Molecular epidemiological study of variegate porphyria (VP) to determine the frequency of the founder gene mutation in South Africa

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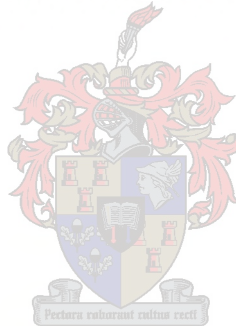

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Declaration

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

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Abstract

Variegate porphyria (VP; OMIM 176200) is caused by mutations in the protoporphyrinogen oxidase gene (*PPOX*) and is inherited as an autosomal dominant trait, displaying incomplete penetrance. Manifestation of VP includes photosensitivity and potentially fatal acute porphyric attacks. The incidence of VP in South Africa is the highest in the world due to a founder effect. In 1960 Geoffrey Dean estimated the frequency of VP to be 0.3% in the Caucasian South African population. However, this estimate is questionable on the grounds of genealogical and biochemical methods used, and as indicated by more recent records from diagnostic laboratories reported here.

The aim of this study was to determine the frequency of the VP founder mutation (R59W) within two South African populations (Caucasian and mixed ancestry) by means of a highly specific DNA test. Various methods of blood sample collection, DNA isolation and R59W mutation detection, suitable for a large-scale population-screening study, were evaluated. Blood samples were obtained from 4 072 participants at blood transfusion clinics, pathology clinics and maternity wards from three provinces within South Africa. Blood sample collection on FTA cards (Whatman BioScience) with subsequent DNA isolation and SSCP analysis were found to be the most appropriate methods.

Four of the participants tested were positive for the R59W mutation. All four were from the 3 233 Caucasian individuals tested while three of these were Afrikaans-speaking, confirming the high prevalence of the founder mutation in the South African Afrikaans-speaking Caucasian population (Afrikaner), as previously reported. One of the two adult R59W positive participants was unaware of her carrier status, in accordance with incomplete penetrance of the trait recently estimated as 60%. The mother of one of the newborn babies found to be R59W

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positive was also not aware of VP in the family, indicating that ignorance regarding VP status in South Africa is a matter of some concern.

The estimated frequency of 0.12% (4 / 3 233) for the R59W mutation is significantly lower than the frequency of 0.3% estimated previously, and analysis of the different populations sampled yielded interesting results. Only one of 2 093 (0.05%) participants in the Caucasian blood transfusion sample was mutation-positive. It is possible that the sample from this group was not unbiased, as porphyrics were discouraged to donate blood in the past. One of 761 (0.13%) Caucasian participants from pathology clinics was mutation-positive. This sample was probably not unbiased either, since suspected porphyrics are referred to these clinics by physicians. Two of 379 (0.53%) Caucasian newborn individuals tested positive, yielding a much higher frequency than the previous estimate by Dean. Although the sample size was very small, it was probably unbiased and therefore provides the best estimate. It is suggested that the sample size of this group be increased in order to improve the accuracy of the founder mutation frequency estimation in South Africa and to determine whether the issue of underdiagnosis and the resulting risk of potentially fatal acute attacks should be addressed by appropriate genetic testing in the future.

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Opsomming

Variegate porfirie (VP; OMIM 176200) word veroorsaak deur mutasies in die protoporforinogeen oksidase geen (*PPOX*) en vertoon 'n outosomaal dominante oorerwingspatroon met onvolledige penetransie. Die manifestasie van VP sluit fotosensitiwiteit en potensieël noodlottige akute aanvalle in. Die voorkoms van VP in Suid-Afrika is die hoogste in die wêreld as gevolg van 'n stigtereffek. In 1960 het Geoffrey Dean die frekwensie van VP in die Suid-Afrikaanse Kaukasiese populasie beraam as 0.3%. Hierdie beraming word egter bevraagteken op grond van die genealogiese en biochemiese metodes wat gebruik is, soos aangedui deur meer onlangse bevindinge van diagnostiese laboratoria.

Die doel van hierdie studie was om die frekwensie van die VP stigtermutasie (R59W) in twee Suid-Afrikaanse populasies (Kaukasiese en van gemengde herkoms) deur middel van 'n hoogs spesifieke DNS toets te beraam. Verskeie metodes van bloedmonsterinsameling, DNS isolasie en R59W mutasie deteksie, geskik vir 'n grootskaalse populasiesifting studie, is ondersoek. Bloedmonsters van 4 072 vrywilligers by bloedoortappingsklinieke, patologieklinieke en kraamsale van drie provinsies binne Suid-Afrika is verkry. Daar is gevind dat bloedmonsterversameling op FTA kaartjies (Whatman BioScience) met gepaardgaande DNS isolasie en SSCP analise die mees effektiewe metodes is.

Vier van die individue wat getoets is, was positief vir die R59W mutasie. Al vier was afkomstig van die 3 233 Kaukasiese monsters, terwyl drie Afrikaanssprekend was, en dus die hoë voorkoms van die stigtermutasie in die Suid-Afrikaanse Afrikaansspekende Kaukasiese bevolking (Afrikaner) bevestig. Een van die twee volwasse R59W positiewe individue was onbewus van haar draer status, wat ooreenstem met die onlangse beraming van 'n 60% penetransie vir VP. Die ma van een van die R59W positiewe pasgebore babas

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was ook onbewus van VP in die familie. Hierdie uitslae impliseer dat onkunde aangaande VP status 'n rede tot kommer in Suid-Afrika mag wees.

Die beraamde frekwensie van 0.12% (4/3233) vir die R59W mutasie was baie laer as die vorige beraamde frekwensie van 0.3%. Analise van die verskillende monsterpopulasies het egter interessante resultate gelewer. Slegs een van 2 093 (0.05%) deelnemers in die Kaukasiese bloedoortappingsgroep was mutasie-positief. Dit is moontlik dat die bloedmonsters verkry vanaf hierdie bron nie ongeselekteerd was nie, aangesien porfirielyers in die verlede afgeraai is om bloed te skenk. Een van 761 (0.13%) Kaukasiese individue verkry van patologieklinieke was mutasie-positief. Hierdie groep was ook nie ongeselekteerd nie, aangesien geneeshere moontlike porfirielyers na hierdie klinieke verwys. Twee van 379 (0.53%) Kaukasiese pasgebore babas het positief getoets, wat 'n baie hoër frekwensie is as wat deur Dean beraam is. Alhoewel hierdie monsterpopulasie baie klein was, was dit ongeselekteerd en het dit waarskynlik die mees akkurate beraming gelewer. Daar word dus voorgestel dat die aantal monsters in hierdie groep vermeerder word om 'n meer akkurate beraming van die stigtermutasie in Suid-Afrika te verkry en sodoende te bepaal of onderdiagnose en die gepaardgaande risiko van noodlottige akute aanvalle in die toekoms deur gepaste genetiese toetsing aangespreek behoort te word.

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List of Abbreviations

µl	microlitre
µM	micromolar
°C	degrees Centigrade
A	adenine
AIP	acute intermittent porphyria
ALA	δ-aminolevulinic acid
ALAD	δ-aminolevulinic acid dehydratase porphyria
APS	ammonium persulphate
ARMS	amplification refractory mutation system
bp	base pairs
C	cytosine
CEP	congenital erythropoietic porphyria
dATP	deoxyadenosine-5'triphosphate
dCTP	deoxycytidine-5'triphosphate
ddH_2O	double distilled water
dGTP	`deoxyguanosine-5'triphosphate
DHPLC	denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
EC	enzyme commission
EDTA	ethylenediaminetetra-acetic acid
EPP	erythropoietic protoporphyria
FH	familial hypercholesterolemia
FISH	fluorescence *in situ* hybridization
g	grams
G	guanine

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gDNA	genomic deoxyribonucleic acid
h	hour(s)
HCP	hereditary coproporphyria
HFE	hemochromatosis
HMB	hydroxymethylbilane
HPLC	high-performance liquid chromatography
IGF2	insulin-like growth factor 2
kb	kilobase
M	molar
MC	Medi-Clinic
$MgCl_2$	magnesium chloride
min(s)	minute(s)
ml	millilitre
mM	millimolar
nm	nanometre
OMIM	Online Mendelian Inheritance in Man
PAA	poly-acrylamide
MRC	Medical Research Council
PBG	porphobilinogen
PCR	polymerase chain reaction
PCT	porphyria cutanea tarda
PPO	protoporphyrinogen oxidase
PPOX	protoporphyrinogen oxidase gene
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
sdH_2O	single distilled water
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
T	thymine
Taq	*Thermus aquaticus*
TBE	tris, boric acid, EDTA

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TEAA	triethylammonium acetate
TLC	thin-layer chromatography
TOGA	Tygerboland Onafhanklike Ginekologiese Assosiasie
UK	United Kingdom
V	volts
v/v	volume:volume ratio
VP	variegate porphyria
w/v	weight:volume ratio

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Chapter One – Introduction

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1.1 The Porphyrias

The porphyrias are a group of disorders of porphyrin or porphyrin-precursor metabolism in the haem biosynthesis pathway, resulting from the inherited or acquired dysfunction of the enzymes controlling the porphyrin-haem biosynthetic pathway, leading to overproduction of porphyrins and their precursors (Bissell, 1979; Bickers *et al.*, 1993). Haem is one of the most versatile prosthetic groups in the cell. Along with specific associated protein structures, it mediates oxygen transport and storage, generates cellular energy and the formation of cGMP, H_2O_2, NO, prostaglandins, bile salts and steroid hormones. It performs some reduction, degradation and detoxification reactions and also induces protein synthesis and cell differentiation. Some of the better known haem containing proteins are hemoglobin, myoglobin, some of the cytochromes, cytochrome P450 oxidases and peroxidases (Dean, 1963; Bissell, 1979).

There are eight enzymes involved in the synthesis of haem, the first one and last three located in the mitochondria, while the rest are present in the cytosol. The synthesis pathway of haem is illustrated in Figure 1.1. All cells are able to produce haem, however, in man most haem is synthesized in the liver and bone marrow. Haem synthesis is initiated in the mitochondrion by the condensation of succinyl-CoA and glycine to form δ-aminolevulinate acid (ALA). In the cytosol two ALA molecules condense to form the monopyrrole porphobilinogen (PBG). Four molecules of PBG are assembled and then rearranged to form the first tetrapyrrolic porphyrinogen intermediate, uroporphyrinogen-III. Stepwise decarboxylation of uroporphyrinogen to coproporphyrinogen-III follows. Coproporphyrinogen-III then enters the mitochondrion where it undergoes oxidated decarboxylation to yield protoporphyrinogen-IX which is in turn oxidized

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to protoporphyrin-IX. This is followed by the insertion of ferrous iron to yield haem (Elder, 1982).

Haem synthesis is normally an extremely efficient, tightly controlled process. Less than 2.5% of the ALA entering the pathway is lost and the amount of haem produced closely matches the need of the body (Neuberger, 1980). This implies that the pathway is subject to some form of feedback control, principally at the level of ALA synthase, the rate-determining enzyme of the pathway. Any defect in haem synthesis which results in a relative shrinking of the haem pool will derepress ALA synthase in order to achieve a new steady state of haem synthesis. This increase in ALA synthase, while partially restoring the haem pool, also stresses the synthetic chain to a point where there is an additional accumulation of substrate proximal to the partial block (Kappas *et al.*, 1989).

A defect in any of the eight enzymes normally leads to a 50% decrease in activity of the deficient enzyme (Bissell, 1979; James and Hift, 2000). This causes an accumulation of the porphyrin precursors and/or the porphyrins themselves. These metabolites can often be detected in the body or after excretion in the urine and/or faeces. Before the advent of molecular diagnostic tests for some of the porphyrias, biochemical assays were the methods commonly used for diagnostic purposes. The type of porphyria that would affect an individual was determined by the specific enzymatic defect in the metabolic pathway (Bissell, 1979).

Except for congenital erythropoietic porphyria (CEP) and δ-aminolevulinic acid dehydratase porphyria (ALAD), all of the porphyrias have a dominant mode of inheritance. Porphyria cutanea tanda (PCT) is thought to be either dominant or acquired from external effects such as excessive alcohol consumption, iron overloads, polychlorinated hydrocarbons and oestrogens (Meissner *et al.*, 1987).

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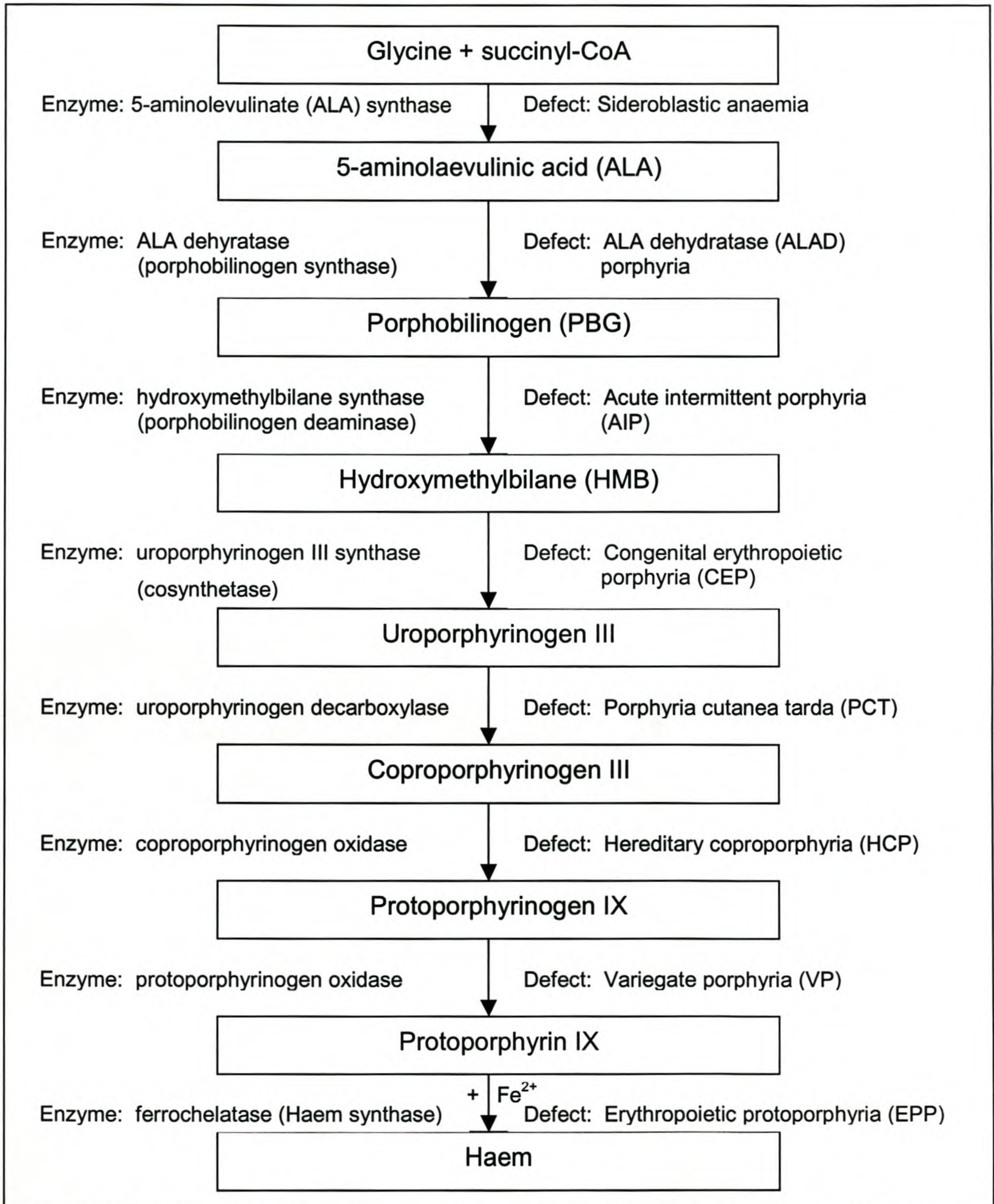

