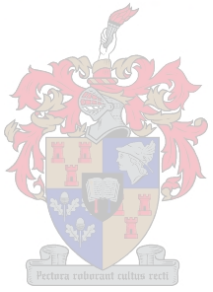
Investigation of the possible influences of candidate modifier genes on the clinical expression of variegate porphyria (VP)

By

Ilse Steyn



Thesis presented in partial fulfillment of the requirements for the degree of Magister Science (M.Sc) in Genetics at the University of Stellenbosch.

Study leader: Prof. L. Warnich

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Abstract

Variegate porphyria (VP, MIM 176200) is a low penetrance autosomal dominant disorder that stems from mutations in the protoporphyrinogen oxidase (*PPOX*) gene. VP is found in most populations, but has a high prevalence in the South African Afrikaner population with most patients inheriting the same *PPOX* mutation (R59W) from a common ancestor. The clinical manifestations of the disease include acute neurovisceral attacks and/or cutaneous photosensitivity. Great variation in the clinical presentation of VP is observed; even in members of the same family that share a common genetic background and that have been exposed to similar environmental factors.

Candidate genes that may have an influence on phenotypic variation due to the regulatory function in the haem biosynthetic pathway include the two delta-aminolevulinic acid synthase (*ALAS*) genes and the porphobilinogen deaminase (*PBGD*) gene. Sequence homology searches between different species indicated that the *ALAS-1, ALAS-2* and *PBGD* genes are highly conserved, indicating that these genes have an important function to fulfill in the haem biosynthetic pathway.

The study population of 25 R59W individuals were divided in four categories according to their clinical presentation. The distribution of clinical symptoms observed in this study corresponds with results from previous studies.

Conformation sensitive gel electrophoresis (CSGE), conventional single stranded conformation polymorphism analysis (SSCP) and two buffer SSCP analysis were implemented to screen for possible sequence variants. The exons of all three genes as well as the adjacent intronic sequences were investigated. A total of six sequence variation sites were identified of which five had previously been described single nucleotide polymorphisms (*ALAS-1*: 4713 T>C; *PBGD*: -64 C>T, 3581 A>G, 6479 G>T, 7064 C>A)] and a novel 8bp deletion (*PBGD*: 4582_4589del). No sequence variant was identified in the *ALAS-2* gene.

The CSGE method proved to have the highest sensitivity (83%), identifying five of six sequence variant sites. The conventional SSCP method identified only three (50%)

sequence variant sites, while the two buffer system detected two (33%) of the sequence variants.

The 4713 T>C SNP in exon 4 of the *ALAS-1* gene and the -64 C>T SNP in the *PBGD* gene were selected for further investigation due to their location in the respective genes. These sequence variants were typed in 50 patients and 50 control subjects matched for ethnic background. The relationship between variation at these loci and clinical features was investigated. No statistical significant association was observed for either of the 4713 T>C SNP (P= 0.717) or the -64 C>T SNP (P= 0.931).

Genetic modifying factors make a variable contribution to the total clinical picture and are difficult to identify in small populations. Due to the fact that we only had a limited number of VP samples, association cannot be ruled out. This study does, however, provide insight into investigational approaches that should be undertaken in future research concerning the *ALAS* and *PBGD* genes. Further knowledge concerning the haem biosynthetic pathway could ultimately lead to the understanding and assessment of the clinical expression observed in individuals with VP.

Opsomming

Variegate porfirie (VP, MIM 176200) is 'n lae penetrasie outosomaal dominante siekte wat veroorsaak word deur mutasies in die protoporfirienogeen oksidase (*PPOX*) geen. VP word gevind in die meeste populasies, maar het 'n hoë voorkoms in die Suid-Afrikaanse populasie waar meeste pasiente dieselfde *PPOX* mutasie (R59W) van 'n gemeenskaplike voorouer oorgeërf het. VP word gekenmerk deur akute neuroviserale aanvalle en/of fotosensitiewe vel. Groot variasie word egter waargeneem in die kliniese uitdrukking van VP, selfs in lede van dieselfde familie wat 'n gemeenskaplike genetiese agtergrond deel en wat blootgestel is aan dieselfde omgewingsfaktore.

Kandidaat gene wat as gevolg van hulle regulatoriese funksie in die heem biosintetiese padweg 'n effek op die ekspressie van VP mag hê, sluit in die twee delta-aminolevuliniese suur sintase (*ALAS*) en die porfobilinogeen deaminase (*PBGD*) gene. Homologie ondersoeke van die *ALAS-1, ALAS-2 en PBGD* gene in verskillende spesies dui daarop dat die gene hoogs gekonserveerd is en dus gevolglik 'n belangrike funksie in die heem biosintetiese padweg vertolk.

Die studie populasie van 25 R59W individue is verdeel in vier kategorieë op grond van hulle kliniese simptome. Die verspreiding van die kliniese simptome wat waargeneem is tydens hierdie studie stem ooreen met die resultate van vorige studies.

Konformasie sensitiewe gel elektroforese (CSGE), konvensionele enkelstring konformasie polimorfisme analise (SSCP) en twee buffer SSCP analise is gebruik vir die identifisering van genetiese variasie. Die eksons van al drie gene, sowel as die aangrensende intron volgordes, is ondersoek. 'n Totaal van ses areas van genetiese variasie is geïdentifiseer, waarvan vyf reeds beskryfde polimorfismes is (*ALAS-1:* 4713 T>C; *PBGD:* -64 C>T, 3581 A>G, 6479 G>T, 7064 C>A) en 'n nuwe 8bp delesie (*PBGD*: 4582_4589del). Geen genetiese volgorde variasie is gevind in die *ALAS-2* geen nie.

Die CSGE metode het die hoogste sensitiwiteit getoon (83%) en het vyf van die ses volgorde variasies geïdentifiseer. Die konvensionele SSCP metode het slegs drie volgorde variasies geïdentifiseer (50%), terwyl die twee buffer deteksie-sisteem twee variasies geïdentifiseer (33%) het.

Die 4713 T>C polimorfisme in ekson 4 van die *ALAS-1* geen en die –64 C>T polimorfisme in die *PBGD* geen, is geselekteer vir verdere ondersoek as gevolg van hulle posisie in die respektiewe gene. Die volgorde variasies is getipeer in 50 R59W pasiënte sowel as in 'n kontrole groep van 50 individue met dieselfde etniese agtergrond. Die verband tussen die variasie by die lokusse en die kliniese kenmerke is ondersoek. Geen statisties beduidende assosiasie is waargeneem vir hetsy die 4713 T>C SNP (P= 0.717) of die -64 C>T SNP (P= 0.931).

Genetiese modifiserende faktore word moeilik geïdentifiseer in klein populasies omdat hulle afsonderlike bydra tot die geheelbeeld van die kliniese simptome so varieerbaar is. 'n Relatiewe klein groep van VP pasiënte was tydens die studie beskikbaar en dus kan assosiasie nie uitgesluit word nie. Die studie verskaf egter insig in verband met toekomstige benaderings wat volg kan word in verdere ondersoeke van die *ALAS* en *PBGD* gene. Verdere kennis in verband met die heem biosintetiese padweg kan uiteindelik lei tot die verduideliking en assesering van die kliniese uitdrukking in VP individue.

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List of abbreviations and symbols

μ	Micro (10^{-6})
χ^2	Chi squared
μg	Microgram
μl	Microlitre
μM	Micromolar
3' UTR	3' untranslated region
5' UTR	5' untranslated region
A	Adenine
ADL	Activities of daily living
ADPKD	Autosomal dominant polycystic kidney disease
$AgNO_3$	Silver nitrate
AIP	Acute intermittent porphyria
ALA	5-aminolevulinic acid
ALAD	5-aminolevulinic acid dehydratase
ALADP	5-aminolevulinic acid dehydratase deficiency porphyria (Plumboporphyria)
ALAS	5-aminolevulinate synthase
ALAS-1/ALAS-N	Housekeeping isoform of 5-aminolevulinate synthase
ALAS-2/ALAS-E	Erythroid isoform of 5-aminolevulinate synthase
AP1	Activating protein 1 (Transcription factor)
AP4	Activating protein 4 (Transcription factor)
***Apa* I**	Restriction enzyme with recognition sequence 5'-GGGCC↓C-3', Source: *Acetobacter pasteurianus*
***Apa* LI**	Restriction enzyme with recognition sequence 5'-G↓TGCAC-3', Source: *Acetobacter pasteurianus*
APS	Ammonium persulphate $(NH_4)_2S_2O_8$
Asn	Asparagine
Asp	Aspartic Acid
ATG	Initiator codon
***Ban* II**	Restriction enzyme with recognition sequence 5'-G(A,G)GC(T,C)↓C-3', Source: *Bacillus aneurinolyticus*
BAP	1,4 Bis(acryloyl)piperazine

BIS	N'-N'-methylene-bisacrylamide
Bp	Base pair(s)
BRCA 1	Breast cancer type 1
BRCA 2	Breast cancer type 2
BSA	Bovine Serum Albumin
***Bsm* AI**	Restriction enzyme with recognition sequence 5'-GTCTCn↓nnnn-3', Source: *Bacillus stearothermophilus*
***Bsr* I**	Restriction enzyme with recognition sequence 5'-ACTGGn↓-3', Source: *Bacillus stearothermophilus*
***Bss* SI**	Restriction enzyme with recognition sequence 5'-C↓TCGTG', Source: *Escherichia coli strain that carries the cloned BssSI gene from Bacillus stearothermophilus*
***Bst* NI**	Restriction enzyme with recognition sequence 5'-CC↓(A,T)GG-3', Source: *Bacillus Stearothermophilus N*
C	Cytosine
c	Centi (10^{-2})
CBAVD	Congenital bilateral absence of the vas deference
CCND1	Cyclin D1
cDNA	Complementary deoxyribonucleic acid
CEP	Congenital erythropoietic porphyria (Günters disease)
CEPH	Centre d'Etude du Polymorphisme Humain
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CJD	Creutzfeldt-Jakob disease
COL 3A1	Type III procollagen
CPGO	Coproporphyrinogen oxidase
CSGE	Conformation sensitive gel electrophoresis
Cy5	Cyanin 5
d	Depth
Da	Dalton
ddH_2O	Double distilled water
del	Deletion
DGGE	Denaturing gradient gel electrophoresis

D-HPLC	Denaturing ion pair reverse-phase high performance liquid chromatography
DNA	Deoxyribonucleic acid
DNase 1	Deoxyribonuclease I
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
E.C.	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid: $C_{10}H_{16}N_2O_8$
EMG	Electromyography
ENH	Core enhancer element
ENOS	Endothelial nitric oxide synthase gene
eNOS	Endothelial nitric oxide synthase
EPP	Erythropoietic protoporphyria
ESRD	End stage renal disease
EtBr	Ethidium bromide: $C_{21}H_{20}BrN_3$
FBN-1	Fibrillin-1
FC	Ferrochelatase
FFI	Familial fatal insomnia
fPCT	Familial porphyria cutanea tarda
G	Guanine
gDNA	Genomic deoxyribonucleic acid
GI	GenInfo identifier
Glu	Glutamic acid
GT-buffer	Glycerol tolerant buffer
h	Height
***Hae* III**	Restriction enzyme with recognition sequence 5'-GG↓CC-3', Source:*Haemophilus aegyptius*
HCl	Hydrochloric acid
HCP	Hereditary coproporphyria
HD or HDA	Heteroduplex analysis
Hemox	Haem oxygenase-1
HEX-SSCP	Heteroduplex SSCP analysis
HFE	Hemachromatosis
HH	Hereditary hemochromatosis

***Hha* I**	Restriction enzyme with recognition sequence 5'-GCG↓C-3', Source: *Haemophilus haemolyticus*
***Hinf* I**	Restriction enzyme with recognition sequence 5'-G↓AnTC-3', Source: *Haemophilus influenzae*
HNPCC	Hereditary nonpolypotic colorectal cancer
***Hpa* II**	Restriction enzyme with recognition sequence 5'-C↓CGG-3', Source: *Haemophilus parainfluenzae*
HPLC	High performance liquid chromatography
***Hsp* 92 II**	Restriction enzyme with recognition sequence 5'-CATG↓-3', Source: *Haemophilus influenzae 92*
IRE	Iron responsive element
k	Kilo (10^3)
kb	Kilo base pair
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium phosphate
$KHCO_3$	Potassium bicarbonate
M	Molar: moles per litre
m	Milli (10^{-3})
Met	Methionine
MFS	Marfan syndrome
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
MMR	Mismatch repair
***Mnl* I**	Restriction enzyme with recognition sequence 5'-CCTC$(n)_7$↓-3', Source: *Moraxella nonliquefaciens*
mRNA	Messenger ribonucleic acid
***Msp* I**	Restriction enzyme with recognition sequence 5'-C↓CGG-3', Source: *Moraxella species*
mt	Mitochondria(l)
n	nano (10^{-9})
N/A	Not available
Na_2HPO_4	Sodium phosphate

NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NE Buffer 3	New England Biolabs Buffer 3 (100mM NaCl, 50mM Tris-HCl [pH 7.9], 10mM $MgCl_2$, 1mM dithiothreitol)
NF-E2	Nuclear factor erythroid 2
ng	Nanogram
NH_2	Amino group, indicating the N-terminal of a protein molecule
NH_4Cl	Ammonium chloride
nm	Nanometre
NMD	Nonsense-mediated decay mechanism
NO	Nitric oxide
NRF-1	Nuclear respiratory factor 1
nt	Nucleotide
OMIM/MIM	Online Mendelian Inheritance in Man/ Mendelian Inheritance in Man
p	Short arm of chromosome
P	Probability
PAA	Polyacrylamide
PAC	Bacteriophage P1 based artificial chromosome
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PCT	Porphyria cutanea tarda
PDA	Piperazine diacrylamide
PKD 1	Polycystic kidney disease 1
PKD 2	Polycystic kidney disease 2
PKU	Phenylketonuria
PPOX	Protoporphyrinogen oxidase
PRPN	Prion
***Pst* I**	Restriction enzyme with recognition sequence 5'-CTGCA↓G-3', Source: *Providencia stuartii*
q	Long arm of chromosome
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic Acid
SDS	Sodium dodecyl sulphate
Sfc I	Restriction enzyme with recognition sequence 5'-C↓T(A,G)(C,T)AG-3', Source: *Streptococcus faecium*
SMA	Spinal muscular atrophy
SMN 1	Survival motor neuron one
SNP	Single nucleotide polymorphism
SNPs	Single nucleotide polymorphisms
snRNPs	Small nuclear ribonucleoproteins
sPCT	Sporadic porphyria cutanea tarda
SSCP	Single strand conformation polymorphism
***Sty* 1**	Restriction enzyme with recognition sequence 5'-C↓CAAGG-3', Source: *Escherichia coli* strain that carries pST27
T	Thymidine
TBE	Tris-Borate EDTA buffer
TEMED	N',N,N',N',-tetramethylethylenediamine
T_m	Melting temperature
TOPO	Topoisomerase
TRE	Thyroid responsive element
Tris	Tris(hydroxymethyl)aminomethan:2-Amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
T-tract	Polythymidyl tract
u	Enzyme activity unit
U.K	United Kingdom
UCT	University of Cape Town
UPGIIIS	Uroporphyrinogen III synthase
UROD	Uroporphyrinogen decarboxylase
UV	Ultra violet
V	Volt
v/v	Volume per volume
Val	Valine
VNTR	Variable number of tandem repeats
VP	Variegate porphyria
w	Width

W	Watt
w/v	Weight per volume

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Chapter One

1 Introduction

1.1 Variegate porphyria

1.1.1 The disorder

Variegate porphyria (VP [MIM 176200]) is a low clinical penetrance autosomal dominantly inherited disease. It is classified as one of the three acute hepatic porphyrias, due to the fact that the overproduction and accumulation of the porphyrins mainly occur in the liver (Dowdle *et al.*, 1967; Meissner *et al.*, 1986). The clinical manifestations of the disease include acute neurovisceral attacks and cutaneous photosensitivity that either occur separately or in combination with each other. VP is found in most populations throughout the world, but has a prevalence of 0.003 in the Afrikaans speaking population of South Africa (Dean, 1972). This high VP frequency is the result of a founder gene mutation effect. The majority of VP patients in SA are therefore descendants of a couple who came to the Cape of Good Hope and married in Cape Town in 1688 (Dean, 1972; Meissner *et al.*, 1996; Warnich *et al.*, 1996).

VP is characterized by a partial deficiency of protoporphyrinogen oxidase (PPOX [E.C. 1.3.3.4]) encoded by the *PPOX* gene. In all the affected tissues studied to date, which include fibroblast (Brenner and Bloomer, 1980), bone marrow (Deybach *et al.*, 1981a), leucocytes (McColl *et al.*, 1985) and Epstein-Barr virus transformed lymphocytes (Meissner *et al.*, 1986), a 50% decrease in the PPOX enzyme activity was found. This enzyme deficiency results in the increased activity of 5-aminolevulinate synthase (ALAS [E.C. 2.3.1.3.7]) and the inhibition of porphobilinogen deaminase (PBGD [E.C. 4.3.1.8]), which in turn result in the overproduction and increased excretion of porphyrin and porphyrin precursors, formed prior to the enzyme defect.

1.1.2 The biochemical profile of VP

The typical biochemical profile observed in most VP patients during the acute and latent stages is an increase in the excretion of urine porphyrins, uro- and coproporphyrins, and an increase in faecal copro- and protoporphyrins as well as

pentacarboxylic porphyrins. An increase of uro-and protoporphyrins is also found in the plasma. In the acute stage an increase in urinary excretion of porphyrin precursors, 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) may be seen, but these values usually return to normal in the latent phase (Eales, 1963; Herbert, 1966; Hamnström *et al.*, 1967; Fromke *et al.*, 1978; Mustajoki, 1980). VP is usually diagnosed by the measurement of faecal porphyrin concentrations; however, variation in the biochemical profiles of patients makes diagnosis problematic. Adult individuals with the gene mutation have shown normal biochemical results. It is also difficult to distinguish between normal and "VP levels" as food and bacterial metabolism in the intestine contribute to the porphyrin content. A high excretion of porphyrins in the stool has also been reported in patients with ulcerating lesions and carcinoma of the gastro-intestinal tract, which could lead to false positives (Dean, 1972; Eales, 1979). To rule out these environmental influences it was concluded that biliary porphyrin levels provide a more accurate reflection of the intercellular porphyrin concentrations than the faecal porphyrin level content. Normal faecal porphyrin excretion has been demonstrated despite elevated biliary porphyrin and porphyrinogen (Logan *et al.*, 1991; Kirsch *et al.*, 1998). The testing of biliary porphyrin levels is however, not routinely performed since the bile can only be collected through duodenal aspiration, which is an invasive procedure (Logan *et al.*, 1991).

A specific plasma porphyrin compound, unique to VP, shows a fluorometric emission at 626 nm when the excitation wavelength is set at 405 nm. This biochemical procedure has proven a valuable tool in the diagnostic identification of symptomatic VP adults, although the detection rate of asymptomatic adult carriers and children is not precise (Poh-Fitzpatrick, 1980; Da Silva *et al.*, 1995).

1.1.3 The clinical symptoms associated with VP

Skin lesions and acute attacks that occur separately or in conjunction with each other clinically characterize VP. Usually the disease does not present in heterozygotes before puberty (Kauppinen and Mustajoki, 1992). The clinical expression in VP patients varies considerably; even patients with the same mutation from a single family were found to show different symptoms (own unpublished results; Frank *et al.*, 2001a). Many individuals with a mutation associated with VP, that expresses in other heterozygote individuals, remain

clinically and biochemically latent. An individual can also show biochemical abnormalities without expressing any associated clinical features (Kappas *et al.*, 1983). The full expression of VP may thus require genetic or environmental factors in addition to a single mutation (Poh- Fitzpatrick, 1998).

Photocutaneous manifestations observed in VP individuals appear to be the delayed result of sunlight exposure and the accumulation of hydrophilic oxidized porphyrins. Skin symptoms are usually found in the areas, which are exposed to sunlight such as the face, neck, hands and legs. These porphyrins, especially protoporphyrin and coprotoporphyrin, absorb long ultraviolet and visible light energy, which via an energy transfer process mediate the photochemical reaction responsible for the skin damage (Mauk and Girotti, 1973). Clinical features include photosensitivity, mechanical fragility, subepidermal blistering, milia formation, postinflammation hyperpigmentation, hypertrichosis and scarring (Poh- Fitzpatrick, 1998) (See Figures 1.1 and 1.2).

Symptoms of an acute attack can be explained by dysfunction of the nervous system. Abdominal pain, which can reach severe levels, is observed in most cases. Sympathetic outflow accounts for tachycardia, hypertension, sweating, restlessness and tremor. The autonomic outflow includes vomiting, constipation or diarrhea, hypertension, pathologic cardiovascular reflexes and bladder dysfunction. Catecholamine hypersecretion may be responsible for renal damage and has been implicated in sudden death caused by cardiac arrythmias. Motor neuropathy that starts as distal muscle weakness in the arms or legs can proceed to tetraplegia. The cranial nerves, especially the 7^{th} and 10^{th}, may become affected leading to bulbar paralysis and respiratory failure. Disorientation and psychosis may also feature during the acute attack (Poh-Fitzpatrick, 1998; Thunell, 2000). Known precipitating factors of the acute attack include drugs, for example barbiturates and thiopentone anesthetics, alcohol, hormones, infection, fasting and stress (Moore, 1980). The mechanism for the neuropathic manifestations has not been firmly established, but they all exhibit the same biochemical profile, namely the excess accumulation of ALA and PBG (Dean 1972; Mustajoki, 1978; Meyer and Schmid, 1978). The proposed mechanisms of the acute attack include the induction of cytochromes, including cytochrome P450, through many drugs. This results in the increased utilization

of haem and the further depletion of the haem pool. As a result of a decrease in the negative feedback control, ALAS activity is then derepressed (Meissner *et al.*, 1987). Agents such as sulphonamides may also have a direct effect on the haem pathway by inhibiting the enzyme that controls it (Peters *et al.*, 1980). Alcohol probably acts via both the above-mentioned routes, while the mechanisms utilized with hormones, fasting, stress and infection are still unclear (McColl and Moore, 1981; Thadani *et al.*, 2000).

The homozygous inheritance of VP was first reported by Kordac *et al.* (1984). Symptoms in these individuals are already observed in early infancy and it was established that they indeed had a very low activity of the PPOX enzyme (9-25%) (Kordac *et al.*, 1984; Mustajoki, 1980; Murphy *et al.*, 1986; Mustajoki *et al.*, 1987; Norris *et al.*, 1990). The clinical symptoms include severe photosensitivity accompanied by mental and/or growth retardation, brachydactyly, nystagmus and convulsions (Hift *et al.*, 1993; Roberts *et al.*, 1998). About twelve cases have been reported to date in which most result from heteroallelic mutations (Meissner *et al.*, 1996, Frank *et al.*, 1998; Roberts *et al.*, 1998; Corrigall *et al.*, 2000; Kauppinen *et al.*, 2001; Palmer *et al.*, 2001) and two cases were found to be due to homoallelic mutations (Roberts *et al.*, 1998).

1.1.4 The treatment of VP

Individuals with VP should be made aware of the importance of avoiding agents and circumstances that might trigger an acute attack or aggravate chronic symptoms. Anaesthesia has also been implicated in the triggering of a number of severe porphyric reactions. The total dose of the drug(s) and the length of exposure time may influence the likelihood of an acute attack. Safe anesthetic management includes the identification and avoidance of potentially porphyrinogenic agents (James and Hift, 2000).

Avoiding sunlight exposure and taking great precautionary measures to protect the skin is very important for VP patients (Thadani *et al.*, 2000). No treatment is currently available to improve the skin conditions associated with VP.

