. Urinary PBG is also used in the under-resourced South African environment where less hemin may be given, and increasing levels are an early indicator of clinical rebound. Indeed, the index of individuality values for PBG and ALA suggest that comparison of repeat samples with the reference change value may be superior to reference intervals for clinical interpretation. A 2-fold increase in PBG will be detected with a probability of greater than 80% independent of the baseline concentration, and is of clinical significance (46).

The Watson-Schwartz test is a qualitative screening assay for urinary PBG, which produces a pink/red chromogen after the addition of Ehrlich's reagent (para-dimethylaminobenzaldehyde in strong acid). A solvent extraction step is able to separate the chromogen formed by PBG from similar chromogens formed by urobilinogen and other interferents. The draw-backs of this method are that it lacks sensitivity, is not quantitative and is prone to positive interference. For this reason a quantitative assay, such as an anion-exchange column method (55) (originally described in 56) should be used to verify qualitative results.

Family screening is essential to identify individuals at risk of developing clinically manifest AIP, so that adequate precautions can be taken to prevent acute attacks. The diagnosis of AIP in asymptomatic carriers and family members of AIP sufferers cannot be made using the same approach as for an acute attack, as haem precursors are often normal. The diagnosis therefore

relies on the demonstration of an approximately 50% reduction in PBGD activity in erythrocytes, but low activity does not distinguish between latent gene carriers and clinically manifest AIP. Normal erythrocyte PBGD activity also does not exclude AIP in the case of the non-erythroid variant, where decreased activity may be demonstrated in cultured fibroblasts or lymphocytes (5). Furthermore, the range of activity of PBGD in erythrocytes of normal individuals is wide, and overlaps with that of patients with AIP. For these reasons, therefore, mutation analysis is recommended and often included as a diagnostic modality.

Treatment of the Acute Attack (or AIP)

Although prompt and successful treatment is the primary focus of a patient suffering from an acute porphyric attack and is the primary aim of the attending physician, this review will only address general treatment principles. The first aims should be to identify and remove any potential precipitants of an acute attack, particularly drugs, hypocaloric intake and underlying infections. Pain, hypertension and electrolyte disturbances, particularly hyponatraemia, should be promptly treated and bedside spirometry should be available to detect early pseudobulbar palsy necessitating ventilation. The use of intra-venous haem arginate preparations to repress ALAS1 activity is the most effective way of reducing production of PBG and ALA, and represents the treatment of choice (57). Haem arginate or hemin administration may prevent the onset and progression of neuropathy, but will not reverse already established neuropathy (11). The biochemical response to haem arginate therapy can be monitored with serial urinary PBG measurement but clinical monitoring must also be used as a guide to whether/when repeat haem treatments are perhaps necessary. Prophylactic haem arginate may occasionally be required for patients who have severely compromised quality of life due to recurrent acute attacks. Its use is not licensed for this indication, and complications related to iron overload, venous network toxicity and difficulty with treatment withdrawal occur (16).

Patients should be counseled about avoidance of precipitants, particularly unsafe drugs, smoking, alcohol and the maintenance of a healthy diet while avoiding fasting. As discussed above, liver transplantation has resulted in excellent clinical and biochemical responses, but such treatment should be reserved for select patients with the most severe and seemingly intractable forms of AIP (58). An extension of the theme of the restoration of PBGD activity in the liver to cure AIP

is the intriguing possibility of liver-specific gene therapy. A recent report (59) provides evidence of liver-directed gene therapy using a liver-specific enhancer and promoter to achieve high levels of hepatic PBGD expression in a mouse AIP model. These mice were protected from biochemical induction of acute attacks, and demonstrated significant improvement in neuromotor function. This study provides a rationale for gene therapy in AIP patients with recurrent acute attacks. Indeed a collaborative project, involving seven partners from four European countries, aims to develop the clinical use of the orphan drug AAV5-AAT-*HMBS* for the treatment of AIP (http://www.aipgene.org/; last accessed June 2013). AAV is a replication-incompetent virus that has been modified to deliver genes into human cells. AAV5-AAT-*HMBS* acts by delivering the *HMBS* expression cassette directly into hepatocytes. The use of gene therapy may not be suitable for all patients due to the presence of pre-existing anti-vector antibodies. A new approach, which may be suitable for such patients, is the use of short interfering ribonucleic acid (siRNA) to knock down ALAS activity (60). Using an AIP phenobarbitol induction model, siRNA targeting of ALAS may prevent up regulation of the ALAS1 transcript and metabolite production during acute attacks, and may herald a promising approach for treatment of AIP.

CONCLUSION

AIP belongs to a group of porphyric disorders which may present with a characteristic group of neurovisceral and motor symptoms. Unfortunately, although such attacks are acute, they potentially leave in their wake long-term neurological sequelae which can be progressive with each attack. It is, therefore, imperative to initiate the formal identification of patients in South Africa with clinically expressed AIP at the molecular level, with a view to further screening of family members. This would allow the identification of latent carriers and, importantly, education of carriers with respect to precipitants and how best to avoid them so as to minimise the risk of provoking an acute attack. Although only about 10% of AIP heterozygotes ever become symptomatic, it is not possible to predict which will manifest clinically. To this end a mutational analysis approach should be used to characterise South African AIP patients and their families, initially diagnosed on clinical and biochemical grounds. This work will also help to delineate any possible founder-effect in the South African AIP population, a phenomenon that has been observed in other population groups.

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PART C

Manuscript for Submission to the

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Molecular Characterisation of Acute Intermittent Porphyria in South Africa

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ABSTRACT

Aims

Acute intermittent porphyria (AIP) is a low penetrant autosomal dominant disorder caused by mutations in the hydroxymethylbilane synthase (*HMBS*) gene. Many mutations in this gene have been reported, but knowledge about the spectrum of mutations present in South Africa is limited. Identification of such mutations is important so that latent carriers of affected family members

can be identified, and counselled to avoid precipitating factors. This study presents the molecular analysis of ten South African AIP patients.

Methods

Of the total of 10 patients with clinical and biochemical evidence of AIP, 4 were black, 3 of mixed ancestry and 3 Caucasian. Genomic DNA was isolated from affected probands and family members of one proband, and the *HMBS* gene amplified by PCR and mutations characterised by direct sequencing.

Results

Four new mutations, a missense p.Lys98Glu; a frameshift mutation, p.Asp230Asp fs*19 and two splice site mutations, c.422+3_6delAAGT and c.161-1 G>A, were detected in the South African probands. Three previously described mutations (p.Arg149X, p.Arg116Trp and p.Trp283X) were found, while in two patients no mutations were revealed. Of the latter two, one is deceased and the other demonstrated HMBS enzyme activity approximately 50% of normal controls. Mutation analysis of 5 offspring of one of the probands carrying the p.Trp283X mutation, revealed 2 asymptomatic carriers.

Conclusion

This study comprises the most recent and comprehensive characterisation of mutations in the *HMBS* gene of AIP patients in South Africa. Four of the mutations were novel, and three have been previously reported. One of these mutations, found in a patient of mixed ancestry, probably originated in The Netherlands, where a founder population exists.

BACKGROUND

AIP (MIM # 176000) is an autosomal dominant disorder of haem biosynthesis caused by a partial deficiency of HMBS or porphobilinogen deaminase (EC 2.5.1.61). This enzyme catalyses the head-to-tail condensation of four molecules of porphobilibogen (PBG) to form hydroxymethylbilane.[1] In AIP, porphyrins and their precursors, PBG and 5-aminolaevulinic acid, are produced in excess and are excreted in urine and stool for many years after an acute attack.[2] Biochemical diagnosis of AIP is based the measurement of these urinary precursors, the presence of a plasma fluorescence peak at 619nm (with UV excitation around 400 nm) and the determination of erythrocyte HMBS activity. Molecular analysis of the *HMBS* gene is not

required to make a diagnosis of AIP, but is essential and worthwhile for presymptomatic diagnosis. A great proportion of patients remain clinically asymptomatic throughout life and are thus potentially at risk of an acute attack. There is, thus, a need for genetic screening of family members potentially at risk.

Clinically, AIP manifests as intermittent attacks of neurovisceral dysfunction, which can be precipitated by various factors such as drugs, hormones and alcohol.[1,4] Indeed, 80-90% of patients who inherit a defective AIP gene remain clinically latent for life,[3] reflecting the poor penetrance of this disease. A recent European study revealed an incidence of symptomatic AIP of 0.13 per year per million population. In northern Sweden, due to a founder effect, the incidence is as high as 0.51 per year per million.[5]

The human *HMBS* gene consists of 15 exons spanning over 10kb of genomic DNA, and is located at chromosome 11q23.3. Located in the 5' flanking region and in intron 1 are two distinct promoters which generate the housekeeping (containing exons 1 and 3-15) and erythroid-specific (containing exons 2-15) transcripts by alternative splicing of exon 1 and 2.[6] Three hundred and ninety mutations in the *HMBS* gene have been reported in the Human Genome Mutation Database (http://www.hgmd.org) including those in several small studies in South Africa.[7-9] To date, ten patients in South Africa have had a diagnosis of AIP confirmed at the gene level, of whom four carry the p.Arg116Trp mutation, described as occurring commonly in the Netherlands, due to a founder effect.[10] The ancestry of three of these patients suggests a mixture of Malay, European and Khoi blood. The European component may well be Dutch, thereby explaining the presence of this mutation in these apparently unrelated patients. The present study expands the cohort of South African patients with a molecular diagnosis of AIP, revealing four novel mutations, and opening the way for family studies.

MATERIALS AND METHODS

Selection of study cohort

The University of Cape Town Lennox-Eales Porphyria Laboratory is a national referral centre for porphyria diagnosis and management. A total of 29 patients with a clinical and biochemical (not shown) diagnosis of AIP were designated as potential participants in this study. Of these, 10

patients were ultimately recruited and of these, 6 previously had DNA stored for further study, and 4 were newly recruited. The remainder of the patients were not traceable. Of the 10 participants, 4 were black, 3 of mixed-race and 3 of Caucasian origin. Two of the Caucasians were siblings, and 5 offspring (all asymptomatic) of one of the siblings were screened for mutations. Informed consent was obtained from the 3 newly recruited patients, while analysis of the *HMBS* gene of the other 7 participants was considered imperative for confirmation of diagnosis. Ethical approval for this study was obtained from the Health Sciences Faculty Research Ethics Committee (HREC/REF: 324/2010), University of Cape Town, South Africa. No member of this cohort is known to be related to South African patients previously investigated[7-9] for *HMBS* mutations.

Molecular analysis

Genomic DNA was isolated from 2.5ml whole blood, using the Wizard Genomic DNA Purification kit (Promega, Madison, USA) as per the manufacturer's protocol. PCR amplification of all 15 exons and flanking intronic sequences was carried out using primers, designed using Primer Designer version 2.0, as detailed in Table 1. PCR products spanning all exons of the entire *HMBS* gene were subjected to direct sequencing, to determine the precise sequence variations. PCR products were purified for sequence analysis using the GFX PCR DNA and Gel Band purification kit (GE Healthcare, Buckinghamshire, UK). In most cases purified samples were then sequenced in both forward and reverse directions on an ABI 3130 XL DNA automatic sequencer using a Big Dye terminator cycle sequencing kit, by the Department of Genetics, University of Stellenbosch, Stellenbosch, South Africa. Sequence data were collected and analysed using BioEdit Sequence Alignment Editor, Version 5.0.9.1 (Tom Hall, North Carolina State University, USA). Sequences in BioEdit were aligned with Genbank data (http://www.NCBI.nlm.nih.gov) using ClustalW (http://dot.imgen.bcm.tmc.edu:9331/ multi-align/Options/clustalw.html).

Restriction Enzyme Digestion

Sequence analysis revealed 4 unpublished mutations (see Results). The absence of these mutations was confirmed in at least 50 race-matched control DNA specimens by restriction enzyme digest analysis (mutation: c.292A>G and enzyme: *Sty1*; mutation: c.689_690delAC and

enzyme: *BstY1*; mutation: c.422 + 3_6delAAGT and enzyme: *XcmI*; mutation c.161-1G>A and enzyme *DdeI*). The DNA of family members of 2 related probands was also interrogated with restriction analysis (mutation: c.848G>A; enzyme: *XbaI*). Restriction enzyme maps for wild type and polymorphic DNA fragments were constructed using Webcutter 2.0 (copyright 1997, Max Heiman).

HMBS Enzyme Assay

HMBS activity was determined according to Piepkorn *et al*.[11] Whole blood (50µl) was incubated with haemolysing reagent (0.2% Triton X-100, 1mM dithiothreitol and Zn ions in the form of $ZnSO_4.7H_2O$; 1ml) for 30min at 37°C. Two and a half ml of 10% trichloroacetic acid was added to one reaction mixture to create a blank, and 4mM ALA in phosphate buffer, pH7.5 (1ml), added to the blank and test specimens and incubated at 37°C for 1h. Ten % trichloroacetic acid (2.5ml) was added to the test specimens, the samples allowed to stand in the dark for 30min, centrifuged and the fluorescence (relative fluorescence units) of the standards (coproporphyrin standard was diluted to 50, 100 and 200ng/ml with 1.5mM HCl), blank and test specimens recorded at an excitation wavelength of 405nm and excitation wavelength of 595nm. HMBS enzyme activity was calculated in $nmol.h.^{-1}ml^{-1}$ red blood cells (haematocrit).

RESULTS

Sequence analysis of the *HMBS* gene in 10 patients revealed 7 mutations, 4 of which were novel (Table 2). A pair of siblings elaborated the same mutation, while in 2 patients no mutation was found. The p.Arg149X mutation in exon 9 creates a premature stop codon and was identified in a black patient, and has previously been found in Finnish families.[12] Similarly the p.Arg116Trp mutation in exon 8 identified in a patient of mixed race, was revealed in the same Finnish study[12] but has also been commonly found in the Dutch AIP population.[10] Two Caucasian siblings (a male and female) were identified with the p.Trp283X mutation[13] in exon 14, and restriction analysis (using the restriction enzyme *XbaI*, results not shown) of DNA obtained from the offspring of the male sibling showed that 2 of 5 children had inherited this mutation (Table 2), but neither children have had symptoms consistent with AIP. Two patients with a biochemical diagnosis of AIP were found not to harbour exonic *HMBS* mutations in the exons and flanking regions interrogated. One of these patients, a female of mixed ancestry, deceased at the age of

14 years. HMBS enzyme analysis of red blood cells derived from the other patient, a female of black ancestry, showed activity of 13.8nmol/h/ml red blood cells, 49.2% of normal control activity (average activity of 3 controls: 28.0nmol/h/ml). This is consistent with the expected 50% decreased in enzyme activity in patients with AIP.