Figure 1.1: The haem biosynthetic pathway showing the sites of enzymes and their resulting deficiencies in the porphyrias. (Adapted from Deacon and Elder, 2001)

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The porphyrias are subdivided into acute and non-acute forms, based on the occurrence of acute and recurrent neurological attacks. The acute porphyrias include acute intermittent porphyria (AIP) (Goldberg, 1959; Lindberg *et al.*, 1996), hereditary coproporphyria (HCP) (Berger and Goldberg, 1955) and variegate porphyria (VP) (Dean, 1963; Cochrane and Goldberg, 1968; Mustajoki, 1978). The non-acute porphyrias are congenital erythropoietic porphyria (Levin, 1968; Tanigawa *et al.*, 1996), δ-aminolevulinic acid dehydratase porphyria (Doss *et al.*, 1979), porphyria cutanea tarda (Hofstad *et al.*, 1973; Wu *et al.*, 1996) and congenital erythropoietic protoporphyria (Peterka *et al.*, 1965).

The possibility of an acute attack occurring is very difficult to define. Some patients with AIP and VP might never have an acute attack. There is evidence that there are a number of factors that might increase the probability for an acute attack. Drugs, alcohol (acute abuse), physiological hormone fluctuations, infection, certain diets (normally low in caloric intake) and stress may induce an acute attack (Frank and Christiano, 1997; Moore and Hift, 1997).

Many enzyme-inducing drugs may be triggers of an acute attack. These drugs can either demand an increased haem production (by inducing the transcription of ALA synthetase directly through mRNA, interfering with the haem synthetic pathway or increasing utilization), or cause a failure in the haem inhibitory feedback control on ALA synthetase production (Hift *et al.*, 1993; James and Hift, 2000). While accumulation of early haem precursors, ALA and PBG, are generally associated with acute attacks, late intermediates, the porphyrins, are associated with skin lesions. Porphyrin molecules are ring structures that absorb visible light, generating exited states. Excessive concentrations of porphyrins exposed to daylight, more specifically wavelengths of about 400 nm, generate free radicals with consequent lipid peroxidation and protein cross-linking, leading

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to damage to tissues, cells, sub-cellular elements and biomolecules (Sandberg and Romslo, 1981; Lim and Sassa, 1993).

1.2 Variegate Porphyria (VP)

Variegate porphyria (OMIM 176200), one of the acute hepatic porphyrias, is characterized by deficiencies in the protoporphyrinogen oxidase enzyme (PPO; EC 1.3.3.4) and is usually inherited as an autosomal dominant trait displaying incomplete penetrance, as not all persons carrying a mutation in the protoporphyrinogen oxidase gene (*PPOX*) develop the clinical phenotype (Grandchamp *et al.*, 1996; Frank and Christiano., 1997). In heterozygotes, PPO activity is decreased by ~ 50% (Eales *et al.*, 1980). The PPO enzyme, localized on the cytosolic surface of the inner mitochondrial membrane, catalyzes the six-electron oxidation of protoporphyrinogen IX to protoporphyrin IX, using molecular oxygen (Bickers *et al.*, 1993; Dailey and Dailey, 1996).

Apart from HCP, VP is the only porphyria to be associated with both the acute and cutaneous types of clinical features. A substantial, though unknown portion of the gene carriers are asymptomatic throughout their lives, and most gene carriers are asymptomatic before puberty. The most characteristic feature of VP is increased photosensitivity, subepidermal blistering with subsequent milia formation and overly fragility of skin in sun-exposed areas such as the face and the back of the hands, less commonly also the arms, legs and feet (Bickers *et al.*, 1993). When levels of the more hydrophilic porphyrins (uroporphyrin, the 7- to 5-carboxyl porphyrins and coproporphyrin) are predominantly accumulated, less rapid development of porphyrin-mediated phototoxic lesions occurs, as in VP. Mechanical fragility and subepidermal blistering, with subsequent milia formation and scarring of the light-exposed skin are common (Poh-Fitzpatric, 1998). The

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epidermis becomes detached form the dermis even with minor trauma, leading to bulla formation and erosions. These lesions heal slowly and often result in pigmented scars (Day, 1986). Homozygous inheritance of VP, unlike that of heterozygous VP, has an onset of symptoms in early childhood, and mental or growth retardation, chronic skin changes and deformed digits due to cutaneous photosensitivity, as well as seizures, are not uncommon (Bickers *et al.*, 1993; Roberts et al., 1995).

Avoidance of sunlight exposure, as far as possible, is the most effective method of evading the skin conditions associated with VP. Sensible clothing should be worn and only sunscreens containing zinc oxide or titanium dioxide must be used. These sunscreens filter out the longer wavelengths of light (Kirsch et al., 1998).

An acute attack is a life-threatening condition of which presentations have been reported between the ages of 7 and 75 (Jensen *et al.*, 1995). During an acute attack, an increase in the excretion of the porphyrin precursors δ-aminolevulinic acid and porphobilinogen occurs in the urine. In the faeces, an increase in the excretion of protoporphyrin and, to a lesser extent, of coproporphyrin is observed (Dean and Barnes, 1959; Eales, 1963; Da Silva *et al.*, 1995). The symptoms of the acute attack are basically the same for all of the acute porphyrias. Signs and symptoms include severe abdominal pain, neuropsychiatric manifestations, constipation, diffuse pain, vomiting, hypertension, tachycardia, fever, convulsions, sensory loss and neuromuscular weakness which may progress to quadriparesis and respiratory failure that can lead to coma and death. Although rarely seen unless the attacks have been multiple or for a long duration, permanent neurological lesions, especially parasympathetic dysfunction, can occur. During acute attacks a marked decrease in the levels of sodium, chloride,

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calcium and potassium in the blood is also observed (Crimlisk, 1997; Frank and Christiano, 1997; James and Hift, 2000).

Today the observed frequency of acute attacks, and especially resulting fatalities (which were estimated to be as high as 10%), are rare, because public knowledge and healthcare regarding the disease have improved (Lip *et al.*, 1993; Kirsh *et al.*, 1998). Normally acute attacks can be controlled, but this is variable and depends on the time delay from the onset of the attack to the point where treatment starts (Dean and Barnes, 1955; Dean, 1963; Eales *et al.*, 1980; Moore, 1980; Meissner *et al.*, 1987).

For the treatment of acute attacks, the first and most important intervention is to avoid factors that are known to precipitate the attacks (Sassa, 2002). No prophylactic therapy is available, but a number of steps can be taken if an acute porphyric attack does occur: As a first step, any possible precipitating factors should be withdrawn. As nausea and vomiting usually accompany the acute attack, sufficient hydration should be ensured. Oral carbohydrate supplements should be given to the patient, as carbohydrate loading can suppress porphyrin synthesis. If the supplement cannot be given orally, a dextrose-saline infusion can be administered (James and Hift, 2000).

Haematin, a drug found to be effective for an acute attack, can be administered. Haematin is thought to supplement the intracellular free haem pool and thus repress ALA synthetase (James and Hift, 2000). Unfortunately this drug is unstable and can cause renal failure, thrombophlebitis and dose-related coagulopathy (Bissell, 1988; Ashley, 1996). Haem arginate, a drug with similar effect, does not have the adverse effects attributed to haematin (Mustajoki and Nordmann, 1993). Another drug, somatostatin, decreases the tempo at which ALA synthetase is produced, and together with plasmaphoresis, has been used

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successfully in reducing pain and producing complete remission of symptoms in seven porphyric patients with acute attacks (Medenica *et al.*, 1997; James and Hift, 2000).

Opiates can be given to diminish the pain, and the patient can be sedated with phenothiazines. Nausea and vomiting also respond to this drug. Beta-adrenergic blockers can be used to control tachycardia and hypertension, and may also decrease the activity of ALA synthetase. If convulsions occur, diazepam and clonazepam may be used, where most other anticonvulsants are contra-indicated (James and Hift, 2000).

1.2.1 The Protoporphyrinogen Oxidase Gene (*PPOX*)

VP is caused by a molecular lesion in *PPOX*. This gene was first mapped to chromosome 14q32 by Bissbort *et al.* in 1988, but this map position could not be confirmed in a subsequent linkage study (Warnich *et al.*, 1996a). More recently *PPOX* was localized on chromosome 1q22-23 by means of fluorescence *in situ* hybridization (FISH) with a genomic clone of the gene (Taketani *et al.*, 1995). A publication by Roberts *et al.* (1995) supported the map location on chromosome 1, determined by means of microsatellite markers and multipoint linkage analysis, as well as FISH.

PPOX consists of 13 exons and 12 introns and spans ~ 5kb (Roberts *et al.*, 1995; Taketani *et al.*, 1995). The coding region of the gene spans 1 431 nucleotides and its mRNA (including a 5' and 3' untranslated region) spans 1.8kb, encoding a 477 amino acid protein with a calculated molecular mass of 50.8 kilodaltons (Nishimura *et al.*, 1995; Dailey and Dailey, 1996).

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The molecular heterogeneity of VP is vast, of which the mutational cause include single base substitutions (transitions and transversions) as well as deletions and insertions in *PPOX*, often resulting in unique mutations in different families (Frank and Christiano, 1997; Whatley *et al.*, 1999). Von Und Zu Fraunberg *et al.* (2002) reported that 111 mutations in *PPOX* have been reported worldwide and that no mutational hot spots have been identified. Of these mutations, 38 (34%) are small insertions or deletions, 44 (40%) are missense mutations, 17 (15%) are invariant nucleotides at splice sites, 1 (1%) is a gross deletion, and 11 (10%) create stop codons.

1.2.2 Biochemical Testing

The decreased activity of PPO in VP results in an increased activity of the initial and rate limiting enzyme of the haem biosynthetic pathway, ALA synthase in the liver, thus leading to overproduction and increased excretion of porphyrins and porphyrin precursors. Certain drugs, such as barbiturates and sulphonamides as well as a reduced caloric intake during dieting or fasting, lead to an induction of the hemoprotein cytochrome P450 (Moore and Hift, 1997). The increased synthesis of cytochrome P450 leads to an enhanced haem demand, followed by the decrease of the regulatory haem pool and an induction of ALA synthetase. As the induction of ALA synthetase leads to an increase of porphyrins and porphyrin precursors up to the step of the *PPO* deficiency underlying VP, VP usually presents with a distinct porphyrin excretion pattern, making it possible to distinguish it from other types of porphyrias by means of quantitative analyses of blood, stool and urine samples (Dailey and Dailey, 1996).

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The exact nature of a porphyria can be determined by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) of porphyrins extracted from stool, urine and blood. The early haem precursors are largely water-soluble and are therefore found in the urine, while the distal intermediates are hydrophobic and are found in the stool. The diagnosis is made on finding the typical pattern of excretion for VP. See Kirsch *et al.* (1998) for typical stool porphyrin data.

However, biochemical testing is not always accurate, as the extracted porphyrin concentrations vary widely within and between individual patients, and stool porphyrin levels do not reflect intracellular porphyrin levels accurately (Kirsch *et al.*, 1998). Biochemical testing for VP with urine samples are ineffective, as the urinary porphyrin excretion found in non-acute VP patients is highly variable and may be completely normal. The most consistent changes are mild elevations in copro- and uroporphyrin, but these are only significantly raised in about 30% of non-acute VP subjects. There are, however, significant elevations in the concentration of all urinary porphyrins during the acute attack of VP (Frank and Christiano, 1997).

The chromatographic analysis of plasma porphyrins are generally troublesome, but fluorescence emission spectra between 621 and 627 nm, when plasma samples are excited at 405 nm, is a highly specific and sensitive marker for VP (Corey *et al.*, 1980; Poh-Fitzpatrick, 1980; Long *et al.*, 1993). Porphyrin abnormalities may, however, still occur in lead poisoning, sideroblastic and haemolytic anaemia, iron deficiency, renal failure, cholestasis, liver disease and gastrointestinal hemorrhage (Lim *et al.*, 1992).

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1.2.3 History of VP in South Africa

The incidence of variegate porphyria in South Africa is the highest in the world and the disease is one of the most common monogenic inherited disease in South Africa (Eales *et al.*, 1980). During the late 1940s to the mid 1960s, Geoffrey Dean traced the history of VP in South Africa back to the marriage between Gerrit Jansz van Deventer, one of 40 free burghers in the Cape of Good Hope community, and Ariaantje Adriaansse, or Jacobs (personal communication, J van Deventer), an orphan from Rotterdam sent to the Cape as one of a group of young Dutch women as potential wives for the free burghers, in 1688 (Dean and Barnes, 1955; Dean, 1963; Dean, 1971; Eales *et al.*, 1980; Meissner *et al*, 1987; Stine and Smith, 1990).

There was no selective pressure against VP, and because of the healthiness and fecundity of the descendants, the disease was easily established within the families in the closed community (Eales *et al.*, 1980). During the 1800s, the community was almost totally cut off from Europe and in only 85 years the population size increased from ± 1 000 to 17 000 (Jenkins, 1996), thus the disease became common among Caucasian Afrikaans-speaking South Africans (Afrikaners). Today, the disease is not confined to the Afrikaner anymore, but because of admixture it is also present in the English-speaking Caucasian population as well as in the population of mixed ancestry (Coloureds).