Figure 1.1: The hands of a 54-year-old female VP patient showing signs of discolouration

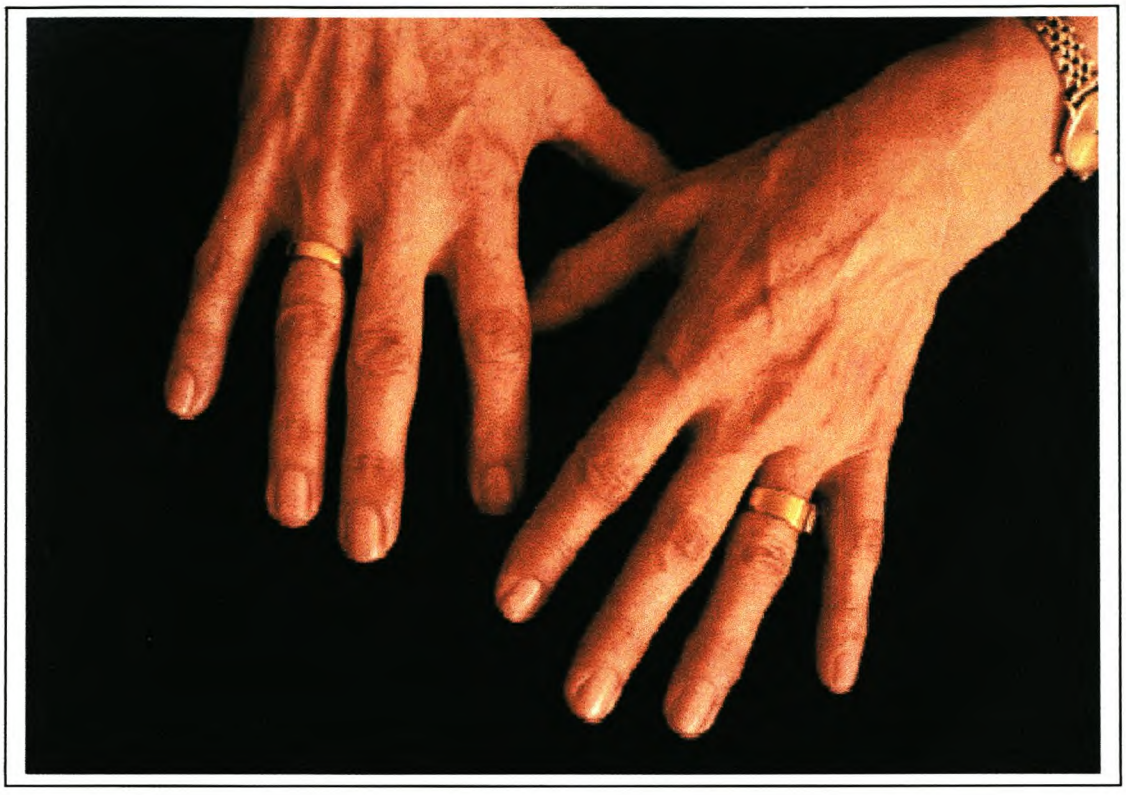
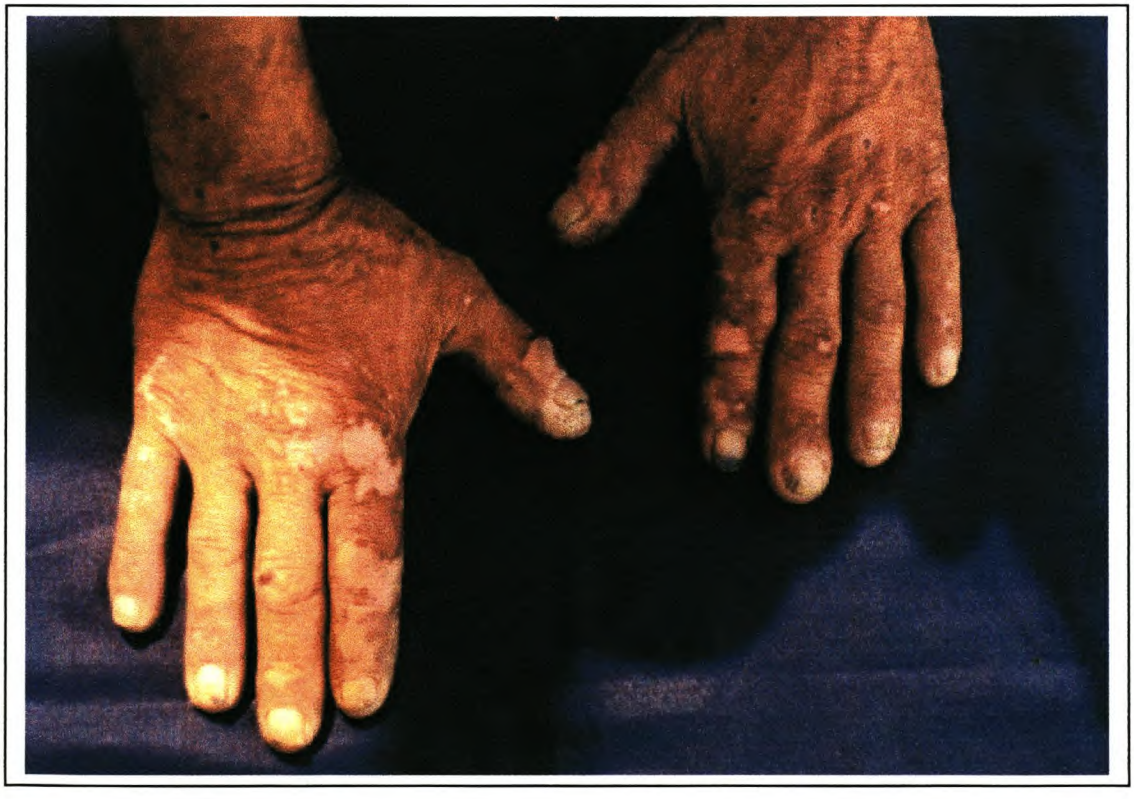


Figure 1.2: The hands of a 59-year-old male VP patient showing severe signs of skin damage



Therapies associated with the acute attack include the administration of safe drugs to alleviate the clinical symptoms present during the attack, the intake of carbohydrate, and haem therapy. Pain can be treated with paracetamol although it is usually not sufficient. In severe cases pethidine or morphine should be used. Nausea and vomiting can be treated with promazine or chlorpromazine. Great care should be taken to ensure adequate fluid and electrolyte (sodium, potassium, magnesium, calcium) intake. The acid-base balance should also be corrected and maintained. Hyponatraemia can be managed with fluid restriction, and hypertension and tachycardia can be treated with β-blockers (Meissner *et al.*, 1987; Thadani *et al.*, 2000).

High oral or intravenous carbohydrate intake throughout the acute attack is very important as it reduces porphyrin synthesis. The mechanism is explained by the effect of haem depletion on gluconeogenesis (Correia and Lunetta, 1989). There is an observed decline in ALA and PBG excretion during glucose therapy, which can be explained by a glucose-mediated repression of ALAS (Moore, 1998).

Haem administration during the early stages of an attack has proven very beneficial to controlling the acute attack and reducing the clinical neurological complications (Tenhunen and Mustajoki, 1998). Haem is intravenously administered as haematin (pH 8.0), the hydroxide of haem, since this is the only aqueous soluble state of haem (Bissell, 1979). The haematin goes to work by restoring the depleted intracellular 'free haem pool' and therefore reducing the activity of ALAS and the overproduction of porphyrin and porphyrin precursors. The normal cellular function is thus restored (Watson *et al.*, 1973; McColl and Moore, 1981). The co-administration of haematin and tin protoporphyrin, an inhibitor of haem oxygenase, have shown to be very efficient in prolonging the working state of the haematin therapy (Kappas *et al.*, 1984; Drummond, 1989; Dover *et al.*, 1991; Dover *et al.*, 1993). Unfortunately tin protoporphyrin has significant side effects viz. cutaneous photosensitivity and toxicity, which should be further investigated before it can be routinely used (Mark and Maines, 1992; Moore, 1998).

1.1.5 The *PPOX* gene

The *PPOX* gene was mapped to 1q22-23 (Taketani *et al.*, 1995) and it was shown that the disorder VP is linked to DNA markers in this region (Roberts *et al.*, 1995). The 5kb gene was shown to be present in single copy by Southern blot analysis and the same transcripts were found in both erythroid and nonerythroid cells (Taketani *et al.*, 1995; Puy *et al.*, 1996). The gene consists of twelve coding exons and one non-coding exon and the human PPOX cDNA (1.7kb) encodes a 477 amino acid protein (Nishimura *et al.*, 1995).

The promoter of the *PPOX* gene is a typical housekeeping promoter with a GC content of 59% and several *cis*-acting regulatory elements. An SP1 site is found at nt-829 to nt-834, a GATA-1 site is described at nt-738 to nt-743 and a CCAAT box is found at nt-505 to nt-509. There is no TATA box present in the promoter (Taketani *et al.*, 1995).

Mutation analysis shows extensive allelic heterogeneity, where some 100 mutations are reported worldwide, except in the South African population where the R59W mutation dominates due to the founder effect (Frank *et al.*, 1999, 2001a, 2001b; Whatley *et al.*, 1999a; Corrigall *et al.*, 2000, 2001; De Siervi *et al.*, 2000a, 2000b; Maeda *et al.*, 2000; Kauppinen *et al.*, 2001; Lam *et al.*, 2001; Palmer *et al.*, 2001; Von und zu Fraunberg *et al.*, 2001; Donnelly *et al.*, 2002; Human gene mutation database http://www.hgmd.org [Krawczak and Cooper, 1997]). Evidence for the existence of a founder mutation was also found in Chile (1239delTACAC) and Finland (R152C) (Von und zu Fraunberg and Kauppinen, 2000; Frank *et al.*, 2001a). To date ten different mutations have been detected in South Africa. These include the founder gene mutation R59W and the H20P, 537delAT, c769delG 770T>A, L15F and Q375Y mutations (Meissner *et al.*, 1996; Warnich *et al.*, 1996; Corrigall *et al.*, 1998; Corrigall *et al.*, 2001). The V290M variant was the only mutation found in a Black South African of indigenous origin (Corrigall *et al.*, 2001). The R168C, R138P and the Y348C mutations were found in compound heterozygotes, heteroallellic to the common R59W mutation (Meissner *et al.*, 1996; Warnich *et al.*, 1996; Corrigall *et al.*, 2000).

In previous studies it was demonstrated that the type of mutation did not influence the clinical presentation, and the distribution of clinical symptoms in studies in the United Kingdom (U.K.) and Western Europe were found to be identical to the results found in South Africa (Eales *et al.*, 1980; Kirsch *et al.*, 1998; Whatley *et al.*, 1999a). It is thus concluded that the genotype of the *PPOX* gene is not a significant determinant of the mode of presentation and that environmental influences and the genetic effects from other loci are likely to be more important in the phenotype-genotype relationship (Elder, 1998; Grandchamp, 1998; Poh-Fitzpatrick, 1998; Whatley *et al.*, 1999a).

To date some 17 single nucleotide polymorphisms (SNPs) have been described in the *PPOX* gene (See Table 1.1). One SNP is described in the promoter region (Warnich *et al.*, 2001), eight SNPs are found in the exonic areas of the gene (Deybach *et al.*, 1996; Warnich *et al.*, 1996, 2001; Kotze *et al.*, 1998; Whatley *et al.*, 1999a), seven SNPs are found in the intronic areas (Puy *et al.*, 1996; Lam *et al.*, 1997; Whatley *et al.*, 1999a; Biochip: http://www.bio.chip.org/biotools/) and one SNP is found in the 3'untranslated area of the *PPOX* gene (Biochip: http://www:bio.chip.org/biotools/).

Table 1.1: The SNPs identified in the *PPOX* gene

Position	Previous numbering	Area in gene	Method of detection	Reference
-1081 A>G		Promoter	SSCP; *Hae III*	Warnich *et al.*, 2001
-420 G>C	-253 (I-22)	Exon 1	HEX-SSCP	Kotze *et al.*, 1998
-414 A>C	-247 (I-26)	Exon 1	SSCP; Direct sequencing	Warnich *et al.*, 1996
-413 G>T	-246 (I-27)	Exon 1	HEX-SSCP	Kotze *et al.*, 1998
-318 G>T	-151 (I-127)	Exon 1	HEX-SSCP	Kotze *et al.*, 1998
-295 C>G	-118 (I-150)	Exon 1	Direct sequencing; *Ban II*	Warnich *et al.*, 1996

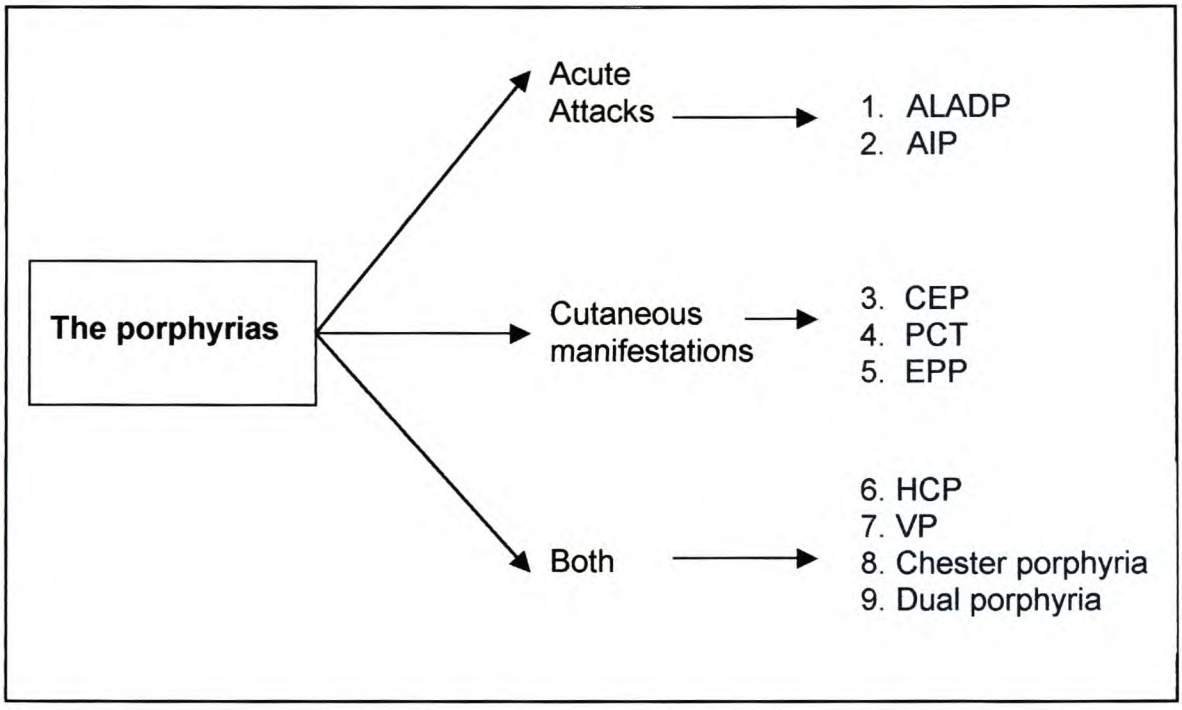
+206 G>C	IVS2-47	Intron 2	HDA; *Msp I*	Lam *et al.*, 1997
+820 G>A	IVS4-327 821G>A	Intron 4	*Hinf I*	Puy *et al.*, 1996
+1498 C>T		Intron 5	Direct Sequencing	Biochip
+1908 A>C	IVS6-237 1909 A>C	Intron 6	Direct sequencing	Puy *et al.*, 1996
+1995 T>C	IVS6-150 1996 T>C	Intron 6	*Sfc I*	Puy *et al.*, 1996
+2296 C>G	767 (P256R)	Exon 7	HDA	Whatley *et al.*, 1999a
+2929 A>C	IVS8+57	Intron 8	DGGE	Whatley *et al.*, 1999a
+3100 G>A	911 (R304H) 1188 G>A	Exon 9	DGGE; *BssSI*	Deybach *et al.*, 1996
+3750 G>C	IVS10-22	Intron 10	DGGE	Whatley *et al.*, 1999a
+3880 A>C	G402	Exon 11	SSCP	Warnich *et al.*, 2001
+4752 C>T		3' UTR	Direct sequencing	Biochip

1.2 The other Porphyrias

The porphyrias can be divided into three main groups based on their clinical expression. The position of the enzyme deficiency in the haem cycle determines the resulting haem precursor accumulation patterns and thus the clinical and biochemical phenotypic expression. The acute porphyrias consist of ALA dehydratase deficiency porphyria (ALADP) and acute intermittent porphyria (AIP) (See Figure 1.3). Both of these disorders are associated with neurological manifestations, due to a surplus of 5- aminolevulinic acid or the deficit of vital haemproteins resulting from reduced haem synthesis. The cutaneous porphyrias include congenital erythropoietic porphyria (Günters disease), porphyria cutanea tarda (PCT) and erythropoietic protoporphyria (EPP) (See Figure 1.3). The cutaneous porphyrias are due to the impairment of the porphyrin processing steps, which in turn give rise to the accumulation of phototoxic porphyrins in the

skin. The last clinical classification groups consist of hereditary coproporphyria and variegate porphyria. These two types of porphyrias express both cutaneous and neurological manifestations (See Figure 1.3). Chester porphyria is clinically classified as a combination of acute intermittent porphyria and variegate porphyria, and therefore exhibits neurological and cutaneous manifestations. Both these clinical symptoms are also found in dual porphyria, which is represented by a combination of variegate porphyria and porphyria cutanea tarda.

Figure 1.3: A summary of the clinical classification groups of the different porphyrias



ALA dehydratase deficiency (ALADP [MIM 125279]), also sometimes referred to as plumboporphyria has only been described in a very few cases. This disease is due to mutations in the ALA dehydratase (ALAD [E. C.4.2.1.24]) gene and only manifests in the homozygous state when a precipitating factor is present (Mercelis *et al.*, 1990). Precipitating factors include estrogen and progesterone, alcohol, drugs that induce the cytochrome P450 system, decreased caloric intake and acute physical and psychological stressors. The initial presentation has ranged from infancy to an individual 63 years of age. The clinical symptoms of this disease mimic those associated with acute intermittent porphyria. Laboratory investigation revealed that these patients have normal PBG excretion, but increased ALA levels in their urine. The complete blood count was also found to

be in the normal range, although there is a marked decrease in the red cell ALA dehydratase activity (Shumate, 2002). Treatment of ALA dehydratase deficiency includes the identification and avoidance of precipitating factors. Intravenous hematin treatment can be administered to relieve the symptoms associated with an acute attack.

Acute intermittent porphyria (AIP [MIM 176000]) (see 1.4.2.2 for detailed discussion) is an autosomal dominant disease due to mutations in the *PBGD* gene. AIP is found in 1 to 2 individuals per 100,000. This disease presents after puberty and the clinical symptoms include acute neurological attacks. An accumulation of porphobilinogen is found in the urine of AIP patients (Kappas *et al.*, 1995).

Erythropoietic protoporphyria (EPP [MIM 177000]) occurs in 0.1 per 10 000 individuals. It is an autosomal dominant disease due to mutations in the ferrochelatase (FC [E.C. 4.99.1.1]) gene, but very rarely also seen to inherited in an autosomal recessive manner (Sassa *et al.*, 1982; Sarkany and Cox, 1995). Light sensitive dermatitis, which is already present in early childhood, is the primary clinical symptom associated with this disease (Peterka *et al.*, 1965; De Leo *et al.*, 1976). Burning and itching of the skin after sun exposure is accompanied by erythema and oedema. The chronic lesions result in the scarring and thickening of the skin. Severe liver disease was found to be present in 10% of EPP patients and protoporphyrin rich gallstones can also be present. Laboratory findings in EPP patients include the deficiency of ferrochelatase, causing protoporphyrin to accumulate in excessive levels in erythrocytes, bile and faeces. Mild microcytic hypochromic anemia is present in 20% to 30% of EPP cases (Rademakers *et al.*, 1993). Beta-carotene can be taken by these patients to increase the tolerance of sunlight exposure. Red blood cell transfusion and the administration of hematin were found to be effective treatment in the reverse of hepatic protoporphyrin accumulation.

Congenital erythropoietic porphyria or Günters disease (CEP [MIM 263700]) is an exceedingly rare autosomal dominant disorder characterized by mutations in the uroporphyrinogen III synthase (UPGIIIS [E.C. 4.2.1.75]) gene (Levin 1968; Romeo and Levin 1969; Romeo *et al.*, 1970). This disease already presents in early

mutations in the HFE gene represent susceptibility factors for PCT but do not explain all iron metabolism disorders observed in sPCT (Lamoril *et al.*, 2002). It should be taken in to account that sPCT is multifactorial in the individual patient and is seldom attributal to a single identifiable cause (Egger *et al.*, 2002). Familial PCT is an autosomal dominant disorder due to mutations in the *UROD* gene (Romana *et al.*, 1987). Hepatoerythropoietic porphyria is a severe form of PCT and already present in infancy. These patients were found to be homozygous for mutations in the *UROD* gene (De Verneuil *et al.*, 1984). The skin symptoms associated with PCT were shown to improve after treatment with phlebotomy (as this reduces excess iron associated with the disease) and antimalarial drugs (the chloroquine forms a complex with uroporphyrin and promotes the release of uroporphyrin from the liver (Kordac *et al.*, 1989)).

Hereditary coproporphyria (HCP [MIM 121300]) is an autosomal dominant disorder characterized by defects in the coproporphyrinogen oxidase (CPGO [E.C. 1.3.3.3]) gene (Martasek, 1998). HCP is rare in all parts of the world and 20 times less common than AIP. The clinical manifestations associated with HCP include both skin and neurological symptoms. Open sores and blisters form especially after exposure to the sun. The neurovisceral symptoms were found to be present in a higher percentage of the patients compared to cutaneous symptoms (Poh-Fitzpatrick, 1998). Elevated porphyrin content is found in the blood, urine and stool. Diagnosis is usually proven by the high coproporphyrin content in the stool. During an acute attack there is usually an increase of ALA and PBG in the urine (Barnes and Whittaker, 1965; Goldberg *et al.*, 1967).

Chester porphyria [MIM 176010] is very rare and only described in the city of Chester, England. The clinical features include those associated with AIP and cutaneous photosensitivity is not a feature of Chester porphyria (McColl *et al.*, 1985). The activity of both PBGD and PPOX is reduced in these patients, but this dual enzyme deficiency is found to be associated with a locus on chromosome 11, which is separate from the *PBGD* locus (Norton *et al.*, 1993). The pattern of PBGD activity in Chester porphyria is lower than the value found in VP patients, but identical to the activity in patients with AIP (Moore, 1998). The excretion patterns of the porphyrins form an intermediate between AIP and VP.

childhood and is associated with light sensitive skin. Cutaneous lesions such as vesicles or bullae formation form on the light exposed skin. After repeated ulceration and erosion scaring occurs which can cause severe deformities. Other symptoms include hypertrichosis, alopecia, conjunctivitis, keratitis and alterations in pigment. Red urine is also observed and in a few cases the teeth are stained red (Dean 1972; Meyer and Schmid, 1978; Deybach 1981b). Porphyrins are deposited in bone, where they cause orange-red fluorescence. The severe loss of bone that results in deformities occurs in most adults with CEP. CEP is associated with the lifelong overproduction of series I porphyrins, which include uroporphyrin I and coproporphyrin I. These porphyrins are circulated and deposited in the plasma, red blood cells, urine and faeces.

Porphyria cutanea tarda (PCT [MIM 176090]) is the most frequently found type of porphyria, but to date no figure for prevalence exist. This disease is clinically defined by light sensitive dermatitis and bullous dermatosis that forms scars and milia during the healing process. Hypertrichosis and areas of increased pigmentation are also observed (Elder *et al.*, 1978; Elder, 1998). The clinical symptoms normally present in later adult life. Large amounts of uroporphyrin are excreted in the urine and the overloading of iron is frequently observed (Elder *et al.*, 1978; Felsher *et al.*, 1982). PCT can be divided into three subgroups; include sporadic (sPCT), familial and hepatoerythropoietic porphyria. Sporadic PCT accounts for 80-90% of all PCT cases and is not due to mutations in the uroporphyrinogen decarboxylase (UROD [E.C. 4.1.1.37]) gene, but the result of the generation of an inhibitor of the UROD enzyme activity, that is restricted to the liver (Garey *et al.*, 1993). Alcohol is the most important agent responsible for the acquired disease, but estrogen steroids, iron overload, viral hepatitis and fungicides such as hexachlorobenzene have been found to be causative agents. Hereditary hemochromatosis (HH) is an inherited disease of excess iron storage in target organs such as the liver and has been implicated in the development of sPCT in some populations (Hift *et al.*, 1997; Roberts *et al.*, 1997; Sherlock and Dooley, 1997; Bonkovsky *et al.*, 1998; Elder and Worwood, 1998; Bulaj *et al.*, 2000). HH is caused by mutations in the hemachromatisis (*HFE*) gene (Feder *et al.*, 1996). Analysis of *HFE* gene indicated that the C282Y, but not the H63D or the S65C genotypes, is a susceptibility factor for the development of sPCT in West European continental patients (Lamoril *et al.*, 2002). Results suggest that

Dual porphyria is found in 25% of all VP patients. The clinical features include those of VP, although they can be present in a more severe form (Day *et al.*, 1982). The activity of both uroporphyrinogen decarboxylase and protoporphyrinogen oxidase is reduced (Sturrock *et al.*, 1989). The urinary and faecal porphyrin profiles represent the superimposition of those found in PCT and VP. Diagnosis is based on the concentrations of faecal protoporphyrin, 7-carboxyl porphyrin, coproporphyrin and isoproporphyrin and the increased concentration of uroporphyrin and 7-carboxyl porphyrin found in the urine.

1.3 The haem pathway

In evolutionary terms, the haem biosynthetic pathway is highly conserved. An eight-step process by which the pigment haem is formed and metabolized is found in almost all species from bacteria to humans (Mauzerall, 1998; Thunell, 2000). The only difference is found in the starting material used. Most of the biosphere, for instance all plants, uses glutamate for the formation of ALA. Mammals use glycine succinate, which on entry into the cycle undergoes a condensation–decarboxylation reaction to form ALA. The haem biosynthesis cycle has two places where it can divaricate. The synthesis of cobalamine, managed by some organisms, is done by the methylation and rearrangement of the carboxylic porphyrinogen formed in the fifth step of the haem biosynthetic pathway (See Figure 1.4). In plants chlorophyll is generated by the incorporation of magnesium, while iron is incorporated to produce haem (Thunell, 2000).