The four novel mutations included a missense mutation (p.Lys98Glu), two splice site mutations (c.422+3_6delAAGT and c.161-1 G>A) and a frameshift mutation (p.Asp230Asp fs*19). The p.Lys98Glu mutation resulted from an A-to-G transition in exon 7 predicting the substitution of positively charged lysine for a negatively charged glutamic acid. The c.422+3_6delAAGT mutation in intron 8 and c.161-1 G>A mutation in intron 4 generate a putative donor and acceptor splice site mutation, respectively. The deletion in exon 12 of the second and third bases of codon GAC (aspartic acid) generates a new codon GAT, also representing aspartic acid. Further, a frameshift occurs which predicts substitution of 19 amino acids in codons 230 to 248 and chain termination at codon 249 (230DILDLVGVLHDPETLLRCI to 230DLGSGGCAARSRDSASLHRX), thereby deleting the last 111 amino acids of the 361 amino acid HMBS enzyme. None of the changes observed in patients with unpublished mutations were detected in the control groups.

DISCUSSION

This report reflects the most current interrogation of HMBS mutations in AIP patients in South Africa. Regrettably this cohort is incomplete, the task being complicated by difficulties with tracing and following up patients in a fragmented health care system and a finite time period for this study. Nevertheless, molecular investigation of AIP patients is an important step in the management of families, where screening is essential to identify those with latent disease so as to prevent acute attacks.[3,14] The sensitivity for mutation detection determined by sequencing is 95%,[15] and increases to 98.1% if gene dosage analysis is included.[16] This provides greater diagnostic accuracy than erythrocyte HMBS activity analysis.[3]

This study identified a total of 7 mutations in the 10 patients screened. It is well known that most AIP mutations are private and limited to the families in which they arise. This is supported by the fact that, to date, 390 mutations have been identified in the *HMBS* gene in AIP. Three of

the mutations found have been previously described. The p.Arg149X mutation creates a premature stop codon and was first identified in two Finnish families,[12] but in this study it was identified in a black male. Indeed, this mutation has also been detected in Spanish probands[17] and occurs at CpG dinucleotides, which are known mutation hotspots. The p.Arg116Trp mutation was found in a patient of mixed ancestry, and the same mutation was revealed in a previous study of 3 South African mixed ancestry patients.[8] A relatively high frequency of this mutation (19/80 families) has been found in the Dutch AIP population and a distinctive haplotype was found to segregate with the p.Arg116Trp mutation in this population.[10] This suggests a founder effect, and as the ancestry of all South African patients with this mutation is mixed, it is likely that the mutation is of Dutch origin, considering the Dutch colonial history of South Africa. A further founder mutation is p.Trp283X, found in 56% of AIP patients in Switzerland and has thus the highest prevalence of any single *HMBS* gene mutation in an AIP population.[18] The two siblings found to harbour this mutation are Caucasian, but are not known to have Swiss ancestry as far as could be determined, and haplotype studies would be required to establish if their mutation is linked to the founder mutation. Indeed, the utility of a molecular diagnosis of asymptomatic family members is highlighted by the revelation that 2 of 5 offspring of one of the siblings were also found to carry the mutation. This allows for appropriate counselling of the parents and children, both those carrying the mutation and those found to be mutation free.

Two patients with clinical and biochemical evidence (results not shown) of AIP were shown not to have coding region mutations or mutations in flanking regions. One of these patients presented peri-pubertally at the age of 11 years, but demised 3 years later. The other patient is still being followed up, and erythrocyte HMBS activity was shown to be decreased by about 50%, consistent with AIP. In such patients, where no mutations can be found, quantitative PCR for gene dosage analysis is necessary to exclude large deletions and insertions or functional intronic mutations caused by nonsense mediated decay.[3] A recent report further suggests mRNA analysis to exclude pseudoexon inclusion as a cause of AIP in mutation negative patients.[19]

This study revealed four novel mutations. Lysine at position 98 has previously been demonstrated to mutate to arginine[12] (p.Lys98Arg) but we reveal a change to glutamic acid

(p.Lys98Glu). This lysine moiety is located within the active site cleft and in close proximity to the dipyromethane cofactor, and is probably critical for substrate docking and catalysis.[20] Bacterial expression of the wild-type and the p.Lys98Glu mutated HMBS proteins by workers in our laboratory (Pienaar pers.com.), facilitated kinetic analysis which revealed a K_M for the mutated enzyme approximately double that of the wild-type enzyme. Furthermore enzyme efficiency, expressed as K_{cat}/K_M, was almost negligible. It was also shown that the p.Lys98Arg mutant lost its ability to bind the dipyromethane co-factor, which was not the case for the p.Lys98Glu mutant. This mutant replaces a positively charged amino acid with one which is negative, suggesting that charge is not important for co-factor binding, whereas the more bulky arginine moiety in the p.Lys98Arg mutant appears to disrupt the binding of the co-factor. Indeed, it appears that charge is important for the ability of the enzyme to interact with its substrate, as reflected by the loss of substrate affinity in the p.Lys98Glu mutant.

Two intronic lesions were detected, and are hypothesised to adversely influence splicing. A mutation in the same position as c.161-1G>A in intron 4 has been described, except that a G to C transition occurs.[21] The p.Asp230Asp fs*19 mutation in exon 12 causes a frameshift, resulting in a truncated protein most likely with compromised enzyme activity. During the preparation of this manuscript, a further *HMBS* mutation was characterised in a Caucasian patient with a biochemical diagnosis of AIP (Pienaar pers. com.). In exon 12, c.771insT introduces a stop codon 33 codons downstream, thereby producing a truncated protein. This mutation has previously been described in South Africa.[8]

There is a dearth of information on AIP in black and mixed ancestry patients and, in particular, the scope of HMBS mutations is almost unknown in the African context. A study of seven West and North African and Afro-Caribbean AIP patients characterised six different mutations, four of which were novel.[22] In this study, three of the four novel mutations were found in black or mixed ancestry patients. Although studies are limited, it appears that AIP is not uncommon in blacks, and the disease-causing mutations are heterogeneous except for, perhaps, p.Arg116Trp which may be of Dutch origin. Furthermore, the spectrum of mutations demonstrated in black patients appears to be different to those observed in the white population. From the data in this study, it is not possible to make inferences about the prevalence of *HMBS* mutations in South

Africa. This could be achieved by a larger, directed prospective study of AIP patients, using this study as a starting point. Further studies are required to delineate the scope of mutations in other regions of Africa, and the continuing emergence of novel mutations and the heterogeneity of mutations demonstrates the usefulness of prior characterisation of *HMBS* mutations in newly diagnosed patients, to allow detection of relatives with latent AIP.

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Funding was provided by the Medical Research Council, self-initiated research grant awarded to PN Meissner, the NRF Incentive award for rated researchers (PN Meissner), The University of Cape Town Research Committee and the National Health Laboratory Service Research Trust.

Table 1: Characteristics of the *HMBS* gene primers used

Amplicon	Primer pair	Size (bp)	Product size (bp)	Tm (°C)	Sequence
Exon 1	*HMBS* F1	20	342	58	5'ggtccactgtcgcaatgttc3'
	HMBS R1	20			5'tagacgactgaggatggcaa3'
Exon 2-3	*HMBS* 2F	19	902	58	5'gcctcttgctggagaaggt3'
	HMBS 3R	19			5'agccacggctcagactctt3'
Exon 4-6	*HMBS* 4F	19	814	58	5'gcctaacctgtgacagtct3'
	HMBS 6R	19			5'ccagcagcctatctgacac3'
Exon 7-9	*HMBS* 7F	19	854	58	5'aggctccaccactgaagta3'
	HMBS 9R	19			5'tgagtggacggatgagtgc3'
Exon 10	*HMBS* 10F	19	598	58	5'agacagactcaggcagagg3'
	HMBS 10R	19			5'ccagcctacggtgttagag3'
Exon 11-12	*HMBS* 11F	19	787	54	5'ctctaacaccgtaggctgg3'
	HMBS 12R	20			5'acagacctgaggacatcact3'
Exon13-15	*HMBS* 13F	20	781	58	5'atgtcctcaggtctgtggtc3'
	HMBS 15R	20			5'cacatactgaggaggcaagg3'

Table 2: Mutation spectrum in the *HMBS* gene in South African patients with AIP

Patient	Gender/Race Ma=male F=female B=Black C=Caucasian Mi=mixed ancestry	Location of mutation	Molecular defect	Predicted protein product	Enzyme activity[d] (nmol/h/ml red blood cells)
1	Ma/B	Exon 9	c.445C>T	p.Arg149X	N/D[e]
2	F/Mi	Exon 8	c.346C>T	p.Arg116Trp	N/D
3	Ma/C[a]	Exon 14	c.848G>A	p.Trp283X	N/D
4	F/C[a]	Exon 14	c.848G>A	p.Trp283X	N/D
5	Ma/C[b]	Exon 14	c.848G>A	p.Trp283X	N/D
6	F/C[b]	Exon 14	c.848G>A	p.Trp283X	N/D
7	F/Mi	No mutation found			N/D (patient deceased)
8	F/B	No mutation found			13.8
9	Ma/C	Exon 7	c.292A>G	p.Lys98Glu[c]	N/D
10	F/B	Intron 8	c.422+3_6delAAGT	Splicing aberration[c]	N/D
11	Ma/Mi	Exon 12	c.689_690 delAC	p.Asp230Aspfs*19[c]	N/D
12	F/B	Intron 4	c.161-1 G>A	Splicing aberration[c]	N/D

[a]Siblings
[b]Siblings, and offspring of patient 3
[c]Novel mutations
[d]PBGD enzyme activity range: 24.0-54.0nmol/h/ml red blood cells[11]
[e]N/D: not determined

Supporting Information

Journal of Clinical Pathology: Instructions for Authors

The text below includes sections on Editorial Policy, Article Types and Manuscript Format from the Instructions for Authors of the *Journal of Clinical Pathology*. The full text can be viewed at: http://jcp.bmj.com/site/about/guidelines.xhtml#original

Editorial policy

The Journal of Clinical Pathology (JCP) is committed to the advancement of all disciplines within the broader remit of human pathology. This also encompasses molecular biology and its applications in the understanding of human biology and pathology. The journal is intended to have world-wide readership and will publish articles that have a wide appeal even though they are regionally based.

Issues with a narrower restricted focus may be submitted as Letters to the Editor or as correspondence. JCP wishes to publish cutting edge, original clinical and laboratory-based articles, especially those with a clear clinical relevance. Provision of an educational platform for trainees, scientists and pathologists is an important function and aim of the journal. As such, state of the art reviews, viewpoints and editorials will be published.

The editorial team wishes to produce a balanced, informative and meaningful journal that is sensitive to the needs of its readership and the specialty at large, as well as being in tune with contemporary issues.

In pursuit of these goals we wish to publish work that is ethical (morally and scientifically), of a high quality and governed by a fair, independent peer review system.

Open Access

Authors can choose to have their article published Open Access for a fee of £1950 (plus applicable VAT).

Colour figure charges

During submission you will be asked whether or not you agree to pay for the colour print publication of your colour images. This service is available to any author publishing within this journal for a fee of £250 per article. Authors can elect to publish online in colour and black and white in print, in which case the appropriate selection should be made upon submission.

Article types and word counts

Original articles

Short reports

Letters to the Editor (original research)

Best Practice

My Approach / Demystified

Reviews

Leading Articles / Editorials

Correspondence

Supplements

Multiple Choice Questions (MCQs)

Take home messages:

To aid understanding and clarity of their paper, Authors are asked to provide three to four key facts that summarise the essence of their work and/or what they intend the reader to focus on. These "take home messages" should be placed at the end of the manuscript, before the references. Please see the current issue for examples.

The word count excludes the title page, abstract, tables, acknowledgements and contributions and the references.

Original articles

Original articles should report original research of relevance to the understanding and practice of clinical pathology. They should be written in the standard form: abstract; introduction; methods; and discussion. **A paragraph about what the paper adds should also be added underneath the abstract.**

The journal uses a structured form of abstract in the interests of clarity. This should be short (no more than 250 words) and include four headings:

Aims - the main purpose of the study

Methods - what was done, and with what material

Results - the most important results illustrated by numerical data but not p values

Conclusions - the implications and relevance of the results

Authors of original articles are required to comply with one of the appropriate reporting guidelines endorsed by the EQUATOR Network. The following are the most commonly used guidelines for this journal. Authors are expected to submit the checklist that is most appropriate for their manuscript type:

Experimental studies - CONSORT Statement

Observational Studies - STROBE Statement

Diagnostic accuracy studies - STARD Statement

Biospecimen reporting - BRISQ

Reliability and agreement studies - GRRAS

If none of the above listed guidelines are suitable for the manuscript, the author is requested to either search for the most relevant set of guidelines supplied by the EQUATOR Network or explain during the submission process why none of the guidelines are appropriate for their study type.

Word count: up to 2000 words.
Structured abstract: up to 250 words.
Tables/Illustrations: at editorial discretion.
References: up to 150.

Short reports

Short technical notes and brief investigative studies are welcomed and usually published in the form of a Short/Technical report. At the discretion of the Editor-in-Chief some short reports will be published in the Correspondence section but will undergo the usual peer review process.

Word count: up to 1200 words.
Abstract: up to 150 words.
Tables/Illustrations: up to 6. If more are required the text must be reduced accordingly.
References: up to 12.

Letters to the Editor (original research)

eLetters provide an opportunity for rapid online publication of correspondence related to articles published in the Journal of Clinical Pathology. The letter must be pertinent/relevant to a recently published article in Journal of Clinical Pathology. Contributors should go to the abstract or full text of the article in question. At the top right corner of each article is a "contents box". Click on the "eLetters: Submit a response to this article" link. Some letters in response to an article may be published in the print version of the journal.

Authors are reminded that eLetters do not serve as a forum for seeking medical/health advice.

References should not exceed a maximum of four.

Best Practice

Best Practice articles are published by editorial invitation. Unsolicited best practice articles are unlikely to be accepted but the editor is always pleased to receive suggestions. The 'Best Practice' series is geared to practising pathologists as well as trainees on how to approach some of the more difficult/contentious issues in Pathology. We are looking for diagnostic algorithms, investigative trees and/or any other useful hint(s) that will facilitate making the best/right diagnosis. These can include molecular techniques which may not be within the remit of every laboratory but certainly something that is doable.