The last quarter of the 19th century witnessed the discovery of modern pharmaceutical agents and changed the nature of VP. The first published description of a South African patient with acute porphyria appeared in the University of Cape Town medical students' journal, I'nyanga, in October 1939. The authors were medical students Lennox Eales and Jack Chait. H.D. Barnes of the South African Institute for Medical Research was the first to describe a

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series of cases of 'porphyrinuria' in South Africa (Barnes, 1951; Jenkins, 1997). Barnes, together with Geoffrey Dean, a Port Elizabeth physician who had recognized that the disease could manifest with skin lesions, acute attacks or both, called it 'porphyria variegata' and 'South African genetic porphyria' (Dean and Barnes, 1955). Dean's genealogical studies subsequently identified the founding couple from which South African VP patients are descended (Dean, 1971).

About 3 in every 1000 Caucasian South Africans, or 1 in every 350 Caucasians of the Eastern Cape, are thought to be affected by the disease (Dean, 1960; Day, 1986; Bickers *et al*, 1993). In a preliminary study by Dean and Barnes (1958), 1 253 Caucasians, consisting of patients from a mental institute and nurses from four hospitals in the Eastern Cape, were tested for VP by means of biochemical analysis of urine and stool samples. They concluded that about 1% of South African Caucasians may be carriers of the VP founder mutation.

Then, in a follow-up study between 1 April 1959 and 31 March 1960, 6 458 stool samples from patients admitted to the Provincial Hospital in Port Elizabeth and St. Joseph's Hospital (also in Port Elizabeth) were biochemically tested for VP prior to administration of thiopentone. A small fragment of stool on a glass rod or wooden stick was inserted into a test tube containing 2 ml of equal parts of amyl alcohol, glacial acetic acid and ether. The solvent was stirred until a light-brown colour was reached, and the liquid was subsequently decanted into a clean test tube. By examining the sample in UV light using a Wood's filter, porphyric stool showed a brilliant pink fluorescence, while a normal sample was green or gray. Stool samples of patients detected by the routine test were sent for quantitive analysis of the stool porphyrins. In this 12-month study, 29 out of 6 458 patients tested were diagnosed with VP, therefore about one in every 223 patients tested. As the number of porphyrics administered to hospital was likely to be higher than

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that in the general population (because of acute attacks), Dean estimated the frequency of VP to be one in 350 among the Caucasian population of the Eastern Cape (Dean, 1960).

It was estimated that 10 000 – 20 000 South Africans were affected by VP in the 1960s (Dean, 1960; Eales *et al.*, 1980). Because of the mixing with the more recent immigrants from Britain and other countries, the VP founder gene can be expected to be present in members derived from these populations too (Jenkins, 1997). It is thus not restricted to the Afrikaner population, which is generally seen as the descendants of the early settlers, any more. Some reports estimate that 80% of VP carriers are undiagnosed, and the disease thus poses a serious risk in terms of potentially fatal acute attacks (Day, 1986; Grandchamp *et al.*, 1996). Up to 40% of these individuals are 'silent' gene carriers, not showing clinical or biochemical signs of the disease other than the reduced activity of protoporphyrinogen oxidase (Day, 1986). Porphyria cutanea tarda and acute intermittent porphyria are also common in South Africa, but not as common as VP (Meissner *et al.*, 1987).

1.2.4 The R59W Mutation

The mutation that either Ariaantje Adriaansse (or Jacobs) or Gerrit Jansz van Deventer brought into South Africa, and which today is regarded as a founder mutation, is the R59W mutation in *PPOX*. It consists of a C to T transition in exon 3 of the gene, resulting in an arginine to tryptophan substitution in codon 59 of *PPOX* (Meissner *et al.*, 1996; Warnich *et al.*, 1996b). By means of linkage disequilibrium and haplotype analysis, Groenewald *et al.* (1998) showed that the R59W mutation in South Africa is not due to multiple mutation events on a common haplotype, but to a common ancestor in the majority of cases.

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The R59W mutation is situated in the FAD dinucleotide-binding motif of the encoding enzyme, resulting in a disruption in the protein and a subsequent virtually undetectable catalytic rate (Dailey and Dailey, 1996; Meissner *et al.*, 1996). The mutation was also found to alter a *β*-amphiphatic region of the wild type protein, which might cause a disruption in the protein structure (Warnich *et al.*, 1996b). The mutation abolishes an *AvaI* restriction site and creates a *StyI* restriction site in exon 3 (Meissner *et al.*, 1996; Warnich *et al.*, 1996b), which allows for a simple diagnostic test.

R59W was found to be present in 26 of 27 South African VP families (96%) investigated by Meissner *et al.* (1996) and in 15 of 17 families (88%) studied by Warnich *et al.* (1996b). Except for the R59W mutation, nine other mutations causing VP have been identified in South Africa, namely H2OP (Warnich *et al.*, 1996b; Hift *et al.*, 1997), R168C (Meissner *et al.*, 1996; Warnich *et al.*, 1996b), 537delAT (Corrigall *et al.*, 1998), Y348C and R138P (Corrigall *et al.*, 2000) and V290M, L15F, c769delG;770T>A and Q375X (Corrigall et al., 2001). These other mutations are very rare and were only detected in single families.

1.3 Population Screening Studies

In any population screening program certain requirements must be met. Strachan and Read (1999) summarize them as follows:

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1. A positive result must lead to some useful action, for example
   a. Preventative treatment, such as a special diet for phenylketonuria.
   b. Review and choice of reproductive options in cystic fibrosis carrier screening.

2. The whole project must be socially and ethically acceptable
   a. Subjects must opt in with informed consent.
   b. Screening without counseling is unacceptable.
   c. There must be no pressure to terminate affected pregnancies.
   d. Screening must not be seen as discriminatory.

3. The test must have a high sensitivity and specificity
   a. Tests with many false negatives undermine confidence in the program.
   b. Tests with many false positives, even if these are subsequently filtered out by a definitive diagnostic test, can create unacceptably high levels of anxiety among normal people.

4. The benefits of the program must outweigh its costs
   a. It is unethical to use limited health care budgets in an inefficient way.

When a large sample is tested, special methodology is also needed for the collection and storage of the sample, the extraction of DNA and the assay used for mutation detection. These methods must take into consideration the cost as well as the time needed to test an individual. The following section contains a few examples of population screening studies.

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1.3.1 Collection of Samples

1.3.1.1 Guthrie Cards

a) Hamvas *et al.* (2001)

To determine the population-based frequency of a rare mutation, the 121ins2 mutation in the surfactant protein B gene, Hamvas *et al.* (2001) obtained a sample of 10 044 on Guthrie cards from the Missouri Department of Health.

b) Olynyk *et al.* (1999)

To assess the prevalence and clinical expression of the hemochromatosis (*HFE*) gene, a population based study in Busselton, Australia was conducted. Hereditary hemochromatosis is associated with homozygosity for the C282Y mutation in the HFE gene on chromosome 6, elevated serum transferring saturation and excess iron deposits throughout the body. Blood from 3011 unrelated Caucasian adults were collected on Guthrie cards to screen for the presence or absence of the C282Y mutation and the H63D mutation (which may contribute to increased hepatic iron levels).

c) Todorava *et al.* (1999)

Four hundred Gypsy newborns from northeast Bulgaria were screened for C283Y, a point mutation in the γ-SG gene (13q12), which among others causes limb-girdle muscular dystrophy type 2C. This autosomal recessive disorder is clinically manifested as an early onset, severe Duchenne-like muscular dystrophy. Blood from the 400 Gypsy newborns were obtained on Guthrie cards from a previous Phenylketonuria mass screening program.

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1.3.1.2 Whole Blood

a) Pastinen *et al.* (2001)

Thirty one rare and common disease mutations underlying 27 clinical phenotypes were monitored in a sample of 2 151 whole blood specimens from four geographical areas representing early and late settlement regions of Finland. Some of these samples were obtained as isolated DNA, 510 from a previous large epidemiological population-based study and 495 samples from the Finnish Twin Cohort Study. The rest of the samples were obtained from random blood donors as whole blood.

b) De Villiers *et al.* (1999)

To determine the spectrum of sequence variants in the haemochromatosis (*HFE*) gene, mutation analysis was performed on DNA of 965 individuals from four different ethnic groups in South Africa. The sample was collected as whole blood.

1.3.2 DNA Extraction

1.3.2.1 Chelex

a) Hamvas *et al.* (2001)

A 3 mm Guthrie card disc of each blood sample was heated in 2% Chelex in a 96 well microtiter plate format to extract the DNA.

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b) Olynyk *et al.* (1999)

DNA was extracted from the blood spots on the Guthrie cards using a Chelex method as described by Singer-Sam and Tanguay (1989).

1.3.2.2 Whole Blood

a) Gaunt *et al.* (2001)

2 743 middle-aged Caucasoid males were screened for 11 markers across the insulin-like growth factor 2 (*IGF2*) gene to determine if there is an association between these single nucleotide polymorphisms and body mass index. The samples used was attained from the NPHSII cohort, a set of previously collected and extracted whole blood samples from nine widespread UK general practices. The sample obtained form the NPHSII cohort was previously extracted from whole blood using a standard lysis method.

b) Han *et al.* (2001)

DNA was previously extracted from whole blood by means of a standard lysis method.

c) Pastinen *et al.* (2001)

The sample of 510 individuals obtained from the previous epidemiological population-based study and the sample of 495 individuals from the Finnish Twin Cohort Study were already extracted from whole blood, and the sample obtained

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from blood donors was extracted using a standard whole blood DNA extraction protocol.

d) De Villiers *et al.* (1999)

Genomic DNA was extracted form whole blood using a standard lysis method described by Miller *et al.* (1988).

1.3.2.3 Others

a) Todorava *et al.* (1999)

No DNA extraction was needed in this study, because PCR was done directly on a disc of the Guthrie card.

1.3.3 Mutation Detection Methods

Various mutation detection methods were used in these studies, such as restriction enzyme (RE) digestion, single strand conformational polymorphism (SSCP) analysis and DNA-arrays. In some of these studies one method was used to detect a mutation and another to confirm the result.

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1.3.3.1 Restriction Enzyme (RE) Digestion

a) Hamvas *et al.* (2001)

Restriction enzyme digestion with *SfyI* was performed on the amplified DNA for detection of mutation 121ins2 in the surfactant protein B gene.

b) Olynyk *et al.* (1999)

After PCR amplification of the area containing the gene in question, restriction-enzyme digestion was used to identify the C282Y mutation in the *HFE* gene. If the C282Y mutation was present, the individual was also screened for the H63D mutation via restriction enzyme analysis.

1.3.3.2 Single Strand Conformation Polymorphism (SSCP) Analysis

a) Gaunt *et al.* (2001)

SSCP analysis of overlapping polymerase chain reaction (PCR) amplicons spanning 200 – 250bp, as well as denaturing high performance liquid chromatography (DHPLC) were performed to identify new SNP markers in the IGF2 gene. Samples displaying a polymorphism were subsequently sequenced. Known polymorphisms were detected by RE digestion or ARMS PCR amplification (including an internal control), both of which were subsequently electrophoresed in 96-well, 192-well or 384-well microplate array diagonal gel electrophoresis (MADGE) gels as described by Gaunt *et al.* (2000).

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b) Todorava *et al*. (1999)

The sample was screened using SSCP analysis, and all heterozygote samples found were digested with *RsaI* restriction enzyme to confirm the presence of the C283Y mutation in the γ-SG gene.

c) De Villiers *et al*. (1999)

This population screening approach utilized a combined heteroduplex and single-strand conformational polymorphism (HEX-SSCP). Where possible, restriction enzyme analysis was performed to verify the sequence changes in PCR amplified DNA, and for mutations not involving restriction enzyme recognition sites, bi-directional sequencing was used.

1.3.3.3 Other

a) Han *et al*. (2001)

DHPLC was performed on a WAVE DNA analysis system by using a DNASep system (Transgenomic). The DNASep columns contain non-porous alkylated polystyrene-divinylbenzene particles that are both electrically neutral and hydrophobic, thus the negatively charged phosphate ions of the DNA molecules cannot bind to the column unaided. Triethylammonium acetate (TEAA) is a positively charged reagent that facilitates interaction between the stationary matrix and the DNA molecules. By altering the ratio of TEAA to acetonitrile, the hydrophobic interaction between the alkyl chains of TEAA and the stationary phase of the column is reduced, thus allowing the DNA fragments to be eluted. DNA molecules eluted from the column are detected by scanning with a UV-C

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detector. At a temperature that only partially denatures heteroduplexes, these molecules are destabilized by the mismatched bases and are therefore slightly more melted than the homoduplexes, resulting in earlier eluation.

Denaturing high performance liquid chromatography (DHPLC) is a modification of the basic gel-based heteroduplex mutation analysis method. It relies upon the rapid separation and visualization of homo- and heteroduplex DNA molecules by using an ion-pair reverse-phase liquid chromatography system. The sensitivity of the analysis is maximized by maintaining the HPLC column at a temperature that favours partial strand denaturation in the presence of base-pair mismatching.

b) Pastinen *et al*. (2001)

DNA samples were screened by means of a DNA-array. This DNA-array was constructed for detection of 31 common mutations in the Finnish population. A pair of allele-specific primers for each of the mutations was spotted on derivatized microscopic glass-slides. Eight replicated arrays were spotted onto each slide. Custom-made reaction chambers were used to analyze up to 80 separate samples per slide, thus 2 480 mutations could be monitored on a single microscopic glass-slide. Allele-specific primer extension reactions provided reliable discrimination between the genotypes. The genotypes of carriers identified by the array-based screening were confirmed using PCR-RFLP digestion or allele-specific PCR with an internal control amplicon.

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1.4 *Motivation for this study*

According to Day (1986) an estimated 10 000 – 20 000 South African individuals are carriers of the R59W mutation, and as many as 80% of these gene carriers have not yet been diagnosed and are therefore at risk of potentially lethal acute neurological attacks. However, during a study conducted at Tygerberg Hospital (1997 – 1998), only 19.3% (81 / 419) apparently unrelated South African patients of European descent, referred for a DNA diagnosis of VP using RE analysis, tested positive for the R59W mutation (De Villiers *et al.*, unpublished results). This relatively low detection rate of the R59W mutation among general referrals is probably a consequence of inappropriate clinical and/or biochemical disease assignment and emphasizes the pivotal role of genetic analysis in disorders characterized by clinical features considered to be non-specific. Forty-two patients previously diagnosed with VP based on biochemical testing of stool, urine and blood tested negative for the founder mutation and were subsequently screened for mutations in the promoter and coding region of *PPOX* using HEX-SSCP analysis (Kotze *et al.*, 1998). No disease-causing mutation could be identified in these patients.