All the mitochondrial containing cells of the body have the capacity to produce haem. Most of the haem synthesis takes place in the bone marrow and muscle tissue. Although less haem is produced in the liver (15% of the total daily production), this takes place at a more rapid and flexible rate. The haem formed in the liver is used as a prosthetic group in enzymes, where more than half are used for the formation of microsomal P450 cytochromes. Haem synthesized in the bone marrow and muscle tissue is used to form red blood cell hemoglobin and muscle tissue myoglobin. The control mechanism for the rate of production differs from hepatic synthesis with the red cell precursors being designed for uninterrupted steady production of great amounts of haem. The synthesis of the enzymes that take part in the haem pathway, in the erythron, is under control of erythropoietin.

Erythropoietin is formed under hypoxic conditions. (The haem biosynthetic pathway is illustrated in Figure 1.4.)

The haem biosynthetic pathway engages four mitochondrial steps, viz. steps 1, 6, 7, 8 and four cytoplasmic reactions steps viz. 2, 3, 4, 5.

The synthesis starts with the two hydrophilic amino acids, glycine and succinate, that condense to form 5- aminolevulinic acid (ALA). The demerization of ALA to porphobilinogen (PBG) that is under the control of the enzyme 5-aminolevulinate dehydratase (ALAD) forms the second step of the cycle. In the third step PBGD catalyzes the stepwise deamination and the condensation of four molecules of porphobilinogen, resulting in the formation of the unstable linear tetrapyrrole pre-uroporphyrinogen. In step four, on enzymatic rearrangement of the side chains, the molecule ring closes. The first porphyrin structure, the highly water-soluble III-isomeric uroporhyrinogen, is thus generated. In parallel, the metabolic inert I-isomeric form of uroporphyrinogen is formed spontaneously and excreted with coproporphyrin-I, the partially decarboxylated product of uroporphyrinogen. In step five, by a sequence of decarboxylations, four acetate groups are removed from the side chains of uroporphyrinogen-III, leaving a less water- soluble molecule coproporphyrinogen-III with four propionate groups. Coproporphyrinogen oxidase, the enzyme that catalyzes the sixth step in the pathway, converts the two propionate groups at positions 2 and 4 of coproporphyrinogen-III to two vinyl groups, thus producing sequentially, harderoporphyrinogen-IX and protoporhyrinogen-IX. PPOX catalyzes the six-electron oxidation of protoporphyrinogen-IX to protoporphyrin -IV that forms the seventh step of the cycle. The final step takes place when ferrochelatase catalyzes the insertion of ferrous iron into the protoporphyrin-IV to form haem.

The initial three enzymes of the pathway, ALAS, ALAD and PBGD, are synthesized in duplicate forms. The one form is ubiquitously expressed in all tissue, while the second form is only expressed in erythroid cells. Tissue specificity is determined by control elements in the promoter regions of these genes. The promoter region of the ubiquitous form contains the transcription factor Sp1, while the erythroid specific form's expression is determined by the activation of the GATA-1, CACCC box and NF-E2 binding sites (May *et al.*, 1995).

The two ALA isoenzymes are encoded by different genes and have different mechanisms of regulation (Bawden *et al.*, 1987; Cox *et al.*, 1991). The ALAD and PBGD isoenzymes arise from only one gene, which contains separate housekeeping and erythroid promoters. The different mRNAs are thus formed through tissue specific alternative splicing (Chretien *et al.*, 1988; Mignotte *et al.*, 1989; Kaya *et al.*, 1994).

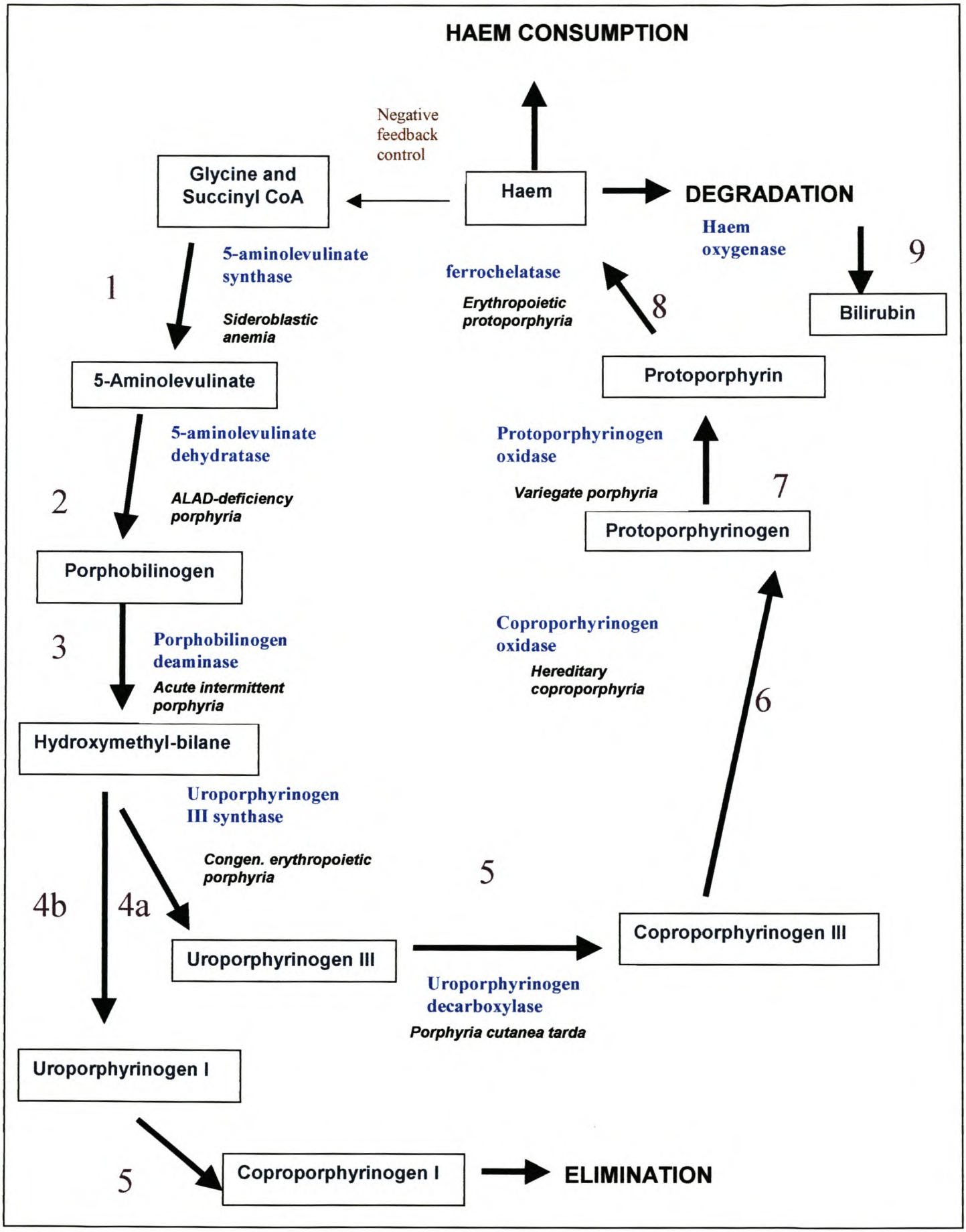
There is no evidence for the existence of isoenzymes in the case of the other four enzymes UPGIIIS, CPGO, PPOX and FC. These enzymes are all coded by a single gene, which is identically expressed in erythroid and other tissue (May *et al.*, 1995). The promoter in the *UROD* gene contains a ubiquitous Sp1 binding site, but to date no erythroid control elements have been identified (Thunell, 2000).

Of all the enzymes, the lowest activity is found for ALAS, the first enzyme in the pathway. ALAS is rate-limiting for the entire synthetic process and its activity is determined by the availability of the amount of free haem that is present within the cell.

The enzyme haem oxygenase-1 (Hemox [E.C. 1.14.99.3]) controls the amount of free haem. Hemox is ubiquitously expressed, but present in higher concentrations in the liver and spleen, the main areas of hemoglobin degradation. After splitting of the porphyrin ring to liberate the iron, the haem molecule is degraded to biliverdin (Step 9, Fig.1.4). In one further step, bilirubin is formed and excreted via the liver-bile route. The liberated iron is then re-utilized in the body (Thunell, 2000).

The pathway normally operates at high efficiency: intermediates are utilized nearly quantitatively, so that less than 1% escapes from the biosynthetic route to appear in blood and undergo excretion.

Figure 1.4: An illustration of the haem biosynthetic pathway



1.4 Modifier genes

1.4.1 The role of modifier genes

The classification of genetic disorders into chromosomal, monogenetic and multi-factorial became insufficient and problematic (Romeo and McKusick, 1994; Scriver and Waters, 1999; Dipple and McCabe, 2000). It became apparent that a consistent correlation between the mutant genotype and the variant phenotype was not possible and certain single gene traits began to be considered as a version of complex traits. The presence of a particular genotype increases the risk of developing a certain phenotype, but is not vital for its expression, nor sufficient to explain the development of the disease (Nadeau, 2001). Three causes of variable phenotypic expression include the presence of alternative alleles, the physiological interactions of environmental factors, and modifier genes (Nadeau, 2001; Van Straaten and Copp, 2001). Multiple genes, each only contributing to a small proportion of the phenotypic difference, seem to operate through modifying susceptibility.

Modifier genes can be traced back to studies done by Bateson *et al.* in 1905 (as cited by Rao, 2001) on the inheritance of colour in flowers. Two versions of modifier genes exist:,one that modifies positively (disease promoting) and one that modifies negatively (disease suppressing) (Nadeau, 2001). Modifier genes influence penetrance, the frequency of expression of an allele, expressivity, and pleiotropy. The understanding of modifying genes and disease loci will solve problems associated with variable disease presentation and lead to disease therapeutics. Modifier factors of genetic origin can be classified into three categories viz. allelic variation involving the disease gene itself, a locus genetically linked to the a disease gene, and disease gene independent locus (Feingold, 2000 as cited by Nadeau, 2001). Examples, discussing each of these categories, will follow.

A) Allelic variation involving the disease gene itself:
Investigations on cystic fibrosis (CF [MIM 219700]), a recessive disease, pointed to a modifying influence of factors other than the respective mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, when

clinical variation was observed among unrelated individuals with the same *CFTR* genotype. Congenital bilateral absence of the vas deference (CBAVD [MIM 219700]) is a form of obstructive azoospermia and the cause of infertility in 1-2% of males (Jequier *et al.*, 1985). CBAVD patients have a distinct but overlapping spectrum of mutations in the *CFTR* gene, comparable with those observed in CF patients. Three known alleles with a variable number of thymidines (5,7,9) in a polythymidyl tract (T-tract) of the acceptor splice site were found in intron 8 of the *CFTR* gene (Chu *et al.*, 1993). A variable level of aberrant *CFTR* mRNA, missing exon 9, which was shown to have defective chloride channel function, is consequently produced (Strong *et al.*, 1993). The amount of incomplete transcripts was found in reverse proportion to the length of the T-tract alleles. The variant with the 5 thymidines (5T) is therefore typically associated with the highest proportion of the aberrant *CFTR* variant and the lowest amount of the normal variant (Chu *et al.*, 1993). The 5T allele rarely causes CF, but can modify a phenotypic effect of other mutations. The R117H missense mutation is present in both CF and CBAVD patients. The R117H allele associated with the 5T variant is found in CF patients whereas it is associated with the 7T variant in CBAVD patients. The R117H mutations on the 7T background affect almost exclusively the male reproductive tract and are not sufficient to produce CF symptoms, whereas the 5T alleles enhances the phenotypic effect of the R117H mutation producing the pancreatic sufficient form of CF. In conclusion, the variation found in intron 8 of the *CFTR* gene influences the effect of the R117H mutation on the phenotype.

A second example of this interaction is illustrated by a mutation in the prion gene (*PRPN*) on chromosome 20. This mutation results in an Asp178Asn (G>A) substitution. The mutation was found to be the cause of two different autosomal dominant inherited prion disorders. The diseases Creutzfeldt-Jakob disease (CJD [MIM 123400]) and familial fatal insomnia (FFI [MIM 176640]) both represent spongiform degeneration of the brain. A widespread degenerative cerebral process characterizes CJD, while FFI is characterized by a severe atrophy of the thalamic nuclei. A common Met/Val (A>G) (with the ratio of 62% to 38%) polymorphism in codon 129 of the *PRPN* gene generates two alleles. The Met 129 and Asn 178 combination results in the disease primarily localized to the thalamus. This phenotype is associated with FFI. The Val 129 and

Asn 178 combination has a more widespread disease process associated with the CJD phenotype (Goldfarb *et al.*, 1992).

A third example includes erythropoietic protoporphyria (EPP) where it was found that an intronic SNP modifIES the degree of penetrance (Gouya *et al.*, 2002) (See section 1.2 for clinical features of disease). EPP is caused by a partial deficiency of ferrochelatase. The disease is transmitted in an autosomal dominant manner with incomplete penetrance (Sassa *et al.*, 1982). Gouya *et al.* (2002) identified an intronic SNP (IVS3-48T>C) that modulates the use of an atypical acceptor splice site. The aberrantly spliced mRNA is degraded by a nonsense-mediated decay mechanism (NMD), producing a decreased level of mRNA (Gouya *et al.*, 2002). By genotyping 25 EPP family members they observed that all the individuals with overt EPP had *in trans* to a specific *FC* mutated allele, the IVS3-48C SNP which cosegregates with the low-expression *FC* allele. The IVS3-48T allele cosegregates with the normal expression allele in all the asymptomatic carriers. Gouya *et al.*, (2002) thus showed that the phenotypic status associated with the disease depends on the co-inheritance of a severe *FC* defect and a common low-expression variant IV3-48C allele.

B) A Locus genetically linked to the disease gene:

Spinal muscular atrophy (SMA [MIM 253300]), a common recessive disorder, is characterized by the loss of motor neurons in the spinal cord. All patients display deletions or gene conversions of the survival motor neuron (*SMNI*) gene (Lefebvre *et al.*, 1995; Rodrigues *et al.*, 1995; Hahnen *et al.*, 1996; Van der Steege *et al.*, 1996). SMA is classified into three types (SMA I, SMAII, SMAIII) based on the age of onset and the severity of the disease. SMA I is the most severe phenotype of the disease followed by SMA II and lastly SMA III. All three classifications of SMA mapped to chromosomal region 5q13 (Brzustowicz *et al.*, 1990; Melki *et al.*, 1990). The majority of SMA I patients display a large-scale 5q13 deletion removing the *SMN1* gene and adjacent microsatellite markers C272 and C212 (DiDonato *et al.*, 1994; Burlet *et al.*, 1996). Additional modifying factors were believed to exist that contribute to the severity of type I SMA. Scharf *et al.* (1998) identified a novel transcript, *H4F5*, which is located closer to the *SMN1* gene than any previously identified genes in the region. A multi-copy microsatellite marker, C212, that is deleted in more than 90% of type I SMA

chromosomes was found to be imbedded in an intron of this *H4F5* gene. The deletion frequency of the C212 marker in type II SMA was found to be between that of type I and the control chromosomes, whereas deletions in type III SMA chromosomes were only slightly more frequent than in the controls. The deletion frequency of the C212 marker in the *H4F5* gene in the three groups of the SMA populations thus correlates with the disease severity. It is therefore concluded that *H4F5* is a candidate phenotypic modifier for SMA (Scharf *et al.*, 1998). The exact function of the *H4F5* gene is still unknown, but futher studies of the H4F5 protein will reveal if it functions in the small nuclear ribonucleoprotein (snRNPs) biogenesis like the SMN protein. It was found that the H4F5 protein shows homology to an snRNP interacting protein that co-localizes with the same proteins as SMN (Scharf *et al.*, 1998).

C) Disease gene independent modifying locus:

The Cyclin D1 (*CCND1*) gene was found to be a genetic modifier in hereditary nonpolyposis colorectal cancer (HNPCC [MIM 114500]) (Bala and Peltomäki, 2001). HNPCC is associated with an inherited predisposition to colorectal carcinoma and a variety of extracolonic cancers. HNPCC is linked with germ-line mutations in one of the five *(MLH 1, MSH 2, MSH 6, PMS 1, PMS 2)* mismatch repair (*MMR*) genes (Lynch and Smyrk, 1996). To date there are more than 300 identified mutations (Peltomäki and Vasen, 1997). Significant phenotypic variation has been reported in HNPCC patients, which could not be explained by the different predisposing mutations. It was thus concluded that other factors beside the mutations in the *MMR* genes influence the phenotypic expression. The *CCND1* gene is a good candidate for a modifier gene locus in cancers. *CCND1* encodes a protein that plays an important role in the cell cycle control in normal cells and neoplasia (Donnellan and Chetty, 1998). It also exhibits alternative mRNA splicing. A G>A polymorphism at the splice donor site of exon 4 was considered to increase alternative splicing (Betticher *et al.*, 1995). In the latter study the different alleles of the G>A polymorphism in codon 242 of the *CCND1* gene was identified through SSCP analysis in patients who had a predisposing mutation in either the *MLH1* or *MSH 2* gene. No correlation was found between the different genotypes and the colonic location of the cancer or the occurrence of extracolonic cancers. No correlation was observed between a particular allele (A versus G) and the age of onset of cancer, but the average age

of onset was higher in heterozygous individuals (AG, 46 years) as compared with homozygous individuals with the normal allele (GG, 41 years) or the variant allele (AA, 39 years) (Bala and Peltomäki, 2001). Therefore the results suggest that the co-existence of A and G transcripts in the *CCND1* gene, modify the age of onset of colon cancer in hereditary nonpolyposis colorectal cancer.

The modifying effect of the endothelial NO synthase (*ENOS*) gene in autosomal dominant polycystic kidney disease (ADPKD [MIM 601313]) was shown by Persu *et al.* (2002). ADPKD, an autosomal dominant disease, is one of the most common monogenic hereditary diseases. ADPKD is characterized by the development of multiple cysts in both kidneys. The cysts continue to grow until renal failure occurs. ADPKD is due to mutations in two major genes, *PKD1* found at chromosome location 16p13.3 and *PKD2* found at chromosome location 4q21-q23 (Pirson *et al.*, 1998). *PKD1* mutations are responsible for 85% of all cases and *PKD2* mutations for the vast majority of the remaining cases. *PKD2* mutations were found to express a clinically milder phenotype than *PKD1* mutations (Hateboer *et al.*, 1999). A substantial variability is observed in the severity of renal phenotype, primarily assessed by the age at end-stage renal disease (ESRD). The release of nitric oxide (NO) by endothelial cells plays a critical role in the control of local haemodynamics and systemic blood pressure (Vallance *et al.*, 1989; Forte *et al.*, 1997). APDKD is associated with an alteration of the endothelium-dependent vasodilation, that has been attributed to a decreased production of NO by endothelial NO synthase (eNOS [E.C. 1.14.13.39]) (Wang *et al.*, 2000). It was thus speculated that small changes in the NO levels may play a role in the progression of renal disease and *ENOS*, the gene coding eNOS, found at chromosome location 7q35-36, could be a modifier gene in ADPKD (Marsden *et al.*, 1993). In the study by Persu *et al.* (2002) the influence of the three most studied polymorphisms (Glu298Asp; intron 4 VNTR; T-786C) of the *ENOS* gene was assessed on the age at ESRD in males and females from a large series of unrelated ADPKD patients. No effect on the age at ESRD was found for the intron 4 and T-786C polymorphisms. The Glu298Asp polymorphism was associated with a significantly lower age at ESRD in the ADPKD males and was over-represented in the subset of ADPKD males reaching ESRD before the age of 50 years. The production of NO through eNOS is regulated by estrogens (Goetz *et al.*, 1994; Chambliss *et al.*, 2000)

which might explain a gender sensitivity to NO (Majmudar *et al.*, 2000) and the observation that the effect of the Glu298Asp polymorphism was restricted to ADPKD males. Expression studies revealed that the *ENOS* activity was systematically lower in patients with the Glu/Asp or Asp/Asp genotype. These results thus show that the ENOS gene can possibly modify the clinical expression found in ADPKD, although further studies in other populations with different genetic backgrounds should be done to support the results. This is particularly true if the Glu298Asp polymorphism is not the causal polymorphism, but only a marker in linkage disequilibrium with the modifier locus (Persu *et al.*, 2002).

1.4.2 Candidate modifier genes considered in this study

Great variation in the clinical expression of VP is found in the South African founder population. Since the majority of VP patients share an identical disease causing mutation, it was concluded that environmental factors or modifier genes contribute to the variation found in the phenotypic expression (Warnich *et al.*, 2002). Variation between sibs could not be explained by environmental factors alone as we expect sibs to be exposed to the same environmental factors. The starting point when searching for modifier genes would include sequence variation found in the *PPOX* gene itself. As the *PPOX* gene is already under investigation as part of a current research project (Warnich *et al.*, 2002; Warnich unpublished results), it was decided that a search for modifier genes should include other genes of the haem pathway that play an important role in regulating the pathway. The rate of the haem synthesis is regulated by a negative feedback control mechanism, through the activity of the initial enzyme, ALA synthase. Secondary control lies at the level of the third enzyme, PBG deaminase, which has a low endogenous activity and is inhibited by proto- and coproporphyrinogen (Kirsch *et al.*, 1998; Moore, 1998).

This study therefore investigated sequence variation in the *ALAS* and *PBGD* genes as possible candidates for modifying the clinical expression in the South African founder VP patients.

1.4.2.1 ALAS

The 5-aminolevulinate synthase genes play a very important role in the haem pathway as they are rate limiting for the entire synthetic process. In humans and other mammals two ALA enzymes are present. These enzymes are encoded by separate genes, *ALAS-1* and *ALAS-2*, and are subject to different forms of regulation. The catalytic domains of both isoforms are similar and highly conserved between species, but their amino terminal regions differ (Maguire *et al.*, 1986; Schoenhaut and Curtis, 1989; Conboy *et al.*, 1992; Yomogida *et al.*, 1993; May *et al.*, 1995; Duncan *et al.*, 1999). This results in 73% sequence conservation between the *ALAS-1* and the *ALAS-2* genes (Ferreira, 1993).

ALA functions in the mitochondrial matrix but is initially synthesized as a larger cytoplasmic precursor protein, which is proteolytically processed during the transport phase into the mitochondria (May *et al.*, 1986). Haem can feed back negatively and regulate its own synthesis by modulating the levels of ALA at probably the translational level, through destabilizing the mRNA and by blocking mitochondrial import (Srivastava *et al.*, 1988; Drew and Ades, 1989; May and Bawden, 1989; Hamilton *et al.*, 1991; Lathrop and Timko, 1993; Zhang and Guarente, 1995).

ALAS-1 or *ALAS-N*, the so-called housekeeping gene, is located on chromosome 3 and consists of 11 exons (Sutherland *et al.*, 1988). This gene is expressed in all cells (Sutherland *et al.*, 1988). The unprocessed precursor ALAS-1 enzyme consists of 640 amino acids and has a molecular weight of 70580Da. ALAS-1 controls the rate of haem synthesis by its activity, which in turn is determined by the amount of free haem in non- erythroid tissue.

The *ALAS-1* promoter contains an array of regulatory elements including a TATA-box. The promoter exhibits basal as well as tissue specific regulation and contains binding sites for the nuclear respiratory factor 1 (NRF-1). This NRF-1 binding site, which is not present in the *ALAS-2* promoter, is necessary for basal expression. NRF-1 coordinates the synthesis of mitochondrial enzymes engaged in the oxidative phosphorylation by regulating the *ALAS-1* gene for the

respiratory chain subunits. By the action of this transcription factor the gene is expressed at a basal level in all tissue, but can be induced by a variety of endogenous and exogenous factors.

No disease has yet been attributed to defects in the *ALAS-1* gene (Elder, 1998; Human gene mutation database http://www.hgmd.org [Krawczak and Cooper, 1997]). To date twenty single nucleotide polymorphisms (SNPs) have been identified through the direct sequencing of the *ALAS-1* gene (Bio-chip: http://www.bio.chip.org/biotools/; Locuslink: http://www.ncbi.nlm.nih.gov/SNP/ [Maglott *et al.*, 2000; Pruitt *et al.*, 2000]) (See Table 1.2).

Table 1.2: The SNPs identified in the *ALAS-1* gene

Nomenclature	Area in gene
-5368 C>T	5' untranslated region
-5112 A>T	5' untranslated region
-4766 A>G	5' untranslated region
-4167 C>G	5' untranslated region
-3582 A>C	5' untranslated region
-2822 A>C	5' untranslated region
-2787 A>T	5' untranslated region
-1077 A>G	5' untranslated region
+604 A>T	Intron 2
+946 C>T	Intron 2
+2820 C>T	Intron 2
+3505 C>T	Intron 3
+4713 A>G	Exon 4
+5399 A>G	Intron 4
+5420 A>G	Intron 4
+7972 A>G	Intron 7
+9645 C>T	Intron 8
+12821 C>G	Intron 9
+13853 C>T	Intron 10
+14057 A>G	Intron 10

The human erythroid isoform *ALAS-2* or *ALAS-E* is located on chromosome X (Cox *et al.*, 1990). The structural organization of the *ALAS-2* gene in the human, mouse and chicken is very similar. The gene consists of 11 exons with the presence of a 5-6kb intron in the 5'-untranslated region, being a unique feature (Lim et al, 1994).