Word count: between 2500 and 3000 words.
Abstract: up to 250 words.
Illustrations: at editorial discretion.
References: up to 150.

My Approach / Demystified

My Approach and Demystified articles are published by editorial invitation. Unsolicited demystified articles are unlikely to be accepted but the editor is always pleased to receive suggestions. These articles are geared to practising pathologists as well as trainees on how to approach some of the more difficult/contentious issues in Pathology.

We are looking for diagnostic algorithms, investigative trees and/or any other useful hint(s) that will facilitate making the best/right diagnosis. These can include molecular techniques which may not be within the remit of every laboratory but certainly something that is doable.

Word count: between 2500 and 3000 words.
Abstract: up to 250 words.
Illustrations: at editorial discretion.
References: up to 150.

Reviews

Any proposals for reviews should be discussed with the editor before submission.

Word count: between 2500 - 3000 words.
Abstract: up to 250 words.
Tables/Illustrations: at editorial discretion.
References: up to 150.

Leading Articles / Editorials

Leaders and Editorials are published by editorial invitation. Unsolicited leaders or editorials are unlikely to be accepted but the editor is always pleased to receive suggestions.

Word count: between 2500 words.
Abstract: up to 250 words.
Tables/Illustrations: at editorial discretion.
References: up to 150.

Correspondence

Single case reports of outstanding interest or clinical relevance are mostly published as Correspondences. The title should be brief. No abstract, keywords or subheadings are required. The correspondence should include a brief

introduction of a few sentences followed by a succinct report and discussion. A detailed literature review is not required.

Letters in response to articles published in the Journal of Clinical Pathology and short technical notes, and brief investigative studies may be published in this section at the discretion of the Editor-in-Chief.

Word count: up to 900 words.
Abstract: Not required. Tables/Illustrations: up to 4.
References: up to 8.

Multiple Choice Questions (MCQs)

MCQs based on submitted manuscripts may be solicited by the editor for publication on the BMJ Online Learning site. An invitation to submit MCQs may be extended to you by the editor at the time of acceptance of your manuscript.

The journal requires between 5-10 multiple choice questions (MCQs) with 5 options each, based on your article for the online learning programme. You may choose to include images as well. The questions need to be submitted to the journal within 4-6 weeks. Please see below for some more helpful guidelines:

Please include in your MCQ:

A separate Word document which also includes the article title and author names.
The title and authors of the article to which the MCQs are associated with must be provided
The author of the MCQs (even if the same) must be clearly stated
The MCQs set must contain at least 5 questions
Each question must have 5 possible answers, with only *one* answer being correct (the correct answer must be marked with an asterisk)
Additional explanation text (for user to see after taking the test) can be submitted for *each individual answer* if appropriate. It is ok to have some answers with explanation and some without.
Figures if applicable can be included in questions (must be submitted as gif/jpg files)

Supplements

The BMJ Publishing Group journals are willing to consider publishing supplements to regular issues. Supplement proposals may be made at the request of:

1. The journal editor, an editorial board member or a learned society may wish to organise a meeting, sponsorship may be sought and the proceedings published as a supplement.
2. The journal editor, editorial board member or learned society may wish to commission a supplement on a particular theme or topic. Again, sponsorship may be sought.

3. The BMJPG itself may have proposals for supplements where sponsorship may be necessary.
4. A sponsoring organisation, often a pharmaceutical company or a charitable foundation, that wishes to arrange a meeting, the proceedings of which will be published as a supplement.

In all cases, it is vital that the journal's integrity, independence and academic reputation is not compromised in any way.

When contacting us regarding a potential supplement, please include as much of the information below as possible.

Journal in which you would like the supplement published

Title of supplement and/or meeting on which it is based

Date of meeting on which it is based

Proposed table of contents with provisional article titles and proposed authors

An indication of whether authors have agreed to participate

Sponsor information including any relevant deadlines
An indication of the expected length of each paper Guest Editor proposals if appropriate

For further information on criteria that must be fulfilled, download the supplements guidelines (PDF).

Manuscript format

Cover letter
Title page
Manuscript format
Statistics
Style
Figures/illustrations
Tables
References
Supplementary files

All material submitted is assumed to be submitted exclusively to the journal unless the contrary is stated. Submissions may be returned to the author for amendment if presented in the incorrect format. It should be in both the manuscript and the details page during submission.

Please note that only the article text (from first word of main text to the last word in reference list) will be used to typeset your article.

All other data (known as the metadata), such as article title, author names and addresses, abstract, funding (etc) statements will be taken from the fields you have filled in at submission, so you must ensure that these are up to date and accurate.

Cover letter

Your cover letter should inform the Editor of any special considerations regarding your submission, including but not limited to:

1. Details of related papers published or submitted for publication.

    - Copies of related papers should be submitted as “Supplementary files not for review” to help the Editor decide how to handle the matter.

2. Details of previous reviews of the submitted article.

    - The previous Editor's and reviewers' comments should be submitted as Supplementary material along with your responses to those comments. Editors encourage authors to submit these previous communications - doing so may expedite the review process.

3. Indication as to whether any of your article (for example, appendices, large tables) could be published as Web only files rather than in the print version of the article. Please label any files for online publication only with this designation.

Title page

The title page **must** contain the following information:

1. Title of the article.
2. Full name, postal address, e-mail, telephone and fax numbers of the corresponding author.
3. Full names, departments, institutions, city and country of all co-authors.
4. Up to five keywords or phrases suitable for use in an index (it is recommended to use MeSH terms).
5. Word count - excluding title page, abstract, references, figures and tables.

Acceptance

Please note: If any of this information is repeated in the final Word document it will be removed by the typesetters and replaced with the information from the submission system. Therefore please check the metadata on ScholarOne Manuscripts carefully and make any changes before submitting the final version of your Word document.

List of the information taken from submission system only:

• Article type
• Title
• Author names

• Author affiliations, and corresponding author's full details
• Abstract (where applicable)
• Keywords
• Study approval
• Patient consent
• Funding statement
• Competing interests
• Contributor statement
• Trial Registration number (for clinical trials)

Manuscript format

Please note, this instruction is for submission only.

The manuscript must be submitted in Word. PDF format is not accepted.

The manuscript must be presented in the following order:

1. **Title page**.
2. **Abstract** (or summary for case reports) (note: references not allowed in abstracts or summaries).
3. **Main text** (provide appropriate headings and subheadings as in the journal. We use the following hierarchy: **BOLD CAPS**, **bold lower case**, Plain text, *Italics*).
4. **Tables** should be in the same format as your article (ie Word) and not another format embedded into the document. They should be placed where the table is cited and they must be cited in the main text in numerical order.
5. **Acknowledgments, Competing interests, Funding**.
6. **Reference list**.

Appendices (these should be Web only files to save space in the print journal; if so, please ensure you upload appendices as Web Only files and ensure they are cited in the main text as such.)

Images must be uploaded as separate files (view further details in Figures/illustrations) All images must be cited within the main text in numerical order.

Do not use the automatic formatting features of your word processor such as endnotes, footnotes, headers, footers, boxes etc. Please remove any hidden text.

Statistics

Statistical analyses must explain the methods used.

Guidelines on presenting statistics.

Guidelines on RCTs: CONSORT, QUORUM, MOOSE, STARD, and Economic submissions.

Style

Abbreviations and symbols must be standard and SI units used throughout except for blood pressure values which are reported in mm Hg.

Whenever possible, drugs should be given their approved generic name. Where a proprietary (brand) name is used,

it should begin with a capital letter.
Acronyms should be used sparingly and fully explained when first used.

View more detailed style guidelines >>

Figures/illustrations

Colour images and charges.
If you wish to publish colour figures in print you will be charged a fee that will cover the cost of printing. The journal charges authors for the cost of reproducing colour images on all unsolicited articles, see the journal web pages for cost information. Alternatively, authors are encouraged to supply colour illustrations for online colour publication and black and white publication in the print. This is offered at no charge.

File type

Ideally, submit your figures in TIFF or EPS format. We can also accept figure files of the following types: BMP, EPI, GIF, JPEG, PNG, PNG8, PNG24, PNG32, PS, PSD, SVG, WMF.
Resolution requirements apply (9cm across for single column, 18cm for double column):
1. For B/W, the format should be either TIFF or EPS. The resolution should be in 300 DPI.
2. For 4-colour, the format should be either tiff or eps in CMYK. The resolution should be 300 DPI.
3. For line-art, vector format is preferable. Otherwise, the resolution should be 1200 DPI.
During submission, when you upload the figure files label them with the correct **File Designation**: for example Mono Image,for black and white figures, and Colour Image for colour figures.
Histograms should be presented in a simple, two-dimensional format, with no background grid.
Figures are checked using automated quality control and if they are below standard you will be alerted and provided with suggestions in order to improve the quality.
All images should be mentioned in the text in **numerical order** and figure legends should be listed at the end of the manuscript.
Please ensure that any specific patient/hospital details are removed or blacked out.
NOTE: we do NOT accept figures which use a black bar to obscure a patient's identity.

Online only material

Additional figures and tables, methodology, references, raw data, etc may be published online only to link with the printed article. If your paper exceeds the word count you should consider if any of the article could be published online only as a "data supplement". These files will not be copyedited or typeset.

All data supplement files should be uploaded using the File Designation: "Web only files".

Please ensure any data supplement files are cited within the text of the article.

Multimedia files

You may submit video and other files to enhance your article (video files should be supplied as .FLV, .F4V, .Mov, .WMV, .AVI, .MP4, .MPG). When submitting video files, ensure you upload them using the File Designation “Video Files”.

Using material already published elsewhere

If you are using any figures, tables or videos that have already been published elsewhere you must obtain permission from the rightsholder (this is usually the publisher and not the author) to use them and add any required permission statements to the legends.

Tables

Tables should be submitted in the same format as your article (Word) and not another format embedded into the document. They should appear where the table should be cited, cited in the main text and in numerical order. Please note: we **cannot** accept tables as Excel files within the manuscript.
If your table(s) is/are in Excel, copy and paste them into the manuscript file.
Tables should be self-explanatory and the data they contain must not be duplicated in the text or figures - we will request that any tables that are longer/larger than 2 pages be uploaded as web only data.

References

Authors are responsible for the accuracy of cited references: these should be checked against the original documents before the paper is submitted. It is vital that the references are styled correctly so that they may be hyperlinked.

Citing in the text

References must be numbered sequentially as they appear in the text. References cited in figures or tables (or in their legends and footnotes) should be numbered according to the place in the text where that table or figure is first cited. Reference numbers in the text must be inserted immediately after punctuation (with no word spacing)—for example,[6] not [6].
Where more than one reference is cited, separate by a comma—for example, [1, 4, 39]. For sequences of consecutive numbers, give the first and last number of the sequence separated by a hyphen—for example, [22-25]. References provided in this format are translated during the production process to superscript type, which act as hyperlinks from the text to the quoted references in electronic forms of the article.
Please note, if your references are not cited in order your article will be returned to you before acceptance for correct ordering.

Preparing the reference list

References must be double spaced (numbered consecutively in the order in which they are mentioned in the text) in the [slightly modified] Vancouver style (see example below). Only papers published or in press should be included in the reference list. (Personal communications or unpublished data must be cited in parentheses in the text with the name(s) of the source(s) and the year. Authors should get permission from the source to cite unpublished data.).

References must follow the [slightly modified] Vancouver style:

12 Surname AB, Surname CD. Article title. Journal abbreviation Year;**Vol**:Start page–End page.

Use one space only between words up to the year and then no spaces. The journal title should be in italic and abbreviated according to the style of Medline. If the journal is not listed in Medline then it should be written out in full. Check journal abbreviations using PubMed.

List the names and initials of all authors if there are 3 or fewer; otherwise list the first 3 and add et al. (The exception is the Journal of Medical Genetics, which lists all authors.)

Example references:

Journal article

13 Koziol-Mclain J, Brand D, Morgan D, et al. Measuring injury risk factors: question reliability in a statewide sample. Inj Prev 2000;**6**:148–50.

Chapter in book

14 Nagin D. General deterrence: a review of the empirical evidence. In: Blumstein A, Cohen J, Nagin D, eds. Deterrence and Incapacitation: Estimating the Effects of Criminal Sanctions on Crime Rates. Washington, DC: National Academy of Sciences 1978:95–139.

Book

15 Howland J. Preventing Automobile Injury: New Findings From Evaluative Research. Dover, MA: Auburn House Publishing Company 1988:163–96.

Abstract/supplement

16 Roxburgh J, Cooke RA, Deverall P, et al. Haemodynamic function of the carbomedics bileaflet prosthesis [abstract]. Br Heart J 1995;73(Suppl 2):P37.

Electronic citations

Websites are referenced with their URL and access date, and as much other information as is available. Access date is important as websites can be updated and URLs change. The "date accessed" can be later than the acceptance date of the paper, and it can be just the month accessed. See the 9th edition of the AMA Manual of Style for further examples.

Electronic journal articles

Morse SS. Factors in the emergency of infectious diseases. Emerg Infect Dis 1995 Jan-Mar;1(1). www.cdc.gov/nciod/EID/vol1no1/morse.htm (accessed 5 Jun 1998).

Electronic letters

Bloggs J. Title of letter. Journal name Online [eLetter] Date of publication. url

eg: Krishnamoorthy KM, Dash PK. Novel approach to transseptal puncture. Heart Online [eLetter] 18 September 2001. http://heart.bmj.com/cgi/eletters/86/5/e11#EL1

Check your citation information using PubMed.

Digital Object Identifiers (DOIs)

DOIs are a unique string created to identify a piece of intellectual property in an online environment; particularly useful for articles which have been published online before appearing in print (and therefore the article has not yet been assigned the traditional volume, issue and page number reference). The DOI is a permanent identifier of all versions of an article, whether raw manuscript or edited proof, online or in print. Thus the DOI should ideally be included in the citation even if you want to cite a print version of an article.

How to cite articles before they have appeared in print

1. Alwick K, Vronken M, de Mos T, et al. Cardiac risk factors: prospective cohort study. Ann Rheum Dis Published Online First: 5 February 2004. doi:10.1136/ard.2003.001234

How to cite articles once they have appeared in print

1. Vole P, Smith H, Brown N, et al. Treatments for malaria: randomised controlled trial. Ann Rheum Dis 2003;**327**:765–8 doi:10.1136/ard.2003.001234 [published Online First: 5 February 2002.
More comprehensive guidance about DOIs.

PLEASE NOTE: RESPONSIBILITY FOR THE ACCURACY AND COMPLETENESS OF REFERENCES RESTS ENTIRELY WITH THE AUTHORS.