This poses a serious question regarding the actual frequency of VP, and specifically the founder gene mutation, in South Africa. Seeing that the original estimated frequency of VP in South Africa (Dean, 1960) was based on clinical expression (known to be highly variable and unspecific) and biochemical tests that are often inconclusive, the question arises whether there are thousands of undiagnosed VP carriers, as genealogical studies and population growth curves have predicted, or is the allele frequency much lower than the 0.3% which is still widely cited in almost every publication on VP (e.g. Sassa, 2002; Von Und Zu Fraunberg, 2002)?

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By re-estimating the frequency of the founder mutation using current rapid and unequivocal DNA diagnostic tests, a more precise estimation can be made. These results can be used to recommend appropriate genetic testing in the future. If found that a large number of South Africans are unaware that they are silent VP carriers, this finding will be publicized widely. By alerting both physicians and individuals form the "at-risk" population groups, and underlining the importance of accurate diagnosis, the risk of life-threatening acute attacks in genetically susceptible individuals could be minimized. If, on the other hand, the incidence of the R59W mutation is less than currently believed, it will also be publicized to avoid inappropriate clinical or biochemical disease assignment ("overdiagnosis") in the future.

1.4.1 Aims of this study

This study had the following objectives:

1. To determine with greater precision the frequency of the R59W founder mutation among the Caucasian South African population.

2. To screen the population of mixed ancestry (Coloureds) and to determine the frequency of the R59W founder mutation in this population. It has previously been demonstrated that founder mutations in the LDL receptor gene provide evidence of Caucasoid admixture and contribute significantly to the familial hypercholesterolemia phenotype in the Coloured population (Loubser *et al.*, 1999).

3. To determine what percentage of the population are silent gene carriers and unaware of their VP carrier status.

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To achieve these objectives, special techniques that are simple, rapid and inexpensive had to be employed. In the words of Leung *et al*. (2001): "The ideal high-throughput mutation screening method should be cheap, not requiring expensive equipment with high running and maintenance cost, it should be simple, no more complex than conventional electrophoresis, and it must be fast." To be statistically meaningful, a large quantity of samples had to be screened. The following specific aims were established:

1. Initial collection of 4 000 – 5 000 blood samples.

2. Development of information forms on VP for patients to read and take home.

3. Development of a shorter informed consent form. In order to collect a large number of blood samples under special conditions (for example in maternity wards) and to simplify the storage of these blood samples, it was necessary to condense the lengthy informed consent form generally used. Other vital information required with the sample was also acquired via this form.

4. Comparison of DNA collection and storage methods appropriate for the large quantity of blood samples to be collected.

5. Comparison of rapid and cost-effective DNA isolation methods.

6. Employment of a simple and inexpensive method for detecting the R59W mutation.

7. Development of a database to record individual samples collected and tested.

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Chapter Two – Material and Methods

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2.1 Ethical Approval

Ethical approval for this study was given by the Ethical Committee of the Faculty of Health Sciences, University of Stellenbosch. The study was also approved by the Tygerboland Onafhanklike Ginekologiese Assosiasie (TOGA), the Medi-Clinic group, the Netcare group, PathCare, Ampath, Lancet Laboratories, and the South African Blood Transfusion Services.

2.2 The Study Populations

The VP gene frequency of 0.3% was initially described in the Caucasoid population of European decent (Dean, 1960), and for comparison purposes, the current study had to use this population as well. Areas with a high concentration of especially Afrikaans-speaking Caucasians were focused on, and for control purposes, at least three geographical areas within South Africa (Western Cape, Gauteng and the Free State) were included. People of mixed ancestry (Coloureds) were also included, however, no persons wanting to participate in the study were excluded on grounds of language preference or race. Collaboration with the South African Blood Transfusion Services, various pathology laboratories and maternity units of hospitals were established and obstetricians, hospital administrators and other personnel at these various organizations were visited to inform them of the project and to discuss possible logistic problems. They were asked to encourage white Afrikaans speaking and Coloured patients to participate. Information forms, informed consent forms and either Guthrie cards (Schleicher and Schuell) or FTA cards (Whatman BioScience) were distributed. Information such as the home language of the family, race and VP status of the parents and the extended family were recorded.

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All samples were obtained anonymously, except in cases where the donor (or the parents of newborns) wished to know the result of the test. In these cases names and addresses of the patients and their physicians were recorded.

2.2.1 Blood Donor Clinics

A total of 2 380 samples were obtained by attending the Blood Transfusion Services clinics in the Western Province, Free State and Gauteng. Blood donors were informed about the project and asked to participate. After the donor completed the informed consent form, some of the excess blood from the finger prick (used for testing blood Fe levels) was spotted on a Guthrie card. The card was allowed to dry (10 – 15 min) and stored in a cool, dry place until DNA extraction could be performed.

Samples obtained from clinics attended were immediately collected, and samples obtained from clinics in Gauteng and the Free State, after the initial three-week personal attendance, were posted to Stellenbosch by the clinic personnel at regular intervals. Samples in the Western Cape were collected between June 2000 and December 2001 and samples in Gauteng and the Free State were collected from February 2002 until May 2002.

2.2.2 Pathology Clinics

Pathology clinics in the Western Cape (PathCare), the Free State (PathCare) and Gauteng (Lancet Laboratories and Ampath) were asked to participate in collection of the 853 samples from patients referred for a routine test, such as an HIV test. Personnel asked the patients for their participation. An EDTA tube of

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blood was drawn from willing participants, and the blood was stored at 4°C until the samples were collected. Western Cape samples were collected fortnightly and samples from the Free State and Gauteng were sent to us within two weeks of collection. All samples were spotted onto Guthrie or FTA cards at the laboratory in Stellenbosch, except for samples obtained from Lancet Laboratories (Gauteng), which were spotted on Guthrie cards before it was sent to Stellenbosch. These samples were sent to us at regular intervals, usually in batches of 100. During our initial visit to clinics in Gauteng and the Free State, samples obtained at the various pathology clinics were spotted and collected within a week after it was obtained.

Samples obtained from pathology clinics in the Western Cape were collected from November 2000 until May 2002 and samples form clinics in Gauteng and the Free State were collected form March 2002 until May 2002.

2.2.3 Maternity Wards

Maternity wards of hospitals in the Western Cape participated in the collection of umbilical cord blood samples from 839 newborn babies. Stellenbosch Medi-Clinic (MC), Somerset-West MC, Durbanville MC, Paarl MC, Worcester MC, Milnerton MC, Panorama MC, Louis Leipoldt MC, N1 City (Netcare) and Tygerberg hospital (Provincial Administrator of the Western Cape) participated in this part of the sample collection. The neonatal ward personnel or the obstetrician, before labour, obtained informed consent from the mother, and some of the blood present in the umbilical cord was spotted on a Guthrie card. The samples were collected fortnightly from the maternity wards from November 2000 until May 2002.

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2.3 DNA Isolation

2.3.1 Chelex

Four discs were removed from a sample on a Guthrie card (Schleicher and Schuell) using a 1.2 mm punch (Whatman BioScience), and inserted into a sterile 1.5 μl microfuge tube. 600 µl TE-buffer (0.1 M TRIS HCl, pH 7.6 and 0.01 M EDTA, pH 8.0) was added to the discs before the contents of the microfuge tube was incubated at room temperature for an hour and occasionally mixed by inversion. Subsequently the microfuge tube was centrifuged for 3 min at 13 500 rpm (16 200 x g) and the supernatant carefully poured off, leaving ~30 μl of TE-buffer and the discs behind. Another 600 μl TE-buffer was added to the microfuge tube. The microfuge tube was inverted occasionally while incubated at room temperature for 10 min before it was centrifuged at 13 000 rpm (16 200 x g) for 3 min. All but ~30 μl of the supernatant was removed and 200 μl of 10% (10 g / 100 ml H_2O) Chelex was added to the pellet. As EDTA is a PCR inhibitor and Chelex is rich in EDTA, the Chelex working stock was washed before being used by removing as much water from the Chelex stock as possible without loosing any beads and replacing it with sdH_2O. The microfuge tube was then incubated in a water bath at 56 °C (±2 °C) for 30 min, where after it was vortexed for 10 sec and centrifuged at 13 500 rpm (16 200 x g) for 20 sec. It was then incubated at 100 °C (±2 °C) for 8 min in a heating block. The lid of the microfuge tube was punctured to prevent it from popping open at this high temperature.

The tube was centrifuged at 13 500 rpm (16 200 x g) for 3 min and subsequently the supernatant contained the DNA, which was now ready to be used as template in a subsequent manipulation. The DNA was stored at 4 °C and used

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within 48 hours after extraction. Care was taken not to carry any Chelex beads over when the DNA was used.

2.3.2 FTA Purification Solution

A clean 1.2 mm punch was used to remove one disc from the middle of the bloodstain on the FTA card (Whatman BioScience) and to place it into a sterile 200 μl microfuge tube. 100 μl FTA purification solution (Whatman BioScience) was added to the disc and vortexed at slow speed for 2 sec, followed by a 5 min incubation at room temperature with another 2 sec low speed vortex halfway through and at completion of the incubation. As much of the FTA solution as possible was removed with a pipette. Another 100 μl FTA purification solution was added to the tube and the above procedure repeated. After a third wash cycle, 100 μl TE-buffer was pipetted into the amplification tube to rinse off any residual FTA purification solution still present. After a 5 min incubation at room temperature, with brief vortexing halfway and at completion, as much of the TE-buffer as possible was removed. The FTA card disc was used in subsequent PCR reactions.

2.3.3 Whole Blood extraction

Whole blood extraction was performed according to a protocol by Miller *et al.* (1988). 5 ml whole blood was placed in a 50 ml Falcon tube and 30ml of cold lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.0001 M EDTA, pH 7.4) was added. The tube was placed on ice for 15 min and shaken every 5 min. Subsequently it was centrifuged for 10 min at 1 500 rpm (201 x g). The supernatant was removed and the pellet washed with 10 ml PBS (0.027 M KCl,

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0.137 M NaCl, 0.008 M Na_2HPO_4, 0.0015 M KH_2PO_4). The Falcon tube was centrifuged at 1 500 rpm (201 x g) for another 10 min before the supernatant was carefully removed and discarded. The pellet was dissolved in 3 ml nuclei lyses solution (0.01 M Tris HCl, 0.4 M NaCl, 0.002 M EDTA, pH 8.2), 10 mg/ml protease K (Roche) and 300 μl 10% SDS (w/v), and incubated at 55°C overnight.

The next morning 1 ml 6M NaCl was added and the Falcon tube was shaken vigorously for 1 min and subsequently centrifuged at 2 500 rpm (559 x g) for 15 min. The supernatant was transferred to a new Falcon tube, taking care not to carry over any of the pellet or foam. Two volumes of 100% EtOH (v/v) were added to precipitate the DNA. The DNA was transferred to a 1.5 ml microfuge tube, 1 ml 70% EtOH was added and the tube was centrifuged at 13 500 rpm (16 300 x g) for 5 min. The supernatant was subsequently removed and the microfuge tube was left uncapped for 10 min to allow the EtOH to evaporate. Depending on the size of the pellet, 200 – 800 μl dH_2O was added, and the DNA was resuspended by placing it on a shaker at low speed for an hour. The DNA was subsequently stored at -20°C.

2.4 PCR amplification

Amplification of the R59W mutation region via the polymerase chain reaction (PCR) was performed to acquire adequate quantities of DNA to perform the different mutation detection techniques. Various primer combinations were utilized in order to obtain fragments best suited for subsequent mutation detection via ARMS, RE analysis and SSCP analysis. These primers are listed in Table 2.1, and their positions indicated on the relevant portion of the PPOX sequence in Table 2.2.

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Special care was taken to prevent contamination of samples before PCR amplification. All the pipettes used were regularly sterilized soaking it in a 20% liquid bleach solution for 20 min. The pipettes were also sterilized by placing it under UV light in a Biocap (Erlab) cabinet for 15 min prior to usage. All pipetting was performed with filter tips, and a positive and negative control reaction was always included in every group of simultaneous amplification reactions. Only a small quantity of primer working solution was prepared at one time, and aliquots of each working solution were made.

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Table 2.1: Summary of primers used in this study

	Primer name	Primer sequence	T_m	Reference
Forward	PPO2F	5' – CTT CTG GAG CGC AGG TTG TC – 3'	73	Warnich *et al.*, 1996(b)
	PPO3F	5' – TGC AGT GTC TCT CCC TCT TGT– 3'	55.6	Warnich *et al.*, 1996(b)
	BF	5' – TGG TCC TAG TGG AGA GCA GT – 3'	51.4	Warnich, unpublished
	PPO10F	5' – AGA GCC CTN TTC CTT CTG ACG CAT G – 3'	81	Warnich *et al.*, 1996(b)
Reverse	PPO3R	5' – GCT CCC CTA AAC TCT ATT CC – 3'	52.2	Warnich *et al.*, 1996(b)
	PPO4R	5' – GAG GGC ACA GTA AAA GGA GC – 3'	56.6	Warnich *et al.*, 1996(b)
	PPOXAR	5' – GGC TGG CCT AAT TCA CCA – 3'	69	This study
	PPO3AR	5' – GCT GGC CTA ATT CCC CG – 3'	47	This study
	PPO10R	5' – TGG CCT TGC CTA CAA TGG AGC AC – 3'	81	Warnich *et al.*, 1996(b)

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Table 2.2: Primer annealing positions indicated on the PPOX sequence. (T) = R59W mutation, a C to T transition.