The unprocessed precursor enzyme consists of 587 amino acids, has a molecular weight of 64693Da, and is induced only during the period of active haem synthesis in the red cells. Its activity in the cells is regulated by the amount of free iron present. In the absence of iron, to be incorporated in the porphyrin synthesized, the mRNA of the *ALAS-2* gene is blocked by the attachment of an iron-responsive element (IRE) binding cytosolic protein. The transcription of this key enzyme is then inhibited. The IRE binding site is found in the 5' untranslated region of the mRNA (May *et al.*, 1990). *ALAS* gene function is thus regulated at the transcriptional and the posttranscriptional level.

The control elements found in the *ALAS-2* promoter region are very similar to that found in the β-globin promoter and the erythroid promoter of the *PBGD* gene. This indicates control elements common to all three genes. The *ALAS-2* gene was found to have a very low GC content and very few repetitive elements (Surinya *et al.*, 1998).

To date eighteen single nucleotide polymorphisms have been identified through the direct sequencing of the *ALAS-2* gene (Bio-chip: http://www.bio.chip.org/biotools/; Locuslink: http://www.ncbi.nlm.nih.gov/SNP/ [Maglott *et al.*, 2000; Pruitt *et al.*, 2000]) (See Table 1.3).

Table 1.3: The SNPs identified in the *ALAS-2* gene

Nomenclature	Area in gene
-5793 C>A	5' untranslated region
-4218 A>C	5' untranslated region
-3945 T>G	5' untranslated region
-3890 T>C	5' untranslated region

-974 T>C	5' untranslated region
+1791 T>G	Intron 3
+3297 T>A	Intron 4
+5750 G>A	Intron 6
+7133 T>C	Intron 6
+7424 A>T	Intron 6
+7630 T>A	Intron 6
+7949 G>C	Intron 6
+8163 T>C	Intron 6
+10602 C>A	Intron 8
+13583 T>C	Intron 10
+14076 T>A	Intron 10
+14449 A>T	Intron 10
+16990 G>A	3' untranslated region

Mutations in the *ALAS-2* gene were found to be the cause of types of X-linked hereditary sideroblastic anemia. The patients present with features of a severe anemia due to the impairment of hemoglobin formation, but show none of the clinical or biochemical features associated with porphyria (Bottomley and Muller-Eberhard, 1988).

1.4.2.2 *PBGD*

The single copy *PBGD* locus is situated on chromosome 11q24 and the complete genomic sequence, including the 5' regulatory, the 3' untranslated and intronic regions has been published (Namba *et al.*, 1991). The 10 024 bp gene contains 15 exons ranging from 39 to 438 bp and 14 introns ranging from 87 to 2913 bp in size (Yoo *et al.*, 1993). *PBGD* cDNA has been cloned from a variety of organisms, from *E.coli* to humans, and a high degree of conservation is evident (Grandchamp *et al.*, 1996). The gene contains two promoter areas, separated by 3kb of DNA, which give rise to two isoenzymes through differential splicing (Lannfelt *et al.*, 1989).

The upstream promoter found at the 5' side of exon 1 is active in all cells and produces mRNA that contains exon 1 and exon 3 through to exon 15.

Translation of this mRNA produces a 44 kDa isoenzyme that differs from the other isoenzyme by the addition of 17 amino acid residues at the NH_2- terminus (Deybach and Puy, 1987; Chretien *et al.*, 1988; Yoo *et al.*, 1993). The minimal non-erythroid promoter sequence for maximal transcription was found to be a 128 bp fragment containing nt-243 to nt-115, relative to the translation start site (Lundin and Anvret, 1997). By implementing computer analysis, Yoo *et al.* (1993) found that this housekeeping promoter contained 10 *Hpa II* sites and had a GC content of 54%. They also found two AP1 binding sites, both in the antisense orientation at nt-491 to nt-487 and nt-126 to nt-119, and a AP4 binding site in the sense orientation at nt-461 to nt-456. A thyroid responsive element (TRE) in the antisense orientation at nt-45 to nt-38 and two core enhancer element (ENH) binding sites in the antisense orientation at nt-828 to nt-821 and nt-16 to nt-89 were also found. Two SP1 elements at nt-201 to nt-191 and nt-190 to nt-181 were present but no CAAT and TATA like promoter elements were found. A CAC box at nt-33 to nt-29 in the sense orientation was found and the presence of a 13 base repeat nt-243 to nt-217 was also confirmed.

Erythroid-specific mRNA is produced from a promoter downstream from exon 1 and encodes exon 2 through to exon 15. A 42 kDa isoenzyme is formed (Deybach and Puy, 1987; Chretien *et al.*, 1988). This promoter shows some homology with the β-globin gene promoter and suggests that some common transacting factors co-regulate the transcription of these genes during erythroid development (Mignotte *et al.*, 1989). Studies done on the erythroid promoter previously reported an AP1 site in the sense orientation at nt-2781 to nt-2788, a CAAC motif in the sense orientation at nt-2839 to nt-2847, and a CAAT like box in the sense orientation at nt-2855 to nt-2863. A TATA like element in the sense orientation at nt-913 to nt-2918, two NF-E1 sites in both the sense orientation nt-2760 to nt-2765, and the antisense orientation nt-2782 to nt-2790, and a NF-E2 binding site at nt-782 to nt-2790 in the antisense orientation, have also been investigated (Chretien *et al.*, 1988; Mignotte *et al.*, 1989; Frampton *et al.*, 1990). Yoo *et al.* (1993) found evidence of an additional four NF-1 binding sites, three in the sense orientation (nt-2675 to nt-2679, nt-2713 to nt-2717, nt-2724 to nt-2728) and one in the antisense orientation (nt-2827 to nt-2831). They also found two topoisomerase (TOPO) binding sites, one in the sense

orientation (nt-2332 to nt-2346), and one in the antisense orientation (nt-2329 to nt-2342). One AP4 binding site (nt-2830 to nt-2835) and one NF-E1 site (nt-2573 to nt-2580), both in the sense orientation, were also present.

The PBGD enzyme is unique in the way that it uses two molecules of its own substrate, porphobilinogen, covalently attached to the apoenzyme as a prosthetic group (Shoolingin-Jordan *et al.*, 1997). The PBGD reaction becomes rate limiting when ALAS activity is increased (Moore *et al.*, 1987). Mutations usually cause the PBGD activity to decrease to about 50% in all tissue, but cases have been reported were the low enzyme activity was restricted to non-erythropoietic tissue (Kappas *et al.*, 1995). These cases could be explained by mutations in the area upstream from the erythroid promoter that therefore influences the housekeeping or ubiquitous form, but not the erythroid form.

Acute intermittent porphyria is the most common of the acute hepatic porphyrias and is caused by mutations in the *PBGD* gene. AIP is an autosomal dominant disorder and characterized by attacks of neurological dysfunction with abdominal pain, hypertension, tachycardia and peripheral neuropathy (Kappas *et al.*, 1995). These attacks are caused by environmental factors such as drugs and alcohol or endogenous factors, for example stress and hormones. In the general population AIP specific mutations are found in about 0.6 per 1 000 individuals (Nordmann *et al.*, 1997) but clinical manifestation of the disease is less prevalent at about 1-2 per 100 000 (Kappas *et al.*, 1995; Elder *et al.*, 1997). Of these individuals with an established AIP diagnosis, only about 10% show a clinically manifest form, the remaining 90% remains latent throughout their lives.

Extensive allelic heterogeneity is found in AIP patients and to date more than 165 mutations have been described (Grandchamp, 1998 for review; Whatley *et al.*, 1999(b); Human gene mutation database http://www.hgmd.org [Krawczak and Cooper, 1997]). Found to be clustered in exons 10, 12 and 14 were 52% to 60% of the described mutations (Kaupinnen *et al.*, 1995; Puy *et al.*, 1997). Only exon 2 has no reported mutations, because the transcript of the housekeeping promoter does not contain exon 2, and exon 2 of the erythroid specific transcript encodes no amino acids. Three mutations were also found

to be relatively common in specific geographical areas and founder mutation effects could explain this. These include the W198X mutation in Sweden, The R116W mutation in Holland and the G111R mutation in Argentina (Lee and Anvret, 1991; Gu *et al.*, 1993; De Siervi *et al.*, 1999). Homozygous AIP has been described where the enzyme activity is less than 10% of the normal value. No clear relationship between the type of mutation and the severity of the clinical symptoms in AIP exists (Puy *et al.*, 1997; Elder, 1998).

To date, sixteen polymorphic sites have been identified in the human *PBGD* gene. Thirteen are characterized as intragenic SNPs and one is found in exon 10 but does not alter the amino acid sequence. Two polymorphisms exist in the nonerythroid promoter region. Two polymorphic sites, a single base pair G deletion at position 3167 in intron 2, and an A>G dimorphism at position 7052 in intron 10, were only identified in a population consisting of African and Afro-Caribbean individuals and never encountered in Caucasian subjects (Robreau-Fraolini *et al.*, 2000). The allele frequencies of the other 14 polymorphisms found in the normal Caucasian population were almost identical to the allele frequencies found in African and Afro-Caribbean control populations (Robreau-Fraolini *et al.*, 2000). A study of the allele frequencies of the four polymorphic sites in intron 1, and the one polymorphic site in intron 3, concluded that they exist in linkage disequilibrium (Yoo *et al.*, 1993). The nine remaining polymorphic sites found in the Caucasian population were shown to segregate independently (Robreau-Fraolini *et al.*, 2000). These polymorphisms have proven useful for heterozygote diagnosis in informative families whose mutations were still unknown.

Table 1.4: The SNPs identified in the *PBGD* gene

Nomenclature	Area in gene	Method of detection	Reference
-235 A>T	Promoter	Direct sequencing	Lundin and Anvret, 1997
-64 C>T	Exon 1	*Apa I*	Picat *et al.*, 1991
+245 G>A	Intron 1	*Msp I*	Llewellyn *et al.*, 1987
+400 T>C	Intron 1	*Pst I*	Lee and Anvret, 1987

+1277 C>A	Intron 1	*ApaL I*	Lee and Anvret, 1987
+2478 A>G	Intron 1	*BstN I*	Lee and Anvret, 1987
+3119 G>T	Intron 2	DGGE	Daimon *et al.*, 1993
+3167 G>delG	Intron 2	DGGE	Robreau-Fraolini *et al.*, 2000
+3581 A>G	Intron 3	*BsmAI*	Daimon *et al.*, 1993
+3982 T>C	Intron 4	*HhaI*	Whatley *et al.*, 1999b
+6479 G>T	Exon 10	DGGE	Gu *et al.*, 1991
+7052 A>G	Intron 10	DGGE	Robreau-Fraolini *et al.*, 2000
+7064 C>A	Intron 10	*Hinf I*	Yoo *et al.*, 1993
+7539 C>T	Intron 12	Direct sequencing	Whatley *et al.*, 1999(b)
+7998 G>A	Intron 14	*MnLI*	Yoo *et al.*, 1993
+8578 G>A	3'UTR	*BsrI*	Law *et al.*, 1999

1.5 Sequence variation detection techniques

We rely on screening techniques to increase the efficiency in identifying unknown mutations. To date quite a few protocols and procedures for the detection of sequence variation exist, but none have been shown to be the ideal screening procedure. The ideal screening procedure should have a 100% detection rate, be inexpensive, not require expensive equipment with costly maintenance, should be simple to the point that it is no more complex than conventional electrophoresis, and it should allow for rapid analysis of large numbers (Ganguly, 2002).

1.5.1 Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism (SSCP) analysis is to date the most widely used mutation detection technique and is based on the principle that single stranded DNA assumes a distinct three-dimensional conformation. This conformation is determined by the exact sequence of the fragment. The conformation in return determines the rate at which a strand will migrate through a nondenaturing polyacrylamide gel. Any change in the DNA sequence thus alters the three-dimensional conformation and therefore the rate of migration (Orita *et al.*, 1989). The resolution and the detection rate of mutations are

determined by factors such as the temperature of the gel during electrophoresis, the length of the gel, the percentage of glycerol in the gel matrix, the pH of the buffer(s) and the size of the DNA fragment. The SSCP technique lacks the theoretical background for exact secondary structure prediction (Glavac *et al.*, 2002) and the senisivity of unknown DNA fragments can therefore not be determined in advance. Hayashi and Yandell (1993) estimated the detection rate to be 90% in fragments shorter than 200 base pairs, and approximately 80% in fragments up to 350 base pairs. A literature survey indicates that the detection rate varies from 60% to 100% (Hayashi and Yandell, 1993; Ravnik-Glavac *et al.*, 1994; Markoff *et al.*, 1998). The position of the base substitution was shown to be more important than the precise nature of the base substitution, in determining whether a mutation was detected (Sheffield *et al.*, 1993). Although the detection rate of the SSCP technique is not 100%, it is still a rapid procedure that can be automated. SSCP analysis does not require too highly specialized skills and can also be used for high throughput applications (Kristensen *et al.*, 2001).

1.5.2 Heteroduplex analysis

Heteroduplex analysis is based on the formation of DNA duplexes that do not match perfectly. DNA is heated and slowly cooled, so that the individual strands may reanneal to complementary strands from either copy of the gene. If the copies are identical, a perfect double helix (homoduplex) forms, but if the two copies differ, as is the case when one copy harbors a mutation, a heteroduplex forms. The migration rate of a heteroduplex and a homoduplex diffes in polyacrylamide gels and a mutation can therefore be detected (Schreiber *et al.*, 1995).

1.5.3 Conformation sensitive gel electrophoresis (CSGE)

Conformation sensitive gel electrophoresis is based on the proven assumption that a system of mildly denaturing solvents can amplify the tendency of a single base mismatch to produce conformational changes. The differential migration of DNA homoduplexes and heteroduplexes during gel electrophoresis is thereby increased (Ganguly *et al.*, 1993). CSGE analysis is simple, requires no special preparation of the PCR product, has a large capacity, and does not require

radioactivity. CSGE was also shown to have a better detection rate than SSCP analysis in a study by Markoff *et al.* (1998). Furthermore, CSGE detected all previous mutations detected by denaturing gradient gel electrophoresis (DGGE) analysis (Körkko *et al.*, 1998). Mutations in fragments between 200 to 800 base pairs in length were shown to have a high detection rate with CSGE, although mutations in unusually GC rich areas, and mutations at the 50 base pair ends of heteroduplexes, showed a decline in their detection rate (Ganguly *et al.*, 1993). One previous drawback of CSGE was the inability to screen for homozygotes. This problem was solved when Leung *et al.* (2000) developed a two-fold strategy to detect homozygous sequence variants. This strategy is based on the creation of artificial heterozygotes by preparing a mixture of the homozygous and wild type PCR products in the ratio of 1:1 or 1:2.

1.5.4 Other screening techniques not used in this study

Direct sequencing of fragments was shown to be the most sensitive screening technique available and, was even described as the 'gold standard' (Kirstensen *et al.*, 2001). In most laboratories manual sequencing, which is very time consuming and labor intensive, has been replaced by automated sequencing (Wen, 2001). Different automated systems are available and can either use four different dyes to label each of the DNA bases, as in the case of ABI sequencing systems, or alternatively in the Pharmacia-ALF sequencing technology, employ a single dye chemistry format in which Cy5 is used to label DNA fragments for the detection of laser-induced fluorescence (Kirstensen *et al.*, 2001). Both these automated sequencing systems increased the turnaround time involved in sequencing, but involve very high costs. This technology is therefore not suited for small research laboratories, where the main aim is to seek sequence variation in entire genes (Ganguly, 2002).

Denaturing gradient gel electrophoresis (DGGE) analysis is based on the melting behaviour of double stranded DNA when exposed to increasing concentrations of a denaturing agent (Lerman and Silverstein, 1987). Any change in the DNA sequence of the fragment can affect the point at which the duplex will begin to denature, which in turn slows its mobility in a polyacrylamide (PAA) matrix. The PAA gel contains an increasing gradient of urea or formamide and is maintained at a fixed temperature higher than room temperature. During the amplification of

the fragment a 'GC-clamp', which consists of 30 to 40 bases of guanine and cytosine, is added to improve the detection rate (Bourgeois *et al.*, 1992). DGGE was shown to be more sensitive than HD and SSCP analysis, but is technically very demanding.

Denaturing ion pair reverse–phase high performance liquid chromatography (D-HPLC) is found to be rapid, robust and inexpensive (Oefner and Underhill, 1998). This technique is based on the separation of heteroduplexes from homoduplexes on a stationary phase under partially denaturing conditions. The D-HPLC procedure can be automated and allows for the separation of ds DNA molecules of up to 1kb in length. This technology is very useful to investigate genes with a high frequency of point mutations at a variety of sites. The sensitivity of D-HPLC was found to be between 95% and 100%, thus making it an extremely accurate method for the detection of sequence variation (Liu *et al.*, 1998; O'Donovan *et al.*, 1998; Ellis *et al.*, 2000; Le Maréchal *et al.*, 2001). Homozygous mutations do not generally alter the stability of DNA fragments and therefore a mixture of PCR products from the patient and a normal control is necessary to create heteroduplexes for detection of different alleles in this procedure.

In the cases of DGGE and D-HPLC, a close prediction of the sensitivity and the behaviour of the mismatches can be made from the theoretical melting curves and the melting theory.

1.6 Statistical association analysis

Allelic association studies have proven to be a very effective way for locating genes responsible for complex traits (Owen and McGuffin, 1993; Risch and Merikangas, 1996). Association studies test whether a genetic marker (polymorphism or allele) occurs more frequently in certain disease cases/phenotype than in controls. The genetic marker can be associated with either an increased or decreased risk. Association studies are better suited for detecting genetic effects of low penetrance with higher resolution, and have recently been successfully used to identify association between sequence variation and different disease phenotypes in cases of Myasthenia gravis

(Sciaccaet *al.*, 2002), late onset Parkinsons disease (Martin *et al.*, 2001) and cutaneous basal cell carcinoma (Ramachandran *et al.*, 2001).

1.7 Aim of the study

The identification of modifier genes responsible for phenotypic variation in VP could lead to a better understanding of the disease pathogenesis. Early diagnosis and calculation of risk can lead to improved counseling and treatment and thus be of great importance to VP individuals as well as clinicians attending to these patients.

Attempts to identify variation in the *PPOX* gene itself, that could explain the phenotypic variation observed in VP patients, have so far failed. Environmental factors, for instance the influence of sunlight exposure, are very difficult to identify and study due to the fact that it cannot be enforced or measured. In this study it was thus decided to investigate sequence variation found in the genes that exhibit a regulating function in the haem biosynthetic pathway. The hypothesis is therefore that a mutation in the *PPOX* gene in combination with a mutation in one of the rate limiting enzymes of the haem biosynthetic pathway would cause a more severe phenotype. The rate of the entire pathway is determined by the activity of the first enzyme ALA synthase, which is under negative feedback control by the free haem. Secondary control is found at the level of PBG deaminase, which is inhibited by proto- and coproporphyrinogen. The *ALAS-1*, *ALAS-2* and *PBGD* genes were thus investigated for their possible modifying effect on the clinical symptoms found in VP patients.

To ensure a high detection rate of sequence variation in our population, three different screening techniques, found to show high sensitivity in recent literature reviews, were decided on (Ganguly *et al.*, 1993; Ravnik-Glavac *et al.*, 1994; Körkkö *et al.*, 1998; Liechti- Gallati *et al.*, 1999). Sequence variants identified would be subjected to statistical analysis to determine the existence of any association.

Should any modifying influences or effects be found in the *ALAS-1*, *ALAS-2* and *PBGD* genes, their role in other porphyrias with low penetrance could also be

determined. Ultimately this research could lead to the better understanding of the haem pathway.

Specific aims of this study were:

1. To classify the R59W VP patients into four clinical groups (acute attack, skin symptoms, acute attack and skin symptoms and asymptomatic).
2. To determine and compare the mutation detection rate of the three screening methods SSCP, two buffer SSCP/HD and CSGE.
3. To compare the allelic frequencies of sequence variants in the *ALAS-1*, *ALAS-2* and *PBGD* genes in symptomatic and asymptomatic VP patients, and a healthy control group
4. To investigate the existence of a possible statistical association between sequence variants found in the *ALAS-1*, *ALAS-2* or *PBGD* genes and the clinical phenotypes of the South African VP population.

Chapter Two

2 Materials and Methods

2.1 Patients

This study included 50 VP patients (37 women; 13 men; mean age 44; range 11-71 years) of Afrikaner origin (white Caucasian ancestry). The diagnosis of VP was based on the presence of the heterozygous R59W founder mutation. A neurologist and dermatologist characterized 25 patients (group A) on their clinical presentation. Group A also completed a clinical questionnaire attached as Appendix A. The remaining 25 patients (group B) were characterized on the basis of information contained in the clinical questionnaire previously completed by themselves and a genetic counselor. Four groups were compiled consisting of a) patients with skin symptoms, b) patients that had an acute attack (in clinical remission at time of study), c) patients that show both skin symptoms and had a previous acute attack and d) asymptomatic patients. Blood samples were collected in tubes containing EDTA. Half of each blood sample collected was sent, together with urine and stool samples, to a chemical pathology laboratory to be analyzed for porphyrin and porphyrin precursors. The control group consisted of 50 healthy unrelated Afrikaner individuals. Informed consent was obtained from all participants under the guidance of the Ethics Committee of the Faculty of Health Sciences, University of Stellenbosch.

2.2 Molecular Methods

2.2.1 Genomic DNA extraction from whole blood

Genomic DNA was extracted from whole venous blood using a standard protocol adapted from Miller *et al.* (1988).

The blood was received in a 5ml EDTA (vacutainer) tube. The whole blood was mixed with cold lysis buffer (0.155 M NH_4Cl; 0.01 M $KHCO_3$; 0.0001 M EDTA; pH 7.4) and left on ice for 15 minutes. The blood and lysis buffer mixture was centrifuged for 10 minutes at 1500 g and 4°C. The supernatant was discarded and the pellet mixed with PBS (phosphate buffered saline) (0.027 M KCl; 0.137

M NaCl; 0.008 M Na_2HPO_4; 0.0015 M KH_2PO_4). After the solution was centrifuged for 10 minutes at 1500 g and 4°C, the pellet was re-suspended in nuclei lysis buffer (0.01 M Tris-HCl; 0.4 M NaCl; 0.002 M EDTA; pH 8.2), 10 mg/ml protease K and 20%(w/v) SDS. After an overnight incubation period at 37°C, 6 M $NaCl_2$ was added and the tube shaken vigorously for one minute. The mixture was centrifuged for 15 minutes at 2500 g and 21°C and the supernatant transferred to a clean tube, while leaving the pellet and foam behind. The supernatant was again centrifuged for 15 minutes at 2500 g and 21°C. In a fresh tube the gDNA in the supernatant was precipitated with two volumes of ice cold 100%(v/v) ethanol. The gDNA was subsequently removed with a plastic needle and placed in a clean 1.5 ml Eppendorf tube containing 70%(v/v) ethanol. To wash the pellet, the Eppendorf tube was centrifuged at 14000g for 5 minutes at 4°C. Afterwards the ethanol was removed and the pellet left to dry at room temperature. The pellet was then dissolved in 500 µl of ddH_2O.

The concentration of the DNA was determined by loading an aliquot of the DNA on a 1%(w/v) agarose gel in the presence of a low mass DNA ladder (Gibco). 1XTBE (5 X TBE: 89 mM Tris; 89 mM borate; 2 mM EDTA; pH 8.3) was used as the running buffer. The gel underwent electrophoresis for an hour at 100V. The fragments were visualized and captured using the Biorad 1000 Geldoc system (Bio-Rad Laboratories) and the Scion Image software after staining with ethidium bromide (EtBr) (1µg/ml).

2.2.2 *Styl* enzyme digestion

Most of the patients were identified from a database and were already characterized as heterozygous for the founder R59W mutation. New patients were molecularly characterized by using the PCR-RFLP protocol first decribed by Warnich *et al.* (1996) incorporating the restriction enzyme *Sty I* (Promega).

A 234 bp fragment encompassing exon 3 of the PPOX gene was amplified by PCR in a total volume of 25 µl. The reaction mixture contained 30 ng template DNA, 15 pmol of each primer, 200 µM of each dNTP, 2.5 µl 10X PCR amplification buffer and 2 mM $MgCl_2$. Half a unit (0.1 µl) of BIOTAQ DNA polymerase (Bioline) was added to each reaction. The forward primer 5'-TGCAGTGTCTCTCCCTCTTG-3' and the reverse primer 5'-

GCTCCCCTAAACTCTATTCC-3' were synthesized by Whitehead scientific. The PCR amplification reactions were performed in a Perkin Elmer GeneAmp 9700 thermal cycler. The amplification profile involved 60 s at 95°C, followed by 10 cycles at 95°C for 30 s, 65°C for 45 s, 72°C for 30 s, and 30 cycles at 95°C for 30 s, 60°C for 45 s, 72°C for 30 s, with a final extention step at 72°C for 8 min.