Supplementary material

You may submit supplementary material which may support the submission and review of your article. This could include papers in press elsewhere, published articles, appendices, video clips (please see Multimedia files instructions), etc.
All supplementary material files should be uploaded using the File Designation: Supplementary material.

Online only material

Additional figures and tables, methodology, references, raw data, etc may be published online only to link with the printed article. If your paper exceeds the word count you should consider if any of the article could be published online only as a "data supplement". These files will not be copyedited or typeset.
All Appendices should be considered Online only material.
All data supplement files should be uploaded using the File Designation: Web Only files.
Please ensure any data supplement files are cited within the text of the article.

Multimedia files

You may submit video and other files to enhance your article (video files should be supplied as .avi, .wmv, .mov .mp4 or .H264). When submitting video files, ensure you upload them using the File Designation “Video Files”.

Part D

University of Cape Town Dissertation Guidelines

MINIMUM REQUIREMENTS FOR DISSERTATIONS FOR MMED AND MPHIL FOR SUBSPECIALITIES DEGREES

Following extensive discussion with Heads of Divisions, Dr S Kalula and Prof S Kidson recommend the following minimum criteria for dissertations for MMed and MPhil (subspeciality) degrees:

The MMed minor dissertation (or the MPhil dissertation in the case of subspecialities) is one of three examination components of the MMed/MPhil degree. This minor dissertation carries one third of the weight of a full master's dissertation in terms of its credit weighting. The dissertation must be a study containing the results of an analytical, quantitative, or epidemiological study carried out by the candidate (for certain disciplines, the candidate may chose instead to do a qualitative study, an audit cycle or a formal systemic review). A case report is not acceptable for the dissertation.

The dissertation must be the result of independent work of the candidate conducted under the guidance and direction of a supervisor(s) and should demonstrate evidence of an ability to undertake research, to adequately interpret results and to comprehensively and critically review the relevant literature. Although the findings of the research need not necessarily be original, they must be seen to advance scientific understanding. The topic and scope of research will depend on the particular disciplines and must be agreed upon in consultation with the supervisor(s).

Research Protocol

Candidates intending to register for the MMed/MPhil Part III are required to submit a full research protocol for approval to their respective Departmental Research Committee (DRC). The candidate must also obtain FHS UCT Ethics approval prior to conducting their research. This full research protocol (together with a copy of the ethics approval letter) must be submitted to the

postgraduate administration for approval by the Board of the Faculty of Health Sciences, prior to commencement of the research. For most disciplines, submission of the research protocol should be made no later than the end of year 2. The research protocol should outline the scope and content of the dissertation and must include the title of the proposed dissertation, name of the supervisor(s) and their brief curriculum vitae.

Submission of Dissertations

On completion, the dissertation should be submitted to the Faculty Postgraduate Officer. The candidate should inform the Faculty Officer one month in advance of the intention to submit.
Submission deadlines:

1. February 17th for June graduation
2. August 15th for December graduation

Supervisors will be requested by the Faculty Postgraduate Officer to submit a letter supporting submission. This letter should be supplied by the primary supervisor. If this supervisor is external, the internal supervisor must be kept informed at every stage of the process. Specific submission requirements may be set by individual disciplines.

Note on fees: To avoid attracting fees, dissertations need to be submitted before the beginning of the first quarter (first day of academic year), and before the start of the second semester (mid July) to qualify for a 50% fee rebate.

Supervisors

One cannot overemphasize the importance of identifying a dissertation supervisor as early as possible. The supervisor should be an individual who can relate to the candidate's research project, be available for frequent and regular discussion and advice, and someone with whom the candidate can develop a good working relationship. Where specialised equipment and/or laboratory work is required for the study, the supervisor should assist in facilitating such access to such facilities. Supervisors may assist candidates in developing scientific communication skills but they are not required to do detailed editing or correction of spelling, grammar, or style. They may refer candidates to the UCT Writing Centre for this purpose.

The primary supervisor may be based outside the candidate's home department, faculty or university. In such a case, an internal (or secondary) supervisor will be required in addition to the primary supervisor, to serve as a guide and link to discipline-specific procedures. Primary supervisors retain responsibilities to the candidate and the university until the dissertation process is complete.

Please note: in order to assist a candidate with a master's research topic the supervisor needs to hold a master's degree or higher, or have relevant research experience. If the primary supervisor does not hold a higher degree or equivalent (such as a Fellowship of The College of Medicine of South Africa), then a secondary supervisor who has a higher degree will need to be appointed in addition to the primary supervisor.

Candidates are strongly encouraged to publish the study with the supervisor(s) as co-author(s). This may require work beyond the graduation date. Such arrangements should be discussed and documented in advance.

The Dissertation

Submission of the dissertation should satisfy the following criteria:

1. The title page should contain the candidate's name, dissertation title and the name of the university. It must also state the degree, e.g. Master of Medicine (MMed) in Public Health Medicine, Occupational Medicine, Family Medicine, Surgery, etc. The title page should also include a statement to the effect that the research report is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. It must also state that this work has not been published prior to registration for the abovementioned degree.

2. The body of the dissertation, which must be structured in 4 parts, should include the following:

   Part A: The *protocol* (as approved by the Departmental Research Committee and Faculty Research Ethics Committee). The protocol should not exceed 4000 words.

Part B: A *structured literature review* appropriate to the subject matter and methods of the dissertation. The literature review must, amongst other things, show that the student is sufficiently acquainted with the relevant literature and is able to perform a critical appraisal and, if appropriate for the topic, show a good understanding of evidence-based medicine.

The review should be between 3 000 and 4 000 words.

A suggested structure for the literature review is as follows:

a) Objectives of literature review
b) Literature search strategy, including inclusion and exclusion criteria
c) Quality criteria - some leeway will be allowed here, as candidates will vary in their ability to appraise studies. This will also vary with the nature of the dissertation
d) Summary or interpretation of literature
e) Identification of gaps or needs for further research
f) References (which will overlap with but will not be the same lists as in the journal article and protocol)

Part C: The results of the study must be presented in the form of a *manuscript* of an article for a named peer reviewed journal, meeting all the requirements set out in the "Instructions for Authors" of that journal, including the word count and referencing style. (Unless specially motivated, the journal chosen will need to allow for *at least* 3000 words excluding abstract, tables, figures and references). The "Instructions to Authors" of the journal must be appended. The journal chosen for publication must be appropriate to the subject matter of the dissertation and accredited by the Department of Education or listed in the citation index of the Institute for Scientific Information (ISI). Important note: the candidate need not have submitted the article, not is the acceptance of the article and requirement for passing the degree. The norm of practise is to publish the study with the supervisor(s) as co-author(s) and candidates are strongly encouraged to submit their manuscript either before or after examination of the mini-dissertation.

Part D: All supporting documents including:

- Questionnaire/data capture instrument
- Consent forms and any related participant information sheets
- Technical appendices, including, if considered necessary, any additional tables not included in the main manuscript for the examiner to have available. These should be accompanied by a brief narrative
- Official Ethics approval letter from the Faculty Research Ethics Committee

3. The article does not have to be submitted to the journal in order to meet academic requirements.

4. A candidate must submit 2 copies of the dissertation in temporary binding, and an electronic copy on compact disc in a universally readable format (e.g. pdf).

Examiners

The full dissertation will be submitted for examination through the Postgraduate office of our Faculty to two external examiners (nominated by the supervisors and HOD).Three examiners will be nominated, two of which are invited to examine, and one held as an alternate. All examiners must be external to UCT. These nominations are circulated to the Faculty Dissertation Committee. It is the *supervisor's (or co-supervisor's)* responsibility to submit names of potential examiners to the Faculty Officer when the candidate is ready to submit. The examiners will be well briefed regarding the specific requirements and criteria for submission and examination of the mini-dissertation. Such criteria will clearly explain the difference between the mini-dissertation and a Master's degree by dissertation alone. Details required for each examiner are: academic qualifications, postal and/or physical address, telephone and fax numbers and e-mail address, and one paragraph description of their standing in the relevant field (drawn from their CV if need be). *The candidate may not be informed of the identity of the examiners*. After the outcome of the mini-dissertation has been finalised, the examiners' identities are made known if the examiners have indicated that they do not object to this.

Patient Information and Consent Forms

PATIENT INFORMATION (English)

ACUTE INTERMITTENT PORPHYRIA

Introduction

The porphyrias are a group of genetic diseases in which the haem pathway is disturbed, and are reasonably common in South Africa. This occurs in the liver in some forms of porphyria, and in the red blood cells in others. These are not liver or blood diseases and these organs function normally. Large amount of substances called porphyrins accumulate in the body and this may cause two problems- abnormal sensitivity of the skin to sunlight, and a complication called the acute attack.

Acute intermittent porphyria (AIP) is not a common form of porphyria in South Africa. People with AIP develop only one complication of the disease viz. the acute attack (see below) Many people with AIP never have any problems and they are referred to as silent carriers of the disease. They remain susceptible to an acute attack and should take the necessary precautions detailed in our porphyria information booklet

Inheritance of AIP

AIP is inherited as an autosomal dominant condition. This means that the disease is transmitted from one generation to the next, with each child (irrespective of sex) having a 50% chance of inheriting the defective gene. However, when a defective gene is inherited the person quite commonly does not show the disease at all. In other words, one might inherit the disease without showing any sign of it. They may, however, pass the mutation on to their children.

We suspect that there are many different mutations in South African patients with AIP (the 4 mutations identified to date are different). This means that the various families are not related to one another, and it is chance that they have developed the disease.

Clinical manifestations:

Patients with AIP do not develop skin disease. This means that the skin is not particularly susceptible to exposure to the sun, i.e. is not more so than an individual without the disease.

They are, however, susceptible to an acute attack. A few particularly unfortunate patients suffer attacks at short intervals. These patients require specialised treatment and their doctors are advised to contact our centre for help and advice (www.porphyria.uct.ac.za). In the case of a clinical emergency contact Groote Schuur Hospital (021 404 9111) and ask for the Liver registrar/ consultant on duty. However, many patients who inherit a mutation never have any problems (silent carriers).

Making a diagnosis of AIP

As AIP is not common in South Africa (it is much less common than variegate porphyria) it is often missed or misdiagnosed.

To confirm a diagnosis of AIP the relevant tests must be performed in a reliable laboratory. In those families in whom the mutation is not known AIP will be diagnosed by routine tests of urine and stool and blood from puberty to 22 years (usually manifests in this time period), the interpretation being assisted by clinical and family information.

DNA testing is not routinely available for AIP. However, once a family's mutation has been identified, other family members can be tested at any age.

Precaution for individuals with AIP

Avoid the acute attack. Avoid all medication that is dangerous in porphyia (see booklet). Wear a medic alert bracelet stating that you are porphyric. Inform your doctor, dentist and pharmacist that you have porphyria to avoid being given unsafe medicines. Advise all blood relatives to be tested as they may carry the mutation and develop symptoms in the future.

What is an acute attack?

Patients with AIP are always at risk of an acute attack of porphyria. This may be very dangerous thus it is important that they understand how they can prevent such an attack. The acute attack occurs when the level of porphyrin precursors (ALA and PBG) reach very high levels-far higher than in porphyria. Full details of the acute attack and how to avoid one are described in our porphyria booklet.

This study

This study is to identify the DNA mutation that you have which is causing the AIP. You will be required to have blood drawn from you (2x 4.5ml). The DNA will be extracted from the blood and analysed to identify the exact mutation. A screening test will then be set up to enable easy testing of other members of your family. This is important as unidentified family members are at risk of an acute attack. Once the mutation has been identified and providing you wish to know, you will be informed of the result and genetic counseling made available to you.

PATIENT INFORMATION (AFRIKAANS)

AKUUT-INTERMITTERENDE PORFIRIE

Inleiding

Die porfirieë is 'n groep genetiese siektes waarin die metabolisme van heem versteur is, en is redelik algemeen in Suid-Afrika. Sommige vorms van porfirie kom in die lewer voor en ander in die rooibloedselle. Porfirie is nie ’n siekte van die lewer of die bloed nie. Hierdie organe funksioneer normaal. ‘n Groot hoeveelheid stowwe, genaamd porfiriene, hoop in die liggaam op en dit kan twee probleme veroorsaak - verhoogde sensitiwiteit van die vel tot sonlig; en 'n komplikasie wat ‘n akute aanval van porfirie genoem word.

Akuut-intermitterende porfirie (AIP) is nie die mees algemene vorm van porfirie in Suid-Afrika nie. Mense met AIP ontwikkel slegs een komplikasie van die siekte, en dit is die akute aanval van porfirie (sien hieronder). Baie mense met AIP het nooit enige probleme en na hulle word verwys as draers van die siekte. Hulle bly vatbaar vir 'n akute aanval en moet die nodige voorsorgmaatreëls tref. Hulle word beskryf in ons porfirie inligtingstuk.

Oorerflikheid van AIP

AIP word oorgeërf as 'n outosomaal dominante toestand. Dit beteken die siekte word oorgedra van een generasie na die volgende en elkeen van die nasate het ‘n 50% kans om die defektiewe geen te hê. Wanneer ’n enkele kopie van die defektiewe geen geërf word, word die persoon gewoonlik nie siek nie – ’n mens moet twee kopieë van die betrokke geen hê. Met ander woorde, ’n mens kan die geen erf met geen teken van die siekte nie. Kinders kan egter die mutatie weer na hul eie kinders oordra.

Ons vermoed dat daar baie verskillende mutasies in Suid-Afrikaanse pasiënte met AIP is (die 4 mutasies wat tot op hede geïdentifiseer is, verskil van mekaar). Dit beteken dat

die verskillende families nie aan mekaar verwant is, en dit kan net deur kans wees dat hulle die siekte ontwikkel het.

Kliniese manifesterings:

Pasiënte met AIP ontwikkel nie velsiekte nie. Dit beteken dat hul vel nie meer sensitief vir die son as gewoonlik is nie.

Hulle is egter vatbaar vir 'n akute aanval. Sommige pasiënte kry kort-kort aanvalle. Hierdie pasiënte benodig spesiale behandeling en hul dokters word aangeraai om ons sentrum te kontak vir hulp en advies (www.porphyria.uct.ac.za). In ’n noodgeval, kontak Groote Schuur Hospitaal (021-404 9111) en vra vir die lewer-dokter / -konsultant aan diens. Daar is egter pasiënte wat 'n mutasie erf en nooit enige probleme ontwikkel nie (draers).

Diagnose van AIP

Omdat AIP nie algemeen in Suid-Afrika is nie (dit is baie minder algemeen as Porfirie Variegata), word dit dikwels nie gediagnoseer nie.