961 ccaccagccc atccgtgcac acttagtttc ccctaaagca gtgagtggcc gggatagaac
1021 tcaaaaccgg cggggcttct ggagcgcagg ttgtcccgg tctgcctgtc catatcgccc
1081 cctttccccc aggtttccgc atgggccgga ccgtggtcgt gctgggcgga ggcatcagcg
1141 gcttggccgc cagttaccac ctgagccggg ccccctgccc ccctaaggtg agtgctccac
1201 ttgtgccaga gggagcttca tttaatgctc ttcccatttc catcaaaagc tagatggatc
1261 ctggccctct gaatatgcct cttacccctc cctactgacc tctcggcggc ggctacaggc
1321 ggtgcgtgca gtgtctctcc ctcttgtcgc caggtggtcc tagtggagag cagtgagcgt
1381 ctgggaggct ggattcgctc cgttcgaggc cctaatggtg ctatctttga gcttggacct
1441**(T)**ggggaatta ggccagcgggagccctaggg gcccggacct tgctcctggt gagaggcttg
1501 tgggatgtct aggagaggtt gtggaggggg cttccattgg ggaatagagt ttaggggagg
1561 aagtatgttt ggtgggtcag atcttccctt agtttctcct cttctgaggg catgtggaga
1621 gcaggtttct gagcttggct tggattcaga agtgctgcct gtccggggag accacccagc
1681 tgcccagaac aggttcctct acgtgggcgg tgccctgcat gccctaccca ctggcctcag
1741 gtaacaccag cacctccgct ccttttactg tgccctcatc ctcatatgcc ttccatttct

4321 tgtaatccca gcattttggg aggccgaggt gggcagataa caaggtcagg agttcgagac
4381 cagcctggcc aacatggtga aaccccatct ctatgaaaaa tacaaaaatt agccaggtgt
4441 ggtggcttgc acctgtaatc tcagctactc gggaggctaa ggcaggagaa tcgcttgaac
4501 ccaggaggtg gaggttgcag tgagctgaga tctcgccatt acactccagc ctgggtgaca
4561 gagtgagact ctgtcccccc caccccccca aaaaaatggg aaggagagac agcctcagct
4621 agagcccttt ccttctgacg catgaatgtc cttctctcca gggatttgga catttggtgc
4681 catcttcaga agatccagga gtcctgggaa tcgtgtatga ctcagttgct ttccctgagc
4741 aggacgggag cccccctggc ctcagagtga ctgtgaggag gaggaaactt tgcctagtgg
4801 catttccaga gggctcctct gtgctccatt gtaggcaagg ccagactgat cagtgctata

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PCR primer stock solutions of 100 pmol/μl were diluted to 15 pmol/μl working solutions. The following PCR program was used for DNA amplification in a GeneAmp PCR System 9 700 PCR cycler (Applied Biosystems)(Table 2.3).

Table 2.3: PCR program used for amplification of DNA

PCR step	Description	#of cycles	Temperature	Duration
1	Denaturation	1	95	7min
2	Denaturation	0 - 10	95	30sec
	Annealing 1		55 – 65	30sec
	Extension		72	30sec
3	Denaturation	30 - 40	95	30sec
	Annealing 2		55 – 60	30sec
	Extension		72	30sec
4	Extension	1	72	7min

$MgCl_2$ concentrations of between 0.5 mM and 2.5 mM were tested. The total reaction volume for all reactions was 25 μl, unless stated otherwise. PCR reactions were performed in either 200 μl microfuge tubes or microtiter plates.

The PCR mixture for optimal amplification using the PPO2F and PPO4R primer pair contained the following: 1x PCR buffer (Bioline), 1.0 mM $MgCl_2$, 200 μM of each dNTP [2'-deoxyadenosine-5'triphosphate (dATP), 2'-deoxycytidine-5'triphosphate (dCTP), 2'-deoxyguanosine-5'triphosphate (dGTP) and 2'-deoxythymidine-5'-triphosphate (dTTP)], 0.32 mM of each of the PPO2F and PPO4R primers, 1 U Bioline Biotaq DNA polymerase and 1μl gDNA extracted from whole blood or Guthrie cards. The PCR program used for optimum amplification is shown in Table 2.3, with annealing temperature one as 62°C (10 cycles) and annealing temperature two as 57°C (40 cycles).

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The PCR mixture for optimal amplification using the PPO3F and PPO3R primer pair contained the following: 1x PCR buffer, 0.75 mM $MgCl_2$, 250 μM of each dNTP, 0.75 mM of the each of the PPO3F and PPO3R primers, 1 U Bioline Biotaq DNA polymerase and 1 μl Chelex extracted DNA or the disc prepared for PCR with FTA purification reagent. A reaction volume of 20 μl was found to be sufficient for SSCP analysis. Annealing temperatures of 64°C (10 cycles) and 60°C (30 cycles) provided the best amplification.

For optimum amplification with the PPO3F and PPO4R primer pair, the following reagent concentrations were used: 1x PCR buffer, 1.0 mM $MgCl_2$, 200 μM of each dNTP, 0.6 mM of the each of the PPO3F and PPO3R primers, 1 U Bioline Biotaq DNA polymerase and 1 μl Chelex extracted DNA or the disc prepared for PCR with FTA purification reagent. A reaction volume of 50 μl was initially used, but was later downscaled to 25 μl. A PCR annealing temperature of 64°C for cycle one (10 cycles) and 59°C for cycle two (30 cycles) were used.

2.5 Mutation Detection Methods

2.5.1 Restriction enzyme (RE) analysis

PCR for RE analysis was performed on whole blood and Chelex extracted DNA, with the PPO2F and PPO4R or the PPO3F and PPO4R primer pairs.

The PCR product of both the PPO2F and PPO4R and PPO3F and PPO4R primer pairs were cut with *StyI* by adding 5 U (0.5 μl) *StyI* (Roche), 2 μl buffer (Roche) and 7.5μl H_2O to 10μl amplified DNA and incubating the mixture overnight at 37°C. The digested fragments were separated on a 20 ml 12% PAA

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gel (6 ml 40% poly-acrylamide, 6 ml 5x TBE and 4 ml H_2O, polymerized with 50 μl APS (25% w/v) and 20 μl TEMED). 5 μl loading buffer (0.25% w/v Bromophenol Blue in dH_2O) was added to 10 μl of the digested DNA and loaded onto the gel. The gel was run at 200 V for 1.5 h and visualized under UV light subsequent to EtBr staining.

2.5.2 Single Strand Conformation Polymorphism (SSCP) analysis

2.5.2.1 SSCP Procedure

The PCR product of the PPO3F and PPO3R primers, of either Chelex or FTA purification reagent extracted DNA, was used for SSCP analysis.

DNA amplified for SSCP analysis was run on 20% poly-acrylamide (PAA) gels (99 acrylamide: 1 bis-acrylamide, 1% cross linking). 5 ml 40% PAA stock, 3 ml 5x TBE and 2 ml H_2O, polymerized with 1.33 μl 10% APS (w/v) and 13.3 μl TEMED, were used for one Mighty Small (Hoefer Scientific Instruments) SSCP gel. Ten samples were loaded per gel in 1.5x TBE buffer.

6 μl 80% (w/v) formamide loading buffer was added to 14 μl PCR product. The sample was then denatured by keeping it at 97°C for 7 min in a PCR cycler and was subsequently placed on ice to keep it denatured until it could be loaded onto the gel. 3.5 μl of a sample was loaded per well, and the gel was run at 200 V for 2.5 hours. The fragments were subsequently visualized by silver staining.

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2.5.2.2 Silver Staining

The gel was covered with a fixing solution (10% (v/v) EtOH, 0.5% (v/v) acetic acid) for 10 min and placed on a belly dancer (Stovall Life Science, Inc.) at low speed, where after the fixing solution was discarded and the gel was rinsed twice by covering it with ddH_2O and placing it on the belly dancer for 1 min. The gel was then stained by covering it with a solution of 0.1% (w/v) $AgNO_3$ and shaking it on the belly dancer for a minimum of 10 min. The staining solution was discarded into a marked container for proper detoxification and the gel was rinsed by covering it with H_2O and shaking it for 5 sec. The gel was covered with 1.5% (w/v) NaOH solution to which 0.405% (v/v) formaldehyde was added just before use, as a developing solution. The developing solution was discarded into a marked container as soon as any bands became visible on the gel (after ~ 6 min), and the gel was rinsed at least 3 times with H_2O to prevent further development. The water used for rinsing was also discarded into the marked container and the gels were sealed between transparencies for storage.

2.5.3 The Amplification Refractory Mutation System (ARMS)

ARMS utilizes a primer designed to anneal to gDNA only if a specific mutation is present (or absent) (Newton *et al.*, 1989). Two ARMS primers, both with their 3' nucleotide (critical for amplification) on the C to T transition of the R59W mutation, were designed using the program "Primer" 5.0. An ARMS primer was used as a nested primer or in conjunction with another primer pair, so that an internal control for successful amplification was always present.

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PPO3AR was designed to anneal to PPOX if the R59W mutation is present. This primer was tested in conjunction with PPO2F and PPO3R as a nested primer, with PCR reagents as follows: 1x PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 mM of each of the 3 primers, 1 U Bioline Biotaq DNA polymerase and 1 μl gDNA (extracted from whole blood). The PCR program as described in Table 2.3 was used, and best amplification was obtained with annealing temperatures of 64°C (10 cycles) and 61°C (40 cycles).

PPOXAR was based on PPOX3R and also designed to anneal to gDNA if the R59W mutation is present. To increase the annealing stringency of this primer, the fourth nucleotide from the 5' side was changed from a cytosine to an adenine, creating a deliberate mismatch, as suggested by Newton *et al.*, 1989.

PPOXAR was used as a nested primer in conjunction with PPO2F and PPO3R primers with PCR reagent concentrations as described in the previous paragraph, and annealing temperatures of 64°C (10 cycles) and 59°C (40 cycles) yielded the best amplification.

The PPOXAR primer was also used as a nested primer in conjunction with the BF and PPO3R primers, with the following PCR reagent concentrations: 1x PCR buffer, 1.5 mM $MgCl_2$, 50 μM of each dNTP, 0.5 mM of the each of the 3 pimers, 1 U Bioline Biotaq DNA polymerase and 1 μl gDNA. The best amplification was obtained with annealing temperatures of 64°C (10 cycles) and 61°C (40 cycles).

PPOXAR, together with the PPO2F forward primer, was also used in conjunction with the PPO10F and PPO10R primer pair. The PCR reaction mixture yielding the best result was as follows: 1x PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 3mM of the each of the PPOXAR and PPO2F primers, and 1.5 mM of each of the PPO10F and PPO10R primers, 1 U Bioline Biotaq DNA polymerase

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and 1μl of Chelex extracted DNA. Optimum amplification was obtained with annealing temperatures of 65°C (10 cycles) and 62°C (40 cycles).

Amplification products were detected on a 1.5% agarose gel, with EtBr, under UV light.

2.6 Database Design

A database for collection of the information obtained from the informed consent forms were designed using Microsoft Excel, and updated regularly. The result of each sample's R59W mutation test was also added to the database.

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Chapter Three - Results and Discussion

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3.1 Development of Information Form

Information forms in Afrikaans (Appendix A) and English (Appendix B) were developed to inform potential sample donors in easily understandable terms what variegate porphyria (VP) is, why the incidence of the disease is so high in South Africa, what the symptoms associated with VP are and what the aims of the study are. The potential donor was also informed that the blood sample donated would only be used for the VP research project and that the results of his or her test for the founder mutation VP would be disclosed to him/her via their physician, if so desired. The form could be taken home and contains contact details if the donor requires any additional information.

3.2 Development of Informed Consent Form

A shorter, more compact informed consent form (compared to the three to seven page copy that is usually used in genetic studies) in Afrikaans (Appendix C) and English (Appendix D) were developed to simplify the filling in of the form, (especially for use in maternity wards where the regularly used extended form would have been unfeasible). The Guthrie or FTA cards were attached to the forms and the subsequent storage of the form together with the sample was thus also simplified. Information vital to the project, such as the patient's gender, race, home language, age group and known inherited diseases were also recorded on this form.

Anonymous samples did not require informed consent, but if the patient chose to be notified regarding the result of the test, his/her name, address and telephone

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number as well as the name, address and telephone number of his/her doctor were required, and informed consent had to be signed.

3.3 Development of a Database for Samples Collected

Information obtained from the informed consent form was entered into a Microsoft Excel database. The name of the hospital or clinic where each sample was obtained was also added to the database to simplify finding a specific sample in case of a query and to compare the number of samples obtained from each hospital of clinic. The result of each R59W mutation test was also added to the database.

3.4 Collection of Samples

3.4.1 Samples Collected

From June 2000 to May 2002 a total of 4 072 samples were collected from blood transfusion clinics, pathology clinics and maternity wards in the Western Cape, and from blood transfusion clinics and pathology clinics in Gauteng and the Free State. The number of samples obtained from the three geographical areas as well as the different main collection points are listed in Table 3.1.

Table 3.1: Summary of total number of samples collected

	Western Cape	Gauteng	Free State	**Total**
Blood Transfusion Clinics	1879	420	81	**2380**
Pathology Clinics	437	327	89	**853**
Maternity Wards	839	0	0	**839**
Total	**3155**	**747**	**170**	**4072**

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As collection of samples initially started in the Western Cape in June 2000 and only by March 2002 in Gauteng and the Free State, the majority of the samples were obtained from the Western Cape.

Table 3.2 depicts the gender, language and race distribution of the samples obtained from the three provinces. The race distribution of the study population is also depicted in Figure 3.1. As the previous frequency determination of the R59W mutation in South Africa was done on a Caucasian population and the descendants of the founder couple was to be mainly from the Afrikaner population, areas with a high concentration of Afrikaans-speaking Caucasians were targeted, such as the Northern suburbs of Cape Town. Seventy-nine percent (3 233) of the total sample population were White South Africans, 17% (692) were of mixed ancestry (Coloureds) and 2% (79) were black patients. Of the White South Africans, 78% (2 544) were Afrikaans-speaking, thus 62% of the total study population was Afrikaners.

Figure 3.1: Racial distribution of the total study population.

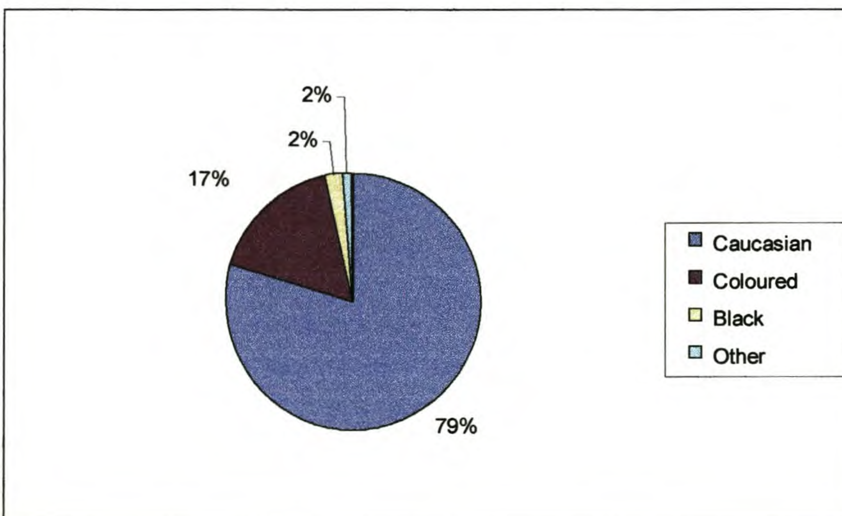

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Table 3.2: Sample population in terms of gender, language and racial distribution

		Gender		Language			Race			
		Male	*Female*	*Afrikaans*	*English*	*Other*	*Caucasian*	*Coloured*	*Black*	*Other*
Western Cape	Blood Transfusion Services	872	1007	1353	505	21	1634	228	8	9
	Pathology Clinics	143	294	370	65	2	360	66	5	6
	Newborn Babies	354	485	638	156	45	379	384	40	36
	Subtotal	**1369**	**1786**	**2361**	**726**	**68**	**2373**	**678**	**53**	**51**
Gauteng	Blood Transfusion Services	219	201	273	133	14	379	9	23	9
	Pathology Clinics	139	188	301	25	1	317	1	1	1
	Subtotal	**358**	**389**	**574**	**158**	**15**	**696**	**10**	**24**	**10**
Free State	Blood Transfusion Services	43	38	68	13	0	80	1	0	0
	Pathology Clinics	33	56	82	5	2	84	3	2	0
	Subtotal	**76**	**94**	**150**	**18**	**2**	**164**	**4**	**2**	**0**
	TOTAL	**1803**	**2269**	**3085**	**902**	**85**	**3233**	**692**	**79**	**61**

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3.4.2 Problems Experienced with Collection of Samples

Even though posters were put up at participating collection points and information and informed consent forms for this project were placed at the most practical and observable positions at the collection points, minimal public participation was obtained when potential donors were not approached and asked for their participation.