The restriction digestion reaction consisted of 20 µl of each PCR reaction, 2.3 µl of reaction buffer and 2µl *Sty I* (10 U/µl) (Promega) enzyme. Digestion was done overnight at 37°C according to the specifications of the manufacturer.

The restricted fragments were fractionated at 200 V on a 12%(w/v) (49:1; acrylamide: bis-acrylamide) Mighty Small (Hoefer) polyacrylamide gel for one hour in the presence of a 100 bp DNA ladder (Promega) in 1.5XTBE (5X TBE Buffer: 89 mM Tris; 89mM borate; 2mM EDTA; pH 8.3). Visualization took place using the Biorad 1000 Geldoc system (Bio-Rad Laboratories) after staining with EtBr (1µg/ml).

2.2.3 PCR amplification of candidate genes

Each of the oligonucleotide primer pairs was designed to span not only the exon of interest, but the flanking intron sequences as well to allow for the inclusion of the splice junction.

Primer sets used for the amplification of the promoter and coding regions of the *ALAS-1*, *ALAS-2* and *PBGD* genes are listed in Tables 2.1, 2.2 and 2.3 respectively. The primer sequences for the *PBGD* gene were obtained from the publication by Lundin and Anvret. (1997). Primers for *ALAS-1* and *ALAS-2* were designed using the Primer Design V1.01 program. Three overlapping sets of primers had to be designed for the promoter regions of both the *ALAS-1* and the *ALAS-2* genes because of the size of these regions.

The PCR conditions were individually optimized for each exon or promoter region and are listed in Tables 2.1, 2.2 and 2.3. The reaction mixture consisted of 15 pmol of each primer, 200 µM of each dNTP, 2.5 µl 10X PCR amplification buffer and 1-2 mM $MgCl_2$. One µl of genomic DNA at a concentration of 30ng/µl was used as the template. Half a unit (0.1 µl) of BIOTAQ DNA polymerase

(Bioline) was added to each reaction. The final volume of the PCR reaction was 25 µl and the PCR amplification reactions were performed in a Perkin Elmer GeneAmp 9700.

The three different PCR cycle programs implemented in the amplification of the different regions were the following: The cycling conditions of program A consisted of denaturation for 7 minutes at 94°C followed by 35 cycles of 94°C for 1 minute, 58°C for 30 seconds and 72°C for 1 minute, and the final step of 72°C for 10 minutes (Lundin *et al.*, 1995). The temperature profile for program B commenced with 5 minutes at 94°C, followed by 30 cycles at 94°C for 30 seconds, 53°C-64°C for 30 seconds (depending on the T_m of the primers), 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The amplification profile for Program C was 5 minutes at 94°C, followed by 10 cycles at 94°C for 30 seconds, 53°C-65°C for 30 seconds (depending on the T_m of the primers), 72°C for 1 minute, and 30 cycles at 94°C for 30 seconds, 50°C-60°C for 30 seconds (depending on the T_m of the primers), 72°C for 1 minute, with a final extension at 72°C for 5 minutes.

The amplified fragments were resolved by electrophoresis on a 1% agarose gel in the presence of a 100 bp DNA ladder (Promega). Only single fragment products, without any background, of the required size were subsequently used for variation screening techniques.

2.2.4 Sequence variation detection methods

2.2.4.1 SSCP/HD protocol

A) The Conventional SSCP protocol:

Four µl of the amplified DNA fragments was denatured at 98°C for 5 minutes in the presence of 3 µl loading dye (for 1 ml, mix 950 µl Formamide, 1 µl 10N NaOH, 40 µl 0.5 EDTA, 2.5 µl 10%(w/v) Bromophenol blue and 2.5 µl 20%(w/v) Xylene cyanol)(Pan and Peng, 1997). The samples were immediately quenched on ice and loaded on a 20%(w/v) poly-acrylamide gel. The ratio of the acrylamide to

Table 2.1: Primer sets and conditions for the amplification of the promoter region as well as the exonic regions of the human ALAS-1 gene from genomic DNA

Fragment	PCR product size (bp)	Annealing Temperature (°C)	$MgCl_2$ (mM)	Program	Forward Primer	Reverse Primer
Promoter area 1	324	N/A	N/A	N/A	5'-GAGACCTCGTCCGCCCAC -3'	5'-CACCTGCGCTGAGGACTG -3'
Promoter area 2	272	N/A	N/A	N/A	5'-TCCATGTCCAAGACACCCAG -3'	5'-GGCAGAGAGGCCCACTTC -3'
Promoter area 3	403	N/A	N/A	N/A	5'-AGCAAGGGGCTTCAGACAAC -3'	5'-AGTTGGGGGCTACAGGGTTG -3'
Exon 1	270	55	1	B	5'-CGAGCGTTTCGTTTGGACT -3'	5'-TAAACGCTGACCTCAAGG-3'
Exon 2	305	55/50	1	C	5'-TGCCTGATTATTAACAACTGTTG-3'	5'-CCCATACCTGCTCCTTCATTG-3'
Exon 3	307	55/50	1	C	5'-AACCAGATCTGATGCTTCAC-3'	5'-CCCTTCACTACCACTAATGG-3'
Exon 4	365	65/60	2	C	5'-GTGAACTAGGCCATAAGC-3'	5'-GGATTACAGGTATGAGCTAC-3'
Exon 5	294	55/50	1	C	5'-TCACAACCATCATTCTGTACTG-3'	5'-CAGTAATATTTGAAAGCCAAGG-3'
Exon 6	287	57/55	1	C	5'-GGGGAAAAAATTCACATGGTG-3'	5'-AAACTCGAAGGCACTCATGC-3'
Exon 7	268	53	1	B	5'-TTTGTCTTCGATGCACATGG-3'	5'-TTTTTCATCTTAGGTTTCAGAGG-3'
Exon 8	298	57/55	1	C	5'-GCTGTCTCCTCACCTGATCC-3'	5'-GCGGCAAAAGCTCTTCAGAG-3'
Exon 9a	222	57/55	1	C	5'-GGCTTCATCTTCACCACCTC-3'	5'-ACGGCAAGTCCAATCAGAGA-3'
Exon 9b	229	58/55	1	C	5'-GGCTCCACAACACCTTGCTG-3'	5'-GCTCTTCAGGATCCGCACAG-3'
Exon10	234	55/50	1	C	5'-GTGACCTTACCTTCTGCTCTC-3'	5'-AACTCCTCAGTGAGGCACCA-3'
Exon 11a	228	53/50	1	C	5'-CAAGTTGGTATCTGCTCAGGC-3'	5'-TAGCCACTGTGTCTAGCCAATG -3'
Exon 11b	283	53/50	1	C	5'-ATGCTGCTATGAACATTTGTGTA-3'	5'-TGAAATAATTGAGGTCATGCTC -3'

Table 2.2: Primer sets and conditions for the amplification of the promoter region as well as the exonic regions of the human ALAS-2 gene from genomic DNA

Fragment	PCR product size (bp)	Annealing Temperature (°C)	$MgCl_2$ (mM)	Program	Forward Primer	Reverse Primer
Promoter area 1	316	53	1.5	B	5'-AGGAAGAGAAGAGTGTGGGTTC -3'	5'-AGATCCCCGGTTGTTGTAAC-3'
Promoter area 2	304	53	1.5	B	5'-AAAGCACTTAGTGAAGGGTCTG-3'	5'-AGAGCTGGGATACCTTGAGC-3'
Promoter area 3	371	53	1.5	B	5'-CCAACTTGTGCCACAGTC-3'	5'-GAGGACGAACGAATGACAGG-3'
Exon 1	221	56	1	B	5'-CTAGCAAGGAAGGGACTGAG-3'	5'-GTACAGCCCTGAGAGACCAG-3'
Exon 2	322	56	2	B	5'-GAAGCTGAAGACGAGGATCA-3'	5'-CCCAGGACCCTAACATTCTC-3'
Exon 3	322	56	2	B	5'-GGGACCAGACTCATCTATCT-3'	5'-GTGACGTAGTGTGTTCAAGC-3'
Exon 4	287	56	2	B	5'-GTAGAAGCTGCCCTTACCTT-3'	5'-CAGAATGCCTTCCCTATTCC-3'
Exon 5	306	54	2	B	5'-GCCAGGGAGAGACTTAAA-3'	5'-CACTGCTGATGGCTGAAA-3'
Exon 6	286	59	2	B	5'-CGATGCCTGACAAGAGTTTC-3'	5'-TGTATTGCAGGATACCAGCC-3'
Exon 7	281	54	1	B	5'-AGGTTGAAGTGGGAGTAC-3'	5'-TTGTGAGACCAACACTAG-3'
Exon 8	281	56	1	B	5'-CATTGGAGATGGAGCTGG-3'	5'-TTGTAAGGGCCTCCTCTC-3'
Exon 9	355	56	1	B	5'-GGGACTGATTATGGGATCTG-3'	5'-AATAGGTGGAGAGGGCAATG-3'
Exon 10	268	55	1	B	5'-CAGAGTGGCAGGTACTGATT-3'	5'-GCTTCTCTTTCAGATCCTGG-3'
Exon 11	413	55	2	B	5'-GGCTCATCTGTACTGTGACA-3'	5'-GGAGTCAGAATGCACTTGTC-3'

Table 2.3: Primer sets and conditions for the amplification of the promoter region as well as the exonic regions of the human PBGD gene from genomic DNA

Fragment	PCR product size (bp)	Annealing temperature (°C)	$MgCl_2$ (mM)	Program	Forward Primer	Reverse Primer
Promoter area 1	661	64	1.5	B	5'-CAACATAGTGAGGCCACCTCCCCGC-3'	5'-ACGACTGAGGATGGCAACCT-3'
Exon 1*	241	58	1.5	A	5'-GTCAGACTGTAGGACGACCT-3'	5'-ACGACTGAGGATGGCAACCT-3'
Exon 3	260	55	1.5	B	5'-TCCTTTCTTCCAAGCCAGTGA -3'	5'-CCACCCCATCTCCTTCATAC-3'
Exon 4	191	58	1.5	A	5'-AAAGAGTCTGAGCCGTGGCT -3'	5'-TGTTCTCTCCTCTCGGGGTA -3'
Exon 5	272	58	1.5	A	5'-CCATCATGAATCGTAGCACAG-3'	5'-TCATTCTTCTCCAGGGCATG -3'
Exon 6	229	57	1.5	B	5'-CCAGTTCTTGTCCCCACTCT -3'	5'-TAAGCCCAGCAGCCTATCTG -3'
Exon 7	286	55	1.5	B	5'-AGGCTCCACCACTGAAGTAG -3'	5'-CTCAGGGCAGAGGAGATAAC -3'
Exon 8	215	58	1.5	A	5'-CCAGTGAGTTGGCCAATCGAGA -3'	5'-CCTGCATCTTCTGGGCACAT-3'
Exon 9	248	58	1.5	A	5'-TGCCCAGAAGATGCAGGGAT-3'	5'-CAGAGCCCTCTAGACCTTGT -3'
Exon 10	308	58	1.5	A	5'-GGGAAAGACAGACTCAGGCA -3'	5'-TGAACGTATATCGCTTTCACAC -3'
Exon 11	213	64	1.5	B	5'-GAACTCCCATCTCACTGCCA -3'	5'-TTCTGGGGAAGCAGATGCAG -3'
Exon 12	254	58	1.5	A	5'-GTCCCATGCTTTCGGCCATT -3'	5'-ATCTTCCCTGCCACCTTTCC-3'
Exon 13	223	57	1.5	B	5'-CAGTGATGTCCTCAGGTCTG -3'	5'-GTCTGAGAGGGGACTACCTA -3'
Exon 14	233	58	1.5	A	5'-CTTCCTGTGCAATCCCAGGT -3'	5'-GGAGGTGGGATTTGGTGAGA -3'
Exon 15a	419	55/52	1.5	C	5'-TGCAGGCTACCATCCATGTC-3'	5'-AGGCTTGAACCCTGCAGTTC -3'
Exon 15b	328	55/52	1.5	C	5'-TGCTGTCCAGTGCCTACATC -3'	5'-GAACTCTGGGCAAAAGTCCC-3'

*Reasons for not including Exon 2 are given in Chapter 1, (1.4.2.2)

the bis-acrylamide was 99:1 and the dimensions of the Mighty Small (Hoefer) gel used were 100 mm [w] x 105 mm [h] x 0.75 mm [d]. The mixture for one gel of 10ml is: 5 ml of 40%(w/v) acrylamide-bisacrylamide stock, 3 ml of 5XTBE, 2 ml of H2O, 25 µl of 25%(w/v) Ammonium persulphate (APS) and 10 µl of N,N,N',N'-tetrametylethylenediamine (TEMED). Electrophoresis was performed for 4 hours at 200 V and 15°C. The running buffer used was 1.5XTBE (89 mM Tris; 89 mM Borate; 2 mM EDTA; pH 8.3). Silver staining (See 2.2.5) was implemented to visualize the SSCP patterns.

B) The Two buffer SSCP/HD Protocol:

This protocol was obtained and adapted from the work done by Liechti-Gallati *et al.* (1999).

Eight µl of the amplification product was mixed with 2 µl of loading dye (See 2.2.4.1. A) and denatured for five minutes at 98°C. The samples were subsequently cooled on ice. A 12%(w/v) non-denaturing polyacrylamide gel with a ratio of 99:1 acrylamide to piperazine diacrylamide, PDA (Bio-Rad Laboratries) was prepared. The mixture for one gel of 40 ml is: 12.5 ml of 40%(w/v) acrylamide-PDA solution, 20 ml Tris-formate buffer (0.75 M, pH 9.0), 7 ml 41%(v/v) glycerol, 565 µl 10%(w/v) APS and 56µl TEMED.

Electrophoresis was performed using a Dual slab Model #DSG-400 (C.B.S. Scientific Co.) unit at 350 V for 2 hours and 15°C. The dimensions of the gel apparatus used are 140 mm [w] x 360 mm [h] x 0.75 mm [d]. The running buffer was 1X Tris-formate buffer (0.75M, pH 9.0) and the trailing ion was Tris-borate buffer (pH 9.0). EtBr staining (1µg/ml) for 12 minutes was followed by UV light exposure using the Biorad 1000 Geldoc System (Bio-Rad Laboratories). The SSCP and HD images were captured using the Scion Image software.

2.2.4.2 CSGE protocol

Fifteen µl of each PCR product was denatured at 98°C for 8 minutes and subsequently incubated at 68°C for one hour to generate heteroduplexes. After incubation the sample was mixed with 2 µl of loading dye (30%(v/v) glycerol, 0.25%(w/v) bromophenol blue, 0.25%(w/v) xylene cyanol) and kept on ice until

loading. A standard 395 mm [w] x 335 mm [h] x 0.75 mm [d] DNA sequencing gel apparatus (Model S2001, Life Technologies) was used in combination with a 40 well square tooth comb. A gel was prepared with 15%(w/v) polyacrylamide (ratio of 99:1 acrylamide to 1,4-bis [acryloyl]piperazine (Fluka)), 10%(v/v) ethylene glycol, 15%(v/v) formamide, 0.1%(v/v) APS and 0.07%(v/v) TEMED with 0.5 X GT buffer (20% stock (250ml), combine 54 g Tris, 18 g Taurine, 1 g Na_2EDTA[pH9.0]). The electrophoresis buffer was also 0.5 X GT buffer. The mixure for one gel of 120 ml is: 45 ml of 40%(w/v) polyacrylamide and 1,4-bis [acryloyl]piperazine solution, 3 ml of 0.5 X GT buffer, 18 ml of formamide,12 ml of ethylene glycol, 40.8 ml of dH_2O, 82.8 µl of TEMED and 1.2 ml of APS. The gel was pre-run at 40W for 15 minutes before loading and the samples resolved at 40W for 6 to 7 hours at room temperature. After electrophoresis the gel was stained with EtBr (1µg/ml) for 12 minutes and visualized and captured using the Biorad 1000 Geldoc System (Bio-Rad Laboratories) and Scion Image software.

2.2.5 Silver Staining

The gel was placed in a fixing solution (10% (v/v) ethanol, 0.5% (v/v) acetic acid) for 10 minutes and afterwards rinsed in dH_2O for approximately 1 minute. This was followed by staining in 0.1% $AgNO_3$ for 10 minutes. A 5 second rinse with dH_2O was performed before developing the gel in a developing solution (1.5% (w/v) NaOH, 0.155% (v/v) Formaldehyde) for about 10 minutes or until clear bands were seen.

2.2.6 DNA sequencing

DNA fragments showing an abnormal SSCP or CSGE pattern were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer, and directly sequenced using the PCR amplification primers and the ABI Prism Dye Terminator Ready Reaction cycle sequencing kit. An ABI Prism 3100 genetic analyzer (Applied Biosystems) was used. All the samples were sequenced in bi-directionally and analyzed using the Sequence analysis 3.7 software (Applied Biosystems).

2.2.7 Restriction enzyme analysis of mutations

Restriction enzyme digestion was used to detect four of the six sequence variations identified.

The *ALAS-1* exon 4 fragment (See primers Table 2.1), 365 bp in length, was digested with the enzyme *Hsp92 II* to identify the different alleles of the polymorphism. The total volume of the reaction was 20 µl and contained 0.2 µl acetylated BSA (10 µg/µl), 2 µl RE 10x Buffer, 10 µl PCR product and 1 µl of enzyme (Promega) (10 U/µl). The final enzyme concentration in the total reaction was thus 0.5 U/µl and the reaction was incubated for 4 hours at 37°C to digest.

The alleles of the polymorphism in exon 1 of the *PBGD* gene could be identified through the restriction of the exon 1 fragment (See primers Table 2.3) with *Mnl I*. The total volume of the digestion reaction was 20 µl and was prepared with 2 µl 10x Universal buffer, 10 µl of PCR product and 0.5 µl of *Mnl I* enzyme (Stratagene) (2U/µl). A final concentration of 0.05U/µl enzyme was thus used in an overnight digestion at 37°C.

In Intron 3 of the *PBGD* gene a polymorphism creates a site for the enzyme *BsmA I*. The exon 4 region (See primers Table 2.3) of this gene which contained this polymorphism, was subsequenctly amplified. The total restriction enzyme reaction was 20 µl and contained 2 µl 10X NE buffer, 10 µl PCR product and 2 µl (5 U/µl) enzyme (New England Biolabs). The final enzyme concentration was 0.5U/µl. The digestion reaction was incubated at 55°C for 4 hours.

A polymorphism found in Intron 10 of the *PBGD* gene deletes a *Hinf I* restriction site. The primers of the exon 11 fragment of the PBGD gene were implemented to amplify the region containing the polymorphism (See Table 2.3). The total volume of the reaction was 20 µl and contained 1 µl (10 U/µl) *Hinf I* enzyme (Roche diagnostics), 2 µl 10x Buffer and 10 µl PCR product. The final enzyme concentration was 0.5U/µl. Digestion took place for 4 hours at 37°C.

All the different fragments of the restriction enzyme digestion reactions were fractionated on a 1.5% agarose gel in 1xTBE (5X TBE Buffer: 89 mM Tris; 89mM borate; 2mM EDTA; pH 8.3) in the presence of a 100 bp DNA ladder (Promega). Afterwards it was stained with EtBr (1μl/ml) and visualized and captured using the Biorad 1000 Geldoc System (Bio-Rad Laboratories) and Scion Image software.

2.3 Nucleotide numbering

In the interest of consistency in nomenclature and numbering, nucleotides are numbered according to the recommendations of Den Dunnen and Antonarakis (2001). The nucleotides of all three genes are numbered according to the cDNA sequence derived from the genomic sequence taking the A of the ATG initiation codon as +1.

The *PPOX* genomic sequence GI 1524086 (Puy *et al.*, 1996) was used for all references made to the *PPOX* gene. The *ALAS-1* genomic sequence was still unpublished at the time of the study and a PAC contig GI 4309923 which was found to contain the *ALAS-1* sequence was used as reference. The *ALAS-2* genomic sequence GI 3220248 (Surinya *et al.*, 1998) was used for all references made to the *ALAS-2* gene and the *PBGD* genomic sequence GI 292384 (Yoo *et al.*, 1993) was used for all references made to the *PBGD* gene.

2.4 Statistical analysis

Allele and genotype frequencies were calculated. The results were statistically analysed by method of standard contingency tables and χ^2 analysis. Allelic and genotypic associations were tested between the alleles/genotypes of the identified sequence variations and the four clinical groups found in VP individuals and a control group. The statistical significance for genotype/phenotype association was taken as $p < 0.05$.

2.5 Assessment of sequence homology

Database searches for homology were performed for the *ALAS-1, ALAS-2. PBGD* and *PPOX* genes using the BLAST website (http://www.ncbi.nlm.nih.gov/ BLAST/; Altschul *et al.*, 1990), as well as the Homologene website (http://www.ncbi.nlm.nih.gov/Homologene/).

BLAST 2 sequences (Tatusova and Madden, 1999) were utilised to compare the exon 4 region (nt-4621 to nt-4771) of the human *ALAS-1* gene (GI 4309923) with the corresponding *ALAS-1* gene sequences of the rat (GI 13324697) and the chicken (GI 63040). The same comparison using BLAST 2 sequences was also performed for the exon 10 region (nt-6372 to nt-6498) of the human *PBGD* gene (GI 292384), comparing it to the rat *PBGD* sequence (GI 6981023) and the mouse sequence (GI 200229).

Chapter Three

3 Results and Discussion

Three genes *ALAS-1, ALAS-2* and *PBGD* were selected as possible candidate modifier genes for the clinical expression of VP on the basis of the regulatory influence they have on the haem pathway. The potential modifier genes were screened for sequence variation by means of three methods. The three screening methods CSGE, conventional SSCP and two buffer SSCP were compared to establish the method with the highest sequence variation detection rate and reproducibility. Two of the six sequence variant sites detected were analysed in the four extended clinicallly evaluated R59W VP patient groups, as well as a control group. The results were statistically analysed to determine whether any association exists between the different alleles of the polymorphic loci and the four clinical groups.

3.1 Patients

At the planning stage of this study it became apparent that a well-defined group of VP patients was crucial and quite a number of problems hindered this characterisation process. The fact that some people had drugs administered while others never came in contact with these medicines was an environmental influence to consider. Some VP patients took precautions to minimize skin ailments earlier than others, while some never applied any precaution to minimize sun exposure/ skin damage. The clinical expression of VP in patients also varies with age as well as with the general clinical condition of the individual. People demonstrate different symptoms while experiencing an acute attack and in certain instances it was difficult to recognize if the individual indeed had had a porphyric attack. With all these potential problems in mind, a dermatologist and a neurologist each established protocols to characterize VP patients.

The neurological examinations assessed the effects of VP on the central and peripheral nervous system. The methods used included a) a standardized clinico-neurological evaluation involving assessment of activities of daily living (ADL) utilizing Barthels index, b) nerve conduction studies of the upper and lower limbs

(EMG) and c) autonomic function assessment. None of the 25 R59W patients fulfilled the minimum criteria for the presence of a polyneuropathy, although 6 patients had a history suggestive of at least one acute visceral crisis, leading to hospitalization in 4 cases (See Table 3.1).

Skin signs of VP include light and dark pathes, blisters, purple discoloration due to bleeding, fragility, scarring and increased hair growth. At least three different skin colours may be present in a single patient, especially in the advanced disease. These are: whitish, shades of brown and purple. Individuals were assessed by means of a medical examination during which the history of the patient was also taken. A patient was concluded as having skin symptoms when he/she showed positive results for any of the above-mentioned symptoms. The degree of the skin symptom was also noted. Only one of the patients was diagnosed with blisters while the other patients diagnosed with skin symptoms showed signs of scarring and discoloration (See Table 3.1).

The 25 patients included in this study could not be characterized by means of their biochemical results as the relevant laboratory has to date not completed the testing of a normal control group to compare their results with. Without this comparison it would be premature to decide what range of values would be taken as a positive diagnosis for VP.

The distribution of clinical symptoms were in the same range when comparing our clinical groups to previous studies performed by Eales *et al.* (1980) and Whatley *et al.* (1999a) (See Table 3.1). This confirms the previous results found by Whatley *et al.* (1999a) that the distrubution of clinical symptoms in the Western European population is the same as the distrubution found in the South African population. Although our R59W cohort is smaller than to the South African VP population studied by Eales *et al.* (1980) the percentages of the clinical groups are very similar. The only significant difference is found in the acute attack group, which may indicate that VP patients are now less exposed to factors that could trigger an acute attack than in 1980. These factors include the diagnosis at an early age with the availability of genetic testing and the avoidance of precipitating factors as the patients and medical docters are more informed about VP.