Om 'n diagnose van AIP te bevestig moet sekere toetse in ‘n betroubare laboratorium gedoen word. In families waar die mutasie nie bekend is nie, sal roetine toetse van urien, stoelgang en bloed gedoen word. Kliniese en familie inligting help om die interpretasie te maak.

DNA toetse is nie beskikbaar vir roetine AIP diagnose nie. Maar as die mutasie in ’n familielid geïdentifiseer word, kan andere familielede getoets word op enige ouderdom.

Voorsorg vir mense met AIP

Probeer om oorsake wat ’n akute aanval kan uitlok, te vermy. Vermy alle medikasie wat gevaarlik is in porfirie (sien boekie). Dra ’n Medic-Alert armband wat sê dat jy aan porfirie ly. Lig jou dokter, tandarts en apteker in dat jy aan porfirie ly, om te voorkom dat jy onveilige medisyne kry. Raai alle familielede aan om getoets te word omdat hulle die mutasie kan hê en in die toekoms simptome kan ontwikkel.

Wat is 'n akute aanval?

Pasiënte met AIP het altyd 'n risiko om 'n akute aanval van porfirie te ontwikkel. Dit kan baie gevaarlik wees, dus is dit belangrik dat ‘n mens verstaan hoe so 'n aanval voorkom kan word. Die akute aanval kom voor wanneer die vlak van porfirie-voorlopers (ALA en PBG) baie hoë vlakke bereik - veel hoër as normaal. Volledige besonderhede van die akute aanval en hoe om dit te verhoed word beskryf in die porfirie boekie.

Hierdie studie

Hierdie studie het ten doel om die DNA mutasie wat jy het en wat AIP veroorsaak, te identifiseer. Ons sal jou vra of ons bloed by jou kan trek (2 x 4.5 ml). Die DNA sal uit die bloed onttrek word en gebruik word om die presiese mutasie te identifiseer. 'n Siftingstoets sal dan opgestel word sodat die ander lede van jou gesin maklik getoets kan word. Dit is belangrik omdat ongeïdentifiseerde familielede die kans het om 'n akute aanval te ontwikkel. Sodra die mutasie geïdentifiseer is, en jy die resultaat wil hê, sal die uitslag en genetiese voorligting aan jou beskikbaar gestel word.

ADULT CONSENT FORMS (ENGLISH)

Molecular characterisation of acute intermittent porphyria in South Africa

CONSENT FOR DNA ANALYSIS AND STORAGE (BY PATIENTS AND FAMILY MEMBERS)

1. I, _____, request that use be made of my DNA to identify the disease causing mutation for acute intermittent porphyria that: I/my child (DELETE WHERE NOT APPLICABLE) have/might have inherited.

2. I understand that the genetic material for analysis is to be obtained from: blood cells/previously frozen transformed lymphoblasts (DELETE WHERE NOT APPLICABLE).

3. I request that no portion of the sample be stored for later use. o (MARK IF APPLICABLE).

Or

I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):

(a) possible re-analysis

(b) other porphyria research, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential

4. The results of the analysis carried out on this sample of stored biological material will be made known to me via GSH/UCT Liver Clinic Doctor or researcher Dr P Fortgens, in accordance with the relevant protocol, if and when available.

5. In addition, I authorise that results may be made known to: (DELETE WHERE NOT APPLICABLE):

a. other doctors involved in my care

b. the following family members: _____

6. I authorise/do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Porphyria laboratories/Liver Clinic GSH /UCT.

7. I have been informed that:

(a) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.

(b) the Porphyria laboratories are under an obligation to respect medical confidentiality.

(c) I may withdraw my consent at any time without this affecting my future medical care.

8. ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:

_____ DATE: _____

Patient signature _____Witnessed consent_____

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Email: Peter.Meissner@uct.ac.za

Mrs L. Emjedi
Faculty of Health Sciences
Human Research Ethics Committee
Groote Schuur Hospital, Observatory
Cape Town, 7925
Tel. 27 21 4066338, Fax. 27 21 4066411
Email: Nosi.tsama@uct.ac.za

CONSENT TO PARTICIPATE IN A STUDY

Study: Molecular characterisation of acute intermittent porphyria in South Africa

Subjects: Individuals with AIP and family members

This study has received ethics approval from the University of Cape Town (UCT), research Ethics Committee no ……..

All individuals participating in this study are asked to read, complete and sign this form on receiving the relevant information on the study.

Lab code …….. DNA sample no. ……

I ……(name of person giving consent),

hospital folder number ……..(if applicable) hereby agree to participate in the study.

Participant: (Relationship to person giving consent if not yourself)……

Name (1)……

Name (2)……

Name (3)……

Name (4)…….

I understand that 2 x 4.5ml blood samples will be taken from me and the sample will be used to investigate the genetic factors involved in acute intermittent porphyria (AIP).

I acknowledge that I have been supplied with information regarding AIP and the nature of the study.

I have been informed that:

- I am encouraged to ask questions regarding porphyria and the present research study
- The only discomfort associated with this study is a needle prick when blood is taken
- I will not receive any reward for taking part in this study
- I am at liberty to refuse to participate/withdraw my consent from this research at any time, and this will not affect my relationship with the UCT/GSH Liver Clinic, my future medical care, or legal rights
- The benefit to my participating in the study is that the specific mutation causing my AIP will be identified, and will enable a simple test to screen for this mutation in my family, allowing an easy diagnosis to be made
- The study will be done in the Porphyria laboratories, Dept. of Medicine, UCT. It will be done by Dr P. Fortgens and Dr A. Corrigall and led by Prof P. Meissner. The study will commence once Ethics permission has been granted (approximately July 2010) and will continue for a period of 3 years.
- If I have any questions about the study I should contact Dr P. Fortgens (tel 021-4044135)

Please initial each of the following paragraphs if you agree:

……My doctor may provide clinical information about my condition to the doctors involved in this study, on request.

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

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

Signature of young person giving consent……..

Date…….

Signature of witness……

Date……..……..

Contact details of witness……..…….

NB: Three copies should be signed for: (1) patient, (2) researcher, (3) hospital folder (if applicable).

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ADULT CONSENT FORMS (AFRIKAANS)

Molekulêre karakterisering van akuut-intermitterende porfirie in Suid-Afrika

TOESTEMMING VIR DNA-ANALISE EN BERGING (DEUR PASIËNTE EN FAMILIE)

1. Ek, _____, gee hiermee toestemming dat my DNA gebruik mag word om die mutasie wat akuut-intermitterende porfirie veroorsaak, te identifiseer; wat: ek / my kind (HAAL DEUR WAAR NIE VAN TOEPASSING) kon erf.

2. Ek verstaan dat die genetiese materiaal vir die analise verkry sal word van: bloedselle / voorheen bevrore getransformeerde limfosiete (HAAL DEUR WAAR NIE VAN TOEPASSING).

3. Ek versoek dat geen gedeelte van die monster geberg mag word vir latere gebruik nie. (KRUIS INDIEN VAN TOEPASSING).

Of

Ek versoek dat 'n gedeelte van die monster vir onbepaalde tyd geberg mag word (HAAL DEUR WAAR NIE VAN TOEPASSING) vir:

(a) moontlike verdere analise

(b) ander porfirie navorsing, onderhewig aan die goedkeuring van die Universiteit van Kaapstad se Etiekkomitee, op voorwaarde dat enige inligting van sodanige navorsing vertroulik sal bly.

4. Die resultate van die analise wat uitgevoer is op die gebergde biologiese materiaal bekend gemaak sal word aan my via die GSH / UK Lewerkliniek se dokter, of navorser, Dr P Fortgens, in ooreenstemming met die betrokke protokol, as wanneer die resultaat beskikbaar is.

5. Verder gee ek toestemming dat resultate bekend gemaak mag word aan: (HAAL DEUR WAAR NIE VAN TOEPASSING):

a. ander dokters wat my behandel

b. die volgende familielede: _____

6. Ek gee toestemming / weier dat my dokter(s) (HAAL DEUR WAAR NIE VAN TOEPASSING) relevante kliniese inligting mag verskaf aan die Porfirielaboratorium / Lewerkliniek GSH / UK.

7. Ek neem kennis dat:

(a) die proses van analise spesifiek is vir die genetiese toestand hierbo genoem; dit kan nie die volledige genetiese samestelling van ’n indiwidu bepaal nie.

(b) die Porfirielaboratorium onder 'n verpligting is om mediese vertroulikheid te respekteer.

(c) ek my deelname aan hierdie studie op enige tydstip kan stop, sonder dat dit gevolge inhou vir my toekomstige mediese sorg.

8. ALLES HIERBO IS AAN MY VERDUIDELIK IN ‘N TAAL WAT EK VERSTAAN EN MY VRAE IS BEANTWOORD DEUR:

_____ Datum: _____

Pasiënt handtekening _____Getuie handtekening_____

Prof P.N. Meissner
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TOESTEMMING OM DEEL TE NEEM AAN DIE STUDIE

Studie: Molekulêre karakterisering van akuut-intermitterende porfirie (AIP) in Suid-Afrika

Deelnemers: Pasiente met AIP en familielede

Hierdie studie het etiese goedkeuring ontvang van die Universiteit van Kaapstad (UK), navorsing Etiekkomitee nr. ... ...

Alle pasiente wat deelneem aan hierdie studie is gevra om die vorm te lees en te onderteken met ontvangs van die inligtingstuk oor die studie.

Lab-kode ... ... DNA-monster nr. ... ...

Ek ... ... (naam van persoon wat toestemming gee),

hospitaalnommer ... ... (indien van toepassing) stem hiermee toe om deel te neem aan die studie.

Deelnemer (Verwantskap aan persoon wat toestemming gee indien nie self)..... .....

Naam (1).....

Naam (2).....

Naam (3).....

Naam (4).....

Ek verstaan dat 2 x 4.5ml bloedmonsters geneem sal word en die monster gebruik sal word om die genetiese faktore betrokke in AIP te ondersoek.

Ek erken dat ek voorsien is met inligting aangaande AIP en die aard van die studie.

Ek is in kennis gestel dat:

• Ek aangemoedig is om vrae te vra oor porfirie en die huidige navorsingstudie.

• Die enigste ongemak wat verband hou met hierdie studie 'n naaldprik sal wees wanneer bloed geneem word.

• Ek sal nie 'n beloning ontvang vir deelname aan hierdie studie nie.

• Ek is vry om te weier om deel te neem/my toestemming om deel te wees van die navorsing enige tyd te onttrek, en dit sal geen invloed op my verhouding met die Universiteit van Kaapstad / GSH Lewerkliniek, my toekomstige mediese sorg, of wetlike regte hê nie.

• Die voordeel van my deelname aan die studie is dat die spesifieke mutasie wat my AIP veroorsaak geïdentifiseer sal word, en 'n eenvoudige toets sal dit moontlik maak om hierdie mutasie in my gesin te diagnoseer.

• Die studie sal gedoen word in die Porfirielaboratorium, Dept van Geneeskunde, Universiteit van Kaapstad. Dit sal gedoen word deur Dr P. Fortgens en Dr A. Corrigall en word gelei deur Prof P. Meissner. Die studie sal begin sodra etiese goedkeuring verkry is (ongeveer Julie 2010) en sal voortgaan vir 'n tydperk van ongeveer 3 jaar.

• As ek vrae het oor die studie het, kan ek kontak maak met Dr P. Fortgens (tel 021-4044135).

Parafeer asseblief elk van die volgende paragrawe:

.....My dokter kan kliniese inligting oor my toestand voorsien aan die dokters wat betrokke is in hierdie studie, op aanvraag.

.....Ek versoek dat resultate wat relevant is vir my en my familie bekend gemaak sal word aan my.

.....My mediese inligting en die laboratorium uitslae mag gehou word in 'n rekenaar databasis. Ek verstaan dat vertroulikheid gehandhaaf sal word en dat my inligting nie gegee sal word aan enige persoon wat nie verbonde is aan die studie nie.

Handtekening van persoon wat toestemming ……..……

Datum……

Handtekening van getuie……

Datum……

Kontakbesonderhede van die getuie…….……

LW: Drie afskrifte moet onderteken word: (1) pasiënt, (2) navorser, (3) hospitaal (indien van toepassing).

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Ethics Approval

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 405 6626 • **Facsimile** [021] 406 6411
e-mail: shuretta.thomas@uct.ac.za

02 July 2010

HREC REF: 324/2010

Prof P Meissner
Division of Medical Biochemistry
Clinical Lab Sciences
Room 5.09, Level 6, Falmouth Building
Medical School

Dear Prof Meissner

PROJECT TITLE: MOLECULAR CHARACTERIZATION OF ACUTE INTERMITTENT PORPHYRIA IN SOUTH AFRICA.

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

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentoned study.

Approval is granted for one year till the 15th July 2011.

Please submit an annual progress report if the research continues beyond the expiry date. Please submit a brief summary of findings if you complete the study within the approval period so that we can close our file.

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

Please quote the REC. REF in all your correspondence.

Yours sincerely

signature removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

S Thomas

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report		
☐ Not approved	See attached comments		
Expiry date	25 July 2013		
Signature Chairperson of the HREC	signature removed	Date	16/6/2012

Principal Investigator to complete the following:

1. Protocol information

RESEARCH ETHICS COMMITTEE
2012 -06- 15
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

Date	14 June 2012
HREC REF Number	324/2010
Protocol title	Molecular characterisation of acute intermittent porphyria in South Africa
Protocol number (if applicable)	
Principal Investigator	PN Meissner
Department / Office Internal Mail Address	Room 6.06, 6th floor Falmouth Building, CLS, FHS

1.1 Does this protocol receive US Federal funding?	☐ Yes	x☐ No
1.2 Has sponsorship of this study changed? If yes, please attach a revised summary of the budget.	☐ Yes	x No

2. List of documentation

3. Protocol status (tick ✓)

☐x	Open to enrolment

Detailed Methods

Selection of Study Cohort

The University of Cape Town Lennox-Eales porphyria laboratory is a national referral centre for porphyria diagnosis and management. A total of 29 patients with a clinical and biochemical diagnosis of acute intermittent porphyria were designated as potential participants in this study. Of these, 10 patients were ultimately recruited and of these, 6 previously had DNA stored for further study, and 4 were newly recruited. The remaining patients were not traceable. Of the 10 participants, 4 were black, 3 of mixed-race and 3 of Caucasian origin. Informed consent was obtained from the 3 of the newly recruited patients, while analysis of the *HMBS* gene of the other 7 participants was considered imperative for confirmation of diagnosis. Ethical approval for this study was obtained from the Health Sciences Faculty Research Ethics Committee (HREC/REF: 324/2010).