Sample collection at any specific blood donation clinic could only be performed for a period of three months. This was to prevent possible double collection of samples, because individuals may donate blood every three months. This implies that collection of blood samples from the donors at any specific clinic had to be as effective as possible within that period. The collection of these blood samples cause more work for the clinic staff, not only for the physical collection of the sample, but also to approach potential sample donors for their participation. The blood transfusion clinics are generally understaffed, not allowing for a staff member to help blood donors fill out forms and to ask the donor to participate in the founder mutation screening project. Only a few donors participated in the project when the information and informed consent forms were placed with the forms of the blood transfusion services, without approaching them for participation. Personal attendance, although very time consuming, proved to be at least four times as effective in the collection of samples from this source.

Blood samples from the umbilical cords of newborn babies had to be obtained by the nursing staff in the maternity ward. It is understandable that the collection of these samples would have the lowest priority when a baby is born. Initially we attempted to distribute information and informed consent forms to the

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obstetricians involved at the various hospitals' maternity wards, so that the informed consent form could be completed before delivery of the baby. This approach was unsuccessful, as too many obstetricians were involved to make it practically feasible. Frequent visits to the maternity wards of the participating hospitals were necessary to call on the nursing staff's renewed enthusiasm in the collection of samples for this project.

The rate of sample collection at pathology clinics was very slow. This was largely due to the large number of patients visiting these clinics, not allowing too much time to be spent per patient by the personnel, and also because an extra blood sample in an EDTA tube had to be collected from sample donors.

3.5 DNA Isolation

3.5.1 Whole Blood DNA Isolation

A total of 56 whole blood DNA isolations were performed. This procedure had to be performed over a period of two days, and the number of samples that could be done at once was limited by the large size of the Falcon tubes – only four tubes could be centrifuge simultaneously in the Beckman CS15R centrifuge, used in our laboratory, for each of the 10 min centrifugation steps.

Even though the procedure is time consuming, whole blood extraction yields 200 – 800 μl of high concentration DNA. As 1 µl of DNA is adequate per PCR, this DNA can be used for numerous reactions. The DNA can also be stored at -80°C for years without degradation, as long as it is not defrosted and refrozen too often. However, for the purposes of this study, where samples are only screened once and subsequent PCR amplification was only done if a R59W mutation was

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detected or if the initial amplification was unsuccessful, the high quantity of DNA isolated is unnecessary, thus not justifying the extraction time or the high cost of this procedure.

3.5.2 Chelex DNA Isolation

The DNA of all samples obtained on Guthrie cards and most of the samples obtained as whole blood (subsequently spotted out on Guthrie cards), were isolated using Chelex. The isolation procedure was found not to be very labour intensive or time consuming and the DNA from 48 samples could be isolated in 1.5 h. Twenty four samples could be centrifuged at one time in the Beckman CS15R centrifuge used in our laboratory, thus not prolonging the extraction procedure too much. The quality of the isolated DNA was adequate for PCR amplification, though the concentration was too low to be determined by either photospectrometry or gel electrophoresis. Approximately 200 μl DNA was available subsequent to Chelex extraction.

The Chelex beads are rich in EDTA (a known PCR inhibitor) and the supernatant containing the DNA should be removed and placed into a new microfuge tube for storage. As these samples were only reused if a sample was positive for the R59W mutation or if the initial PCR amplification was unsuccessful, the DNA could be used directly from the microfuge tube that was used for the extraction, thus saving the cost of a set of storage tubes. We found that DNA left in the container with Chelex beads and kept at 4°C could be used for successful PCR amplification up to 2 weeks after extraction.

In a study done by Hamvas *et al.* (2001), where 10 044 samples obtained on Guthrie cards and DNA isolation was performed with Chelex, it was reported that

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99.2% of the PCR amplifications were successful on the first attempt, and that a successful Chelex extraction was performed on a 6 year old Guthrie card sample. We found an equally high PCR success rate with DNA isolated from Guthrie cards with Chelex. Because DNA can still be successfully isolated from Guthrie cards after such a long time, it is very favourable when compared to DNA isolation from whole blood, which must be performed within 2 weeks after the sample has been obtained if stored at 4°C, and somewhat longer if stored at -20°C. Storage of large quantities of whole blood samples took up a lot of space in these cooling facilities, further adding to the appeal of sample collection on cards.

The Guthrie cards were stored at room temperature, away from dampness and direct sunlight.

3.5.3 FTA Purification Solution DNA Isolation

The DNA from samples obtained on FTA cards was isolated using FTA Purification Solution. This isolation method proved to be fast (one hour for 96 samples) and not labour intensive. No centrifugation was needed and amplification was performed directly on the FTA card disc in the same tube used for the DNA isolation, making the use of microtiter plates ideal. Only one 1.3 mm disc of the blood spotted FTA card was used per PCR, so ample DNA was available on the card for subsequent PCR amplification, if this was found necessary.

The FTA cards were stored at room temperature, away from dampness and direct sunlight.

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3.5.4 Comparison of DNA Isolation Methods

As this project only required DNA from each sample to be used once of twice, whole blood DNA isolation proved to be too expensive and too time consuming.

If one compares FTA purification solution DNA isolation from FTA cards to that of Chelex DNA isolation done on Guthrie cards, the FTA method is more expensive, but the DNA can be isolated in a microtiter plate and subsequently PCR amplified in the same microtiter plate, saving time and costs. DNA isolation and amplification in microtiter plate format also lends itself towards automation for DNA-array based mutation detection (see 1.3.3.3(b)). DNA can also be stored on FTA cards for over 11 years without any loss in PCR efficiency (www.dnatestingcentre.com/FTA_cards.htm).

McCabe *et al*. (1999) as well as Todorova *et al*. (1999) report successful direct amplification of DNA on Guthrie card discs with no prior DNA isolation required. Our attempts at direct PCR amplification of DNA on Guthrie cards were unfortunately unsuccessful. Substantial savings on time and costs could have been achieved if the Chelex DNA isolation procedure could be omitted before PCR amplification of the samples.

3.6 Mutation Detection Systems

3.6.1 Amplification Refractory Mutation System (ARMS)

The ARMS primer PPO3AR, in conjunction with the PPO2F primer, failed to distinguish between samples with and without the R59W mutation. At lower

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annealing temperatures the primer annealed to gDNA, regardless of the presence or absence of the single base mutation, and at higher annealing temperatures it failed to anneal to the gDNA.

PPOXAR, the ARMS primer designed with a deliberate mismatch at the fourth nucleotide from the 3' side, to increase annealing stringency (Newton *et al.*, 1989), was used as a nested primer in conjunction with the PPO2F and PPO3R primers to screen for the R59W mutation. After PCR amplification, the DNA was run on a 1.5% agarose gel. The nested ARMS primer was able to detect the R59W mutation, but amplification of the fragment between the flanking primers was primarily obtained, with little amplification of the fragment between the PPO2F and PPOXAR primers. PPOXAR was also used in conjunction with the BF and PPO3R primer pair, and amplification between the flanking primers were also primarily obtained, with little or no amplification of the region between BF and PPOXAR.

The best results were obtained by multiplexing, using PPO2F and PPOXAR in conjunction with the PPO10F and PPO10R primers, the latter pair serving as an amplification control (see Figure 3.2).

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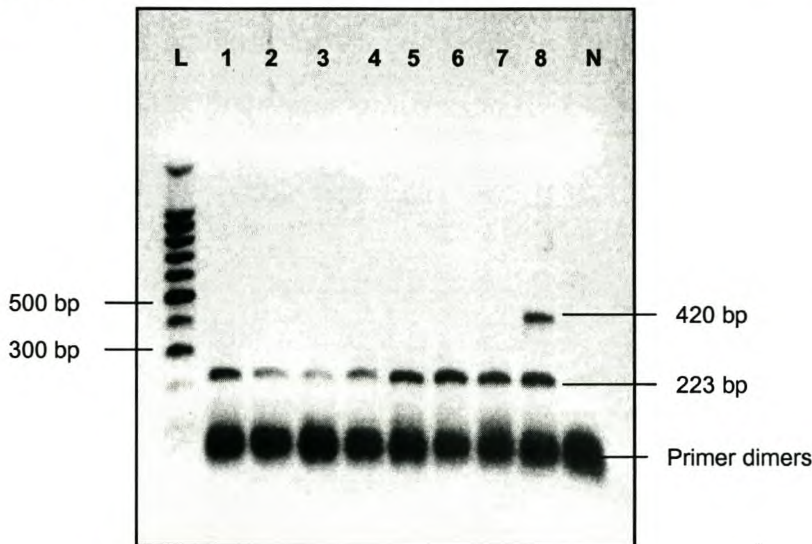
Figure 3.2: ARMS primer PPOXAR and PPO2F in conjunction with PPO10F and PPO10R primer. PPOXAR and PPO2F only amplify the 420 bp fragment if the R59W mutation is present. L = a 100 bp ladder. In samples 1 – 7 the R59W mutation is absent, while the mutation is present in sample 8. N = a negative PCR control.



The 223 bp amplicon of the PPO10F and PPO10R primer pair was present in all successful PCR reactions, and the 420 bp amplicon of the PPO2F and PPOXAR primer pair was only obtained if the R59W mutation was present in a sample. A high quantity of primer dimers was unfortunately obtained at conditions optimized for PPO2F and PPOXAR amplicon amplification.

One thousand and forty-one samples from the Western Province Blood Transfusion Services were screened using PPOXAR and PPO2F, together with PPO10F and PPO10R as a control for successful amplification. PCR amplification was done in microtiter plate format on DNA isolated from Guthrie cards with Chelex. One sample was found positive for the R59W mutation. A subsequent DNA isolation and PCR amplification was performed before digested with *StyI* restriction enzyme to confirm the presence of the mutation.

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Even though this primer combination could be used to detect the R59W mutation, it was often difficult to differentiate between R59W positive and negative samples, because non-specific annealing of the ARMS primer and subsequent amplification of the test fragment (between PPOXAR and PPO2F) were often obtained. Thus, to confirm results obtained with the ARMS test, all these samples were re-extracted and retested using restriction enzyme digestion.

In a study performed by Waterfall and Cobb (2001) two ARMS primers were used in conjunction with two flanking primers in a multiplex reaction to determine the zygosity of patients for sickle cell anaemia. As individuals can be homozygous (HbS/S), heterozygous carriers (HbA/S) or normal (HbA/A), two separate ARMS PCR reactions, one for HbA and one for HBS, were previously needed to screen for this disease. The authors report difficulty optimizing the four primer reaction, but successful distinction between the three genotypes was obtained using HotStart *Taq* DNA polymerase. When this very expensive *Taq* DNA polymerase (Roche) was used, amplification of the large fragment between the flanking primers was not always obtained. Because the PCR product of at least one of the two ARMS primers must always be present (serving as a control for successful amplification), amplification of the fragment between the two flanking primers is not necessary in this case. As experienced in the R59W mutation screening study, the authors also found that amplification of the large fragment was generally done at the expense of the allele-specific fragment, with subsequent decreased specificity. PCR amplification using Amplitaq Gold (Applied Biosystems) yielded improved results, but the high cost made the use of this enzyme unfeasible.

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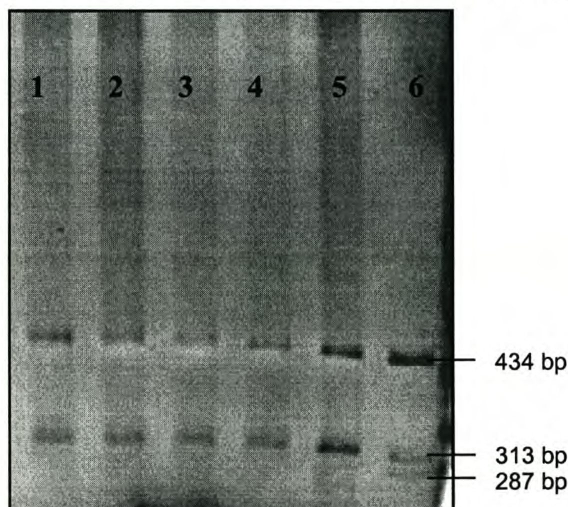
3.6.2 Restriction Enzyme Digestion

Restriction enzyme (RE) digestion with *StyI* was performed on 1 041 samples obtained form the Western Cape Blood Transfusion clinics and 378 samples obtained from newborn babies in the Western Cape. No R59W positive samples, other than the one previously detected with ARMS, were found in the group of 1 041 samples previously tested. None of the 378 samples obtained from newborn babies in the Western Cape were mutation-positive.

Figure 3.3: Restriction enzyme digestion with *StyI* on amplification product of PPO2F and PPO4R. The R59W mutation is absent in sample 1 – 5, and present in sample 6.



RE digestion of the PCR product obtained with primers PPO2F and PPO4R always yielded two fragments, one of 434 bp and one of 313 bp. This served as an internal control for successful RE digestion. When the R59W mutation was present, another RE digestion site was created, 26 bp from the 3' end of the 313 bp fragment, thus yielding a third (287 bp) and fourth (26 bp) fragment. Because

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the 26 bp fragment migrated rapidly through the 12% PAA gel, this fragment was not visible on the gel after EtBr staining, thus a 434, a 287 and a 313 bp fragment were obtained after digestion of a heterozygous R59W sample.