Table 3.1: The comparison of results obtained from three different studies indicating the distrubution of the clinical symptoms associated with VP

Symptom groups	Result obtained during this study.		Results from study conducted by Eales et al. (1980).		Results from study conducted by Whatley et al. (1999a)	
	Number	%	Number	%	Number	%
Asymptomatic	8	32%	N/A	N/A	N/A	N/A
Skin symptoms	11	44%	156	52%	61	59%
Acute Attack	2	8%	51	17%	20	20%
Both skin symptoms and acute attack	4	16%	62	21%	22	21%
Biochemical	N/A	N/A	31	10%	N/A	N/A
Total	**25**	**100%**	**300**	**100%**	**103**	**100%**

3.2 Genomic DNA extraction

Genomic DNA extractions were performed within 24 hours after collection of the blood samples. Samples of all DNA extracted underwent electrophoresis on a 1% agarose gel in the presence of a low mass DNA ladder to determine the concentration and to test for intactness of the samples. Concentrations of between 30 ng/μl and 80 ng/μl were obtained. The original undiluted DNA samples were stored at –80°C and the dilutions of 30 ng/μl were aliquoted and stored at –20°C.

3.3 *Styl* enzyme digestion

The R59W mutation causes a C to T transition in codon 59 of the *PPOX* gene that creates a second *Sty I* enzyme-cutting site in the amplified fragment and results in an arginine to tryptophan substitution (Meissner *et al.*, 1996; Warnich *et al.*, 1996).

Digestion of the amplified 234 bp fragment in normal individuals results in two fragments of 138 bp and 96 bp. In the case of heterozygous R59W VP individuals digestion with the *Sty I* enzyme results in three fragments of 138 bp, 112 bp, 96 bp respectively (See Figure 3.1). This is a rapid screening test, which has proved to be very useful in the identification and genetic counseling of VP patients in South Africa where the R59W mutation is frequent due to a founder effect (Meissner *et al.*, 1996; Warnich *et al.*, 1996; UCT website: http://www.uct.ac.za/dept/liver/index%20porphyria.htm).

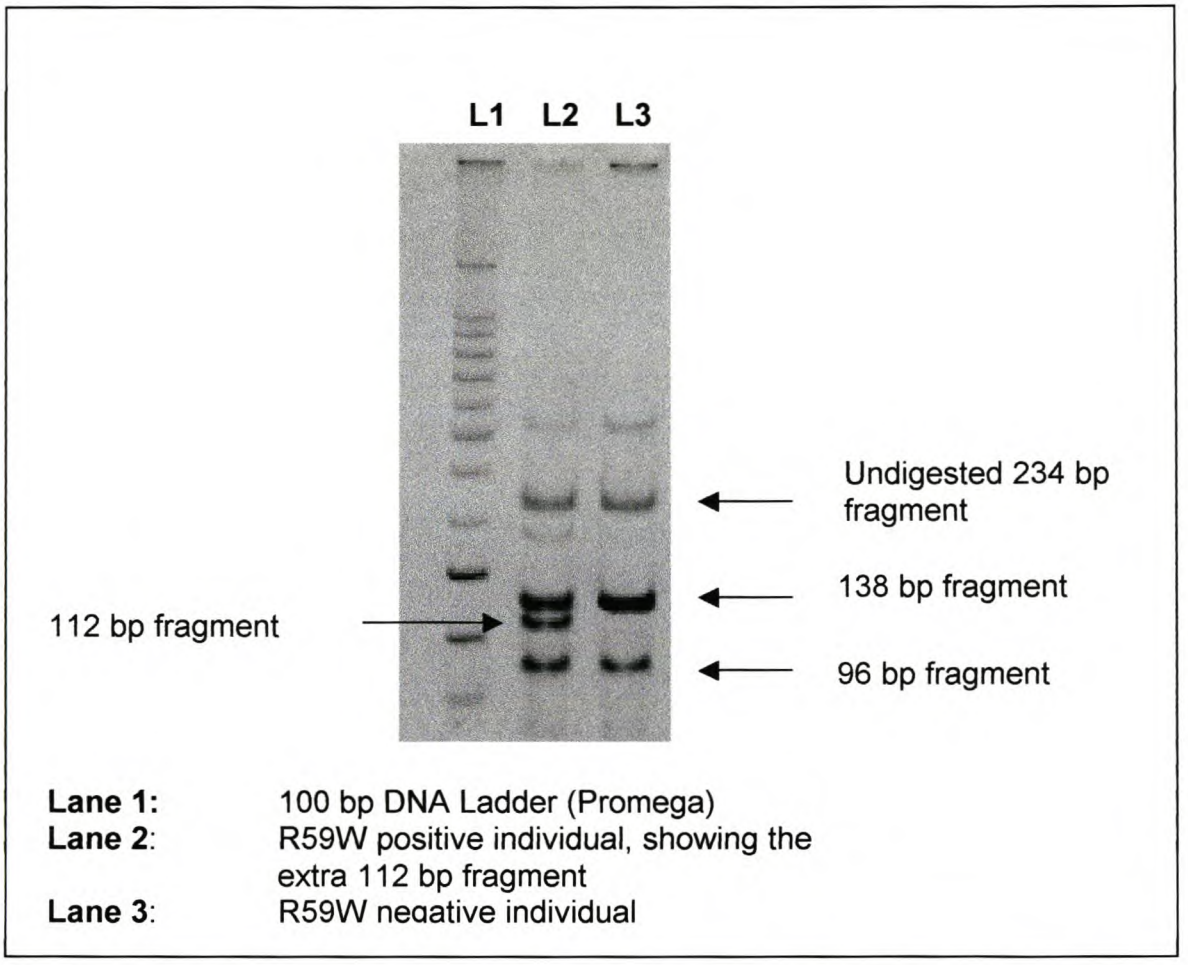
3.4 PCR amplification of candidate genes

Primer sets and conditions for the amplification of the *PBGD* gene were avaiable (Lundin and Anvret, 1997), but primers had to be designed and optimized for the amplification of the exons and promoter areas of the *ALAS-1* and *ALAS-2* genes. These primers were all designed to include the exon/intron boundaries. The amplified regions ranged in size from 191 bp to 661 bp (See Tables 2.1, 2.2, 2.3). The promoter region of the *PBGD* gene was the largest fragment (661 bp) and a SNP in the 5' untranslated area of the gene could be detected with the CSGE method but not with the two SSCP methods. The same SNP was also not detected with both the SSCP methods in a 241 bp fragment (See Table 3.2). The majority of the fragments were smaller than 350 bp which is acknowledged to be optimal for SSCP and CSGE analysis (See Tables 2.1, 2.2, 2.3). The optimal length of fragments for CSGE analysis has been determined to be between 250 and 500 bp (Ganguly, 2002) and the optimal fragment length for SSCP analysis is smaller than 350 bp (Hayashi and Yandell, 1993).

The only primers found to be problematic were those which encompassed the promoter area of the *ALAS-1* gene. The three sets of primers could never be optimized to produce single amplicons. The primers were unsuccessfully attempted in different combinations, including combinations with the primers of exon 1. The genomic sequence for the *ALAS-1* was obtained from a PAC contig (GI 4309923), as no published genomic *ALAS-1* sequence was available at the time of the study. The genomic sequence is still unpublished. Different primer design programs should be implemented when the sequence for this promoter

area becomes available to attempt to amplification of this region with the minimal formation of primer dimers.

Figure 3.1: The *Sty I* enzyme digestion of the fragment containing exon 3 of the *PPOX* gene to identify all R59W patients



Undigested fragments are seen due to the fact that a high amount of PCR product was used during the digestion procedure.

The amplification reactions incorporating the rest of the primers resulted in products of the expected size. The amplified fragments obtained were of good quality and sufficient quantity to perform further sequence variation detection analysis .

3.5 Mutation detection techniques

Three PCR-based mutation-detection approaches were implemented to identify sequence variation in the *ALAS-1, ALAS-2* and *PBGD* genes. The SSCP (Orita *et al.*, 1989) detection technique was used as well as a modified version of the SSCP/HD technique entitled the two buffer SSCP/HD mutation detection

technique (Liechti-Gallati *et al.*, 1999). The third screening technique, CSGE, is based on the differential migration of homoduplexes and heteroduplexes in mildly denaturing solvents (Ganguly *et al.*, 1993).

A total of six sequence variant sites were identified in the three genes (*ALAS-1, ALAS-2 and PBGD*) investigated (See Table 3.2 and 3.3). In the *ALAS-1* gene a previously described single nucleotide polymorphism 4713 T>C was identified in exon 4. (Bio-chip:http://www.bio.chip.org/biotools/; Locuslink: http://www.ncbi.nlm.nih.gov/SNP/ [Maglott *et al.*, 2000; Pruitt *et al.*, 2000]) (See Table 1.2). The remaining of the five sequence variants were identified in the *PBGD* gene and consisted of four previously described SNPs (-64 C>T; 3581 A>G; 6479 G>T; 7064 C>A) and one novel 8 bp deletion (4582_4589del) (See Table 1.4). No sequence variation could be identified in the *ALAS-2* gene using the three screening techniques.

The 3581 A>G SNP and the 4582_4589del, both in the *PBGD* gene, and the 4713 T>C SNP in the *ALAS-1* gene were the only sequence variation sites that could be detected by the conventional SSCP technique. The two buffer SSCP technique only facilitated the identification of two out of the six variation sites, namely the 3581 A>G SNP and the 4582_4589del in the *PBGD* gene. The CSGE method detected the 4582_4589del as well as all the SNPs in both genes except for the 3581 A>G polymorphism in the *PBGD* gene. The latter variation was thus only detected by the conventional SSCP and the two buffer SSCP techniques. The 4582_4589del deletion was the only sequence variation detected by all three techniques.

The only SNP detected by SSCP analysis but which failed detection by the CSGE technique (3581 A>G in the PBGD gene) was the one located nearest to the 5' end of the PCR fragment (See Table 3.2). This sequence variant was found to be less than 50 bp, i.e. 43 base pairs from the 5' end of the amplified fragment. The other SNPs were found to be between 58 and 193 base pairs from either the 5'or the 3' end. The 4582_4589del was detected with the CSGE technique although it was only 37 bp from the 5' end of the fragment. It thus seems as if the position of a deletion is not as important as the position of a SNP when the CSGE technique is used.

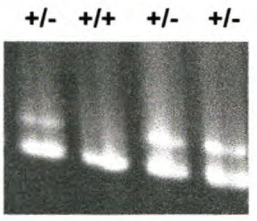
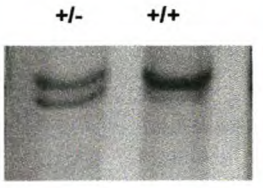
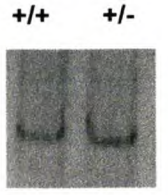
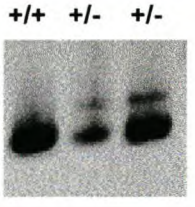
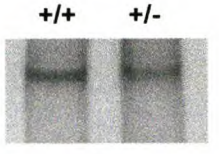
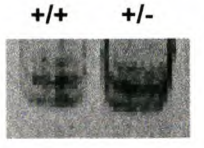
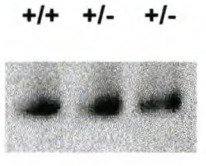
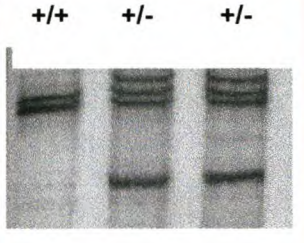
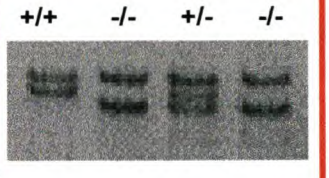
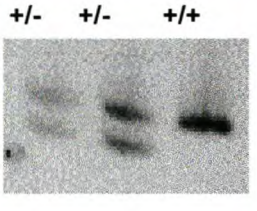
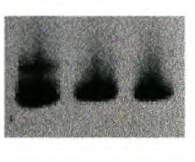
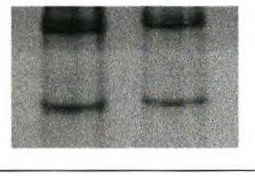
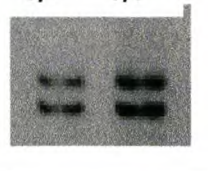
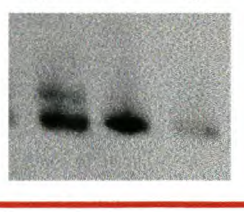
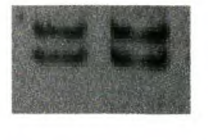
Table 3.2: The information concerning fragments in which sequence variant were identified as well as information concerning the sequence variants

Gene		Type of mutation	Position of mutation	Fragment size	Base pairs from nearest 3' or 5' end
ALAS-1	**4713 T>C**	Transition	Exon 4	367 bp	193 bp from 5' end
PBGD	**–64 C>T**	Transition	Exon 1	241 bp	59 bp from 5' end
PBGD	**3581 A>G**	Transition	Intron 3	214 bp	43 bp from 5' end
PBGD	**4582_4589 del**	Deletion	Intron 6	286 bp	37 bp from 5' end
PBGD	**6479 G>T**	Transversion	Exon 10	308 bp	121 bp from 3' end
PBGD	**7064 C>A**	Transversion	Intron 10	213 bp	58 bp from 5' end

Although not many sequence variant sites were detected in this study, results indicated that the position of the sequence variant had an influence on the CSGE detection rate while the length of the fragment and the detection rate could not be correlated with each other using any of the CSGE, SSCP or two buffer SSCP techniques. From evaluating the three screening techniques, it was concluded that the CSGE procedure had the highest detection rate (Five of six sequence variants, 83%) for the three genes studied. The CSGE method facilitated the identification of transitions and transversions in this study.

The CSGE method makes use of routine laboratory equipment and needs no further manipulation of the PCR product. Although 1,4 Bis (acryloyl) piperazine (BAP) is used, which is more expensive than some other cross-linkers, it was shown to increase the gel conductivity and therefore give the gel more physical strength compared to the same percentage acrylamide gels with the various other cross-linkers, such as N'-N'-methylene-bisacrylamide (BIS) (Ganguly, 2002).

Table 3.3: Results obtained with the three mutation detection techniques

	CSGE	SSCP	Two buffer SSCP/HD
ALAS-1 **4713 T>C**			
PBGD **-64 C>T**			
PBGD **3581 A>G**			
PBGD **4582_4589del**			
PBGD **6479 G>T**			
PBGD **7064C>A**			

+ = wild type allele
- = variant type allele
Red squares indicate the screening method that successfully could discriminate between wild type and variant alleles

The degree of the altered migration of heteroduplexes is defined by both the nature of a mutation and the position of the mutation with respect to the molecule investigated (Ganguly *et al.*, 1993). Research has shown that the detection rate of CSGE thus depends on the size of the PCR product as well as the location of the mismatch in the PCR product (Ganguly *et al.*, 1993; Ganguly, 2002). For most CSGE-based screening protocols, the optimal length of the PCR product was found to be between 250 bp to 500 bp (Ganguly, 2002). Mismatches located within 50 bp of the ends of the fragment were shown to have a low detection rate and should be avoided (Ganguly *et al.*, 1993; Ganguly, 2002). It was also shown that mutations in A:T rich sequences are better resolved than those in G:C rich context. The CSGE method was very successful in detecting mutations/polymorphisms in the breast cancer type one (*BRCA 1)* and breast cancer type two (*BRCA2)* genes, which is both very A:T rich (Markoff *et al.*, 1998). Three mutations in the Type III procollagen *(COL3A1)* gene were missed and later found to be located in G:C rich high-melting domains (Ganguly *et al.*, 1993). By adding a short GC rich clamp to the end of PCR products and not observing any rise in the detection rate, it was shown that the melting profile analysis alone can not predict domains within a sequence that could give rise to false negative results (Körkkö *et al.*, 1998; Ganguly, 2002). Results from this study correspond with the results of Markoff *et al.* (1998) who also found that the CSGE had a higher detection rate than the SSCP techniques.

In detecting sequence variants in the genes investigated, the conventional SSCP technique proved to be superior (three of six sequence variants, 50%) to the two buffer SSCP technique (two of six sequence variants, 33%) in our hands. All the SNP identified with both the SSCP methods were classified as transitions and no transversion were identified with the SSCP techniques during this study. The original SSCP method was introduced by Orita *et al.* (1989) and is the most extensively used mutation detection technique to date. The only other mutation detection technique currently competing with SSCP is denaturing high performance chromatography (D-HPLC) (Underhill *et al.*, 1997; Xiao and Oefner, 2001; Glavac *et al.*, 2002). A disadvantage of the SSCP technique is that it lacks the theoretical background for exact secondary structure prediction making the optimisation procedure very difficult.

3.6 *ALAS-1* as a candidate modifier gene

The *ALAS-1* gene was amplified and subjected to intensive investigation by all three sequence variation detection techniques. Only one SNP was detected of a total of 19 SNPs previously described in the *ALAS-1* gene (See Table 1.2). These 19 SNPs were identified using direct sequencing. This information only became available after we completed our variation screening procedures. Investigation of the positions of the described SNPs revealed that only three (3505 C>T, 4713 A>G, 5420 A>G) were indeed found in the areas amplified during this study, concluding that our detection teqniques failed to identify two of three possible SNPs in the *ALAS-1* gene. These SNPs, 3405 C>T and 5420 A>G were both very close to the ends of the amplified fragments, 30 bp from the 3' end and 10 bp from the 5' end, respectively. This could explain why they were not detected. No information was provided on the allele frequencies of the SNPs or the study population.

CSGE screening of the 367 bp fragment containing the *ALAS-1* exon 4 region, followed by automated sequencing of the PCR amplified genomic DNA, confirmed the presence of a sequence variant site in exon 4 (See Figures 3.2, 3.3, 3.4 and Table 3.4). The T>C sequence variant is located at nucleotide position 4713 in exon 4 of the *ALAS-1* gene. This 4713 T>C sequence variant creates a second *Hsp92 II* restriction site site in the fragment which contains the exon 4 and surrounding intron areas. The fragment containing the mutant C allele is cleaved into 3 fragments (196 bp, 135 bp and 36 bp) while the T allele is only cut once into two fragments (331 bp and 36 bp)(See Figure 3.5).

Figure 3.2: Structure of *ALAS-1* gene and the location of the 4713 T>C SNP

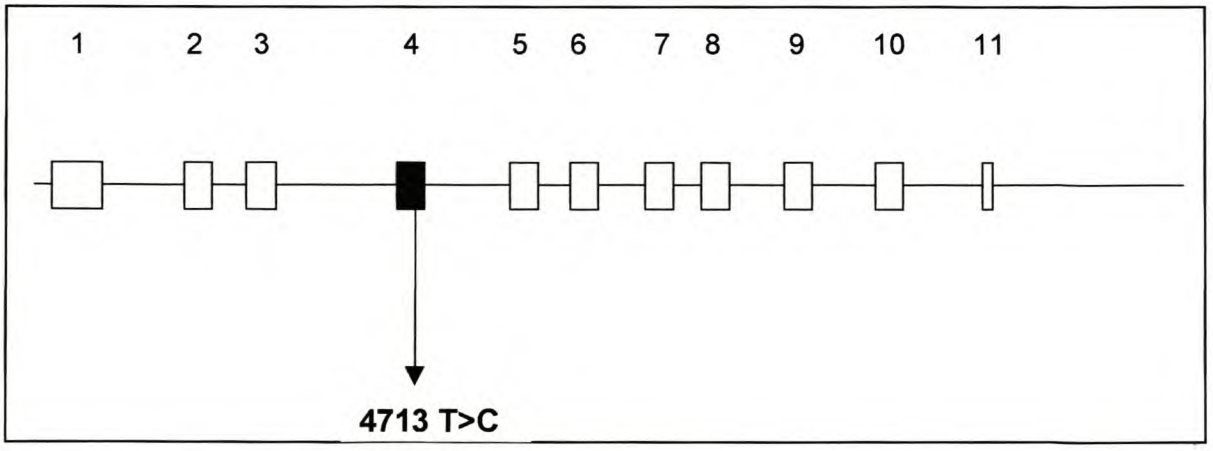
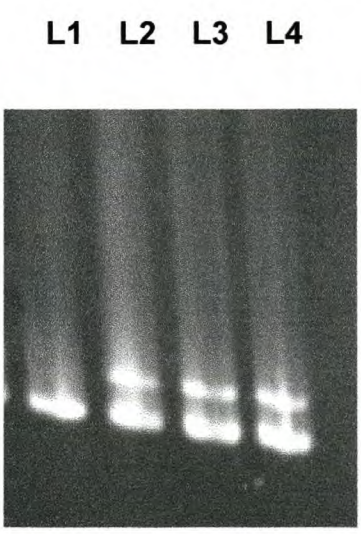


Table 3.4: Partial gDNA sequence of the amplified region containing exon 4 of the *ALAS-1* gene indicating the position of the 4713 T>C SNP

gtgaactagg	ccataagcca	tttttgacaa	tatgtttgga	agatattgtg
aactcagaat	agaaagttgg	tcccatttgt	ttcttgttac	ttttgttcca
gAGGTTGCTG	AAACCTCAGC	AGGCCCCAGT	GTGGTTAGTG	TGAAAACCGA
TGGAGGGGAT	CCCAGTGGAC	TGCTGAAGAA	CTTCCAGGAC	AT**T**ATGCAAA
AGCAAAGACC	AGAAAGAGTG	TCTCATCTTC	TTCAAGATAA	CTTGCCAAAA
Tgtaagtctc	attgttattt	gcctgatgta	gaaaagaatt	tataattcaa
atgtacatta	gattaaatat	aaaattgcat	ggtgaggctg	ggcacagtag
ctcatacctg	ttaatcc			

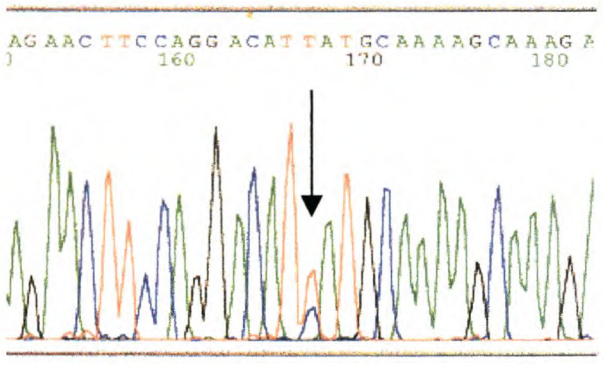
Partial gDNA sequence obtained from PAC contig accession number GI4309923. The exon 4 region is indicated by capital letters while the surrounding intron areas are given in lowercase letters. The sequence of the forward primer and reverse primer are indicated in blue. The site of the 4713 T>C polymorphism is indicated with bold font and underlined.

Figure 3.3: A CSGE gel photo indicating the different migration patterns of the heteroduplexes and homoduplexes of the exon 4 fragment of the *ALAS-1* gene



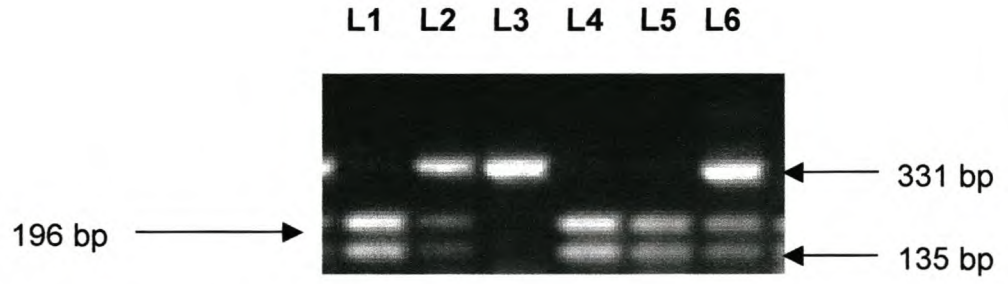
Lane 1: Homozygous individual (TT or CC)
Lane 2 to 4: Heterozygous individuals (TC)

Figure 3.4: Results obtained from the automated sequencing in the sense direction of the *ALAS-1* fragment of a heterozygous individual containing the 4713 T>C polymorphism



The arrow indicates the exact position of the 4713 T>C polymorphism

Figure 3.5: A 1.5% Agarose gel showing the different alleles of the 4713T>C polymorphism in the *ALAS-1* gene after digestion with the restriction enzyme *HSP92 II*



Lanes 1,4,5: CC homozygotes
Lanes 2 and 6: TC heterozygotes
Lane 3: TT homozygote

The 36 bp fragment is not visible on this gel, due to it's increased migration rate.

The allelic frequency for the 4713 T>C sequence variant was found to be T= 0.49; C= 0.51 in 49 VP patients and the frequency found in the normal control group of 50 was T= 0.52; C= 0.48. Statistical analysis was performed for the smaller well characterized group A of 25 as well as for the combined group (A and B)

comprising 49 individuals (See Tables 3.5 to 3.6). The Chi Square value for group A was calculated as 10.136 and the probability p= 0.2556 when comparing the genotypes of the 4713 T>C SNP with the four clinical groups and a control group of 50 individuals. The Chi Square value was calculated to be 5.369 and the probability p=0.717 when comparing the genotypes of the same SNP with the four clinical groups and the control group using the combined group (A and B) (See Tables 3.5 and 3.6). No statistical significant association was demonstrated between the genotypes and any of the VP symptom groups.