Isolation and Storage of DNA

On receipt of blood samples (ethylenediaminetetraacetic (EDTA) acid additive) in the laboratory, each patient was numerically coded, and such codes were further used in the study. The information chain linking patient identity to the coded specimens was known only by the researchers participating in the study.

Genomic DNA was isolated from 3.0ml whole blood, using the Wizard Genomic DNA Purification kit (Promega, Madison, USA) as per the manufacturer's protocol (see Appendix B) and quantified with the Nanodrop 2000 (Wilmington, USA). The purity of DNA was assessed on the basis of the optical density $(OD)_{260/280}$ ratio obtained, and only DNA with an $OD_{260/280}$ ratio of 1.5–2.0 was utilised. Aliquots of the DNA samples were diluted to 100-150ng/µl and stored at -20°C for the duration of the project.

Primer Design and Polymerase Chain Reaction

The genomic sequence of the *HMBS* gene was obtained from both the National Centre for Biotechnology Information website (http://www.NCBI.nlm.nih.gov) and the ENSEMBL website (http://www.ensembl.org/index.html) and annotated using information from both these sites (Appendix A).

PCR primers were designed using Primer Designer version 2.0. Primer Designer allows for the specification of certain parameters such as primer length and melting temperature. OligoAnalyzer was used to confirm melting temperatures and detect homodimers and heterodimers (http://eu.idtdna.com/analyzer/applications/oligoanalyzer). At least 55 base pairs (bp) of the flanking intronic regions were included in the amplicons, as splicing regulatory sites commonly occur within 40 bases from the exon splice sites. In addition, to assess primer specificity for the target sequences, the primer sequences were compared to the rest of the human genome, using the Basic Local Alignment Search Tool (http://www.NCBI.nlm.nih.gov/BLAST) and checked for single product formation by e-PCR (NCBI). Primers were synthesized by Integrated DNA Technologies, Inc (Coralville, USA). Further criteria for the design of primers, as well as primer sequences and information, are listed in Appendix B. PCR reactions were performed (Appendix C) and products subjected to 3% agarose gel electrophoresis (Appendix D) to assess products for purity.

Sequence Analysis

PCR products spanning all exons of the entire *HMBS* gene were subjected to direct sequencing, to determine the precise sequence variations. PCR products (40µl) were purified for sequence analysis (Appendix E) using the GFX PCR DNA and Gel Band purification kit (GE Healthcare, Buckinghamshire, UK). Purified samples were then sequenced in both forward and reverse directions on an ABI 3130 XL DNA automatic sequencer using a Big Dye terminator cycle sequencing kit, by the Department of Genetics, University of Stellenbosch, Stellenbosch, South Africa. Sequence data were collected and analysed using BioEdit Sequence Alignment Editor, Version 5.0.9.1 (Tom Hall, North Carolina State University, USA). Sequences in BioEdit were aligned with Genbank data (http://www.NCBI.nlm.nih.gov) using ClustalW (http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html).

Restriction Enzyme Digestion

Sequence analysis revealed 3 unpublished mutations. The absence of these mutations (Appendix H) was confirmed in at least 50 race-matched anonymised control DNA specimens (previously obtained from the Western Province Blood Transfusion Service laboratories) by restriction enzyme digest analysis (Appendix F). The DNA of family members of 2 related probands was also interrogated with restriction analysis. Restriction enzyme maps for wild type and polymorphic DNA fragments were constructed using Webcutter 2.0 (copyright 1997, Max Heiman) (Appendix F).

HMBS Enzyme Assay

In two patients sequence analysis revealed no mutations. At the time of this study one of these patients had recently deceased, but the other patient was traced and HMBS enzyme activity determined[1] (Appendix G).

[1]Piepkorn MW, Hamernylk P, Labbé RF. Modified erythrocyte uroporphyrinogen I synthase assay, and its clinical interpretation. Clin Chem 1978;24:1751-1754.

Appendix A

Genomic sequence of the *HMBS* gene illustrating the positions of exons and flanking primers. The exon1 forward primer is not represented, and some forward and reverse primers may overlap, but both can be viewed in Appendix B.

```
   1 ccggaagtgacgcgaggctctgcggagaccaggagtcagactgtaggacgacctcgggtc
  61 ccacgtgtccccggtactcgccggccggagcccccggcttcccggggccgggggacctta
 121 gcggcacccacacacagcctactttccaagcggagccatgtctggtaacggcaatgcggc  EXON1
 181 tgcaacggcggtgagtgctgagccggtgaccagcacactttgggcttctggacgagccgt
 241 gcagcgattggccccaggttgccatcctcagtcgtctattggtcagaacggctatctttt
 301 ttttttttttttttttttttttttggtccgagtagcttttaaagggccagtagctcggt
 361 tgccctccggaaggaatggggaaatcagagagcggtgatactgggttaagagtggaagga
 421 ttgtttggaacggaactccggtccctgcgggcatctgggtgggattcccatcaggcctgg
 481 gatgcacggctctagatttagtgacccagaccaagaacgttcgtctacacagacggggtc
 541 ctttcattcgaggctgggctgaggcggatgcagatacggccccttgggaagacacgttc
 601 cacttttgattcataggagagagtatcagccaagcctccgaactgcacacaaacgtctta
 661 gaagtgcgccttctttttgtgttatagtggtctcccagccacagccaacgctccaagtcc
 721 ccagctgtgacacacctactgaattactaccgtgggtgggaggccgccgtgggcctttcc
 781 attacgagcctgcttgccgagccctgggcttgtgcacagacaaactgcagagctggtgga
 841 ggccactgccaggccgagataagaaagagatggggagctgctaatctccccctgtccagc
 901 ctgttggtgagggctgggatctttgctcttgcagtcattccagagccctggactaggagt
 961 aggaagatctgaattgtggccccaactctctttcggttattagctctgtgaccctaggca
1021 agtcacctcatcccttgatgccacccgttgcttctgtaacatggtcccaaaggtgcctgt
1081 cttgtccacctgataggatttttgagacgacaacaatatgcaaaagcaatagcttcaaca
1141 tagaagtgctcagtgttttatttttttaatgaaacggtttgacttggatatgctgtgcaca
1201 ttcaatgaacttaaggaattgtttgaacctagtagttctgggaccttagagtcctttctg
1261 tgggctccctgtggcccagaattttggtggccacgtttaatatcaagcctagcctaattt
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1321 gcaaagggtctcccagggttaatttattggagtgatcacatggagtagaccagagtctga

1381 gggcagaaagctgtcacctgcttcggcaatagaggccccagatgtctgggtgcaaaagaa

1441 ctccatagcaccccgaccaacatggtgaaaccccgtctctactaaaaatataaaaattag

1501 gccgggcacagtggctcatgcctgtaatcctagcactttgggaggccgaggcaggtggat

1561 tgcctgagctcaggagttcgagaccagcctagggaacacagtgaaaccccgtttctacta

1621 aaaatacaaaaaattagccgacgtggtggcatgcgcctgtagtcccagctacttgggagg

1681 ctaagacaggagaatcgcttgaacctgggaggtggaggttgcactgagccgagaccgcgc

1741 cattgcactccagcctgggtgacagagcgcgactccccctcaaaaaaagaaaaaaaaaaa

1801 aaatatatatatatatatacacacacacacatattttagctgggcatggtggtgtgcgtc

1861 tgtagtagtcccagctacttgggaggctgagtcaggagaatcgcttgaacctggaaggca

1921 gtggttgtagttagctgagaacatgccactgcactccagcctgggcaacagagggagact

1981 ctgtctcaaaaaaaaaaaaaaaaggaactacataggatgaacatcccagatcagggaatg

2041 ttgactgtcgacagtatcagtatctacagtggctactgtctgatgtagaaagaaatggga

2101 tcaggctaggcgtggtggctcacgcctgtaatcccagcactttgggaggctggggcagga

2161 ggatcacaagttcgagaccagcctggccaacacagtgaaaccccgtctctactaaaaatg

2221 tgaaaattagctgggcatggtggaacatgcctgtagttccagcttgaacccaggggtgga

2281 ggttgtagtgagcctagatcacgccactgcactccagcctgagcaaaacagtgagactct

2341 gtctaaaaaaaaaaaaaaaaaaagagagaaatgggacctccgtcttagactgaagaattc

2401 agttctacgtgcttagcagtgaatacttttgtccaaggtactctggcaggaggaagaggc

2461 gtgtcctcttgagttcttgacttgggctctggcctgttaatatttccatgttggtgaaac

2521 cagaggcagcactctaggtgcacgaactttaggcagcgcagcctcctagtcttatggaac

2581 atctgaggcagaagaaacctgagtccaacctttcattttatagatgaacaaacagatcc

2641 tggtgggacagtgtacccaaggtcacccagccaagaggctgagcaggactgtacgtcaga

2701 tccgtttacctcagtccttaatgcatgcagtccagccagattaagggacccttaatactg

2761 tcagctttccccactgtgggatcttcatcctcttgacttcttttgtagccagacatctgg

2821 gcctcttgctggagaaggtggcagcttgctgctcttagactctagtctactccatgtggc

2881 atctggatggcactgaaattttctcaagtgccttgtctgttgtagataatgaatctatcc

2941 tccagtgactcagcacaggttccccagtgtggtcctggctgccctgcccctgccagctgc

3001 aggccccacccttcctgtggccaggctgatgggccttatctctttacccacctggctgtg

3061 cacagcactcccactgacaactgccttggtcaaggtgggcttcagggctcagtgtcctgg EXON2

3121 ttactgcagcggcagcaacagcaggtcctactatcgcctccctctagtctctgcttctct

3181 ggatccctgaggagggcagaaggtactgaggaaggttaaagggaccagccttggagtatt

3241 tccccactctgagactcagctggccacaggccaggttctgaagttcctttcttccaagcc

3301 agtgattctggttcttggacaaggtgttgaggaacactagaaacagaggggactgtgacc

3361 tggggacttttctgcaggaagaaaacagcccaaagatgagagtgattcgcgtgggtacc EXON3

3421 cgcaagagccaggtgggtgcaggagccggggtggaggaggtttgtcagaacagttatgat

3481 gctcacagcatcacaaattgggggactcagagggttagttcctagtatgaaggagatggg

3541 gtggctgggcgttaagttccccgggaaatggcagattacattctatggcaagatcatccc

3601 taggctgggaaaattgttggagtgcagagggctcccaagccccttctcatgcccagatgg

3661 aaattccagtcccttcaggatctgcctaacctgtgacagtctaaagagtctgagccgtgg

3721 ctgggaagggcaggactaatccaagtctctacccgcagcttgctcgcatacagacggaca EXON4

3781 gtgtggtggcaacattgaaagcctcgtaccctggcctgcagtttgaaatcagtgagtttt

3841 ctggaaaggagtggaagctaatgggaagcccagtaccccgagaggagagaacacaacatt

3901 tctggctttgcctatagctaaagcccgtcccgctgccccgagattccttctgggctgctc

3961 ccagttctgaaggtgctttcctctgaatacctccagctctgactacctggattagcctgg

4021 catttaacatcttgagctttgggtctttttatgagtgtttctggtcttcctgctcgattg

4081 tatatactcagagggcaggaaccagggattatgtgcctctgtccccatcatgaatcgtag

4141 cacagcgctaggctcagtaaatgctgatcaataatgagcacctgattgattgactctctc

4201 ctcagttgctatgtccaccacaggggacaagattcttgatactgcactctctaaggtaac EXON5

4261 aacatcttcctccccagttcttgtccccactcttctttccttccctgaagggattcactc

4321 aggctctttctgtccggcagattggagagaaaagcctgtttaccaaggagcttgaacatg EXON6

4381 ccctggagaagaatgagtaagtaaagataggagagtgtggtgccctcccagtctcttgct

4441 gggaccctagtatgctaggtctcttgctgggacccggggtgtcagataggctgctgggct

4501 taaaccctcagagaggctgaaggcagctcataggtgggtttttcaggcttcagaaaagg

4561 agagtgtctggttctgagccatctggctgcctggactgcaagaatggctgggggagggag

4621 ggtaggagggagagtaggagggagagtgagaggagagcagttttcatgctcctgagatct

4681 tgagaaggtgtgcttcctgaactgccctaggctccaccactgaagtagaggcaggggtgg

4741 gtggagaaggggtgaaggctggctgctcatacccttttctctttgccccctctcccatct

4801 ctatagagtggacctggttgttcactccttgaaggacctgcccactgtgcttcctcctgg EXON7

4861 cttcaccatcggagccatctgcaagtaagagtcttgcaagtaaggggcttgggcaggggt

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4981 tcctctgccctgaggagtgttgactggtggcagaaaactcaagaaataccagtgagttgg

5041 caatcgagagagaatagaggtgatctgaacttaaatctcttccctcattctgtgcccttc

5101 cctcctcccccaggcgggaaaaccctcatgatgctgttgtctttcacccaaaatttgttg EXON8

5161 ggaagaccctagaaaccctgccagagaagaggtaagtggggcctggataggcagcttggt

5221 gggatgtgcccagaagatgcagggatgggaggaggaggaaaggaacagtgactgcctagt

5281 gttaaaatctcattgtaacttctctctgggcagtgtggtgggaaccagctccctgcgaag EXON9

5341 agcagcccagctgcagagaaagttcccgcatctggagttcaggagtattgtatcctttta

5401 gaagagtgacggatccttttggaagagtgacggagacagcagccaaggaaaaagacaagg

5461 tctagagggctctgggagtccggagagtggaaggggcttccagcaagcagcccgtggggt

5521 cagtggcctgtctgtctttccatgcactcatccgtccactcatttacagtctaatgtttt

5581 cttagccccagacaagtgttcagagtgcaaggcattggggataatggtgagcaagataaa

5641 catttccctgcatatgtagagtttacgtcttacttagggataatgcagttatactgaact

5701 gaatagtgactacttctggagggatagggagtacttccttttttttttttttttttttct

5761 gagacggagtctcgctctgttgcccaggttggagtgcagtggcgcaatctaggctcactg

5821 caacttctgcctcctgagttcaagcaatcttcctgcctcagcctcctaagtagttgggat

5881 tacaggtgccaccacacctggctaatttttgtatttttagtagagactgggtttcaccat

5941 gttagtcaggctggtctcaaactcctgacctcaggtgatccaccagcctcggcctcccaa

6001 agggctgggattacaggcttgagccccgcacccggtcagtacttccatttttatatgcta

6061 ctatattgtcttgacttttacaatgaatatgtagtacatttcataaaactaaatttaaaa

6121 atagtatgtgctaagtgctccaataagtgaagttgggaattttctggaaacttctagttg

6181 gaacatctaaacacagaagtctggggtgtcagggaaggtttctcagaggtcttgtaacct

6241 tggcaagttatttagcctccctatgtcattttccttatctgtaaagtggggataataata

6301 ctaccttcctcacagggttgttgtgaagatgaaatgagctgacatatggaaagtactttt

6361 agagcagtgtctggcatgtagtaagtatgatgtaactgttagctgttaacattaagctga

6421 gagctggaagatgactgaaagtcagccagctagagagggaaagacagactcaggcagagg

6481 gaaccgcacgaggccccagattgcccgacactgtggtccttagcaactctccacagcggg EXON10