3.6.3 Single Strand Conformation Polymorphism (SSCP) Analysis

The presence of the R59W mutation causes a conformational change in the PCR amplified DNA. If the amplified fragment is short, (less than 400 bp) this conformational change can be detected by denaturing the double stranded DNA to yield single strands, and running it on a high density gel, as the conformational change affects the migration tempo of the single strand DNA. After PCR amplification with the PPO3F and PPO3R primer pair, SSCP analysis was performed on the majority of the samples, i.e. 1 339 samples obtained from the blood transfusion clinics, 853 samples obtained from pathology clinics and 461 samples obtained from maternity wards. Three R59W positive samples were detected by SSCP analysis.

SSCP analysis can be seen as a mutation screening method as opposed to a mutation detection method, as any change in the DNA sequence will be observed. Gaunt *et al*. (2001) used SSCP analysis to screen 2 743 individuals in order to detect new SNPs in the insulin-like growth factor 2 (*IGF2*) gene. In this study, where we were only interested in the presence or absence of the R59W mutation, verification of the result with a mutation detection method was essential to ensure that the conformational change detected was indeed caused by the R59W mutation. DNA from the three samples in which a change in DNA sequence was found were re-isolated and digested with *StyI* RE, subsequent to PCR amplification, to verify the presence or absence of the R59W mutation.

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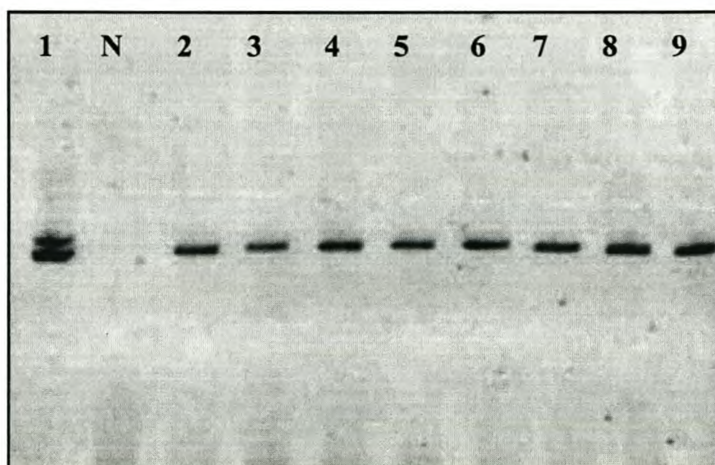
Figure 3.4: SSCP analysis of samples amplified with the PPO3F and PPO3R primer pair. The R59W mutation is present in sample 1 and absent in samples 2 – 9. N = a negative control for PCR.



3.6.4 Comparison of Mutation Detection Systems

Of the three mutation detection systems used, ARMS is the quickest and most cost effective method, as only a PCR reaction and subsequent agarose gel electrophoresis is required. By adding EtBr to agarose gel mix, the gel can be stained while electrophoresis is in progress, and visualization is done under UV light upon completion of electrophoresis, eliminating the need for $AgNO_3$ staining. No special equipment is necessary, and the relatively few steps in the process minimize the likelihood of mistakes being made. Unfortunately, with the conditions used in this study, this method was not as robust as would be expected and subsequent testing with RE digestion was deemed necessary to confirm the results obtained. However, retesting of the 1 419 samples with RE digestion did not reveal any false positive or negative results, and the ARMS test can thus be considered as a valid option for large scale mutation detection studies. In future studies the sensitivity and specificity of the ARMS test can be

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improved by the use of high fidelity *Taq* polymerases, although this would increase the cost of the test.

RE digestion with *StyI* is a reliable method to detect the R59W mutation, as the PCR amplicon used in this study included a control site to confirm successful digestion, regardless of the R59W mutation status of the sample. Olynyk *et al.* (1999) tested 3 011 samples for the hemochromatosis gene using RE digestion. However, we found the *StyI* enzyme to be too expensive to use for the screening of such a large quantity of samples. Furthermore the incubation step for RE digestion was usually done overnight to ensure complete digestion. Mutation detection via this system is thus time-consuming. There are also many pipetting steps needed when performing RE digestion, rendering this procedure labour intensive and increasing the possibility of mistakes to occur.

After initial screening of samples with *StyI* RE for comparative purposes, this method of mutation detection was only used to verify R59W mutations found with other methods. As only a few positive samples were found, it was not too expensive or laborious to confirm these test results with *StyI* RE digestion.

SSCP analysis was found to be an effective method of screening for the R59W mutation. It is a more time consuming method than ARMS (because silver staining was used for visualization of the fragments obtained), but we found the method to be robust and less time consuming than RE digestion. SSCP analysis is also cheaper to perform than RE digestion.

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3.7 Results Obtained with Population Screening

A total of four R59W positive samples were found in this study:

1. A Caucasian male, Afrikaans-speaking, in the age group 21 – 40, obtained from the Western Cape Blood Transfusion clinic cohort, with a known family history of VP,
2. A Caucasian male, Afrikaans-speaking, newborn, obtained from a maternity ward in the Western Cape, with a known family history of VP,
3. A Caucasian female, English-speaking, newborn, obtained from a maternity ward in the Western Cape, with no family history of VP, and
4. A Caucasian female, Afrikaans-speaking, in the age group 41 – 59, obtained from the Gauteng pathology clinics, with no family history of VP.

3.7.1 Screening Methodology

As porphyrin abnormalities may occur, among others, in cases of lead poisoning, sideroblastic and haemolytic anaemia, iron deficiency, renal failure, cholestasis, liver disease and gastrointestinal hemorrhage (Lim *et al.*, 1992) and certain diets, it is possible that false positives can be diagnosed via biochemical testing. Furthermore, even with current biochemical tests, many false positive results are still reported (Kirsch *et al.*, 1998; Kotze *et al.*, 1998). Stool porphyrin levels do not accurately reflect intracellular porphyrin concentrations, as normal fecal porphyrin excretion has been demonstrated in the face of elevated biliary porphyrin and porphyrinogen concentrations (Logan *et al.*, 1991; Kirsch *et al.*, 1998).

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The biochemical method of VP detection used by Dean (1960) cannot distinguish VP caused by the founder mutation and VP caused by other mutations, and thus any individuals in the latter group, even though they are rare, would have been counted as part of the founder mutation group, increasing the estimated frequency of the founder mutation.

Calculation of the VP founder gene mutation frequency by means of a DNA based test is more accurate than estimation by means of biochemical testing, especially the qualitative methods used by Dean for the previous frequency estimation (Kirsch *et al.*, 1998; Kotze *et al.*, 1998). The DNA tests are very specific, as only the R59W mutation will be detected, and the patient's health status or diet cannot influence the result of the test.

3.7.2 Study Populations

The study population used for the previous frequency determination of the founder mutation causing VP was not unbiased. Dean (1960) expected a higher concentration of porphyrics at the hospitals than in the general public because of acute porphyric attacks. After obtaining a frequency of 1 in 250, he estimated that 1 in 350 of the general public could have VP. This could still be an overestimation of the frequency in the general population. Hift *et al.* (1997) report that the frequency of porphyrics presenting with acute attacks have decreased from 17%, prior to 1980, to only 9%. There was also a decline in the total number of acute attacks of VP seen at Groote Schuur Hospital, from 56 in the period 1957 to 1966 to 24 from 1988 to 1997.

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The frequency estimated by Dean *et al*. in 1960 is also only representative of one geographical area within South Africa (Eastern Cape). By obtaining samples from various geographical areas as well as from multiple unbiased sources, a more accurate estimation of the R59W mutation frequency can be made.

In the 1970's, blood donors who suffered from any of the porphyrias were prohibited from donating blood, because fears existed that the disease, or symptoms thereof, may be transferred to the receiver of the blood (personal communication, Sr. Money, Western Cape Blood Transfusion Services). It may be possible that, after these fears have been eradicated and individuals with porphyria are permitted to donate blood again (only for the last couple of years), these individuals, and maybe even their affected progeny, still do not donate blood, making the group of samples obtained from the blood transfusion clinics biased.

Pathology clinics may also not be a completely unbiased source for sample collection, as pathology laboratories perform tests for porphyrias, and physicians would refer patients suspected to have VP to the pathology laboratories to verify the diagnosis.

Blood samples obtained from newborn babies may be the most unbiased group of samples collected, as we strived to collect blood samples from all babies born at the participating hospitals, irrespective of any conditions that the parents of the baby might be suffering from, making this group more representative of the population. If the R59W mutation was detected in a newborn sample and the names and addresses of the patient and physician were provided, the latter was requested to take a second blood sample from the baby, this time by means of a heal prick. This was necessary to rule out the potential problem of motherly contamination when obtaining a newborn's blood sample from the umbilical cord.

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By obtaining blood directly from the baby, the carrier status of the baby could be confirmed.

One of the R59W positive samples obtained from a Caucasian newborn could not be traced as the mother chose to participate anonymously. The parents of the child indicated that their home language is English, but unfortunately the ancestry of the patient could not be traced, nor could the parents be tested for R59W or the test result confirmed in this case.

3.7.3 Screening of the Caucasian Population

In the total South African Caucasian study population, 0.12% (4 / 3 233) were found to carry the R59W mutation (1 / 808). This newly obtained molecular genetic frequency for the South African Caucasian population is remarkably lower than the previously estimated frequency of 0.3% reported by Dean (1960), established with biochemical testing of 6 458 individuals from the same population group in the Eastern Cape.

Of 2 544 Afrikaans-speaking Caucasians screened, 3 (0.12%) were positive for the R59W mutation. Two of these individuals had previous knowledge of their VP carrier status or possible family history of VP.

In two of the four mutation-positive cases, the patient (or the newborn baby's parents) was unaware of any cases of VP in their family. One of the two positive adults had knowledge of his VP status, implying that 50% of the individuals in our adult Caucasian study population were unaware of their carrier status, 30% lower than the previously reported frequency of 80% (Day, 1986; Grandchamp *et al.*, 1996). The 50% penetrance is more in accordance with the figure of 60%

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reported by Hift *et al*. (1997). Even though a family history of VP was known in one of the two VP positive newborn babies, these cases were not included in our penetrance estimation, as expression of VP symptoms only occur after puberty.

The Afrikaner founder mutation, R59W, was detected in one English-speaking patient. This confirms that the mutation is not limited to the Afrikaner, but is also present in the English-speaking population due to admixture with other, more recent, immigrants. Three Afrikaner patients were mutation-positive, confirming that the mutation is still more prevalent in the Afrikaner (Jenkins, 1996).

Of the 308 Afrikaner newborns tested, one (0.32%) was positive for the R59W mutation, and in the total group of 379 Caucasian newborn samples, two (0.53%) were positive for the R59W mutation. The frequency of the R59W mutation obtained in the newborn cohort is even higher than Dean's estimated frequency for the founder mutation. Even though this study population is very small, it is the most unbiased group of samples.

Only one (0.05%) of 2 093 Caucasian samples obtained from blood transfusion services was positive of the R59W mutation. The frequency of the mutation in this group was expected to be lower than in the general population, as mentioned in the previous section.

One (0.1%) of 761 samples obtained from Caucasians at pathology clinics tested positive for the R59W mutation. The frequency of the mutation in this group was expected to be higher than in the general population, as mentioned in the previous section.

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3.7.4 Screening of Coloured and Black Populations

Samples from the mixed ancestry (Coloured) population included in this study allowed us to determine the incidence of the R59W mutation in this population. In a study conducted by Loubser *et al.*, (1999), 236 apparently unrelated South African patients of mixed ancestry with clinical features of familial hypercholesterolemia (FH) were screened for the Afrikaner FH founder mutations. Two common founder-related Afrikaner mutations, D206E and V408M, were found in 14% of the patients in this study, and are attributed to admixture of the populations. The R59W mutation has previously been detected in a large VP family of mixed ancestry (Warnich, unpublished results), however, no R59W positives were found in the group of 692 individuals of mixed ancestry screened in this study. It can be expected that the frequency of the R59W mutation in the Coloured population would be much lower than that found in the South African Caucasian population. Because the Caucasian contribution to the Coloured population was approximately 33% (Loubser *et al.*, 1999), the frequency of the R59W mutation is expected to be only a third of that found in the South African Caucasian population. A much larger sample size will therefore be needed to accurately approximate the frequency of the mutation in the population of mixed ancestry.

The R59W mutation was not found in any of the 79 samples obtained from the indigenous black population. VP is not prevalent among the black populations of South Africa, and only one *PPOX* mutation (V290M) has been described in an indigenous black South African (Corrigall *et al.*, 2001). To date the R59W mutation has never been reported in a black patient and admixture from the Caucasian population is not evident at this stage.

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3.8 Ethical Issues

According to Mueller and Young (2001) a population screening program should be presented in a fair and equitable way and should be morally acceptable to a large proportion of the population to which it is offered. Participation to the population screening should be completely voluntary and easily understood information and well-informed counseling should both be readily available. This study met all these requirements.

The positive mutation status of a voluntary blood donor was only revealed to the individual via his or her physician and not directly to that individual (in cases where results were requested). Disclosure of genetic screening results should be accompanied by genetic counseling. If the physician was not qualified to provide counseling, we suggested that the patient be referred to a genetic counselor. If needed, we also offered to connect the physician with a qualified genetic counselor in the area.

An ethical issue which arose in this study (and other similar studies) was that of disclosure of a positive R59W mutation result when the individual, or the parents of the individual (in the case of a newborn baby) did not require feedback regarding the result. According to the Guidelines on Ethics for Medical Research (compiled by the MRC, South Africa), "respect of persons, families and their decisions according to the principles underlying informed consent" should be applied. In the case of newborn babies the decision not to be informed regarding the result of the R59W mutation test was made by the individual's parents, and they, as legal guardians, speak on behalf and for the baby. In this study the abovementioned guidelines were strictly followed and the decision of the participants respected. However, lack of informed consent is sometimes justified

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if there is a clear benefit for genetic screening, e.g. all babies in the UK are tested for phenylketonuria a few days after birth. No informed consent is required as babies diagnosed with phenylketonuria can be effectively treated (Smith, 1993).

A sample obtained from a newborn baby girl from one of the maternity wards in the Western Cape tested positive for the R59W mutation, and the child's parents indicated that they did not require to be informed regarding the result of the test. It is my believe that by doing so the parents of this child did not distinctly request that the result of the test be withheld from them, but rather to save time (the second part of the informed consent form only had to be completed if a result was required) or because of ignorance. Although information sheets were provided to every participant, it is difficult to assess whether he/she understood everything, especially the benefits associated with the test. This problem will always be an issue when the geneticist conducting the research, or a genetic counselor, is not present when the participants are approached. Early attempts to introduce a sickle-cell carrier detection program in the black population of North America had to be abandoned because of public criticism. The information pamphlets tended to confuse the sickle-cell carrier status (which is usually harmless) with the homozygous disease, which causes severe morbidity, leading to employers and insurance companies discriminating against sickle-cell carriers (Mueller and Young, 2001).

As knowledge regarding the positive R59W test result, accompanied by genetic counseling, can prevent the individual from suffering a potentially fatal acute attack caused by inappropriate drugs or other factors, every measure should be taken to inform and educate patients in order to enable them to make informed decisions.

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Chapter Four – Conclusion

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This study was conducted to estimate the incidence of the R59W founder mutation in South Africa by means of molecular testing. The requirements for a population screening study (listed on page 15 and 16), and the specific aims (listed on page 26) have all been fulfilled:

- We believe that the benefits that follow from this study warrant the effort and cost. Autonomy is enhanced, as individuals are better informed about the genetic risks of VP (Mueller and Young, 2001). A positive test result can be communicated by a genetic counselor and the necessary action be taken. Skin symptoms can be prevented by taking the necessary precautions and acute attacks can be evaded by avoiding known precipitating factors. The prevention of morbidity and alleviation of the suffering that the disease could impose is thus addressed.

- The project was ethically and socially acceptable, and approval of the study, including sample collection, information forms and informed consent forms, were obtained.

- VP information forms were designed to inform potential participants of the project and to provide background information on VP in South Africa. A shortened informed consent form was developed not to discourage potential participants with the lengthy informed consent forms generally used, to shorten the time needed to complete the form by maternity ward personnel (in the case of sample collection from newborn babies) and also to minimize the space needed for storage of the blood samples obtained. These forms can be valuable when forms have to be designed for similar screening projects.

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- Blood samples collected on FTA cards (Whatman BioScience) with subsequent DNA isolation using FTA purification solution were found to be the best collection and isolation methods to be used for this population screening study. SSCP analysis was found to be the best suited mutation detection system for this study, and positive results were confirmed using RE analysis. We are thus confident that the DNA tests used are both sensitive and specific.

- 4 072 samples were obtained from blood transfusion clinics, pathology clinics and maternity wards in three provinces. A database was compiled to record details obtained from the informed consent forms.

- The frequency of the VP founder mutation in the South African Caucasian population is estimated as 0.12%, less than half of what was previously estimated by Dean (1960) and in accordance with recent studies by De Villiers *et al.* (unpublished results). However, different frequencies were obtained within the various population samples. It is suggested that a newborn baby population sample be used in the future, as it is the most unbiased sample source. Interestingly, the highest frequency for the R59W mutation was obtained in this sample.

- No R59W mutation-positive patients were found in the population of mixed ancestry. The Caucasian contribution to this population is expected to be ± 33%, therefore the incidence of the R59W mutation in this population is expected to be a third (0.04%) of the incidence in the South African Caucasian population. Our preliminary results thus confirm the low incidence of the R59W mutation in this population group, but a much

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larger sample size will be needed to accurately estimate the frequency of the mutation in the population of mixed ancestry.

- Results obtained in this study also indicate that many individuals are ignorant regarding their carrier status and are therefore at risk of suffering potentially lethal acute attacks.

Future Study

These conclusions are based on a limited sample size, and in order to obtain a more accurate estimation of the R59W frequency in South Africa, we suggest that the number of individuals screened for the mutation should be increased to include at least 10 000 individuals. In order to obtain a population sample that is representative of the entire country, blood samples must be obtained from as many geographical areas in South Africa as possible. It will be interesting to compare the frequency of the R59W mutation in the various provinces of South Africa. As the previous estimation of the founder mutation frequency was performed on individuals living in the Eastern Cape, genetic screening of patients residing in this province will be useful in assessing the accuracy of the biochemically based study done by Dean (1960).

The infrastructure for collection of blood samples have been created during this study, and the most effective collection, DNA isolation and molecular analysis methods have been determined, ensuring rapid screening of numerous samples. This estimated frequency of VP among South African Caucasians is still much higher than in any other part of the world. It is thus important that the sample size be increased in order to improve the accuracy of the founder mutation frequency estimation in South Africa and to determine whether the issue of

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underdiagnosis and the resulting risk of potentially fatal acute attacks should be addressed by appropriate genetic testing in the future.

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Appendix A

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Variëgate Porfirie (VP) Navorsingsprojek

Wat is variëgate porfirie?

Variëgate porfirie, of VP, is 'n oorerflike siekte wat veroorsaak word deur 'n defek in een van die liggaam se essensiële ensieme. Simptome ontwikkel gewoonlik eers na puberteit en sluit velskade, akute aanvalle en sensitiwiteit t.o.v. sekere geneesmiddels in. Daar is beraam dat Suid-Afrika tussen 10 en 20 duisend VP lyers het, wat die hoogste voorkoms van VP in die wêreld is.

Hoekom is die voorkoms van VP so hoog in Suid-Afrika?

Genealogiese navorsing het getoon dat die oorgrote meerderheid van VP lyers in Suid-Afrika afstammelinge is van 'n egpaar (Adriana Jacobs, 'n weesmeisie van Holland, en Gerrit Janz van Deventer, 'n vryburger) wat in 1688 aan die Kaap in die huwelik bevestig is. Een van hulle twee was vermoedelik die draer van die mutasie verantwoordelik vir die oorerflike siekte en het dit na sommige van hulle agt kinders oorgedra, wat dit weer na hulle kinders oorgedra het, ens. Suid-Afrikaanse wetenskaplikes het onlangs hierdie mutasie (die R59W mutasie) in meer as 90% van VP gevalle in ons land geïdentifiseer, en die teenwoordigheid van die mutasie kan nou opgespoor word nog voordat enige simptome sigbaar is. Dit beteken dat voorsorgmaatreëls in sulke gevalle getref kan word.

Wat is die simptome van VP?

Die uitdrukking van VP wissel aansienlik van pasiënt tot pasiënt, maar die mees algemeen is velsimptome en akute aanvalle. Velsimptome kom veral voor op die dele wat aan die son blootgestel is. Die vel kan baie sonligsensitief en broos wees, en dus maklik beseer word. Tydens 'n akute aanval word erge buikpyne, naarheid en spierswakheid ondervind. Die inname van sekere geneesmiddels, waar onder sommige narkosemiddels, anti-epileptiese middels, voorbehoedmiddels en antibiotika, kan hierdie potensieel gevaarlike of selfs noodlottige akute aanvalle meebring. Ander faktore soos hormonale veranderinge en verkeerde dieet kan egter ook 'n akute aanval ontlok. **Dit is belangrik om te weet dat baie mense wat die VP-mutasie dra nooit enige simptome van die siekte vertoon nie.** Hierdie mense kan egter wel die siekte na hulle kinders oordra en sommige van hulle mag wel simptome vertoon.

Wat is die doel van hierdie studie?

Met hierdie populasiesiftingstudie word beoog om die aantal VP lyers in ons land met meer akkuraatheid te beraam sonder om, soos in die verlede, 'n moontlike sydige beraming te doen deur net gesertifiseerde kliniese gevalle te gebruik. Daar sal veral gefokus word om te bepaal hoeveel mense in Suid-Afrika onbewus daarvan is dat hulle die porfirie mutasie dra en dus onder meer die gevaar loop om geneesmiddels te gebruik wat tot akute aanvalle kan lei. Daar sal gepoog word om ongeveer 10 000 bloedmonsters van die naelstringe van pasgebore babas sowel as van donors by bloedbanke te neem en te ontleed vir die teenwoordigheid van die R59W mutasie, ander VP mutasies en mutasies wat 'n uitwerking op die kliniese simptome van die siekte mag hê.

Watter rol kan u speel?

Ons wil u vriendelik versoek om asseblief aan hierdie belangrike studie deel te neem. Op die invulstrokie kan u aandui of u anoniem aan die projek wil deelneem **of** die uitslag van die R59W toets via u geneesheer wil bekom, in welke geval u u persoonlike inligting sowel as die van u geneesheer moet invul en die toestemmingstrokie moet teken.

Hierdie projek is deur die Etiese Komitee van die Fakulteit Geneeskunde van die Universiteit van Stellenbosch goedgekeur, en word finansieel deur die Mediese Navorsingsraad ondersteun. Die bloedmonster sal tans slegs gebruik word vir die variegate porfirie navorsingsprojek, en die uitslag van die toets en enige verdere toetse sal uiters konfidensieel hanteer word. Vir enige verdere inligting aangaande die projek, kontak gerus die studieleier, VP navorsingsprojek, by onderstaande adres.

- See reverse side for English version -

DEPARTEMENT GENETIKA, UNIVERSITEIT VAN STELLENBOSCH, J.C. SMUTS-GEBOU,
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Appendix B

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Variegate Porphyria (VP) Research Project

What is variegate porphyria?

Variegate porphyria, VP, is a heritable disease caused by a defect in one of the body's natural essential enzymes. Symptoms develop fairly late in life after puberty and include various skin abnormalities, acute attacks and sensitivity to certain medicines. It is estimated that South Africa has some 10-20 thousand VP sufferers, which is also the highest incidence of VP known in the
world today.

Why is the incidence of VP so high in South Africa?

Genealogical research shows that most VP sufferers in South Africa are probably descendants of Adriana Jacobs, a Dutch orphan, and Gerrit Janz van Deventer, a free burgher, who married at the Cape in 1688. One of the two was a carrier of the heritable mutation responsible for the disease and passed it on to future generations of the family through one or more of their eight children. Scientists in South Africa have recently identified the mutation (the R59W mutation) in more than 90% of VP cases occurring in South Africa and its presence in carriers can now be detected even before the onset of disease symptoms. This means that precautionary measures can be taken in such cases.

What are the symptoms of VP?

The expression of VP varies substantially from patient to patient, but generally it includes skin symptoms and acute attacks. The skin is mostly affected in areas that are exposed to the sun, is very sensitive to UV light and extremely fragile, so it is easily damaged or bruised. During acute attacks severe abdominal pains, nausea and muscle weakness are experienced. The use of certain medications, among others certain types of anesthetics, anticonvulsants, contraceptives and antibiotics can mediate these potentially dangerous or even deadly acute attacks. Other factors, for example hormonal change or an excessive diet can also mediate acute attacks. **It is important to know that it is possible for carriers of the VP mutation not to experience any symptoms of the disease.** These individuals can, however, carry the disease over to their children, who may suffer from the VP symptoms.

What is the goal of this study?

The aim of this population survey is to obtain a better estimate of the frequency of VP sufferers and carriers of the VP mutation in South Africa without the danger of bias from having to rely on certified clinical cases as in the past. The main focus will now be on individuals who are unknowingly carriers of the porphyria mutation and for whom precautionary measures to reduce the risk of suffering symptoms may be prescribed. The intention is to collect blood samples from the umbilical cords of newborn babies as well as donors at blood banks and to screen samples for the presence of the R59W mutation, as well as other VP mutations and mutations that may influence the clinical symptoms of the disorder. A total sample size of around 10 000 is envisaged.

What role can you play?

We would very much appreciate your participation in this very important study. On the submission form you can indicate whether you would like to participate anonymously **or** be notified of the results of the R59W test via your doctor, in which case you have to provide your personal details, as well as that of your doctor, and sign the consent slip.

This project was approved by the Ethics Committee of the Faculty of Medicine of the University of Stellenbosch, and is financially supported by the Medical Research Council. The blood samples will currently only be used for the variegate porphyria research project, and the results of this test and any other possible tests will be treated as highly confidential. For any further information regarding this project, please contact the study leader, VP research project, at the address below.

- Sien keersy vir Afrikaans -

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Appendix C

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Variëgate Porfirie Projek

Departement Genetika
Universiteit van Stellenbosch

Naam van Hospitaal/ Kliniek: ______________________________

Geslag: ☐ Manlik ☐ Vroulik

Ouderdom: ☐ Pasgebore ☐ 0–20 ☐ 21–40 ☐ 41–60 ☐ 60+

Huistaal: ☐ Afrikaans ☐ Engels
☐ Xhosa ☐ Ander ______________________

Ras: ☐ Blank ☐ Kleurling ☐ Swart
☐ Indiër ☐ Ander ______________________

Enige gevalle van porfirie bekend in die familie? ☐ Ja ☐ Nee

Enige ander oorerflike siektes bekend in die familie? ☐ Nee ☐ Ja* * Indien ja, spesifiseer asb.

____

Verlang u die resultate van hierdie toets? ☐ Nee ☐ Ja* *Indien ja, voltooi keersy asb.

Hierdie kant hoef slegs voltooi te word indien u die uitslag van die toets verlang

Pasiënt inligting:

Naam: ____

Adres: ____

____

Tel no.: ____

Dokter aan wie resultate gestuur moet word:

Naam: ____

Adres: ____

____

Tel no.: ____

Ingeligte toestemming:

Ek is tevrede dat ek ingelig is oor die variegate porfirie mutasieopsporingsprojek. Hiermee verklaar ek my bereidwilligheid om deel te neem aan die studie, en ek gee toestemming dat die bloedmonster deur my of my kind geskenk vir hierdie navorsing gebruik mag word.

____________________ ____________________
Handtekening Datum

Hierdie projek is deur die Etiese Komitee van die Fakulteit Geneeskunde van die Universiteit van Stellenbosch goed gekeur. Monsters sal slegs vir die opspoor van mutasies gebruik word. Alle inligting verskaf op die vorm, sowel as inligting bekom vanaf die monsters sal konfidensieel hanteer word.

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Appendix D

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Variegate Porphyria Screening Project

Department of Genetics
University of Stellenbosch

Name of Hospital / Clinic: ______________________________

Gender: ☐ Male ☐ Female

Age: ☐ Newborn ☐ 0–20 ☐ 21–40 ☐ 41–60 ☐ 60+

Home language: ☐ Afrikaans ☐ English ☐ Xhosa ☐ Other ______________________

Race: ☐ White ☐ Coloured ☐ African ☐ Indian ☐ Other ______________________

Any known cases of porphyria in the family? ☐ Yes ☐ No
Any other inherited diseases In the family? ☐ No ☐ Yes* * If yes, please specify

____

Results of this test required? ☐ No ☐ Yes* *If yes, fill in reverse side of this form as well

---

This page has to be completed only if a result is required

Patient's information:

Name: ____

Address: ____

____

Tel no.: ____

Medical practitioner to whom results must be sent:

Name: ____

Address: ____

____

Tel no.: ____

Informed consent:

I am satisfied that I have been informed about the variegate porphyria mutation screening project. I hereby voluntarily agree to participate in this study and give my consent that the blood sample I or my child donate(s) be utilised for this research.

____________________ ____________________
Signature Date

This project has been approved by the Ethics Committee of the Medical Faculty of the University of Stellenbosch. Samples will only be used for the screening of mutations. All information provided on form or obtained from samples will be treated confidentially.