Table 3.5: Contingency Table of the 4713 T>C polymorphism data generated for the group A VP patients and the control group

	Observed Genotype			
Group	T/T	T/C	C/C	**Total**
No symptoms	3	2	2	7
Acute Attack	1	0	1	2
Skin lesions	4	5	2	11
Acute attack and Skin lesions	0	4	1	5
Controls	9	34	7	50
Total	17	45	13	75

Chi Square value: 10.136
Probability: 0.256

Table 3.6: Contingency Table of the 4713 T>C polymorphism data generated for the group A and group B VP patients and the control group

	Observed Genotype			
Group	T/T	T/C	C/C	**Total**
No symptoms	4	7	3	14
Acute Attack	1	3	1	5
Skin lesions	5	8	4	17
Acute attack and Skin lesions	1	8	4	13
Controls	9	34	7	50
Total	20	60	19	99

Chi Square value: 5.369
Probability: 0.717

A homology search of the human *ALAS-1* gene (GI 4309923) and *Rattus norvergicus* aminolevulinic acid synthase 1 mRNA (GI 13324697) was conducted using the BLAST 2 sequences program (http://www.ncbi.nlm.nih.gov/blast; Tatusova and Madden, 1999)(See Figure 3.6). The same homology search was applied to the human *ALAS-1* gene and the chicken gene for mt 5-aminolevulinate synthase exons 1-7 (GI 63040)(See Figure 3.6). The area under investigation, exon 4 nt 4621 to nt 4771 of the human *ALAS-1* gene, revealed significant homology between both the rat and chicken *ALAS-1* genes.

Sequence alignment analysis revealed that the rat *ALAS-1* gene 448 to 599 had an 81% homology with the human *ALAS-1* gene nt 4619 to nt 4770, while the *ALAS-1* gene of the chicken 3057 to 3149 had 86% homology with the human *ALAS-1* gene nt 4684 to nt 4776.

The region encompassing surrounding the exon 4 polymorphism is conserved with the C allele present in the rat sequence and the T allele present in the chicken sequence. The high degree of conservation found between these three species probably points to the biological significance of the *ALAS-1* gene in the haem biosynthetic pathway.

Figure 3.6: Sequence alignment of the human exon 4 region of the *ALAS-1* gene with the rat *ALAS-1* mRNA and the chicken *ALAS-1* mRNA

```
Rattus norvegicus:(448)      cagaggttgctcaaagccagtgctccccagcttggtcaatgcaaaaagggatggagaag (507)
Homo sapiens:     (nt 4619)  cagaggttgctgaaacctcagcaggccccagtgtggttagtgtgaaaaccgatggagggg (nt 678)

Rattus norvegicus:(508)      gtccaagcccactgctgaagaacttccaggacatcatgagaaagcaaaggccagaaagag (567)
Homo sapiens:     (nt 4679)  atccagtggactgctgaagaacttccaggacattatgcaaaagcaaagaccagaaagag (nt 4738)
Chicken:          (3055)         agtggcttgctcaagaagtttaaggatattatgctgaagcaaagaccgaaaagtg (3110)

Rattus norvegicus:(568)      tgtctcatcttcttcaggataacttgccaaa  (599)
Homo sapiens:     (nt 4739)  tgtctcatcttcttcaagataacttgccaaaa  (nt 4770)
  Chicken:        (3111)     tgtctcatctgcttcaggataacttgccaaaa  (3143)
```

Regions of homology with the human *ALAS-1* sequence are indicated in blue and highlighted in grey. The polymorphism identified in the human *ALAS-1* gene at position 4713 is highlighted in purple.

This sequence variant changes codon 173 from AUU to AUC, both encoding the amino acid isoleucine.This nucleotide substitution is thus regarded as a silent mutation because both the wild-type and the mutant alleles encode the same amino acid. However, silent mutations can cause phenotypic variability by influencing the splicing accuracy or efficiency (Cartegni *et al.*, 2002). A silent mutation has been shown to induce post-transcriptional exon skipping in the case of phenylketonuria (PKU [MIM 261600]) where the c1197 A>T substitution results in the skipping of exon 11, leading to a PKU phenotype (Choa *et al.*, 2001). The exon 11 skipping event, induced by the SNP, results in a frame-shift mutation with the replacement of the C-terminal 97 residue segment with 21 missence codons, followed by a premature stop codon in exon 12.

The exon skipping mutation mechanism is usually caused by changes in the consensus sequence at splice sites or the lariat branch point region, but sequences outside these regions may also effect the inclusion or exclusion of exons. The silent mutation C6354T in the Fibrilllin –1 (*FBN-1*) gene found in individuals with Marfan syndrome (MFS) is unlikely to affect known binding sites as it is located 26 bp upstream of the 3' splice site and 6 nucleotides downstream from a purine rich potential exon recognition sequence. The results of different types of expression constructs, however confirmed that the skipping of exon 51 is solely due to this silent mutation (Liu *et al.*, 1997).

To demonstrate the influence of penetrance, or the disease-causing potential of a silent mutation through abnormal splicing, *in vivo* studies need to be carried out by using an exon-trapping expression vector, which falls outside the scope of this study.

3.7 *ALAS-2* as a candidate modifier gene

No sequence variation was identified in the *ALAS*-2 gene in our VP cohort using the three mutation screening techniques. A total of 18 SNP has been described in previous studies through direct sequencing (See Table 1.4). Only one (8163 T>C) of these decribed polymorphims was situated in the region of the *ALAS-2* gene that we amplified and subjected to sequence variation analysis in this study. None of the three sequence variation detection techniques, however, detected this polymorphism. This SNP is located 43 bp from the 5' end of the amplified

fragment that contained exon 7. Once again this SNP will likely not be detected by the CSGE method according to Ganguly *et al.*, (1993). No information is available on the allele frequencies of the SNPs or the study population.

Introns 1, 3 and 8 correspond to Dnase I hypersensitivity sites in the structurally related mouse *ALAS-2* gene (Schoenhaut and Curtis, 1989). Dnase I hypersensitive sites are indicative of nucleosome free regions of DNA associated with transcription regulatory factors (Elgin, 1988; Gross and Garrard, 1988). Surinya *et al.* (1998) investigated the effect these three regions might have on the expression of the gene. Preliminary results indicated that the intron 3 (850 bp) region had an inhibitory effect and both the intron 1 (4.9 kb) and the intron 8 (460 bp) regions had a stimulatory effect on the promoter activity. Intron 8 had the most significant increase in the promoter activity and was thus chosen for further investigation. The erythroid specific enhancer region in intron 8 was found to be localized to a 239 bp region. Results illustrated that a GATA-1 binding site and two CACCC boxes are located within this region. These binding sites are conserved across species and are critical for the erythroid-specific enhancer activity in transfected erythroid cells. To date no sequence variations, have been described in the intron 1 region, but at the end of 2001, a list of polymorphisms was published on the internet websites Bio-chip:http://www.bio.chip.org/biotools/ and LocusLink: http://www.ncbi.nlm.nih.gov/SNP describing SNPs in both the intron 3 (1791 T>G) and the intron 8 (10602 C>A) regions. It will be very interesting to investigate in future studies whether these two polymorphisms are present in our population and whether they are indeed associated with a clinical phenotypic group of VP.

3.8 *PBGD* as a candidate modifier gene

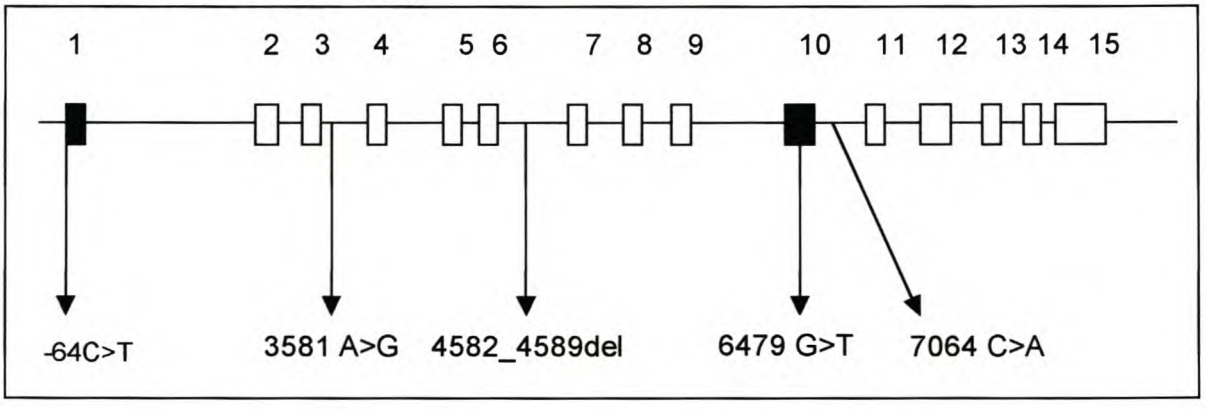
Twelve of a total of 16 described polymorphisms in the *PBGD* gene were located in the areas that were investigated. Of these twelve (-235 A>T, -64 C>T, 3119 G>T, 3167 D>Del, 3581 A>G, 3982 T>C, 6479 G>T, 7052 A>G, 7064 C>A, 7539 C>T, 7998 G>A, 8578 G>A) (See Table 1.4 for detection techniques) only four (-64 C>T, 3581 A>G, 6479 G>T, 7064 C>A) were detected in this study. Seven of the eight SNPs not detected were located closer than 50 bp from either the 5' or 3' end of the amplified fragment. The 3119 G>T SNP was located 1 bp from the 3' end, the 3167 D>Del was 47 bp from the 5' end, the 3982 T>C SNP was 22 bp

from the 5' end, the 7052 A>G SNP was 46 bp from the 5' end, the 7539 C>T SNP was located 10 bp from the 3' end, the 7998 G>A SNP was located 48 bp from the 5' end and the 8578 G>A SNP was located 23 bp from the 3' end of the amplified fragment. The –235 A>T SNP was 380 bp from the 5' end and 281 bp from the 3' end, but the amplified fragment may have been too large to detect this SNP. The SNPs not detected were previously described by means of direct sequencing, DGGE analysis and through the use of specific restriction enzymes.

The 3167 D>Del in intron 2 and the intron 10 polymorphism, 7052 A>G were only identified in an Afro-Caribbean and an African population group. These two polymorphisms were not encountered in 750 unrelated Caucasian individuals, which could explain why they were not found in our population (Robreau-Fraolini *et al.*, 2000). The four described intron 1 SNPs (1345 G>A, 1500 T>C, 2377 C>A, 2479 A>G) were not located in the areas that were amplified and investigated, but were shown to be in marked linkage disequilibrium with the intron 3 polymorphism 3581 A>G in both the Northern American Caucasian and the Northern European population (Lee and Anvret, 1991; Yoo *et al.*, 1993). The intron 2 polymorphism of the *PBGD* gene 3119 G>T has demonstrated a wild type allele frequency of 0.95 and a mutant allele frequency of 0.05 in 78 Caucasians and a wildtype allele frequency of 0.97 and mutant allele frequency of 0.03 in an Afro-Caribbean and an African population population group of 98 individuals (Robreau-Fraolini *et al.*, 2000). The allele frequencies of the intron 12, 7539 C>T, polymorphism in these two populations was found to be C=0.87 and T=0.13 and C=0.97 and T=0.03 respectivily (Robreau-Fraolini *et al.*, 2000). The common G allele of the 7998 G>A polymorphism in intron 14 was found at an allele frequency of 0.97 in 96 normal American Caucasians. The allele frequency of the A allele is thus 0.03. The possibility therefore exists, that our screening population was likely too small to detect these three rare polymorphisms. It could also be that the non-identified polymorphisms are not present/found in the specific population that was investigated. The close proximity to the ends of the amplified fragments of some of the non-detected SNPs could explain the false negative results found with the CSGE protocol.

Four previously described polymorphic sites were identified in the VP patient-screening group (See Figure 3.7). They include the –64 C>T polymorphism in exon 1 (Picat *et al.*, 1991), the 3581 A>G polymorphic site in intron 3 (Daimon *et al.*, 1993), the 6479 G>T polymorphic site in exon 10 (Gu *et al.*, 1991) and the 7064 C>A polymorphism in intron 10 (Yoo *et al.*, 1993). A novel 8 bp deletion, 4582_4589del, was also identified in our population (See Figure 3.7). Homology searches to determine the conservation of areas containing the sequence variation sites found in the *PBGD* gene, could only be done for the exon 10 6479 C>T polymorphism. The other sequence variation sites were found in intronic areas or, in the case of the exon 1 –64 C>T polymorphism, in the 5' untranslated region for which sequences of other species are still unavailable or not yet released.

Figure 3.7: Structure of the *PBGD* gene and locations of sequence variant sites identified in this study



3.8.1 The -64 C>T polymorphism in exon 1 of the *PBGD* gene

Screening of the 241 bp fragment containing exon 1 of the *PBGD* gene resulted in the detection of the –64 C>T SNP. After detection with both the CSGE and SSCP methods, the presence of the SNP was confirmed with sequencing (See Table 3.7 and Figure 3.8).

The C>T polymorphism upstream from exon 1 was previously detected by the use of a 5'-primer, with a mutated sequence. Together the mutated primer and the polymorphic base C create an *Apa I* restriction site (Picat *et al.*, 1991). Our research proved that the restriction enzyme *MnI I* instead of the preceding mutated primer could be used for identifying this polymorphic site (See Figure

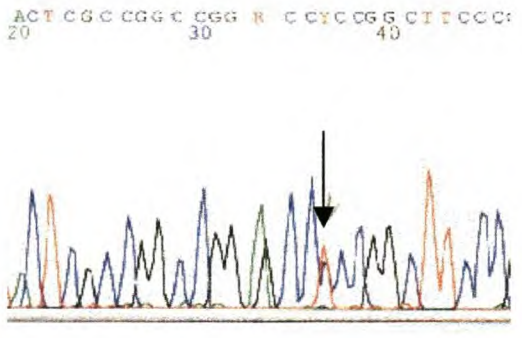
3.9). The fragment containing the wildtype allele has no recognition site for the *MnI I* enzyme, whereas the mutant allele has a recognition site for the *MnI I* enzyme resulting in the cleaving of the 214 bp fragment into fragments of 175 bp and 39 bp. This polymorphism is found close (-64) to the 3' splice site of the promoter area, but thorough investigation by Yoo *et al.*, (1993) not form part of any transcription factor binding or splice site (See Chapter 1.4.2.2).

Table 3.7: Partial gDNA sequence of the amplified region containing exon 1 of the *PBGD* gene indicating the position of the –64 C>T SNP

gtcagactgt	aggacgacct	cgggtcccac	gtgtccccgg	tactcgccgg
ccggagcc**t**c	cggcttcccg	gggccggggg	accttagcgg	CACCCACACA
CAGCCTACTT	TCCAAGCGGA	GCCATGTCTG	GTAACGGCAA	TGCGGCTGCA
ACGGCGgtga	gtgctgagcc	ggtgaccagc	acactttggg	cttctggacg
agccgtgcag	cgattggccc	caggttgcca	tcctcagtcg	t

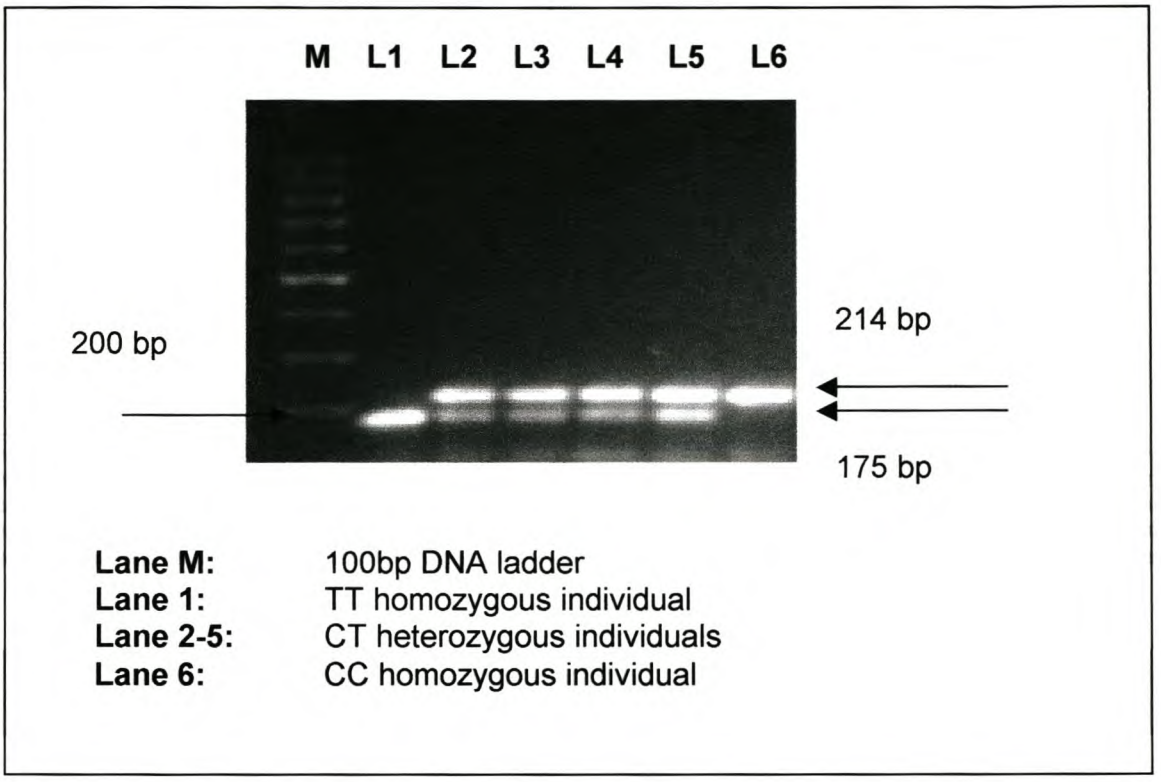
Partial gDNA sequence obtained from genomic sequence Accession number GI292384. The exon 1 region is indicated by capital letters while the surrounding intron areas are given in lowercase letters. The sequence of the forward primer and reverse primer are indicated in blue. The site of the –64 C>T polymorphism is indicated with bold font and underlined.

Figure 3.8: Results obtained from the automated sequencing in the sense direction of the *PBGD* gene fragment containing the –64 T>C polymorphism in a heterozygous individual



The arrow indicates the exact position of the -64 T>C polymorphism

Figure 3.9: A 1.5 % agarose gel showing the banding patterns of the *PBGD* exon 1 polymorphism after digestion with restriction enzyme *MnlI*



The allelic frequency for the –64C>T polymorphism from 35 unrelated individuals of European Caucasoid origin T=0.37; C=0.63 (Picat *et al.*, 1991) were identical to that of 78 non-porphyric Caucasoid unrelated individuals from the Centre d'Etude du Polymorphisme Humain (CEPH, Paris, France) (Robreau-Fraolini *et al.*, 2000). The allelic frequency found in our 42 VP patients (group A and B combined), differed only slightly T=0.42; C=0.58. No association of statistical significance could be demonstrated between the –64 C>T polymorphism genotype and any of the clinical symptoms associated with VP (See Tables 3.8 to 3.9). (χ^2= 2.82; p= 0.945). The Chi square value for Group A and B was 3.054 and the probability 0.931. These results could be due to the limited number of VP patients, especially the acute attack group. However, if the polymorphism had a major influence on the clinical expression, it would most likely have been noticable in this small group.

Table 3.8: Contingency Table of the –64 C>T polymorphism data generated for the group A VP patients and the control group

	Observed Genotype			
Group	C/C	C/T	T/T	**Total**
No symptoms	3	3	1	7
Acute Attack	1	1	0	2
Skin lesions	3	5	3	11
Acute attack and Skin lesions	3	1	1	5
Controls	17	20	7	44
Total	27	30	12	69

Chi Square value: 2.821
Probability: 0.945

Table 3.9: Contingency Table of the –64 C>T polymorphism data generated for the group A and group B VP patients and the control group

	Observed Genotype			
Group	C/C	C/T	T/T	**Total**
No symptoms	5	4	2	11
Acute Attack	2	2	0	4
Skin lesions	4	6	4	14
Acute attack and Skin lesions	4	7	2	13
Controls	17	20	7	44
Total	32	39	15	86

Chi Square value: 3.054
Probability: 0.931

3.8.2 The 3581 A>G polymorphism in intron 3 of the *PBGD* gene

Both the SSCP methods facilitated the detection of the 3581 A>G polymorphism in the exon 4 amplified fragment of the *PBGD* gene (See Table 3.10 and Figure 3.10). The 191 bp fragment was subjected to sequencing to confirm the presence of this SNP.

When the mutant G allele is present, *Bsm AI* cuts the 191 bp fragment containing the 3581 A>G polymorphism. This results in two fragments of 144 bp and 47 bp (See Figure 3.11).

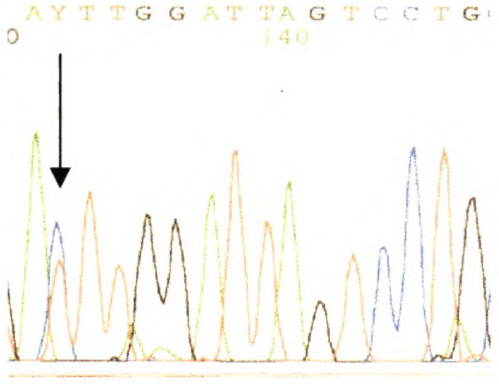
The allele frequency in a normal American Caucasian group of 100 individuals was found to be A=0.59 and G=0.41 (Yoo *et al.*, 1993). The allele frequency in 78 Caucasians (non-porphyric white unrelated families from the Centre d' Etude du Polymorphisme Humain, CEPH, Paris, France) was G=0.75 and A=0.25. The frequency in 30 Afro Caribbean individuals were G=0.79 and A=0.21 and the frequency in 68 African individuals were found to be G=0.85 and A=0.15 (Robreau-Fraolini *et al.*, 2000). Due to a time and financial constraint this SNP was not investigated in our extended VP group or in the control group.

Table 3.10: Partial gDNA sequence of the amplified region containing exon 4 of the *PBGD* gene indicating the position of the 3581 A>G SNP. (In 5'- 3' direction)

aaagagtctg	agccgtggct	gggaagggca	ggactaatcc	aa**a**tctctac
ccgcagCTTG	CTCGCATACA	GACGGACAGT	GTGGTGGCAA	CATTGAAAGC
CTCGTACCCT	GGCCTGCAGT	TTGAAATCAg	tgagttttct	ggaaaggagt
ggaagctaat	gggaagccca	gtaccccgag	aggagagaac	a

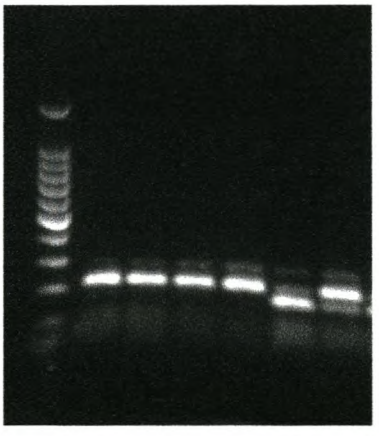
Partial gDNA sequence obtained from gDNA sequence Accession number GI 292384. The exon 4 region is indicated by capital letters while the surrounding intron areas are given in lowercase letters. The sequence of the forward primer and reverse primer are indicated in purple. The site of the 3581 A>G polymorphism is indicated with bold font and underlined.

Figure 3.10 : Results obtained from the automated sequencing in the antisense direction (3'-5') of the *PBGD* gene fragment containing the 3581 A>G polymorphism in a heterozygous individual



The arrow indicates the exact position of the 3581 A>G polymorphism

Figure 3.11: A 1.5% agarose gel showing the various homozygous and heterozygous banding patterns of the intron 3, 3581 A>G polymorphism after digestion with *BsmAI*



M:	100 bp DNA ladder
Lane 1 to Lane 4:	AA homozygous individuals
Lane 5:	GG homozygous individual
Lane 6:	AG heterozygous individual

3.8.3 The 4582_4589del in intron 6 of the *PBGD* gene

During the course of this study a novel 8 bp deletion was detected in intron 6. (See Table 3.11). The deletion was observed with all three sequence variantion detection techniques and confirmed by the sequencing of the 286 bp fragment (See Figures 3.12 and 3.13). The 4582_4589del site was initially detected in 3 of 25 individuals. Two of the three individuals with the deletion were related (mother-daughter). We were not able to analyze more individuals during this study for the 4582_4589del, due to finacial and time constraints. When a larger characterized VP group is available the influence of this deletion should be further investigated. The two related patients were, while the third individual had skin symptoms. All three were from Group A (characterized by the neurologist and dermatologist).

In higher eukaryotes at least three sequence elements participate in the initiation of the splicing reaction viz. the 5' splice site, the 3' splice site consensus sequence and the RNA branchpoint (Ruskin and Green, 1985). Mutations of these sequences may cause abnormal gene expression (Cooper and Mattox, 1997). The 4582_4589del deletion is located close to the 3' splice site (-51) but does not contain sequences that are considered to be involved in the splicing process. However, further expression studies will have to be performed to determine with confidense wheter or not this mutation has any influence on the splicing process.