```
6541 gaaacctcaacacccggcttcggaagctggacgagcagcaggagttcagtgccatcatcc
6601 tggcaacagctggcctgcagcgcatgggctggcacaaccgggtggggcaggtagggcctg
6661 cccctatcctctccccagctcatctgcatctcctttctgccttacagtcatccccaatttt
6721 aggatttttagactttatgattgtgtgaaagcgatatacgttcagtagaaactgtactta
6781 gtacccatacagccattctgtttttttactttcagtacagtattcattacatgagatattc
6841 actttattgtaaaacaggcttggtgtcagatgattttgtccaactataataggctaatct
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6961 tcaacttaacaatgggtttatcaggatgtaacccctattgtaagtcaaggaccatctgtct
7021 tcacttcttgaccaccccacctctaacaccgtaggctggaagattgtgaatcagaggcc
7081 agactctaggctttcatggagaaaatttacaaaaaaaaaaaaaaaagaggccagactcaca
7141 cttaggcctacccaggctttctagatgatagggaactcccatctcactgccaggtgcttt
7201 tagacacccccgtgtccacccttttgactccctgttccgcctccacagatcctgcaccct  EXON11
7261 gaggaatgcatgtatgctgtgggccaggtacacttgaccagggaagccacatggtgacat
7321 atgccttccctttgttctcaaccaagaagcttgtctcacaaccttctgcatctgcttccc
7381 cagaatagcattctcagggaggggcagaccttgggatgctaccggtccaaaaggcgctgg
7441 ggagcaagtagatagaggtggtcccatgctttgcgccattggttggggaaagatcaggcc
7501 tgatgtcctaggatgtttttccatcaggggggccttgggcgtggaagtgcgagccaaggac  EXON12
7561 caggacatcttggatctggtgggtgtgctgcacgatcccgagactctgcttcgctgcatc
7621 gctgaaagggccttcctgaggcacctggtagggcctgtgctccacctgtggagggctggg
7681 gacttggagagctgggaaaggtggcagggaagatttcttacatgaatgctctgtatacag
7741 tgctaactcattcttgttgaatgttgtgtatggataggaccaggtctgggcccacagttg
7801 cctttcagtgatgtcctcaggtctgtggtcacagggtggtgttaagagcccttgcagct
7861 cacaagaacttcttgttacaggaaggaggctgcagtgtgccagtagccgtgcatacagct  EXON13
7921 atgaaggatgggcaagtaagtgggggggaaatgggcgggaagccagggaaaggaggactgt
7981 ggcatttcttcctgtgcatcccaggtttctaggtagtcccctctcagactgtgctgaggc
8041 aactgttttcttccccagctgtacctgactggaggagtctggagtctagacggctcagat  EXON14
8101 agcatacaagagaccatgcaggctaccatccatgtccctgcccaggtaccaaagctggag
8161 ggcgaggggggtaataaacaagagtgcatataatctcttgttctcaccaaatcccacctcc
8221 ttccctcatacagcatgaagatggccctgaggatgacccacagttggtaggcatcactgc  EXON15
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8281 tcgtaacattccacgagggccccagttggctgcccagaacttgggcatcagcctggccaa
8341 cttgttgctgagcaaaggagccaaaaacatcctggatgttgcacggcagcttaacgatgc
8401 ccattaactggtttgtggggcacagatgcctgggttgctgctgtccagtgcctacatccc
8461 gggcctcagtgccccattctcactgctatctggggagtgattaccccgggagactgaact
8521 gcagggttcaagccttccagggatttgcctcaccttggggccttgatgactgccttgcct
8581 cctcagtatgtggggcttcatctctttagagaagtccaagcaacagcctttgaatgtaa
8641 ccaatcctactaataaaccagttctgaaggtgt
```

Appendix B

PRIMER CRITERIA

- Primer lengths of 18 to 22bp were chosen to ensure specificity. Shorter primers tend to bind non-specifically to the genome
- Amplicon sizes of no more than approximately 900bp were chosen, to enable successful sequencing of the whole amplicon
- Melting temperatures (Tm) of 55 to 65°C were chosen. Primers with melting temperatures above 65°C have a tendency for secondary annealing
- The Tm difference between primer pairs was chosen to be preferably no more than 3°C and definitely no more than 4°C
- Primer dimer formation due to complementarity between primers was avoided
- The possibility of a primer folding back on itself and creating a hairpin loop due to self-complementarity was examined, and where possible avoided
- Sequences containing three or more tandem repeats of a single nucleotide (e.g. GGG) were avoided where possible as they can misprimed
- A GC clamp at the 3' end helps promote specific priming due to the stronger bonding of G and C bases. However, more than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer
- 3’ ends of primers should not be complementary as this may result in primer dimerisation
- Characteristics of the *HMBS* gene primers used are summarised in Table 1

Table 1: Characteristics of the *HMBS* gene primers used

Amplicon	Primer Pair	Size (bp)	Product Size (bp)	Tm (°C)	Sequence
Exon 1	*HMBS* F1	20	342	58	5′ ggtccactgtcgcaatgttc3′
	HMBS R1	20			5′ tagacgactgaggatggcaa3′
Exon 2-3	*HMBS* 2F	19	902	58	5′ gcctcttgctggagaaggt3′
	HMBS 3R	19			5′ agccacggctcagactctt3′
Exon 4-6	*HMBS* 4F	19	814	58	5′ gcctaacctgtgacagtct3′
	HMBS 6R	19			5′ ccagcagcctatctgacac3′
Exon 7-9	*HMBS* 7F	19	854	58	5′ aggctccaccactgaagta3′
	HMBS 9R	19			5′ tgagtggacggatgagtgc3′
Exon 10	*HMBS* 10F	19	598	58	5′ agacagactcaggcagagg3′
	HMBS 10R	19			5′ ccagcctacggtgttagag3′
Exon 11-12	*HMBS* 11F	19	787	54	5′ ctctaacaccgtaggctgg3′
	HMBS 12R	20			5′ acagacctgaggacatcact3′
Exon13-15	*HMBS* 13F	20	781	58	5′ atgtcctcaggtctgtggtc3′
	HMBS 15R	20			5′ cacatactgaggaggcaagg3′

Appendix C

POLYMERASE CHAIN REACTION

Reagents

- Distilled water (dH_2O)
- Mg-free PCR buffer (Promega, Madison, USA)
- $MgCl_2$ 25mM (Promega, Madison, USA)
- Deoxynucleotide triphosphates (dNTPs; Promega, Madison, USA)
- Primers (forward and reverse) (Appendix B)
- Go taq flexi (Promega, Madison, USA)

Method

- All work was done on ice
- Gloves were used at all times to prevent contamination
- A PCR mastermix was prepared in a sterile 1.5ml microcentrifuge tube (Table 2)
- The mixture was aliquotted into 0.2ml microcentrifuge tubes (49µl per tube)
- 1µl (100 to 150ng) of genomic DNA was added to each reaction (for one reaction DNA was replaced with 1µl dH_2O to serve as a negative control)
- Tubes were briefly vortexed and centrifuged
- PCR reactions were performed in the thermocycler (Labnet Multigene Optimax) as follows:
    - denaturation at 95°C for 1min

- 35 Cycles of denaturation at 95°C for 30s, annealing at the specified temperatures (Appendix B, Table 1) for 30s and elongation at 72°C for 30s
- Final elongation at 72°C for 7min
- PCR products were stored at 4°C until further use

Table 2: PCR reagent mixture per reaction

Reagent	Stock Concentration	Volume (µl)	Final Concentration
dH_2O	-	32.8	-
Buffer	5X	10.0	1X
$MgCl_2$	25mM	3.0	1.5mM
dNTPs (Each)	2.5mM	1.0	50µM
Primers (forward and reverse)	25µM	1.0	0.5µM
Go Taq Flexi	5U/µl	0.2	1Unit

Appendix D

AGAROSE GEL ELECTROPHORESIS

Solutions

50X Tris acetate-EDTA (TAE) buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 7.6)

- Tris base 242g
- Glacial acetic acid 57.1ml
- 0.5M EDTA 100ml
- Reagents were diluted to a final volume of 1l with dH_2O
- Prior to use 50X stock buffer was diluted 1:50 with dH_2O

Method

- The gel tray of the gel electrophoresis apparatus (Sub-Cell GT Agarose Gel Electrophoresis System; Bio-Rad) was assembled onto a level gel caster and a 15-well comb engaged
- Agarose (1.8g) (Agarose MP, Roche) was weighed into a 250ml Ehrlenmeyer flask and 60ml of TAE buffer added
- This mixture was heated in a microwave to boiling point, with intermittent swirling, till all agarose was dissolved
- Ethidium bromide (6µl of 10mg/ml) was added to the dissolved agarose with swirling, poured into the gel tray and the agarose allowed to set for 30min
- The comb was removed, the gel tray placed into the electrophoresis apparatus, submerged in TAE buffer (approximately 1 500ml), and the samples (10µl) and 1kb ladder (5µl; Promega, Madison, USA) loaded into wells with a 20µl pipette

- The lid was secured and electrophoresis performed at 110v for approximately 45min
- PCR fragments were visualised using the Uvipro gel documentation system and photographed for future use

POLYACRYLAMIDE GEL ELECTROPHORESIS

Solutions

Acrylamide-bisacrylamide (ABA) solution

- Acrylamide (Promega, Madison, UK) 30% (60g)
- Bisacrylamide (Promega, Madison, UK) 0.8% (1.6g)
- Made up to 200ml with dH_2O

10X Tris borate-EDTA, pH 8.3

- Tris 540g
- Boric Acid 275g
- EDTA 37g
- Made up to 5l with dH_2O and pH adjusted to 8.3
- Autoclaved for 30min

10% Ammonium Persulphate

- Ammonium persulphate 1g
- Made up to 10ml with dH_2O

Bromophenol blue loading dye

- Bromophenol Blue 0.25% w/v (0.25g)
- Sucrose 40% w/v (40g)
- 0.5M EDTA 4ml
- Made up to 100ml with dH_2O

6% Acrylamide Gel

- 10X Tris borate-EDTA 4ml
- ABA 8ml
- Made up to 40ml with dH_2O

  Added to this:

- Ammonium persulphate 400µl
- N,N,N´,N´-tetramethylethylenediamine 40µl
- Mixed well

Ethidium bromide staining solution

- Ethidium bromide (1mg/ml) 200µl
- dH_2O 200ml
- Mixed by swirling gently

Materials

- Hoefer Vertical Slab Gel Unit, SE600 series with gel apparatus (Hoefer, GE Healthcare, Vienna, Austria)
- Hoefer PS1500 DC Power Supply (Hoefer, GE Healthcare, Vienna, Austria)

- Hamilton Microliter Syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)
- Gel Documentation system, UVItech (UVItech Limited, Cambridge, UK)
- Mitsubishi P90E(B) Video copy processor (Mitsubishi Electric Corporation, Tokyo, Japan)

Reagents

- 6% Acrylamide Gel
- Tris borate-EDTA running buffer (1X)
- Ethidium bromide (1µg/ml)
- Bromophenol blue loading dye
- 100bp molecular weight marker (Promega, Madison, USA)

Method

- The gel apparatus was assembled by placing two glass plates, separated by 1.5mm spacers and secured with clamps, vertically onto a gel stand and secured using cams
- A 6% acrylamide solution was made up and poured between the glass plates
- All air bubbles were removed
- A 20 well 1.5mm gel spacer comb was inserted and the gel allowed to set for at least 30min
- The comb was carefully removed from the gel
- The upper buffer chamber was assembled on top of the gel and clamped into place
- 500ml of 1X Tris borate-EDTA buffer was poured into the upper buffer tank
- 5µl DNA was mixed with an equal amount of PCR loading dye
- Samples were carefully loaded into the wells using a Hamilton syringe

- To one of the wells 10µl 100bp DNA step ladder (diluted 1:2 with bromophenol blue loading dye) was added to serve as a size standard
- Samples were run in 1X Tris borate-EDTA running buffer at 270V for 1 to 1.5h
- The gel was removed and stained in ethidium bromide solution for 10min
- PCR fragments were visualised using the gel documentation system and photographed for future reference

Appendix E

PURIFICATION OF PCR PRODUCTS FOR SEQUENCING

Method

- Capture buffer (500µl) was added to a 1.5ml microcentrifuge tube
- PCR product (up to 100µl) was added to the capture buffer and the solution mixed by vortexing
- The solution was added to the GFX microspin column already assembled in a collecting tube and centrifuged (10 000g; 30s)
- The flow-through was discarded and the GFX column placed back inside the collection tube
- Wash buffer (500µl) was added to the column and centrifuged (10 000g; 30s)
- The collection tube was discarded and the GFX column transferred to a 1.5ml microcentrifuge tube
- Approximately 30µl of elution buffer type 6 was applied directly to the surface of the matrix in the GFX column, allowed to incubate for 1min at room temperature and centrifuged (10 000g; 1min) to recover purified DNA
- Note: recovery is reduced when using low elution volumes to concentrate DNA

Appendix F

RESTRICTION ANALYSIS

Method

- A restriction enzyme mixture consisting of the appropriate enzyme and corresponding buffer (Table 3) was made up in a 1.5ml microfuge tube to a final volume of 10µl per reaction with dH_2O

Table 3: Restriction enzyme reagent mixture per reaction

Polymorphism	Enzyme	[E] (U)	Buffer 10X (µl)	Digestion Time (h)
c.292A>G	*Sty1* (Fermentas, Hanover, Maryland, USA)	1	2	3
c.689_690delAC	*BstY1* (Thermo Scientific, Vilnius, Lithuania)	1	2	3
c.422 + 3_6delAAGT	*XcmI* (New England Biolabs, Ipswich, MA, USA)	1	2	3
c.848G>A	*XbaI* (Thermo Scientific, Vilnius, Lithuania)	1	2	3
c.161-1G>A	*DdeI* (Thermo Scientific, Vilnius, Lithuania)	1	2	5

[E] = enzyme concentration

U = units

- Restriction enzyme master mix (as per Table 3) was vortexed and spun down briefly in a microcentrifuge
- 10µl of the above mixture was aliquoted into 0.5ml microcentrifuge tubes and 10µl of PCR product added
- After vortexing, a drop of mineral oil was added to the tube followed by a brief spin in a microfuge
- A positive control was included with each set of digests to confirm complete digestion of product
- Restriction digests were incubated at 37°C as indicated (Table 3)
- Restriction enzyme digests were visualised by agarose or polyacrylamide gel electrophoresis (Appendix D)

RESTRICTION MAPS

Sty1 (Echo130I)

- Primers: 7F/8-9R
- Fragment sizes: 854bp = 735bp + 119bp (wild type (WT))

  854bp = 119bp + 616bp + 119bp (mutated)

A C T C|C T T G A A G G A C C WT sequence

A C T C|C T T G **G** A G G A C C mutated sequence (c.292A>**G**)

- The WT sequence contains one recognition site: c | c w w g g (between the 735bp and 119bp fragments) where w = a or t
- The mutation creates a recognition site: c | c w w g g (between the 119bp and 616bp fragments of the 735bp fragment)

- A second cutting site exists in the mutated sequence (between the 616bp and 119bp fragments)

BstY1 (PsuI)

- Primers: 11F/12R
- Fragment sizes: 787bp = 207bp + 325bp + 255bp (WT)

787bp = 207bp + 318bp + 7bp + 255bp (mutated)

deletion

mutated sequence (c.689_690del**AC**)

- The WT sequence contains two recognition sites: r | g a t c y (between the 207bp and 325bp fragments; and the 325bp and 255bp fragments)

  where r = g or a, and y = c or t

- The deletion in the mutated sequence introduces a cutting site (in the 325bp fragment)

XcmI

- Primers: 7F/8-9R
- Fragment sizes: 854bp (WT)

850bp = 480bp + 370bp (mutated)

deletion

mutated sequence (c.422 + 3_6del**AAGT**)

- The WT sequence contains no cutting site
- The mutated sequence introduces a cutting site between the 480bp and 370bp fragments: c c a n n n n n | n n n n t g g where n = any base pair

XbaI

- Primers: 13F/15R
- Fragment sizes: 785bp = 278bp + 507bp (WT)

  785bp = 271bp + 7bp + 507bp (mutated)

A G G A G T C T G G A G T│C T A G A C WT sequence

A G G A G T│C T **A** G A G T│C T A G A C mutated sequence (c.848G>**A**)

- The normal sequence contains one recognition site: t | c t a g a (between the 278bp and 507bp fragments)
- The mutated sequence introduces a cutting site 7bp 5' of the cutting site in the normal sequence

DdeI

- Primers: 4F/6R
- Fragment sizes: 814bp = 27bp + 377bp + 66bp + 48bp + 50bp + 67bp + 179bp (WT)

  814bp = 27bp + 377bp + 66bp + 98bp + 67bp + 179bp (mutated)

T C T C T C C│T C A G T T G C T A WT sequence

T C T C T C C T C A **A** T T G C T A mutated sequence (c.161-1G>**A**)

- The normal sequence contains six recognition sites: c | t n a g (which produces seven fragments) where n = any base pair
- The mutated sequence abolishes the cutting site between the 48bp and 50bp fragments to create a 98bp fragment

Appendix G

HMBS ENZYME ASSAY

Reagents

- Haemolysing reagent: 0.2ml Triton X-100 and 28.8mg $ZnSO_4.7H_2O$ were dissolved in 100ml dH_2O, and stored at 4°C. Just prior to use 4mg dithiothreitol was dissolved in 25ml of this solution (giving a final concentration of Zn ions and dithiothreitol of 1mM)
- Reaction buffer: 4.4g $Na_2HPO_4.2\ H_2O$ was dissolved in about 80ml of H_2O, pH adjusted to 7.5 with 0.25M citric acid (5.25g citric acid monohydrate dissolved in dH_2O and made up to 100ml) and made up to 100ml with dH_2O
- 4mM ALA substrate: 7mg ALA.HCl (Sigma-Aldrich, St Louis, Mo., U.S.A.) was dissolved in 10ml reaction buffer
- Stopping reagent (10%, w/v, trichloroacetic acid): 10g trichloroacetic acid was made up to 100ml with dH_2O
- Coproporphyrin standard: 1.98mg coproporphyrin (Sigma-Aldrich, St Louis, Mo., U.S.A) was dissolved in 50ml of 1.5M HCl with warming, filtered and the final concentration (2008ng/ml) determined after measuring the absorbance at 399.5nm and using an extinction co-efficient for coproporphyrin ($489cm.^{-1}mM^{-1}$)

Samples

- Controls: on the day of the assay blood was drawn into heparinised phlebotomy tubes from one healthy volunteer. The sample was transported at room temperature overnight and subsequently stored at 4°C for a further 24hrs. The total time from phlebotomy till assay was 48hrs

- Patient specimen: blood was drawn into a heparinised phlebotomy tube, transported and stored as described for the controls
- The haematocrit was measured on all whole blood specimens
- Coproporphyrin standard was diluted to 50, 100 and 200ng/ml with 1.5mM HCl

Method

- Test specimens (patient and three controls) were assayed in duplicate with one blank sample:

	Blank	Test
Haemolysing reagent	1ml	1ml
Whole blood (well mixed)	50µl	50µl
Mix and pre-incubate at 37°C for 30min		
10% trichloroacetic acid	2.5ml	nil
ALA	1ml	1ml
Mix and incubate at 37°C for 1h		
10% trichloroacetic acid	nil	2.5ml
Stand at room temperature in the dark for 30min		

- Samples were centrifuged to produce a clear supernatant and the fluorescence (arbitrary fluorescence units) of the standards, blank and test specimens recorded at an excitation wavelength of 405nm and excitation wavelength of 595nm
- For each of the standards a ratio of concentration (in nmol/l) over arbitrary fluorescence units was calculated and an average (S) of the three values generated. Blank fluorescence

was subtracted from each of the average of the duplicates for the patient and control fluorescence values (F)

- HMBS enzyme activity was calculated in $nmol.h.^{-1}ml^{-1}$ red blood cells:

  (F x S x 4.55 x 100) / 0.05 x haematocrit

Appendix H

RESULTS OF CONTROL SUBJECTS' RESTRICTION DIGESTS

Restriction digests, revealing each of the novel mutations discovered during sequence analysis, were performed on at least 50 race-matched control specimens: purified DNA was subjected to PCR (Appendix C), restriction enzyme digestion (Appendix F) and either agarose or polyacrylamide gel electrophoresis for visualization (Appendix D) of digested products. In all cases no mutations were found in the control groups. Representative electrophoresis gels are presented (Figures 1-4).

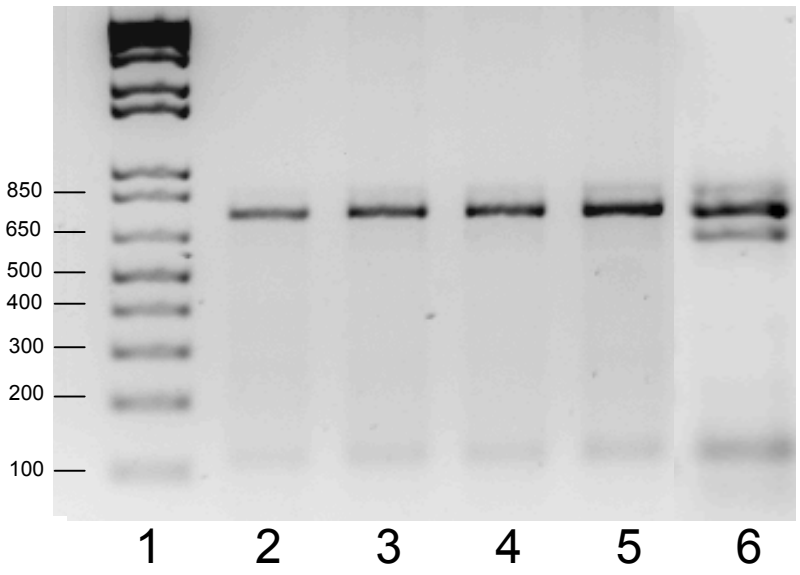

Figure 1: Restriction digest showing representative control specimens (lanes 2-5) and patient specimen (lane 6; c.292A>G) after digestion with *Sty1*. DNA size ladder is measured in base pairs (lane 1); 6% polyacrylamide gel.

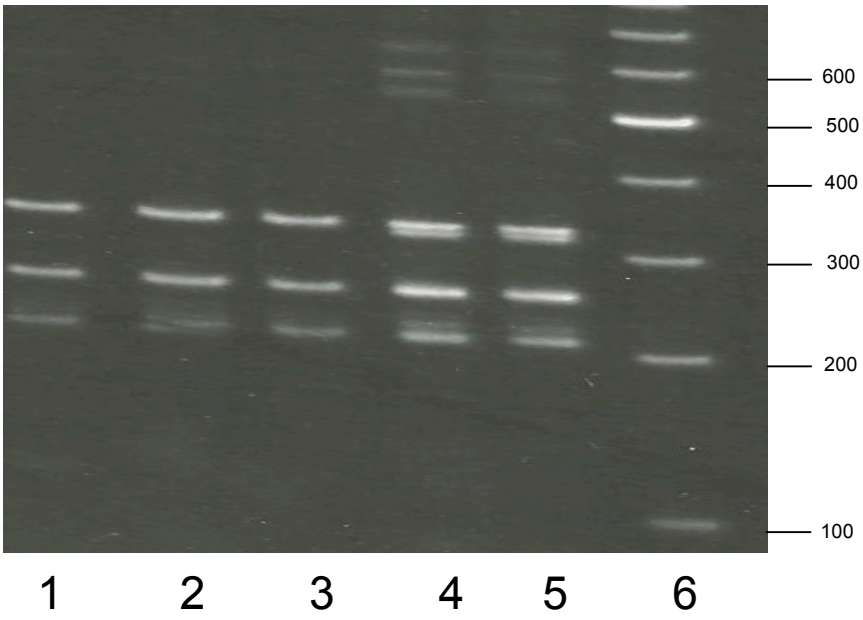

Figure 2: Restriction digest showing representative control specimens (lanes 1-3) and patient specimens (lanes 4 and 5; c.689_690delAC) after digestion with *BstY1*. DNA size ladder is measured in base pairs (lane 6); 6% polyacrylamide gel.

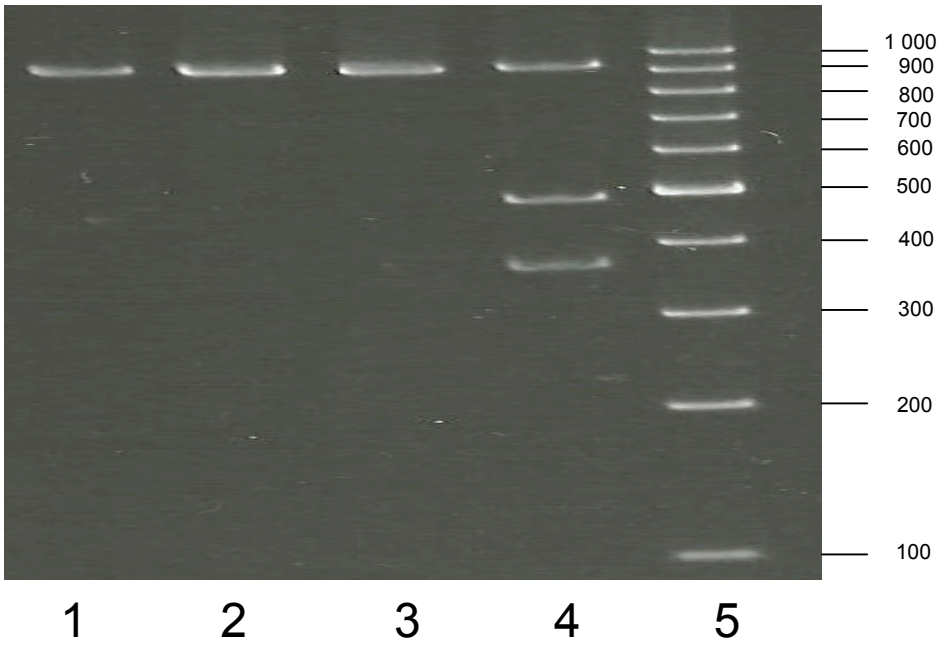

Figure 3: Restriction digest showing representative control specimens (lanes 1-3) and patient specimen (lane 4; c.422 + 3_6delAAGT) after digestion with *XcmI*. DNA size ladder is measured in base pairs (lane 5); 6% polyacrylamide gel.

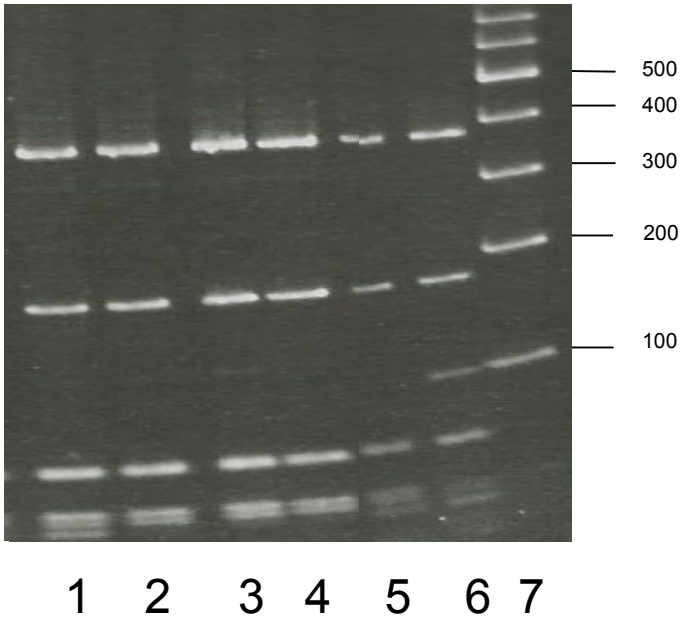

Figure 4: Restriction digest showing representative control specimens (lanes 1-5) and patient specimen (lane 6; c.161-1G>A) after digestion with *DdeI*. DNA size ladder is measured in base pairs (lane 7); 6% polyacrylamide gel.