Table 3.11: Partial gDNA sequence of the amplified region of exon 7 of the *PBGD* gene indicating the position of the 4582_4589del

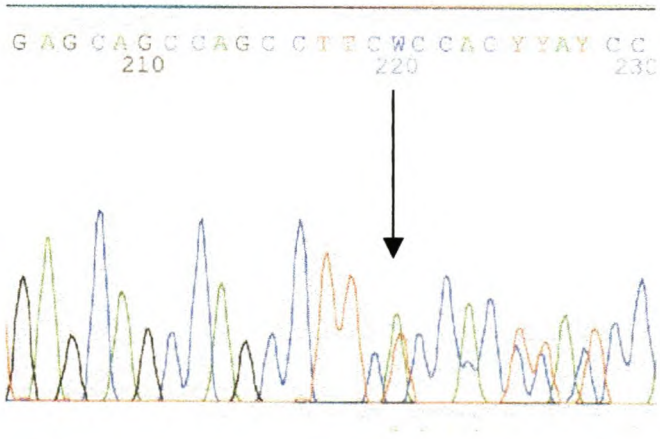
aggctccacc	actgaagtag	aggcaggggt	gggtgga**gaa**	**ggggt**gaagg
ctggctgctc	ataccctttc	tctttgcccc	cctctcccat	ctctatagAG
TGGACCTGGT	TGTTCACTCC	TTGAAGGACC	TGCCCACTGT	GCTTCCTCCT
GGCTTCACCA	TCGGAGCCAT	CTGCAAgtaa	gagtcttgca	agtaaggggc
ttgggcaggg	gtaggcatca	tgtgaacctt	tgcctttccc	tttggggcct
gaccctctgc	ttcagggtta	tctcctctgc	cctgag	

Partial gDNA sequence obtained from gDNA sequence Accession number GI 292384. The exon 7 region is indicated by capital letters while the surrounding intron areas are given in lowercase letters. The sequence of the forward primer and reverse primer are indicated in blue. The site of the 4582_4589del deletion is indicated with bold font and underlined

Figure 3.12: The results obtained from the two buffer SSCP/HD system indicating the 4582_4589del polymorphic site

Lane 1: Individual with 4582_4589del
Lane 2: Control individual without deletion

Figure 3.13 : The results obtained from the automated sequencing in the antisense direction of the *PBGD* gene fragment containing the 4582_4589del 8 bp deletion in a heterozygous individual



The arrow indicates the location of the deletion in the antisense orientation

3.8.4 The 6479 G>T polymorphism in exon 10 of the *PBGD* gene

The CSGE screening method identified the 6479 G>T SNP in a 308 bp fragment (See Table 3.12). This fragment contained exon 10 and the presence of the SNP was confirmed with sequencing (See Figure 3.14). No restriction enzyme is currently available to discriminate between the two alleles of this polymorphism.

The different allele frequencies of this SNP was found to be G=0.69 and T=0.31 in a Caucasian group of 78 individuals, G=0.76 and T=0.24 in a group of 30 Afro-Caribbean individuals and G=0.70 and T=0.30 in a group of 68 Africans (Robreau-Froalini *et al.*, 2000). Due to financial and time constraints this polymorphism was not investigated in the extended VP group or the control group.

Table 3.12: The Partial gDNA sequence of the amplified region of exon 10 of the *PBGD* gene indicating the 6479 G>T SNP. (In the 5'-3' direction)

gggaaagaca gactcaggca gagggaaccg cacgaggccc cagattgccc

gacactgtgg tccttagcaa ctctccacag CGGGGAAACC TCAACACCCG

GCTTCGGAAG ATGGACGAGC AGCAGGAGTT CAGTGCCATC ATCCTGGACA

ACAGCTGGCC TGCAGCGCAT GGGCTGGCAC AACCGGGT**T**G GGCAGgtagg

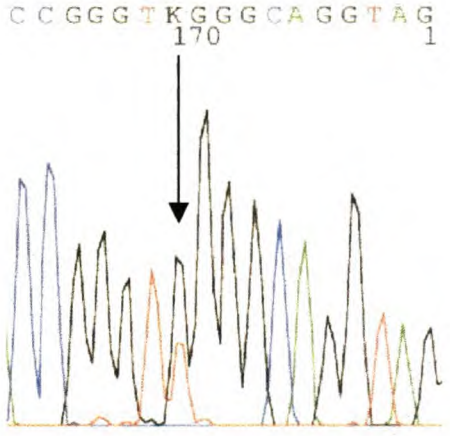
gcctgcccct atcctctccc cagctcatct gcatctcctt tctgccttac

agtcatcccc aatttaggat ttttagactt tatgattgtg tgaaagcgat

atacgttca

Partial gDNA sequence obtained from gDNA sequence Accession number GI 292384. The exon 10 region is indicated by capital letters while the surrounding intron areas are given in lowercase letters. The sequence of the forward primer and reverse primer are indicated in blue. The site of the 6479 G>T polymorphism is indicated with bold font and underlined

Figure 3.14: The results obtained from the automated sequencing, in the sense direction, of the *PBGD* gene fragment in a heterozygous individual containing the 6479 G>T polymorphic site



The arrow indicates the exact position of the 6479 G>T polymorphism

A homology search of the human *PBGD* gene (GI 292384) and *Rattus norvergicus PBGD* mRNA (GI 6981023) was conducted using the BLAST program (http://www.ncbi.nlm.nih.gov/blast)(See Figure 3.15). The same homology search was also performed for the human *PBGD* gene and the Mouse porphobilinogen deaminase gene, exon 5 (GI 200229)(See Figure 3.15). The area under scrutiny, exon 10 nt-6372 to nt-6498 of the human *PBGD* gene, revealed significant homology with both the rat and mouse *PBGD* genes.

Sequence alignment analysis revealed that the rat *PBGD* gene 504 to 617 showed a 87% homology with the human *PBGD* gene nt-6372 to nt-6485, while the *PBGD* gene of the mouse 402 to 528 had 86% homology with the human *PBGD* gene nt-6372 to nt-6498.

The area surrounding the 6479 G>T polymorphism in exon 10 is conserved with the G allele present in the rat and mouse sequence and the T allele present in the human sequence. The high degree of conservation identified in these three species probably points to the biological importance of the PBGD gene in the

haem biosynthetic pathway and specifically this base position in a highly conserved exon.

Figure 3.15: Sequence alignment of the human exon 10 region of the *PBGD* gene with the rat *PBGD* mRNA and the mouse PBGD mRNA. (In 5'-3' direction)

Regions of homology with the human *PBGD* sequence are highlighted in purple. The G>T polymorphism found in the human *PBGD* gene at position 6479 is highlighted in yellow.

3.8.5 The 7064 C>A polymorphism in intron 10 of the *PBGD* gene

The 213 bp fragment containing exon 11 was showed to habour a SNP (7064 C>A) when subjected to CSGE analysis (See Table 3.13). This was confirmed by sequencing the fragment (See figure 3.16).

The restriction enzyme *Hinf* I, cleaves the 213 bp fragment resulting in a 159 bp and 54 bp fragment when the C allele is present. When the T allele is present

there is no recognition site for the *Hinf I* enzyme and therefore no cleavage of the fragment (See Figure 3.17).

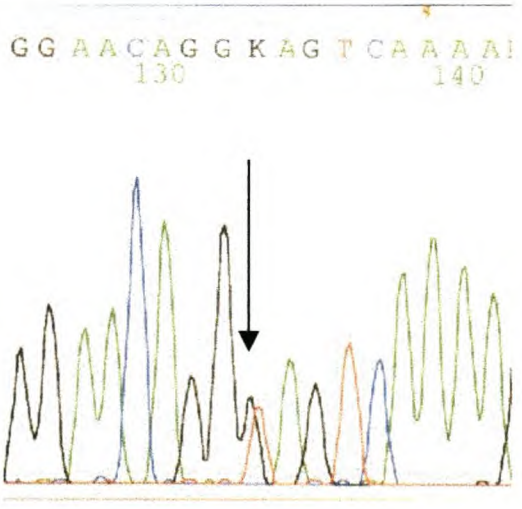
The allelic frequencies of the 7064 C>A polymorphism was reported to be C=0.75 and A=0.25 in a Caucasian group of 78 individuals (Robreau-Fraolini *et al.*, 2000) and in a normal group of 92 American Caucasians (Yoo *et al.*, 1993). The 7064 C>A SNP was not investigated in the extended group of VP patients due to financial and time constraints.

Table 3.13: Partial gDNA sequence of the amplified region containing exon 11 of the *PBGD* gene indicating the position of the 7064 C>A SNP

gaactcccat	ctcactgcca	ggtgctttta	gacacccccg	tgtccaccct
tttgact**c**cc	tgttccgcct	ccacagATCC	TGCACCCTGA	GGAATGCATG
TATGCTGTGG	gCCAGgtaca	cttgaccagg	gaagccacat	ggtgacatat
gccttcccct	tgttctcaac	caagaagctt	gtctcacaac	cttctgcatc
tgcttcccca	gaa			

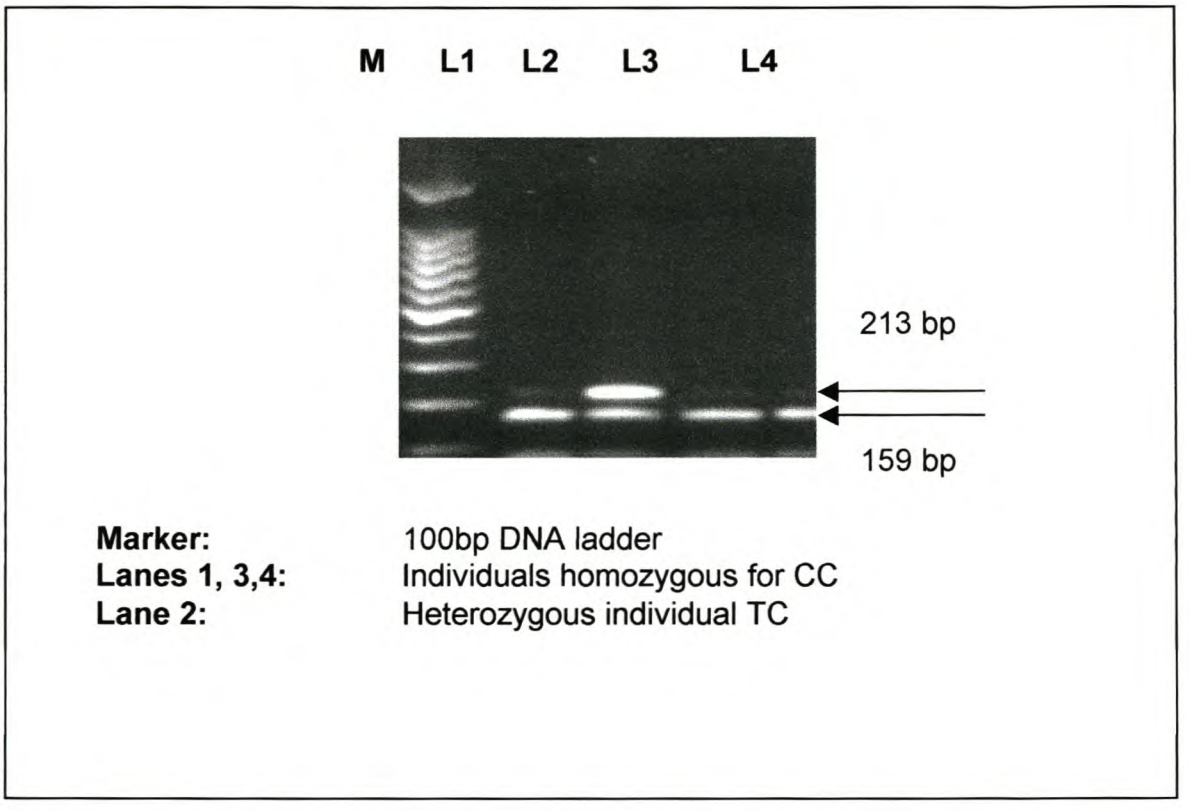
Partial gDNA sequence obtained from gDNA sequence Accession number GI 292384. The exon 11 region is indicated by capital letters while the surrounding intron areas are given in lowercase letters. The sequence of the forward primer and reverse primer are indicated in blue. The site of the 7064 C>A polymorphism is indicated with bold font and underlined

Figure 3.16 : Results obtained from the automated sequencing in the antisense direction of the PBGD gene fragment containing the 7064 C>A polymorphic site in a heterozygous individual



The arrow indicates the exact position of the 7064 C>A polymorphism

Figure 3.17: A 1.5% agarose gel demonstrating the *Hinf I* restriction enzyme cleavage of a 213bp product of intron 10 of the PBGD gene



3.9 A comparison of the studied genes

Both the ALAS and PBDG enzymes are synthesized in duplicate forms, the difference being, that ALAS is encoded by two separate genes, while PBGD is encoded by a single gene that undergoes differential splicing. The two genes encoding the human ALAS isoenzymes evidently evolved from a primitive ancestral gene and their divergence resulted in different regulating domains (May *et al.*, 1990). Comparison of the *Homo sapiens* mRNA with the mRNA of the rat showed that high conservation exists between species for the *ALAS-1, ALAS-2* and *PBGD* genes. The *ALAS-1* mRNA for the two species show 86% homology, the mRNAs for *ALAS-2* show 87% homology and the mRNAs for the *PBGD* gene show 85% homology.

Among all the genes in the haem biosynthetic pathway, the *PBGD* gene has the most identified mutations (Thunell, 2000). AIP caused by mutations in the *PBGD* gene, is one of the porphyrias with the highest frequency. To date, sixteen

polymorphic sites have been identified in the *PBGD* gene (See Table 1.4). Thirteen are characterized as intragenic SNPs, one in an exon and two SNPs in the non-erythroid promer region.

The *ALAS-1* gene has no reported mutations and in the *ALAS-2* gene only a few mutations, causing X-linked sideroblastic anemia, have been identified (Human gene mutation database; Krawczak and Cooper, 1997). To date 20 SNPs have been identified in the *ALAS-1* gene (Bio-chip: http://www.bio.chip.org /biotools/; Locuslink: http://www.ncbi.nlm.nih. gov/SNP [Maglott *et al.*, 2000; Pruitt *et al.*, 2000](See Table1.2). One SNP is located in the exonic area, eight are found in the 5' untranslated region and eleven in the intronic areas. Eighteen SNPs have been identified in the *ALAS-2* gene (Bio-chip: http://www.bio.chip.org/biotools/; Locuslink: http://www.ncbi.nlm. nih.gov/SNP [Maglott *et al.*, 2000; Pruitt *et al.*, 2000] (See Table 1.3). None of the identified SNPs are found in the exonic areas, five of the SNPs are found in the 5'untranslated area, one SNP is found in the 3' untranslated area and twelve SNPs are located in the intronic areas.

A homology comparison using the HomoloGene website (http://www.ncbi.nlm.nih.gov/HomoloGene) revealed that all three genes investigated (*ALAS-1, ALAS-2* and *PBGD*) are greatly conserved among species. The human ALAS-1 gene (UniGene Cluster: Hs.78712) shows 87.2% alignment with the rat sequence (Unigene Cluster: Rn 6274) and 87.2% alignment with the mouse sequence (UniGene Cluster: Mm 19143). The human *ALAS-2* gene sequence (UniGene Cluster: Hs.323383) shows 99.7% alignment with the rat sequence (UniGene Cluster: Rn 58391) and 87.9% alignment with the mouse sequence (UniGene Cluster: Mm 140509). The human *PBGD* gene sequence (UniGene Cluster: Hs 82609) shows 88.8% alignment with the rat sequence (UniGene Cluster: Rn 11080) and 87.8% alignment with the mouse sequence (UniGene Cluster: Mm 1710).

All this information supports the theory that these three genes are very important in the haem biosynthetic pathway. The significance of the ALAs genes, known to rate-limit the whole haem biosynthetic pathway is re-inforced by their conservation, the lack of mutations and the presence of few SNPs. The *ALAS*

genes may therefore be so important that mutations mau not be tolerated i.e. they may be incompatible with life.

Chapter Four

4 Conclusion

In the South African population, a high prevalence of VP exists due to a founder gene effect (Meissner *et al.*, 1996; Warnich *et al.*, 1996). The majority of VP patients in South Africa therefore have the same R59W mutation in the *PPOX* gene. However, great variation in the clinical expression of the R59W patients is observed and even members of the same family who would be expected to share a common genetic background, and that have been exposed to similar environmental factors, exhibit great clinical variation.

An attempt was therefore made to identify modifying factors that could contribute to the clinical presentation of these individuals. The study population consisted of 25 individuals with the R59W mutation considered ideal for this study because the contribution of the disease causing mutation could be ignored. The patients were classified by a dermatologist and neurologist into four clinical groups. These clinical groups consisted of a) asymptomatic individuals, b) individuals who had previous symptoms of an acute attack (in clinical remission at time of study), c) individuals who display skin symptoms, d) individuals who had both an acute attack and skin symptoms. The distribution of the clinical symptoms found in our patients corresponded with the results from previous studies done by Eales *et al.* (1980) and Whatley *et al.* (1999), even though the number of our patients was relatively small. Although the current study population is very small, a good foundation has been established for the clinical classification of VP patients which will be very valuable in further studies concerning the clinical expression of VP. It is thus suggested that more R59W patients be subjected to the same established clinical classification process in order to assemble a larger VP study group.

The rate of the entire haem biosynthetic pathway is determined by the activity of the first enzyme ALA synthase, which is under negative feedback control by the free haem. Secondary control is found at the level of PBG deaminase, which is inhibited by proto- and coproporphyrinogen. The hypothesis is therefore that a mutation in the *PPOX* gene in combination with a mutation in one of the rate limiting enzymes of the haem biosynthetic pathway would cause a more severe phenotype. The *ALAS-1, ALAS-2*

and *PBGD* genes were thus investigated for their possible modifying effect on the clinical symptoms found in VP patients.

After comparing recent literature on the detection of sequence variation it was decided to use two different SSCP techniques, one being a conventional method and the other a method develop by Liechti-Galliti *et al.* (1999), entitled the two buffer SSCP protocol. In combination with the two SSCP protocols a CSGE method was employed. The conventional SSCP method was previously used in our laboratory, but the two buffer SSCP protocol and the CSGE protocol were set up during this study. The CSGE method had the highest success rate (83%), detecting 5 out of 6 sequence variants identified during this study. The conventional SSCP method failed to identify 3 of the sequence variants detected and the two buffer method failed to detect 4 of the sequence variants. Both SSCP methods did, however, identify a SNP not detected by the CSGE method. The SSCP methods only identified transition type SNPs and failed to identify any transversion during this study.

Markoff *et al.* (1998) and Ganguly (2002) also found the CSGE method to be superior to the SSCP method. We failed to detect 11 known SNPs (8 SNPs in the *PBGD* gene; 2 SNPs in the *ALAS-1* gene and 1 SNP in the *ALAS-2* gene), which in the case of the *ALAS-1* and *ALAS-2* genes were published in December of 2001. The failure to detect these SNPs could be due to either the frequency of these SNPs, that vary between the populations in which they were described and the current study population, or the location of the SNPs in the relevant PCR amplified fragment (See sections 3.6, 3.7 and 3.8). Primers can in future be redesigned to allow for a longer fragment between the SNP and the end of the amplification product.

No variation detection technique developed to date has proven to have a 100% sensitivity rate. Direct sequencing is still the superior method to use to identify sequence variants, but is too expensive to use on a routine basis in the smaller laboratory. The results of this study therefore indicate that two different techniques (e.g. CSGE and conventional SSCP) based on different principles are more successful in identifying sequence variation than one method which could miss a sequence variation due to the limitation of the relevant procedure.

Six sequence variant sites (*ALAS-1*: 4713 T>C; *PBGD*: -64 C>T, 3581 A>G, 4582_4589del, 6479 G>T, 7064 C>A) were identified in the *ALAS-1* and *PBGD* genes using a screening population of 25 VP patients. Five of these variants were identified in the *PBGD* gene. These five include four previously described polymorphisms and a novel 8 bp deletion. One polymorphic site was identified in the *ALAS-1* gene. No sequence variation could be found in the *ALAS-2* gene. Current literature indicates that all three genes studied have the same amount of SNPs (See section 3.9). After the investigation of the exon and the flanking intronic regions we speculate that the *ALAS* genes may well be more conserved than the *PBGD* gene as we found more sequence variation in the *PBGD* gene than in the two *ALAS* genes. More disease causing mutations have also been identified in the *PBGD* gene than in the *ALAS* genes. The conservation of the *ALAS* genes would coincide with the important regulatory function these genes have to fulfill in the haem biosynthetic pathway.

Only two sequence variant sites were identified in exonic regions. The 4713T>C SNP was found in exon 4 of the *ALAS-1* gene and the 6479 G>T SNP in exon 10 of the *PBGD* gene. The remaining four sequence variant sites were all identified in intronic areas, except for the -64 C>T SNP in the 5' untranslated area of the *PBGD* gene. Recent studies involving the successful identification of genetic modifying genes all identified sequence variation in either an exon (Goldfarb *et al.*, 1992; Persu *et al.*, 2002) or promoter region of a gene or in an intronic region where it was shown to influence splice sites (Bala and Peltomäki, 2001; Chu *et al.*, 1993; Gouya *et al.*, 2002). All six sequence variants could not be tested in an extended group of VP individuals, due to financial and time constrains. We therefore selected two sequence variant sites (*ALAS-1*: 4713 T>C; *PBGD*: -64 C>T) due to their location in the respective genes. These SNPs were tested in an extended group of 50 R59W VP patients (25 extensively classified) and in a normal control group matched for ethnic background. Both of these polymorphisms did not show any association of statistical significance with any of the clinical groups. However, individual genetic modifying factors make a small contribution to the total clinical picture and are therefore difficult to identify, even more so in a small population. To rule out any possibility of association with one of the clinical groups, the six sequence variant sites will need to be typed in future in a larger well-defined VP patient group.

The identification of modifier genes is further complicated by the involvement of environmental factors in the disease progression. These difficulties are minimized in animal models that allow for the extensive experimental control of both the genetic and environmental variables. Recently researchers have developed a mouse model with the R59W VP mutation and they are currently in the process of characterization. Urine and faecal samples obtained from these mice indicate that they indeed have biochemical abnormalities similar to those observed in human VP subjects (Medlock *et al.*, 2002). This mouse model could therefore prove very valuable in studies to identify modifier genes that contribute to the clinical expression of variegate porphyria.

All the aims of this study as stated on page 36 have been met. However, due to the fact that a limited number of individuals were available for this study, we could not prove any positive association. Although we cannot prove our hypothesis until the sequence variants are studied in a larger population, we can conclude that neither the 4713 T>C in the ALAS-1 gene or the -64 C>T in the PBGD gene are probably a major locus contributing to the clinical expression. In the event of these sequence variants being a major locus we would most likely have detected association even in our small population. This study did, however, provide insight into future investigational strategies concerning these three important genes. Knowledge concerning these genes could lead to a better understanding of the haem biosynthetic pathway and ultimately to the understanding and assessment of the clinical expression found in VP and other porphyrias.

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Appendix A

Variegate Porphyria

Clinical and Biochemical Data

Name:	Maiden name:	Sex:

Address: _____

_____

_____

_____

Telephone number: _____ (H) _____ (W)

Date of Birth:	Age:	Origin:

	Positive	Negative	None	
Biochemical test (mark x)				
Acute attack (mark x)				How frequently? _____
Sun/light sensitivity (mark x)				
Skin lesions (mark x)				

Were you also tested for any other types of porphyria ?

_____

Please enclose copies of any laboratory- and/or doctors reports, if available.

Details of general practitioner to whom the results must be sent:

_____

_____

_____

Postal code: _____ Telephone number (W): _____

Please complete to the best of your ability:

1. Medication:

Have you ever had anaesthesia administered ? _____

Did you experience any problems while being under the anaesthetic or thereafter ?_____

Did you inform your anaesthetist that you or any members of your family are porphyria suffers?_____

Are you currently using any medication ?

-Contraceptives ☐ name: _____

-Disprin ☐ name: _____

☐ name: _____

☐ name: _____

☐ name: _____

Have you ever used any of the above mentioned medications ? _____

Did you show any reaction after these medications have been administered?_____

2. Abdominal pain:

Have you ever been hospitalized for an acute attack ?_____

Have you ever experienced serious bouts of abdominal pain?_____

Could the cause be determined ?_____

Specify / Describe the pain _____

Do you regularly experience abdominal pain ? _____

3. Sun exposure

Is your work of such a nature that you are daily exposed to sunlight? _____

Do you suntan ? _____

Do you take precautions against sun exposure ? _____

4. Skin

Does your skin easily get hurt or bruised? _____

Is your skin prone to form blisters or leave scars? _____

Please specify: _____

General:

- Dark urine _____
- Emotional disturbances _____
- Constipation _____
- High / low blood pressure _____
- Food sensitivity _____
- Alcohol sensitivity _____
- Iron supplements? _____
- Iron related defects _____

Family history:

- Porphyria _____
- Multiple Sclerosis _____
- Hemochromatosis _____
- Other _____

For office use:

Date form received: Date of DNA extraction: DNA sample no.: