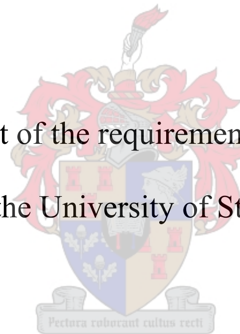
ANALYSIS OF GENES IMPLICATED IN IRON REGULATION IN INDIVIDUALS PRESENTING WITH PRIMARY IRON OVERLOAD IN THE SOUTH AFRICAN POPULATION

BY

FADWAH BOOLEY



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (MSc) at the University of Stellenbosch.

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March 2007

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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SUMMARY

Hereditary haemochromatosis (HH), a common autosomal recessive disease, is characterized by increased iron absorption leading to progressive iron accumulation in organs such as the liver, heart and pancreas. In the South African population the disease is prevalent in individuals of Caucasian origin, with a carrier frequency of one in six for the C282Y mutation in the *HFE* gene.

We investigated the role of genes implicated in iron metabolism, including the high-iron gene (*HFE*), haem oxgenase-1 gene (*HMOX1*), solute carrier family 40 (iron-regulated transporter) member 1 gene (*SLC40A1*), cytochrome b reductase gene (*CYBRD1*), hepcidin antimicrobial peptide gene (*HAMP*) and the hemojuvelin gene (*HJV)* in a patient cohort with non-*HFE* iron overload. DNA analysis was performed on samples from 36 unrelated South African Caucasian patients presenting with primary iron overload, who tested either negative or heterozygous for C282Y. In this study, mutation screening was performed by PCR amplification and HEX-SSCP analysis.

Sixteen previously described and two novel variants were identified by semi-automated DNA sequencing. Common variants identified in the *HFE* gene included C282Y, H63D, IVS2+4T→C, IVS4-44T→C, IVS4+48G→A and IVS5-47G→A. The Q127H mutation in exon 3 of the *HFE* gene was identified in one patient, who tested negative for both C282Y and H63D. Mutation S65C was identified only in the population-matched controls and was absent in the patient group. Other previously described polymorphisms identified included the

IVS5+51delTGGCTGTCTGACT deletion in *HMOX1*, I109 and V221 in *SLC40A1*, IVS1-4C→G, IVS2+8T→C and S266N, in the *CYBRD1* gene and, S264 and A310G in the *HJV* gene.

The novel variants, -89C→T, in the promoter region of the *CYBRD1* gene, was detected in only one patient, while S333 in exon 4 of the *HJV* gene was present in three patients. These variants were not identified in any of the population-matched controls screened and could explain the non-*HFE* iron overload presented by these patients. This study clearly demonstrates the importance of modifier genes in patients with iron overload that cannot be explained by the common C282Y mutation. Studies on iron-related genes and the identification of mutations in these genes in non-*HFE* patients could lead to improved diagnosis and counselling of South African patients presenting with primary iron overload.

OPSOMMING

Oorerflike hemochromatose (HH), 'n algemene outosomale resessiewe siekte word gekarakteriseer deur verhoogte yster absorpsie wat lei tot yster akkumulasie in organe soos die lewer, hart en pankreas. In die Suid-Afrikaanse populasie kom die siekte algemeen voor in Kaukasiese individu, met 'n draer frekwensie van een uit ses vir die C282Y mutasie in die *HFE* geen.

Ons het die rol van gene geïmpliseer in yster metabolisme, insluitend die hoë yster geen (*HFE*), heme oksigenase (*HMOX1*) geen, oplosbare-draer familie 20 (yster gereguleerde vervoerde) lid 1 geen (*SLC40A1*), sitochroom b reduktase 1 geen (*CYBRD1*), hepsidien anti-mikrobe peptide geen (*HAMP*) en die hemojuvelien geen (*HJV)* in 'n pasiëntgroep met yster oorlading nie verwant aan C282Y homosigositeit nie. DNA analise is uitgevoer op monsters van 36 onverwante Suid-Afrikaanse pasiënte met primêre yster oorlading, wat negatief of heterosigoties vir die C282Y mutasie getoets het. In hierdie studie is mutasie sifting uitgevoer deur gebruik te maak van PKR amplifikasie en HEX-SSCP analise.

Sestien bekende en twee nuwe variante is geïdentifiseer. Algemene variante insluitend C282Y, H63D, IVS2+4T→C, IVS4-44T→C, IVS4+48G→A, en IVS5-47G→A is in die *HFE* geen geïdentifiseer. Die Q127H mutasie in ekson drie van die *HFE* geen is in een pasiënt gekry wat negatief vir beide C282Y en H63D getoets het. Mutasie S65C is net in die kontrole groep geïdentifiseer en is afwesig in die pasiënte groep. Ander bekende variante geïdentifiseer het die

IVS5+51delTGGCTGTCTGACT delesie in *HMOX1*, I109 en V221 in *SLC40A1*, IVS1-4C→G, IVS2+8T→C en S266N in the *CYBRD1* en S264 en A310G, in die *HJV* geen ingesluit.

Die nuwe variant, -89C→T, in die 5' ongetransleerde area van die *CYBRD1* geen, is in net een pasiënt gekry, terwyl S333 in ekson 4 van die *HJV* geen in drie pasiënte gekry is. Hierdie variante is nie in enige van die kontroles gevind nie en mag yster oorlading in pasiënte met die nie-*HFE* fenotipe verduidelik. Hierdie studie het duidelik die belangrike rol van modifiseerende gene in pasiënte met yster oorlading, wat nie deur die algemene C282Y mutasie verduidelik kan word nie, aangetoon. Studies oor yster-verwante gene en die identifikasie van mutasies in hierdie gene in sulke pasiënte kan lei tot verbeterde diagnose en genetiese voorligting vir Suid-Afrikaanse pasiënte met die siekte.

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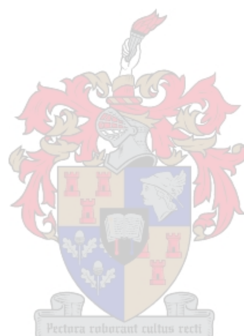

ALPHABETICAL LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
A	Adenine (in DNA sequence)
AA	Acrylamide
A (Ala)	Alanine
$AgNO_3$	Silver nitrate
APS	Ammonium persulphate
β	Beta
BAA	Bisacrylamide
bp	Base pair
B.C.	Before Christ
BLAST	Basic Local Alignment Search Tool
χ^2	Chi-square
C (Cys)	Cysteine
C	Cytosine (in DNA sequence)
˚C	Degrees Celsius
cDNA	Complementary deoxyribonucleic acid
CH_3COOH	Acetic acid
$C_{19}H_{10}Br_4O_5S$	Bromophenol blue
$C_{31}H_{28}N_2Na_4O_{14}S$	Xylene cyanol
CYBRD1	Cytochrome b reductase

D (Asp)	Aspartic acid
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dH_2O	Distilled water
ddH_2O	Double distilled water
Del	Deletion
dGTP	2'-deoxy-guanosine-5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DMT1	Divalent metal transporter 1 gene
dTTP	2'-deoxy-thymidine-5'-triphosphate
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
F	Forward primer
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FPN1	Ferroportin-1
Fre1	Ferric reductase transmembrane component 1
g	Gram
G	Guanine (in DNA sequence)
HAMP	Hepcidin antimicrobial peptide

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H_3BO_3	Boric acid
HCHO	Formaldehyde
$HCONH_2$	Formamide
HEPC	Hepcidin gene
HEX-SSCP	Heteroduplex single strand conformation polymorphism
HFE	High-iron gene
HFE1	Haemochromatosis type 1
HFE2	Haemochromatosis type 2
HFE3	Haemochromatosis type 3
HFE4	Haemochromatosis type 4
HH	Hereditary haemochromatosis
HHSA	Haemochromatosis society of South Africa
HMOX1	Haem oxygenase 1
HLA	Human leukocyte antigen
I (Ile)	Isoleucine
Inc	Incorporated
IRE	Iron regulatory element
IREG1	Iron-regulated transporter 1 gene
IRP	Iron regulatory protein
JH	Juvenile haemochromatosis
kb	Kilobase
KCl	Potassium chloride
$KC_2H_30_2$	Potassium acetate

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$KHCO_3$	Potassium hydrogen carbonate
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MTP1	Metal transporter 1 gene
n	Sample size
N (Asn)	Asparagine
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information, USA
ng	Nanogram
NH_4Cl	Ammonium chloride
NTBI	Non-transferrin bound iron
%	Percent
%C	Mass of the cross-linkers/mass of all monomers and cross-linkers per 100 ml volume
p	Short arm of chromosome
P	Probability value
PAA	Polyacrylamide

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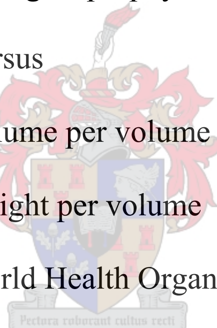
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Picomole
q	Long arm of chromosome
®	Registered trademark
R	Reverse primer
R (Arg)	Arginine
RGD	Repulsive guidance domain
RGM	Repulsive guidance molecule
S (Ser)	Serine
SDS	Sodium dodecyl sulphate
SLC40A1	Solute carrier family 40 (iron-regulated transporter) member 1 gene
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism
T	Thymine (in DNA sequence)
TBE	Tris-borate/EDTA buffer
TBI	Transferrin bound iron
TEMED	N,N,N'N',-tetramethylenediamine
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
Tris-HCl	Tris hydrochloride [2-Amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloride]

T	Thymidine
T_A	Annealing temperature
Tm	Melting temperature
™	Trademark
U	Units
UTR	Untranslated region
UV	Ultraviolet
V	Volts
V (Val)	Valine
VP	Variegate porphyria
vs	Versus
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
Y (Tyr)	Tyrosine



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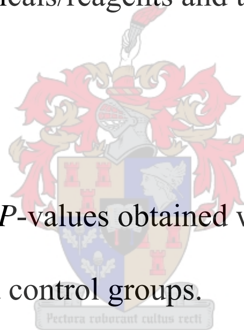

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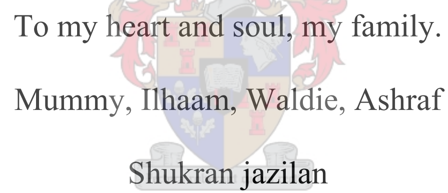
"Not everything that can be counted counts, and not everything that counts can be counted". Albert Einstein



To my heart and soul, my family.

Mummy, Ilhaam, Waldie, Ashraf

Shukran jazilan

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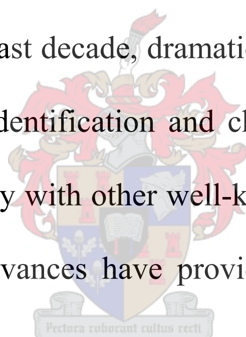
CHAPTER ONE: INTRODUCTION

1.1. INHERITED DISORDERS OF IRON METABOLISM

PREFACE

Enhanced knowledge of the molecular and cellular mechanisms encompassing absorption, utilization and storage of iron in the body enabled the development of laboratory methodology for the detection of abnormalities of iron metabolism, including iron deficiency and overload (Brittenham et al. 2000). Over the past decade, dramatic advances have been made in studies of iron. Such studies lead to the identification and characterization of novel proteins that interact in the iron metabolic pathway with other well-known proteins such as transferrin and ferritin (Andrews 1999). These advances have provided greater insight into the intricate discipline of iron metabolism.



Iron is the most common metal in the crust of the earth and is essential for the maintenance of mammalian cells (Andrews 2005). Most genetic disorders of iron metabolism are caused by iron overload rather than iron deficiency. The accumulation of excessive amounts of iron in the body results in damage to various organs such as the liver, pancreas, heart and other endocrine organs (EASL International Conference on Haemochromatosis 2000; Limdi and Crampton 2004; Beutler et al. 2005). Some iron overload conditions occur without any other underlying disorder and are classified as primary, synonymous with genetic, hereditary haemochromatosis (HH). Others are secondary to another disease. Among the latter are β-thalassemia (associated with ineffective erthropoiesis), porphyria cutanea tarda, African iron

overload, neonatal iron overload, sideroblastic anaemia and aceruloplasminaemia. Due consideration should be given to the causes of secondary iron overload to elucidate the many questions surrounding the regulation of iron homeostasis. The scope of the present study, however, is limited to primary iron overload.

Studies on diseases and proteins of iron homeostasis have brought about the development of genetic diagnostic tests for HH (Feder et al. 1996; Potekhina et al. 2005). Genetic testing not only permits disease diagnosis, it has a broad spectrum of applications (Burke 2005). These include the identification of future health risks, the prediction of responses to drug treatment and risk assessment of the progeny of affected individuals.

HH is accompanied by a myriad of clinical symptoms some of which can be prevented and controlled by early diagnosis (Niederau et al. 1996). Initial screening with serum transferrin and serum ferritin saturation tests is vital for detection of abnormal iron parameters, but these procedures do not provide a definitive diagnosis for HH. It is highly recommended that such biochemical tests be performed in conjunction with DNA analysis before resorting to an invasive procedure such as liver biopsy. Once a diagnosis of HH has been made, management of iron excesses can be achieved using the ancient practice of phlebotomy, a method used well before the time of Hippocrates in the fifth century B.C. (Wilbur 1987). HH is a remarkable example of a disease in which clinical benefit has been achieved from a basic discovery at the molecular level (Brittenham et al. 2000). Treatment of the disease is seemingly paradoxical combining practices from the middle ages with diagnostic procedures of the twenty-first century.

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HH is one of the most common inborn errors of iron metabolism affecting Caucasian populations of northern European descent worldwide (Sheldon 1935; Edwards et al. 1988). Within the South African population an estimated one in 100 individuals of European descent are affected (Meyer et al. 1987; de Villiers et al. 1999a). Two mutations (C282Y and H63D) in the *HFE* gene cloned in 1996 are the cause of HH in more than 80% of Caucasian HH cases, allowing efficient DNA-diagnostics for patients of European ancestry (Feder et al. 1996; Potekhina et al. 2005). However, in Asian, Australasian, Amerindian and African populations the *HFE* C282Y mutation occurs very rarely or not at all (Beckman et al. 1997; Chang et al. 1997; Merryweather-Clarke et al. 1997; Agostinho et al. 1999; Rochette et al. 1999; Sohda et al. 1999; Barut et al. 2003; Zorai et al. 2003; Karimi et al. 2004; Kotze et al. 2004; Sassi et al. 2004; Leone et al. 2005). In South Africa the carrier frequency for the C282Y mutation was estimated to be at approximately one in six in the Caucasian population (de Villiers et al. 1999b). Increasingly novel mutations are being identified in a number of genes implicated in iron homeostasis and varying forms of HH (Beutler 2005). The development of mutation detection tests is vital for the rapid identification of the underlying causes of HH in patients not displaying the typical C282Y homozygous status (Kotze et al. 2004).

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1.2. LITERATURE REVIEW

1.2.1. HISTORY

HH was first reported by Trousseau in 1865 in a patient with liver cirrhosis, diabetes mellitus, and bronze skin pigmentation (reviewed by Pietrangelo 2003). Von Recklinghausen is accredited with coining the term haemochromatosis in 1889 and for recognizing it as a distinct disease characterized by a progressive increase in body iron stores. The hereditary nature of the disease was defined in 1935 (Sheldon 1935). Autosomal recessive inheritance of HH and linkage of the disease to the major histocompatibility complex (MHC) class 1 molecule HLA-A3 (human leukocyte antigen) on the short arm of chromosome 6 was described four decades later (Simon et al. 1977). The gene responsible for most HH cases was localized to chromosome 6p and subsequently called *HFE* (Feder et al. 1996). The *HFE* gene was identified in 1996 and shown to be an MHC class I-like gene, mapping over 5Mb from HLA-A. Two mutations in the *HFE* gene, i.e. C282Y and H63D, were present in 83% of the 178 HH patients analysed in this study.

The C282Y mutation may have originated approximately 2000 years ago in a single Celtic (or Viking) ancestor (Merryweather-Clarke et al. 1997; Rochette et al. 1999 Today the distribution of this mutation is widespread indicating that it posed no deleterious threat to reproduction as it was passed on and spread by population migration. It may have conferred certain advantages such as resistance to dietary iron deficiency and infectious diseases.

1.2.2. GENETIC CLASSIFICATION

Four different types of disease expression were recognized by Muir et al. (1984), suggesting that more than one gene involved in iron metabolism can cause HH. Group I, the classic

form, is associated with elevated serum transferrin saturation, serum ferritin levels, and liver iron content; group II with severe iron overload and accelerated disease manifesting at an early age; group III with elevated total body iron stores, normal serum transferrin saturation and serum ferritin levels; and group IV with markedly elevated findings on serum biochemical tests, i.e., serum transferrin saturation and serum ferritin, with minimal elevation in total body iron stores.

Four types of HH, each resulting from mutations in different genes all thought to be involved in regulating iron homeostasis have been identified. Type 1 or classic haemochromatosis (HFE1) is the most common of the primary HH conditions caused by mutations in the *HFE* gene (Feder et al. 1996). Type 2 haemochromatosis also known as juvenile haemochromatosis (JH) or HFE2, has two genetic forms, i.e. subtypes 2A and 2B (Roetto et al. 1999). Subtype 2A is the most common of the two and is caused by mutations in the haemojuvelin (*HJV*) gene located on chromosome 1q21 (Papanikolaou et al. 2004). Subtype 2B is caused by mutations in the hepcidin antimicrobial gene (*HAMP*) located at chromosome 19q13 (Roetto et al. 2003). Type 3 haemochromatosis or HFE3 results from mutations in the transferrin receptor 2 gene (*TFR2*) and type 4 haemochromatosis or HFE4 is caused by mutations in the solute carrier family 40 (iron-regulated transporter) gene (*SLC40A1*) (Camaschella et al. 2000; Montosi et al. 2001; Njajou et al. 2001).

Most experts in the field of iron metabolism do not support the OMIM classification because (a) it is a combination of genotypic and phenotypic descriptions (b) recently identified atypical cases related to combinations of sequence variations has no place in this description; (c) hypothetical genes, e.g., HFE1, HFE2, HFE3, etc., are created even though these genes do not belong to the same gene family; and (d) other forms of HH exists that are not included in

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this classification (Levy et al. 2000; Merryweather-Clarke et al. 2003; Bensaid et al. 2004; Jacolot et al. 2004; Le Gac et al. 2004; Pietrangelo et al. 2005; 148-152; Swinkels et al. 2006). Pietrangelo (2004) recently suggested that a definition of the disease should be based on the pathophysiologic entity and not on the responsible genes. The associations of the various genes with either an adult (*HFE*, *TFR*2, *SLC40A1*) or a juvenile onset (*HJV* and *HAMP*) are useful for genetic testing (Swinkels et al. 2006).

1.2.3. CLASSIC HEREDITARY HAEMOCHROMATOSIS

Type 1 or classic haemochromatosis (HFE1) is an autosomal recessive disease (Simon et al. 1977). It is characterized by excessive absorption of iron from the gut, leading to progressive iron accumulation in organs such as the liver, heart and pancreas (Bothwell et al. 1995). If left untreated, damage to and alteration of the structure and function of these organs will take place (Powell et al. 1998). Three stages are distinct to HH progression, these are: latency, biological expression (arises before the age of 20 years and corresponds to elevated iron parameters, such as serum iron, transferrin saturation, and serum ferritin) and clinical expression. The disease is associated with mutations in the *HFE* gene located on chromosome 6p (Feder et al. 1996). The majority of HH patients are either homozygous for the C282Y mutation in this gene or compound heterozygotes for the C282Y/H63D mutations (Feder et al. 1996).

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1.2.3.1. CLINICAL FEATURES

The most common clinical features of HH are cutaneous hyperpigmentation, diabetes mellitus and hepatomegaly (Sheldon 1935). Other symptoms include fatigue, abdominal pain, abnormal liver tests, hepatocellular carcinoma, cardiomyopathy, cardiac conduction defects, hypogonadism, impotence and arthropathy. In men clinical symptoms appear in the fourth or

fifth decade (Bothwell and MacPhail 1998). Females on average present with symptoms about a decade later than men because they are spared iron accumulation by physiological blood loss during menstruation and childbirth. Females usually have different symptoms than males such as fatigue and pigmentation although they present with the complete phenotype (Moirand et al. 1997). Symptoms typically exhibited by males include cirrhosis and diabetes. Due to this difference females may be underdiagnosed.

Fibrosis and cirrhosis generally develop when there is an excess of 400 µmol^{-1} dry weight of iron in the liver (Bassett et al. 1986; Adams et al. 1997). Primary hepatocellular carcinoma presents in 30% of cirhhotic patients, a fact that has to be orne in mind when patients display clinical deterioration, rapid liver enlargement, abdominal pain and ascites (Deugnier et al. 1993; Niederau et al. 1996). Hepatomegaly may occur in 95% of symptomatic patients and around 56% of these patients may experience abdominal pain (Niederau et al. 1996). Diabetes mellitus occurs in 30-60% of patients with a hereditary predisposition, cirrhosis or iron deposition in the pancreas (Powell and Yapp 2000). Athropathy of the hands is seen in 20-70% of patients. Loss of libido and testicular atrophy are common in symptomatic males. Amenorrhoea presents in 15% of symptomatic female cases (Niederau et al. 1996). Abnormal ECG readings are present in up to 35% of symptomatic patients presenting with cardiomyopathy. Cardiac complications result from iron deposition in the myocardium and conducting system.

1.2.3.2. TREATMENT

The treatment of HH is achieved through weekly regimens of phlebotomy of 500 ml whole blood (Tavill 2001). Venesections of 500 ml of whole blood removes approximately 250 mg of iron. A serum transferrin saturation value of greater than 45% and a serum ferritin

concentration of greater than 200 μg/l and 300 μg/l, in women and men respectively, is usually confirmation of iron overload (Burke et al. 1998). Hence, serum ferritin and transferrin saturation levels should be monitored and measured every three months. As soon as the serum ferritin and transferrin saturation levels are reduced to normal the procedure can be repeated every three to four months to maintain iron stores at the normal level.

Evidence shows that phlebotomy, when introduced before the onset of cirrhosis, reduces HH morbidity and mortality (Niederau et al. 1996). Clinical symptoms such as malaise, fatigue, skin pigmentation, insulin requirements in diabetics, and abdominal pain have been shown to improve with phlebotomy (Tavill 2001). However, for more severe clinical manifestations like arthropathy, hypogonadism and cirrhosis, phlebotomy has no effect. HH has båMn found to be rare in non-cirrhotics; which justifies the rationale of introducing phlebotomy before the development of cirrhosis (Deugnier et al. 1993).

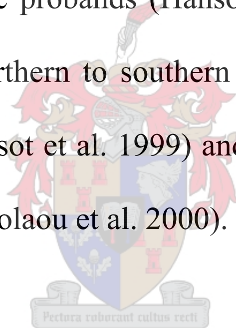
Patients undergoing phlebotomy are not required to follow a strict dietary regimen (Cunningham 2003). However, reduced intake of iron-containing and iron-fortified foods can prevent excess accumulation of dietary iron. Tannin, found in tea has been shown to inhibit iron absorption (blocking agent), but cannot be used as a substitute for phlebotomy. HH patients are usually recommended against consumption of uncooked seafood as they are susceptible to *Vibrio vulnificus* infection (Barton et al. 1998). Vitamin C supplementation should also be avoided as it could increase iron mobilization to a level that saturates circulating transferrin, resulting in an increase in pro-oxidant and free-radical activity (Tavill 2001). During rapid mobilization of iron, there is an additional risk of developing cardiac dysrhythmias and cardiomyopathy, which are the most common cause of deaths in iron overload cases. Clinical complications from HH should be treated accordingly; diabetes may

require insulin, arthritis should be managed with non-steroidal anti-inflammatory drugs and hypogonadotrophic hypogonadism may require hormone replacement therapy.

1.2.3.3. THE PREVALENCE OF *HFE* GENE MUTATIONS

i. C282Y

In the 1996 study by Feder et al., 83% of the HH patients investigated were homozygous for the C282Y mutation. Table 1.1 illustrates the worldwide distribution of the C282Y mutation among iron loaded patients (Camaschella et al. 2002). The C282Y homozygous genotype occurs in 84.5% of European probands, while the C282Y/H63D compound heterozygous genotype is present in 3.8% of these probands (Hanson et al. 2001). The incidence of the C282Y mutation decreases from northern to southern Europe, with the highest frequencies occurring in Brittanny (96.3%) (Brissot et al. 1999) and the lowest in Italy (64%) (Carella et al. 1997) and Greece (30%) (Papanikolaou et al. 2000).



In North America C282Y homozygosity is present in 60 to 83% of patients (Beutler et al. 1996; Feder et al. 1996; Barton et al. 1997). Merryweather-Clarke et al. (1997) reported a global allele frequency of 1.9% for this mutation in 2978 normal subjects from 42 different populations. The highest C282Y frequencies were found in Northern Europe, an observation which supports the theory that the mutation has a Celtic origin. C282Y is rare and has never been found in the homozygous state in indigenous populations of Asia, Africa, the Middle East and Australasia (Merryweather-Clarke et al. 1997; Hanson et al. 2001).

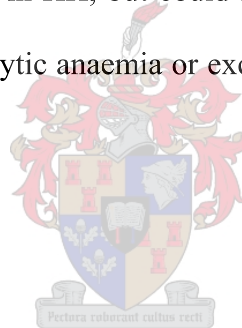
Table 1.1. The worldwide distribution of C282Y and H63D mutations

Population	Number of probands	C282Y/C282Y n (%)	C282Y/H63D n (%)	H63D/H63D n (%)	Heterozygotes or wildtype n (%)	References
Brittany	217	209 (96.3)	4 (1.8)	1 (0.5)	3 (1.4)	Brissot et al. (1999)
Germany	92	87 (94.6)	4 (4.3)	0 (0)	1 (1.1)	Nielsen et al. (1998)
Sweden	87	80 (92)	3 (3.4)	1 (1.2)	3 (3.4)	Cardoso et al. (1998)
United Kingdom	115	105 (91.3)	3 (2.6)	1 (0.9)	6 (5.2)	UK Haemochromatosis Consortium (1997)
Ireland	78	70 (89.7)	3 (3.8)	0 (0)	5 (6.5)	Ryan et al. (1998)
Spain	31	27 (87.0)	2 (6.5)	0 (0)	2 (6.5)	Sanchez et al. (1998)
France	94	68 (72.3)	4 (4.3)	2 (2.1)	20 (21.3)	Borot et al. (1997)
Italy	186	120 (64.5)	10 (5.4)	3 (1.6)	53 (28.5)	Carella et al. (1997)
Greece	10	3 (30)	2 (20)	0 (0)	5 (50)	Papinikolau et al. (2000)
Total Europe	910	769 (84.5)	35 (3.8)	8 (0.9)	98 (10.8)	
Canada (Ontario)	128	122 (95.3)	0	0	6 (4.7)	Adams and Chakrabarti (1998)
USA	147	122 (83)	8 (5.4)	2 (1.4)	15 (10.2)	Beutler et al. (1996)
USA (Alabama)	74	44 (59.5)	4 (5.4)	3 (4.1)	23 (31)	Barton et al. (1997)
South Africa	22	17 (77.5)	3 (13.6)	0	2 (9.1)	de Villiers et al. (1999a)
Australia	64	64 (100)	0	0	0	Jazwinska et al. (1996)

Camaschella et al. 2002

ii. H63D

The distribution of the H63D mutation is more widespread than C282Y, with the highest occurring among Basques (Merryweather-Clarke et al. 1997). In the Caucasian population the frequency of H63D carriers is almost twice that of C282Y mutation carriers (20% *vs* 10%) (Hanson et al. 2001). In the absence of C282Y, H63D has little effect on HH disease expression. When inherited with C282Y to produce a compound heterozygous genotype, it may contribute to a mild phenotype (Bacon et al. 1999). C282Y/H63D has a low penetrance, with only approximately 1.5% of individuals with this genotype developing iron overload. Only one case has been reported where C282Y and H63D was found in cis; this confirms that they are almost mutually exclusive (Feder et al. 1996; Spriggs et al. 1999). The H63D homozygous genotype rarely results in HH, but could in the presence of complications such as ineffective erythropoiesis, haemolytic anaemia or excess alcohol intake cause HH (Spriggs et al. 1999).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iii. S65C

A third mutation in the *HFE* gene, S65C was identified by Mura et al. (1999) and accounted for almost 8% of HH cases that harbour neither the C282Y nor H63D mutations. An allele frequency of the S65C mutation ranging from 1.6 to 5.5% in Caucasians has been reported (Rochette et al. 1999). S65C appears to be a benign polymorphism. In the presence of C282Y it may confer a slight increase in disease risk resulting in a mild disease phenotype (Mura et al. 1999).

iv. RARE *HFE* MUTATIONS

In addition to C282Y, H63D and S65C, other missense, nonsense and splice site mutations have been identified in the *HFE* gene including G93R (Barton et al. 1999), I105T, Q127H (de

Villiers et al 1999a), E168X (Piperno et al. 2000) and W169X. The effects of rare mutations on HH and population frequencies for these mutations still need to be determined (Lyon and Frank 2001). Pointon et al. (2000) estimated that approximately 2-10% of HH cases are attributed to mutations other than C282Y in the *HFE* gene.

Mutations in other genes causing non-HFE related forms of HH have been discovered (Camaschella et al. 2000; Montosi et al. 2001; Njajou et al. 2001; Roetto et al. 2001; Roetto et al. 2003; Franchini and Vineri 2005). Significant progress has been made in understanding the genetic transmission and molecular basis of HH. However, many questions remain to be answered regarding the regulation of iron metabolism before this intricate disease can be fully understood.

1.2.3.4. THE PENETRANCE OF THE *HFE* GENE MUTATIONS

The penetrance of a mutation is defined as the degree to which a specific phenotype is expressed in individuals that harbour the mutation (Beutler et al. 2003a). HH was originally described as a rare iron overload disease, but since then it has undergone several redefinitions; resulting in varying estimates of its prevalence (Waalen et al. 2005). The discovery of high frequencies of the C282Y in Northern European populations suggested that HH was more common than previously thought. However, today the penetrance of HH is a rather contentious issue (Beutler et al. 2003a).

i. CY282Y HOMOZYGOSITY

a. Biochemical penetrance

Few studies exist where an unbiased population has been screened for the C282Y mutation and, the transferrin saturation and ferritin levels of the homozygotes were determined (Beutler et al. 2003a). In one study of over 9000 individuals (3367 men and 6029 women) screened for C282Y; only 10 homozygous men and 44 homozygous women were identified (Deugnier et al. 2002). When iron parameters were measured, 80% of the men were found with transferrin saturations over 55% and 44% of women had transferrin saturations over 50%. These results support the observed increase in transferrin saturation and serum ferritin levels usually associated with C282Y homozygosity (Beutler et al. 2003a). This, however, is not true for all individuals, as a large number of C282Y homozygotes do not present with typical iron overload. Thus, even on a biochemical level the homozygous genotype is not always expressed, demonstrating that C282Y homozygosity is a necessary but not sufficient factor in causing HH.

b. Clinical penetrance

Numerous large population-based studies have demonstrated no significant difference between the prevalence of symptoms associated with iron overload in C282Y homozygotes and age-and sex-matched controls (Waalen et al. 2005). In 2002, Beutler et al. undertook a study of more than 41 000 individuals to compare the penetrance of clinical symptoms associated with HH between patient and control subjects. Results from this study showed that classic clinical symptoms of HH such as poor general health, diabetes, arthropathy, impotence and skin pigmentation were no more prevalent in the 152 identified C282Y homozygous individuals than they were in homozygous wildtype matched controls. The only significant difference found between homozygous C282Y patients and controls was a higher prevalence of abnormal liver function tests. Among the 152 homozygotes only a single patient was

identified with clinical symptoms typical of HH. They deduced that the penetrance of the homozygous genotype is about 1%.

ii. COMPOUND HETEROZYGOSITY

Compared to wild-type individuals, compound heterozygotes display notably higher transferrin saturation and ferritin levels (Beutler et al. 2003 a). Due to the fact that the H63D mutation is so ubiquitous, compound heterozygosity is also frequent. In patients diagnosed with HH using biochemical parameters, a greater number of compound heterozygotes were found (Beutler 1997). The biochemical penetrance of this genotype was estimated to be about 1% of that of the homozygous genotype. Severe cirrhosis and other clinical symptoms of HH are usually uncommon in these patients (Beutler et al. 2003 a).

iii. THE HETEROZYGOUS GENOTYPE

Individuals heterozygous for either the C282Y or H63D mutations have only mildly raised transferrin saturation and ferritin levels (Beutler et al. 2003a). Certain studies suggest that patients with these mutations are at a greater risk of developing diabetes (Kwan et al. 1998; Moczulski et al. 2001), heart disease (Roest et al. 1999; Tuomainen et al 1999), and cancer (Geier et al. 2002, Shaheen et al. 2003). However, no evidence exists to confirm these suggestions (Bozzini et al. 2002; Waalen et al. 2002a; Waalen et al. 2002b; Halsall et al. 2003). These patients usually do not experience poor health, although there is one exception. It has been shown that patients are at increased risk of developing porphyria cutanea tarda if they carry either C282Y or H63D (Bulaj et al. 2000; Tannapfel et al. 2001). Common mutations in the genome may provide an advantage; perhaps they represent a balanced polymorphism (Datz et al. 1998; Beutler et al. 2003b).

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1.2.3.5. FAMILY AND POPULATION SCREENING

All first-degree relatives (parents, siblings and children) of an affected subject should be screened for the C282Y and H63D mutations (Tavill 2001). Before testing, individuals should be counselled about risks, benefits and options by a qualified professional (Adams et al. 2000). Those family members testing homozygous for the C282Y mutation or heterozygous for the C282Y/H63D mutations should have their serum ferritin, fasting transferrin saturation and liver enzymes assessed (Guyader et al. 1998; Bacon et al. 1999; Tavill 2001; Morrison et al. 2003). Phlebotomy should be initiated in those subjects with raised ferritin levels and transferrin saturation levels greater than 45%. A liver biopsy should only be considered in homozygous subjects, especially those over the age of 40, with clinical evidence of liver disease and serum ferritin levels greater than 1000 µg/l.

HH meets all the criteria set out by the World Health Organisation (WHO) for population screening such as latent period for disease development, the availability of a screening test, and safe, cost-effective treatment (Burke et al. 1998; Adams et al. 2000). However, a clear consensus about the value of universal population screening for HH remains a controversial issue. Two reasons are uncertainty still exists about the penetrance of the disease and which test, biochemical or genetic, should be used to diagnose at risk individuals (Njajou et al. 2004).

1.2.4. NON-*HFE* RELATED FORMS OF HAEMOCHROMATOSIS

Juvenile haemochromatosis or type 2 haemochromatosis is a rare autosomal recessive condition characterized by severe iron overload (De Gobbi et al. 2002). It affects both males and females at an early age, usually presenting in the second and third decades of life. The most common clinical symptoms are cardiomyopathy and hypogonadism.

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Type 3 haemochromatosis is an autosomal recessive adult onset form of HH, characterized by gradual iron loading, a relatively late onset of parenchymal iron deposition, and predominantly hepatic organ damage (Camaschella et al. 2000; Kawabata et al. 2004; Nemeth et al. 2005). Presently it is unclear as to how the transferrin receptor 2 (TfR2) relates to HFE or hepcidin. It has been speculated that transferrin-bound iron (TBI) uptake via TfR2 or TfR2 protein expression in the liver plays a crucial role in hepcidin expression. Reduced hepcidin levels were shown in patients with type 3 haemochromatosis and TfR2 mutant mice.

Type 4 haemochromatosis, an autosomal dominant condition, was first recognized in three Italian families with iron overload not linked to the *HFE* gene (Pietrangelo et al. 1995). The clinical symptoms of this disease are milder than that of type 1 haemochromatosis (Montosi et al. 2001; Njajou et al. 2001). Minimal sinusoidal fibrosis is the predominant clinical feature of type 4 haemochromatosis. Patients usually present with anaemia in early life despite increased serum ferritin levels and iron accumulation in reticuloendothelial cells. It has been speculated that mutations in the *SLC40A1* gene causes ferroportin 1 (FPN1) protein to lose its iron export function in macrophages. This leads to iron retention, impaired iron recycling in the reticuloendothelial cells and reduced iron availability for circulating transferrin. Patients with type 4 haemochromatosis have inappropriately low serum transferrin saturation levels compared to their serum ferritin levels (Pietrangelo 2003). This results in abnormal increases in duodenal iron absorption contributing to iron overload. During phlebotomy treatment patients may develop anaemia and low transferrin saturation levels despite elevated serum ferritin levels.

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1.2.5. DIGENIC INHERITANCE AND MODIFIERS

Increasing evidence suggest that multiple genes cause HH (Camaschella 2005). Digenic inheritance was initially described in a severe juvenile phenotype with heterozygous mutations in both *HFE* (C282Y) and *HAMP* (ATGG deletion in exon 2) (Merryweather-Clarke et al. 2003). Hfe $^{-/-}$ mice deficient of a single hepcidin allele were shown to have much higher liver iron accumulation than mice that are only Hfe $^{-/-}$ (Nicolas et al. 2004). A more severe biochemical phenotype was reported in C282Y homozygous subjects with heterozygous mutations in *HAMP* or *HJV* than subjects with only C282Y homozygosity (Jacolot et al. 2004; Le Gac et al. 2004). This modulatory effect may also be observed in C282Y/H63D compound heterozygotes with *HAMP* or *HJV* heterozygous mutations (Biasiotto et al 2003; Merryweather-Clarke et al. 2003; Biasiotto et al. 2004; Jacolot et al. 2004; Le Gac et al. 2004). The precise effect of these mutations in only C282Y heterozygotes has not been determined. Digenic inheritance has also been illustrated in a juvenile phenotype where the C282Y/H63D compound heterozygous genotype and a homozygous missense mutation in *TFR2*, occurred together (Pietrangelo et al. 2005). The phenotype observed was similar to that resulting from full hepcidin inactivation. Polymorphisms in haemochromatosis-related genes may be modifiers of the main genotype (C282Y homozygous state) and possibly increase the iron burden or provide a protective effect (Roy and Andrews 2001). Modifiers have a greater effect on the milder C282Y homozygous genotype than severe mutations. Multiple polymorphisms may contribute in altering the classic monogenic haemochromatosis disorder into an oligogenic disease (Camaschella 2005). In selected cases mutation screening for all haemochromatosis genes could be beneficial, particularly in cases where a severe phenotype cannot sufficiently be explained by the C282Y/H63D compound heterozygous or C282Y heterozygous genotypes.

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1.2.6. IRON HOMEOSTASIS

Iron is one of the most important nutrients required by the human body. Its bioavailability, however, is limited (Papanikolaou et al. 2004). In states of excess, this vital nutrient poses a major threat to cells and tissues. Thus stringent regulation of iron homeostasis is crucial.

1.2.6.1. IRON DISTRIBUTION IN HUMANS

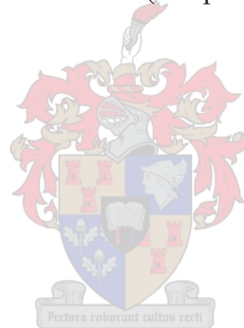
Mammals lack physiological pathways for iron excretion, thus body iron homeostasis is controlled by absorption from the intestine (reviewed by Papanikolaou and Pantopoulos 2005). The human body normally contains between 3-5 g of iron [45-55 mg/kg of body weight in adult women and men, respectively] (Ponka 1997; Andrews 1999). Approximately 60 to 70% of iron in the body is utilised within haemoglobin in circulating red blood cells and in the region of 20%-30% is stored in hepatocytes and in reticuloendothelial macrophages. The rest is contained in myoglobin, cytochromes, and iron-containing enzymes. The daily iron absorption of a healthy individual is about 1-2 mg of iron from the diet. Non-specific iron losses are compensated by cell desquamation in the skin and the intestine. The liver stores approximately 1 g iron under normal circumstances (Bomford and Williams 1976). Upon the clinical presentation of HH this can increase to as much as 40 g.

1.2.6.2. IRON ABSORPTION

Dietary iron occurs in two states, i.e. haem and ionic (non-haem) iron (McKie et al. 2001). These are absorbed at the apical surface of the duodenal enterocytes *via* different mechanisms. Before dietary non-haem iron can be transported across the intestinal epithelium it has to be reduced from its pre-existing oxidised ferric (Fe^{3+}) form (which is not bioavailable) to ferrous iron (Fe^{2+}) by cytochrome b reductase (CYBRD1), a ferrireductase enzyme. Following transport across the intestinal epithelium, Fe^{2+} is carried into the cell by

the divalent metal transporter 1 (DMT1) which facilitates the transport of other metal ions such as zinc, cobalt, copper and lead (Gushin et al. 1997; Fleming et al. 1997).

Haem iron is imported into the enterocyte by an as yet unidentified haem receptor (Quigley et al. 2004). As soon as iron reaches the enterocyte, it is released from haem by haem oxygenase (HMOX). From here it can either be stored or moved out of the enterocyte across the basolateral membrane by mechanisms similar to that of ionic iron. Ferroportin 1 [FPN1, also known as the iron-regulated transporter 1 (IREG1) or metal transporter 1 (MTP1)] transports Fe^{2+} across the basal membrane (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). Fe^{2+} is then oxidised by hephaestin (HEPH), a multicopper oxidase protein, before it is bound by plasma transferrin (Vulpe et al. 1999) (Figure 1.1).



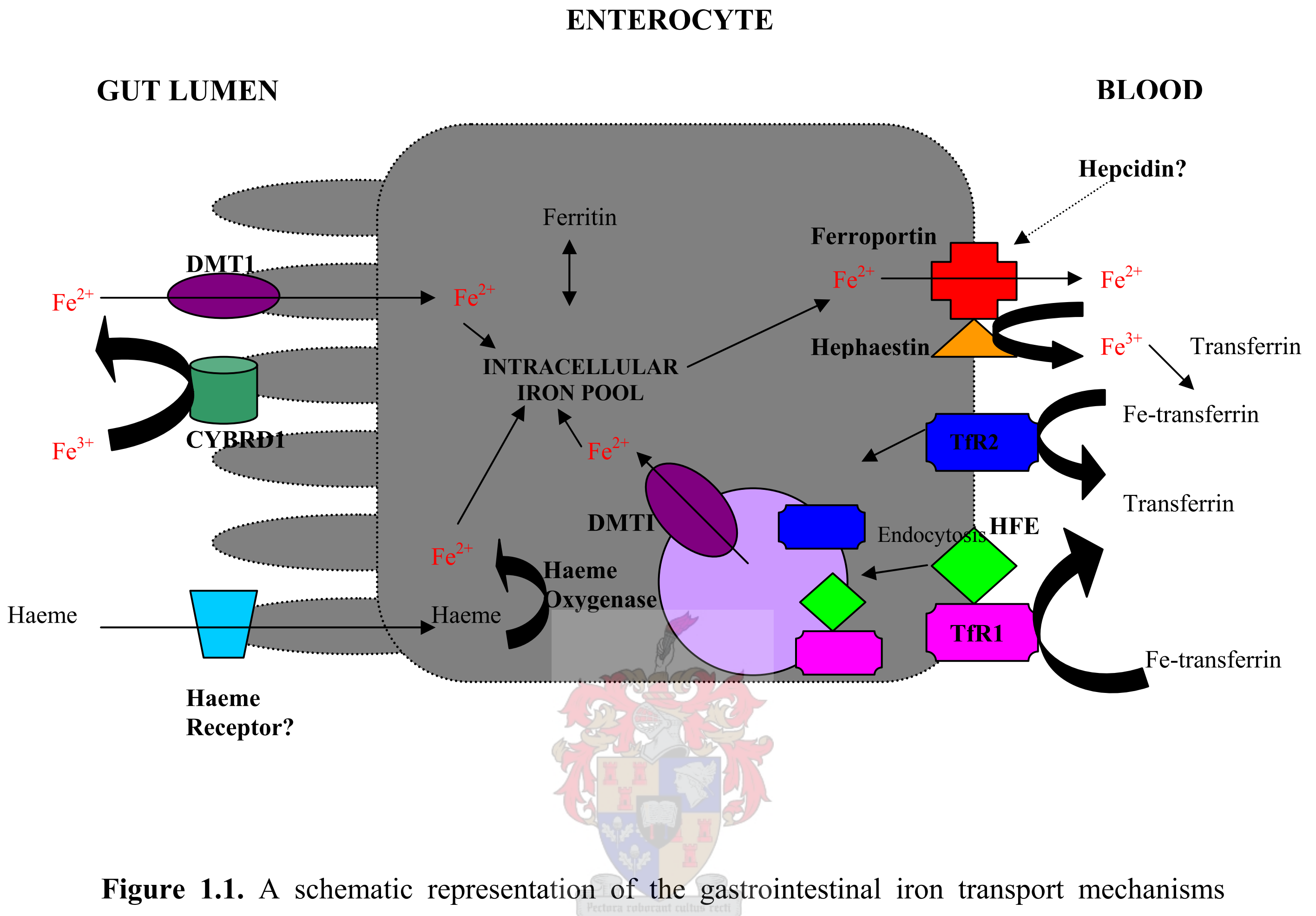


Figure 1.1. A schematic representation of the gastrointestinal iron transport mechanisms (Adapted from Siah et al. 2005)

1.2.6.3. LIVER IRON TRANSPORT AND STORAGE

Iron is predominantly stored in the liver (Sciot et al. 1987; Trinder 1990). In a state of iron overload, free radicals and lipid peroxidation products are usually produced. These end products may cause progressive tissue damage and consequently result in cirrhosis or hepatocellular carcinomas. Ferritin and haemosiderin are the two main forms in which iron is sequestrated in hepatocytes. Liver uptake of TBI is ascribed to two transferrin receptors - TfR1 and TfR2. TfR1 is down-regulated in the hepatocytes in conditions of iron overload and its expression on hepatocytes is completely absent in untreated HH patients. The hepatocytes

also express the HFE protein. Increasingly evidence is being provided to support the notion that HFE probably controls TfR1-mediated uptake of TBI (Kawabata et al. 1999).

TfR2 is abundantly expressed in the human liver and plays a vital role in liver iron loading under conditions of iron overload. Transferrin saturation regulates TfR2 6kotein expression, whereas TfR1 expression is under the control of an iron response element, lacking in TfR2 (Johnson and Enns 2004; Robb and Wessling-Resnick 2004). The TfR2 protein has been shown to be upregulated in a state of iron overload and in a Hfe knockout mouse model of HH. It therefore could most likely play a crucial role in liver TBI uptake in iron overload.

Although TfR2 has a 30-fold lower affinity than TfR1 for TBI, it has a greater capacity to transport TBI into the hepatocyte (Fleming et al. 2000). Studies have demonstrated that in both normal and iron loaded conditions expression of TfR2 is higher than that of TfR1. These findings suggest that TfR2 is essential in hepatic iron loading in HH.

Excess iron also occurs as non-transferrin-bound iron (NTBI). NTBI has been shown to possibly play an important part in hepatocyte iron loading in HH and other iron overload conditions (Wright et al. 1986). Once NTBI is detected in the plasma it is removed by the liver because of its extreme toxic properties. Humans and mice lacking transferrin develop substantial iron overload in non-hematopoietic tissues such as the pancreas and liver (Trinder and Morgan 1997; Trendor et al. 2000). Plasma NTBI is increased and hepatocyte NTBI uptake is increased 2.5 fold in Hfe knockout mice (Chua et al. 2004). A carrier-mediated process consistent with DMTI is responsible for the transport of NTBI across the hepatocyte membrane (Randell et al. 1994; Chua et al. 2004). FPN1 mediates the transport of iron from the hepatocytes, which is oxidised by ceruloplasmin and then bound to transferrin (Harris et al. 1999; Abboud et al. 2000) (Figure 1.2).

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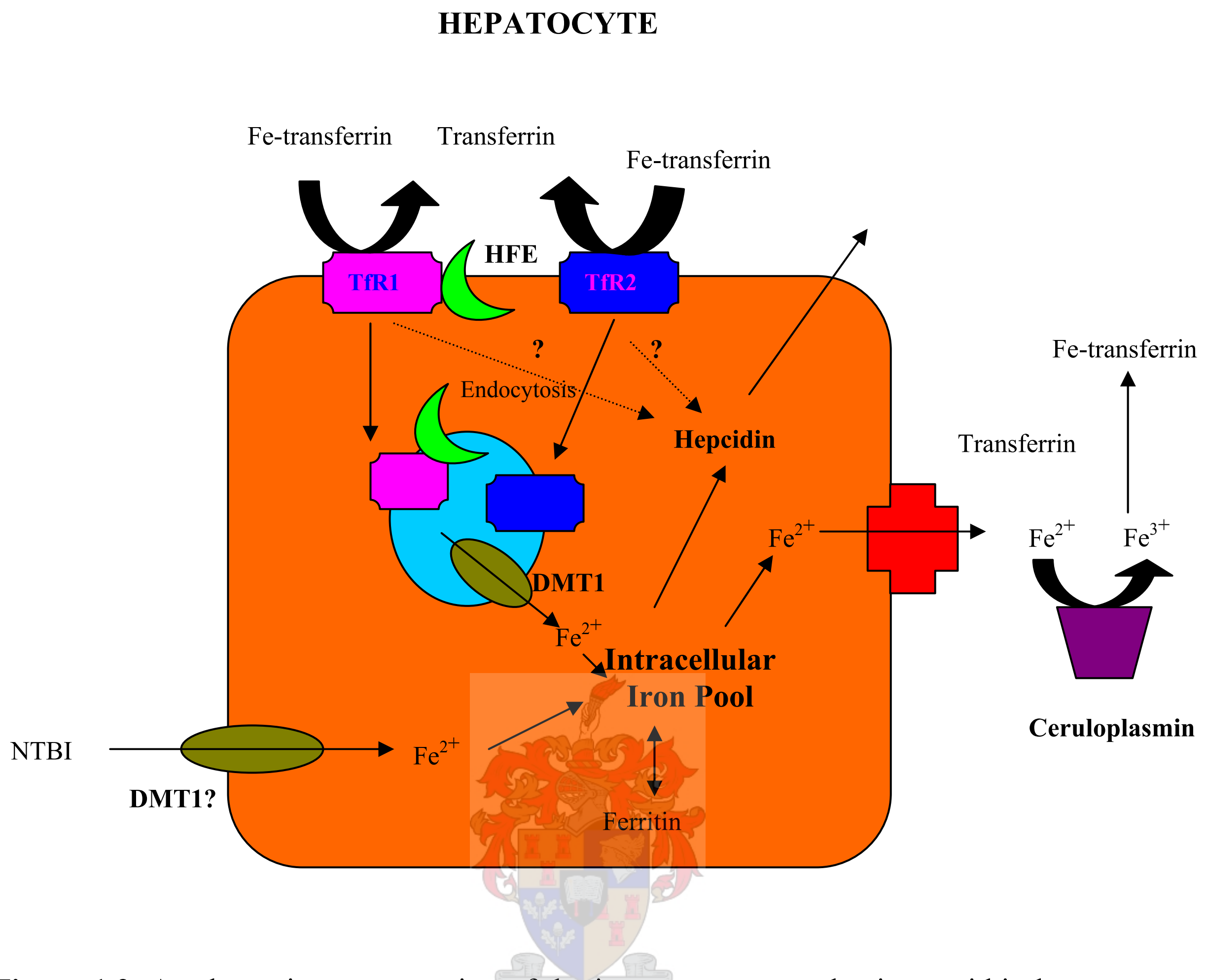
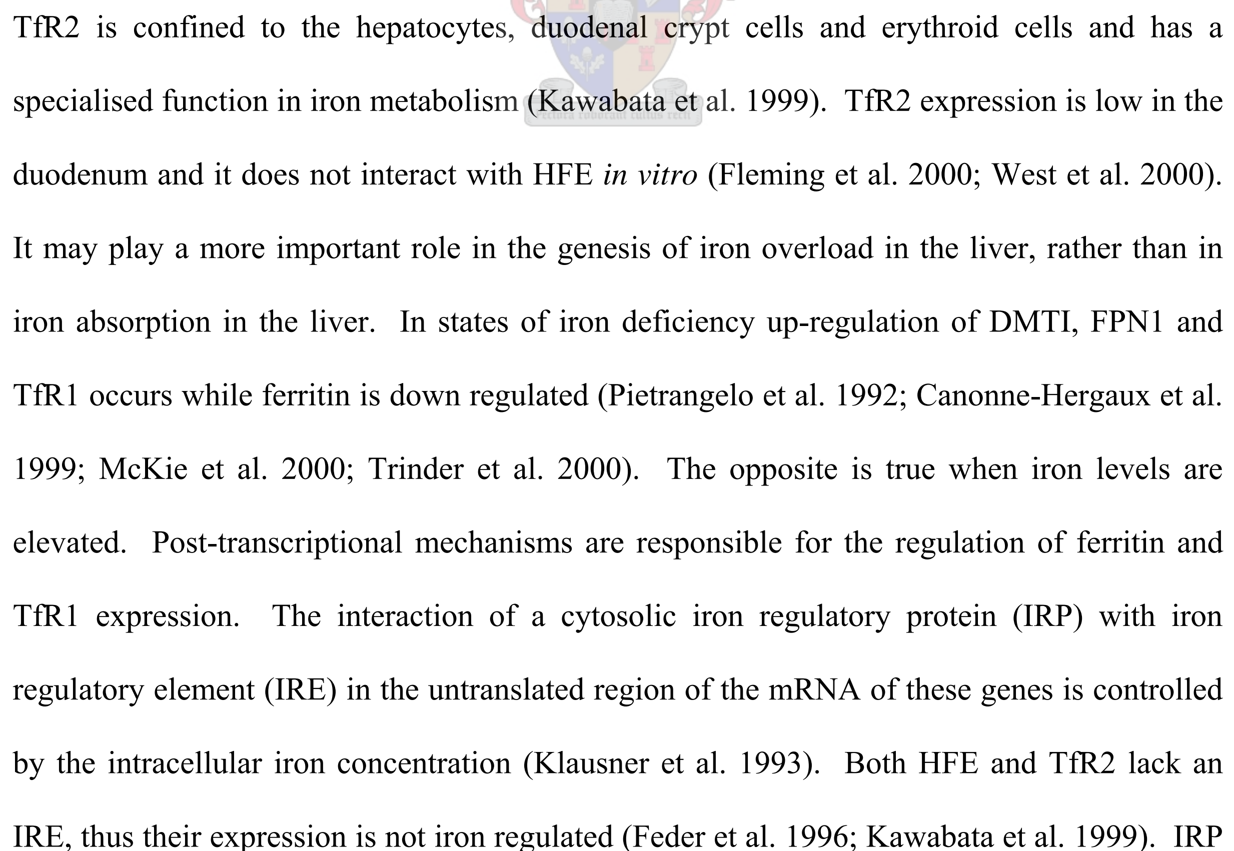


Figure 1.2. A schematic representation of the iron transport mechanisms within hepatocytes (Adapted from Siah et al. 2005)

1.2.6.4. THE REGULATION OF IRON METABOLISM

The regulation of iron absorption is dependant on numerous factors, including the body's iron stores, hypoxia and the rate of erythropoieisis (reviewed by Siah et al. 2005). Enterocytes localized in the crypts of the duodenum are responsible for the uptake of iron from the plasma. An association between the intracellular iron levels of the crypt cells and the body's iron stores has been documented. The body's iron stores determine the amount of iron absorbed from the gut lumen by upward migrating crypt cells, which eventually become absorptive cells at the brush border. Both TfR1 and TfR2 are expressed in the crypt cells

(Hentze et al. 2004). The crypt cells also highly express HFE, which forms a complex with β_2-microglobulin and TfR1 (Parkkila et al. 1997). There is still uncertainty as to what the exact role of the HFE protein is in the regulation of TfR1 mediated TBI uptake (Waheed et al. 1999). Studies have illustrated that HFE competitively inhibits TBI binding to TfR1. It has also been shown that it reduces the cycling time of the HFE/TfR1-TBI complex through the cell and decreases the rate of iron release from transferrin inside the cell. When HFE and β_2-microglobulin were overexpressed in Chinese Hamster Ovary cells, TBI uptake was improved as a consequence of increased recycling of TfR1 through the cell (Waheed et al. 2002). The effect was opposite to that seen under conditions of high intracellular iron concentrations. Lack of HFE in human HH and Hfe knockout mice may cause a decrease in TBI uptake from plasma into the crypt via TfR1 (Trinder et al. 2002). Townsend and Drakesmith (2002) proposed that this may also result in the inhibition of iron release from the cell via FPN1.



TfR2 is confined to the hepatocytes, duodenal crypt cells and erythroid cells and has a specialised function in iron metabolism (Kawabata et al. 1999). TfR2 expression is low in the duodenum and it does not interact with HFE *in vitro* (Fleming et al. 2000; West et al. 2000). It may play a more important role in the genesis of iron overload in the liver, rather than in iron absorption in the liver. In states of iron deficiency up-regulation of DMTI, FPN1 and TfR1 occurs while ferritin is down regulated (Pietrangelo et al. 1992; Canonne-Hergaux et al. 1999; McKie et al. 2000; Trinder et al. 2000). The opposite is true when iron levels are elevated. Post-transcriptional mechanisms are responsible for the regulation of ferritin and TfR1 expression. The interaction of a cytosolic iron regulatory protein (IRP) with iron regulatory element (IRE) in the untranslated region of the mRNA of these genes is controlled by the intracellular iron concentration (Klausner et al. 1993). Both HFE and TfR2 lack an IRE, thus their expression is not iron regulated (Feder et al. 1996; Kawabata et al. 1999). IRP

activity is a crucial regulator of iron absorption. In the crypt cells, IRP activity is an indicator of the body's iron status (Schumann et al. 1999). The level of IRP binding activity is predetermined in the crypts. This is crucial when the crypt cells migrate to the villus region of the duodenum, where the level of IRP activity regulates expression of iron transporters and the rate of iron absorption. The villus cells also respond when a change in dietary iron levels take place (Oates et al. 2000). An iron gavage can result in a decrease in IRP activity, DMTI expression and iron absorption by the villus cells within hours.

Studies have demonstrated that FPN1 and CYBRD1 expression are upregulated in hypoxia and in a hypotransferrinaemic mouse with chronic anaemia as a result of defective erythropoiesis (Raja et al. 1988; McKie et al. 2000; McKie et al. 2001). This clearly illustrates that the rate erythropoeisis and hypoxia regulates iron absorption and that increased expression of these two genes result in increased iron absorption.

1.2.7. GENES IMPLICATED IN THE REGULATION OF IRON HOMEOSTASIS

Over recent years a number of genes have been identified that are involved in iron homeostasis. These include the high-iron (*HFE*) gene (Feder et al. 1996), haem oxygenase 1 (*HMOX1*) gene (Wise et al. 1964), solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2 gene [*SLC11A2*, also known as the natural resistance-associated macrophage protein 2 gene (*NRAMP2*) or divalent metal transporter 1 gene (*DMT1*)] (Gruenheid et al. 1995; Gunshin et al. 1997), hephaestin (Vulpe et al. 1999; Kaplan and Kushner 2000), transferrin receptor 2 gene (*TFR2*) (Camaschella et al. 2000; Roetto et al. 2001), hepcidin antimicrobial peptide gene [*HAMP*, also known as the liver-expressed antimicrobial peptide gene (*LEAP1*) or hepcidin (*HEPC*)] (Krause et al. 2000; Park et al.

2001; Pigeon et al. 2001; Nicolas et al. 2001), solute carrier family 40 (iron-regulated transporter) member 1 gene [*SLC40A1,* also known as the ferroportin 1 gene (*FPN1*), iron-regulated transporter 1 gene (*IREG1*), metal transporter 1 gene (*MTP1*) or solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 gene *(SLC11A3)*] (Donovan et al. 2000; McKie et al. 2000; Abboud and Haile 2000), cytochrome b reductase 1 gene [*CYBRD1,* also known as duodenal cytochrome b gene (*DCYTB*)] (McKie et al. 2001), ceruloplasmin (Cairo et al. 2001) and the hemojuvelin (*HJV*) gene (Papanikolaou et al. 2004). In this study we investigated the role of the *HFE*, *HMOX1, SLC40A1*, *CYBRD1*, *HAMP* and *HJV* genes in patients presenting with primary iron overload in the absence of secondary factors. The ensuing sections will be dedicated entirely to discussing these genes, the proteins they encode (see Table 1.2), and their respective roles in iron homeostasis.

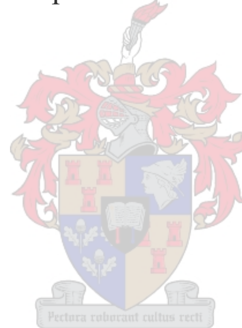


Table 1.2. Proteins involved in iron homeostasis

Protein	Chromosomal Location	Structure	Function
HFE	6p21	MHC class 1-like glycoprotein; forms heterodimer with β_2-microglobulin	Uncertain; the HFE protein β_2-microglobulin heterodimer binds transferrin receptor, reducing its affinity for transferrin
Haem oxygenase-1	22q12	Protein with haem site between α-helical folds	Catalytic oxidation of haem to Fe (II), carbon monoxide and biliverdin
SLC40A1	2q32	Single-chain glycoprotein with at least 10 transmembrane domains	Iron export
CYBRD1	2q31	286 amino acid di-haem protein	Ferric reductase
HAMP	19q13	20-25 amino acid peptide	Regulator of iron transport
Hemojuvelin	1q21	426 amino acid protein with RGM motif	Modulates hepcidin expression
Ceruloplasmin	3q21-24	Single-chain glycoprotein that contains six copper atoms	Probable serum ferroxidase
Hephaestin	Xq11-12	Transmembrane-bound ceruloplasmin homologue	Possible intracellular ferroxidase
Transferrin receptor 2	7q22	Transferrin receptor homologue	Uncertain; possible receptor-mediated endocytosis of ferric transferrin; mutated in a form of non-HFE haemochromatosis

Abbreviations: HAMP = hepcidin antimicrobial peptide; SLC40A1 = solute carrier family 40 (iron-regulated transporter) member 1; CYBRD1 = cytochrome b reductase 1 (Adapted from Sheth and Brittenham 2000)

1.2.7.1. HIGH-IRON GENE *(HFE)*

Since its discovery in 1996 the *HFE* gene has been implicated as the major cause of HH in most Caucasian populations (Feder et al. 1996). It is located on chromosome 6p21.3 and encodes a 343- residue type 1 transmembrane glycoprotein. This protein is homologous to the MHC class 1 protein, HLA-A2 and the non-classical class 1 protein, HLA-G. These molecules share similarity in their sequence and three-dimensional structure. Both HFE and MHC class 1 proteins contain three extracellular domains (α1, α2 and α3), a transmembrane domain and a short cytoplasmic tail (Lebron et al. 1998). The α1 and α2 globular domains form an eight-stranded antiparallel β-sheet platform topped by two α helices. This platform structure is maintained on the surface of an immunoglobulin constant-like α3 domain. The α3 domain binds to β_2-microbulin to form a heterodimer. This interaction is vital for cell surface expression of this molecule. In MHC proteins, α1 and α2 helices create a groove for peptide binding; in contrast HFE does not bind peptides. Crystallographic studies provided evidence that the HFE α1 helix is located close to the α2 helix and forms a shallower, narrower groove than the MHC peptide-binding groove. The differences in physical structures indicate each of these proteins has different roles in cellular Tf-mediated uptake (Feder et al. 1998). A cluster of four histidine residues resembling the composition of iron-binding sites in several proteins occurs on the surface of the α1 domain (Lebron et al. 1998).

The precise molecular mechanism by which HFE regulates iron uptake is being disputed. HFE is thought to form a complex with the transferrin receptor 1 (TfR1) and influence intracellular iron delivery (Parkkila et al. 1997; Feder et al. 1998). The association of HFE with TfR1 has been shown to reduce the binding affinity of TfR1 for transferrin by five-to ten-fold (Feder et al. 1998, Gross et al. 1998; Ikutu et al. 2000).

Studies have sought to establish the effects of the *HFE* gene mutations, C282Y and H63D, on protein structure and function (Feder et al. 1996). The C282Y mutation located in the α3 domain converts a cysteine residue to a tyrosine residue at amino acid position 282. This mutation disrupts the formation of a disulphide bond and alters HFE protein folding (Waheed et al. 1997). As a consequence, the mutant HFE protein cannot bind β_2-microbulin. Interaction of this protein with β_2-microbulin is essential for protein processing, transport and cell surface expression. These processes are impaired in the mutant HFE protein confined to the endoplasmic reticulum and mid-Golgi compartments. Hence it does not undergo late Golgi processing and is degraded rapidly resulting in the loss of protein function.

A second mutation in the *HFE* gene, H63D is localized in the α1 domain and converts a histidine residue to aspartate at amino acid position 63 (Waheed et al. 1997). The amino acid substitution resulting from this mutation hinders the formation of a His-Asp salt bridge and disrupts local protein structure. H63D is expressed at the cell surface and lacks the TfR interaction of the wild-type protein (Feder et al. 1998). Under normal circumstances cells depend on HFE to modulate iron intake, but the mutation results in the deposition of excess iron in the cells. Thus the deduction that H63D mutations disrupt normal protein functioning is supported by strong evidence. Figure 1.3 is a schematic representation of the HFE protein.

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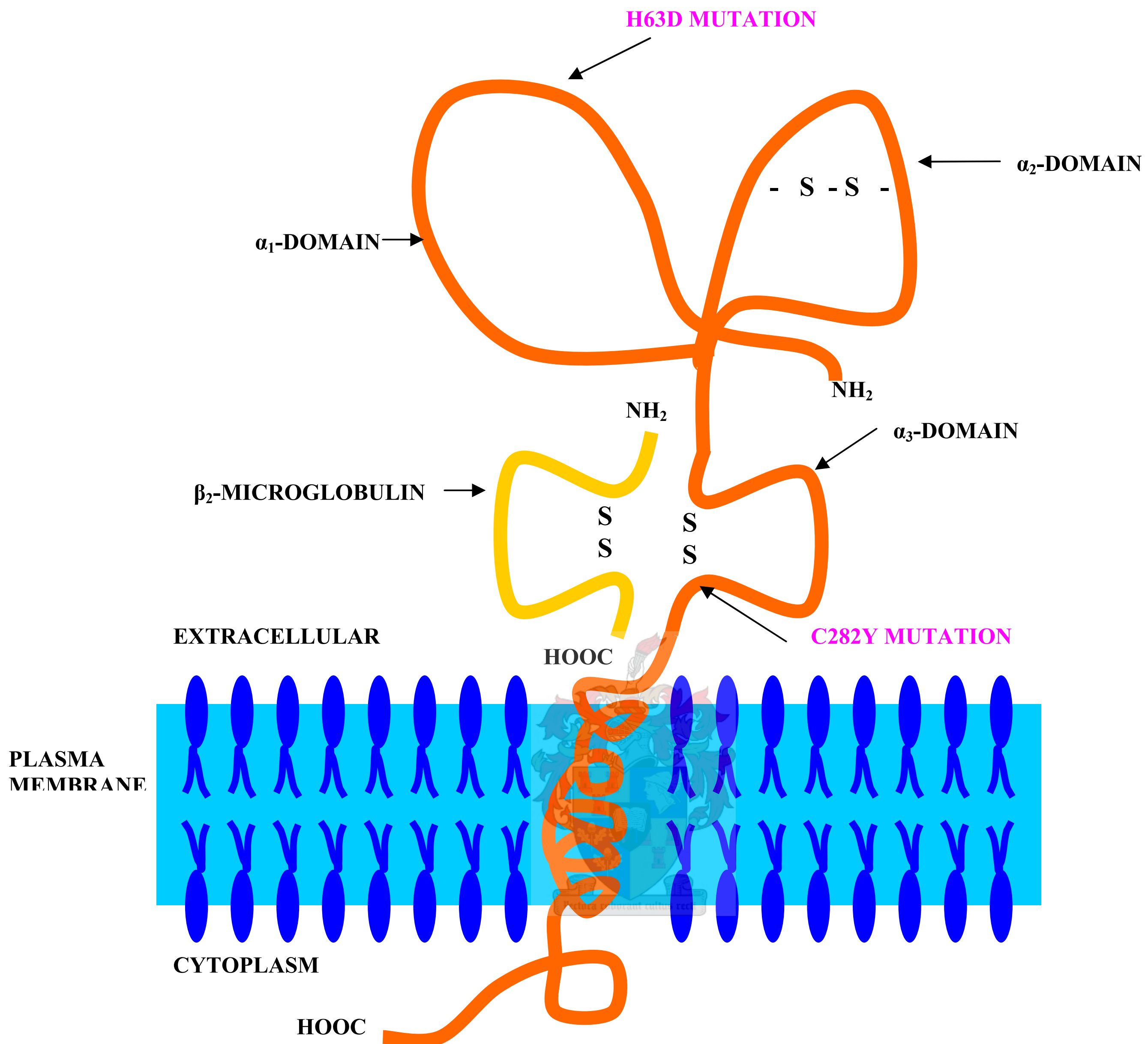
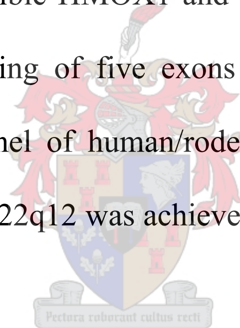


Figure 1.3. A schematic representation of the HFE protein structure (Adapted from Feder et al. 1996). The HFE protein consists of three extracellular domains, a transmembrane domain and a short cytoplasmic tail. Under normal conditions the α3 domain binds to β_2-microbulin to form a heterodimer for cell surface expression. The C282Y mutation, localized in the α3 domain, disrupts the formation of a disulphide bond and the H63D mutation localized in the α2 domain hinders the formation of a His-Asp salt bridge.

1.2.7.2. HAEM OXYGENASE-1 GENE (*HMOX1*)

The HMOX protein is a vital enzyme in haem catabolism, degrading haem to biliverdin, which is converted to bilirubin by biliverdin reductase, and carbon monoxide (Wise et al. 1964). Both haem and non-haem substrates induce the activity of HMOX. The *HMOX* gene was cloned by Yoshida et al. (1988). Human macrophages treated with hemin displayed an elevation in HMOX activity and mRNA levels. Poly (A)-rich RNA isolated from these macrophages was used to construct a cDNA library, from which human HMOX cDNA was isolated when screened with a rat cDNA. The nucleotide sequence of the deduced HMOX consists of 288 amino acids and has a molecular mass of 32,800 Da. The amino acid sequence between rat and human HMOX share 80% homology. The HMOX protein occurs in two isozymic states, i.e. an inducible HMOX1 and a constitutive HMOX2 (Maines et al. 1986). The *HMOX1* gene, consisting of five exons was mapped to chromosome 22 by polymerase chain reaction on a panel of human/rodent somatic cell hybrids (Kutty et al. 1994). Refined mapping to position 22q12 was achieved by fluorescence *in situ* hybridisation (FISH).



The protein was shown to be identical to the heat shock protein 32, which raised the possibility that HMOX may represent a stress-responsive protein (Keyse and Tyrrell 1989). A Wistar rat model was used to study the involvement of haem and its degrading enzyme enzyme HMOX in the inflammatory process during wound healing (Wagener et al. 2003). Haem was shown to accumulate directly at the edges of a wound. This coincided with an increased adhesion molecule expression and the recruitment of leukocytes. Administered intra-dermally 24 hours before injury, resultant haem-induced influx of macrophages and granulocytes were reported. In animals without wounds, HMOX1 was expressed in the mucosa and skin epithelia. In the presence of inflammation, HMOX1 expression increased,

especially in infiltrating cells during the resolution phase. It was suggested that local release of haem might possibly act as a trigger to initiate inflammatory processes, whereas HMOX1 antagonizes inflammation by attenuating adhesion interactions and cellular infiltration. In the skin basal level expression of HMOX could serve as a protection against acute oxidative and inflammatory insults. HMOX1 can be induced by more diverse stimuli than any other enzyme described to date (Maines 1997). Most of these inducers including haeme, hyperoxia, hypoxia, UV light, heat shock, endotoxins, heavy metals, hydrogen peroxide and nitric oxide seem to cause oxidative stress (Keyse and Tyrell 1987; Ewing and Maines 1991; Lautier et al. 1992; Camhi et al. 1995; Eyssen-Hernandez et al. 1996; Lee et al. 1996; Lee et al. 1997; Carraway et al. 1998; Carraway et al. 2000). The nature and extent of mutations in the *HMOX1* gene contributing to HH in humans remain to be determined. However, it is evident that this gene plays an essential role in regulating iron homeostasis.

1.2.7.3. SOLUTE CARRIER FAMILY 40 MEMBER 1 GENE (*SLC40A1*)

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The *SLC40A1* gene maps to chromosome 2q31 and encodes a transmembrane protein involved in cellular iron export from duodenal enterocytes and macrophages (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). It was discovered independently by two different groups; the first of whom identified the gene through positional cloning in *weissherbst zebrafish*, as a cause of hypochromic anaemia (Donovan et al. 2000). This group subsequently isolated mouse and human SLC40A1 from the liver and placenta by RT-PCR. The second approach employed a subtractive cloning strategy and PCR analysis to isolate the gene from mouse and human duodenal cDNAs (McKie et al. 2000).

The *SLC40A1* gene is 20 kb long and comprises of eight exons (Njajou et al. 2001) encoding a 571 amino acid protein (McKie et al. 2000). The predicted protein structure contains ten

transmembrane domains situated in the basolateral membrane in the intestinal enterocyte. An iron regulatory element (IRE) with a hairpin-loop structure is located in the 5' untranslated region of the gene. In humans *SLC40A1* expression was shown to be highest in placenta, liver, spleen and kidney. McKie et al. (2000) observed iron efflux in *Xenopus oocytes* stimulated by *SLC40A1* expression. Type 4 haemochromatosis (HFE4) was found to be associated with mutations in the *SLC40A1* gene (Montosi et al. 2001; Njajou et al. 2001; Devalia et al. 2002; Roetto et al. 2002; Wallace et al. 2002).

1.2.7.4. CYTOCHROME B REDUCTASE 1 GENE (*CYBRD1*)

The *CYBRD1* gene was isolated from hypotransferrinaemic mice by a subtractive cloning strategy (McKie et al. 2001). It is located on chromosome 2q31.1 and comprises four exons, encoding a putative 286 amino acid di-haem protein. The protein is highly hydrophobic containing six predicted transmembrane domains and four conserved histidine residues (McKie et al. 2001, Frazer 2002). CYBRD1 shares 40 to 50% homology with cytochrome b561, an enzyme involved with regeneration of ascorbic acid from dehydroascorbate. It has also been found that the yeast ferrireductase, Fre1 has nucleotide sequence homology to CYBRD1 (Shatwell et al.1996). CYBRD1 is a ferric reductase that is highly expressed in the duodenum, particularly at the intestinal brush border (McKie et al. 2001). It catalyses the reduction of ferric to ferrous ions in the gut lumen for transport across the apical membrane as the first step in intestinal iron absorption. The expression of CYBRD1 is similar to that of DMT1, which is increased when iron absorption is stimulated. Iron is an essential regulator of CYBRD1, although it lacks the motifs for binding conventional cofactors such as nicotinamide adenine dinucleotides, nicotinamide adenine dinucleotide phosphates and flavin adenine dinucleotides. It has been speculated that CYBRD1 probably uses ascorbate as a cofactor.

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1.2.7.5. HEPCIDIN ANTIMICROBIAL PEPTIDE GENE (*HAMP*)

The cDNA encoding HAMP was isolated using a series of biochemical and DNA analysis methods (Krause et al. 2000). Blood ultrafiltrate was purified by cysteine alkylation and mass spectrophotometry. This was followed by micropeptide sequence analysis, RT-PCR analysis and 5' and 3' RACE. HAMP was also cloned by another group employing biochemical purification and amino acid sequence analysis of hepcidin peaks in urine, followed by EST database searching and 5' RACE (Park et al. 2001). The human gene was localized to chromosome region 19q13 (Krause et al. 2000; Park et al. 2001) and the mouse gene to chromosome 7.

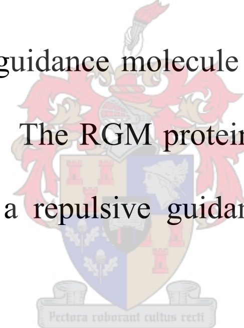
The *HAMP* gene is composed of three exons, with the final exon encoding the active peptide (Krause et al. 2000; Park et al. 2001; Pigeon et al. 2001). It encodes a protein consisting of 84 amino acids containing a 24-residue N-terminal signal sequence and a pentaarginyl proteolysis site, followed by the active C-terminal 25-amino acid peptide (Krause et al. 2000). This protein undergoes enzymatic cleavage into mature peptides of 20, 22, and 25 amino acids (Park et al. 2001). A unique 17-residue stretch with eight cysteines forming four disulphide bridges forms part of the active peptide. The cysteine rich region of the active peptide forms intramolecular bonds that act to stabilize the beta-sheet structure (Pigeon et al. 2001).

Complete inhibition of *Hamp* gene expression was demonstrated in mice exhibiting iron overload following targeted disruption of the upstream stimulatory factor 2 gene (*Usf2*), located close to *Hamp*. Iron overload in these mice was found to be very similar to that seen in human HH states and in Hfe knockout mice (Zhou et al. 1998; Bahram et al. 1999; Levy et al. 1999). It was proposed that hepcidin overexpression might result in phenotypic traits of

iron deficiency. Transgenic mice expressing hepcidin under the control of liver-specific transthyretin promoter displayed reduced body iron levels and severe microcytic hypochromic anaemia (Nicolas et al. 2002). These findings support the proposed role of hepcidin as an iron-regulatory hormone. Mutations in the *HAMP* gene were later identified in humans and found to be associated with JH (Roetto et al. 2003).

1.2.7.6. HEMOJUVELIN GENE (*HJV*)

The *HJV* gene was identified by positional cloning within a previously characterized 1q21 region, linked to JH (Papanikolaou et al. 2004). It spans a region of 2.6 kb and gives rise to five alternatively spliced transcripts. The longest is mainly transcribed in the liver, skeletal muscle and heart. The gene consists of four exons and encodes a protein consisting of 426 amino acids with a large repulsive guidance molecule (RGM) motif, homologous to RGMs involved in neuronal cell migration. The RGM proteins contain various functional domains such as a transmembrane domain, a repulsive guidance domain (RGD) motif and a von Willebrand type D-like domain.



1.3. AIMS OF THE STUDY

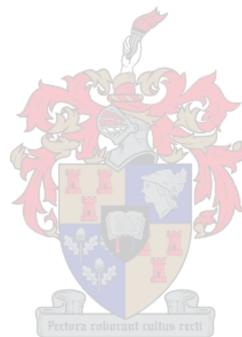
The aim of this study was to investigate the possible role of genes implicated in iron homeostasis in 36 South African Caucasian patients with primary iron overload. The results generated from this study will potentially lead to improved genetic counselling of patients and their families.

SPECIFIC OBJECTIVES:

i. Mutation analysis of the *HFE*, *HMOX1*, *SLC40A1*, *CYBRD1*, *HAMP* and *HJV* genes was performed to identify novel or previously described mutations in these patients.

ii. Subsequent screening of a population-matched control group was carried out for all variants identified in the patient group.

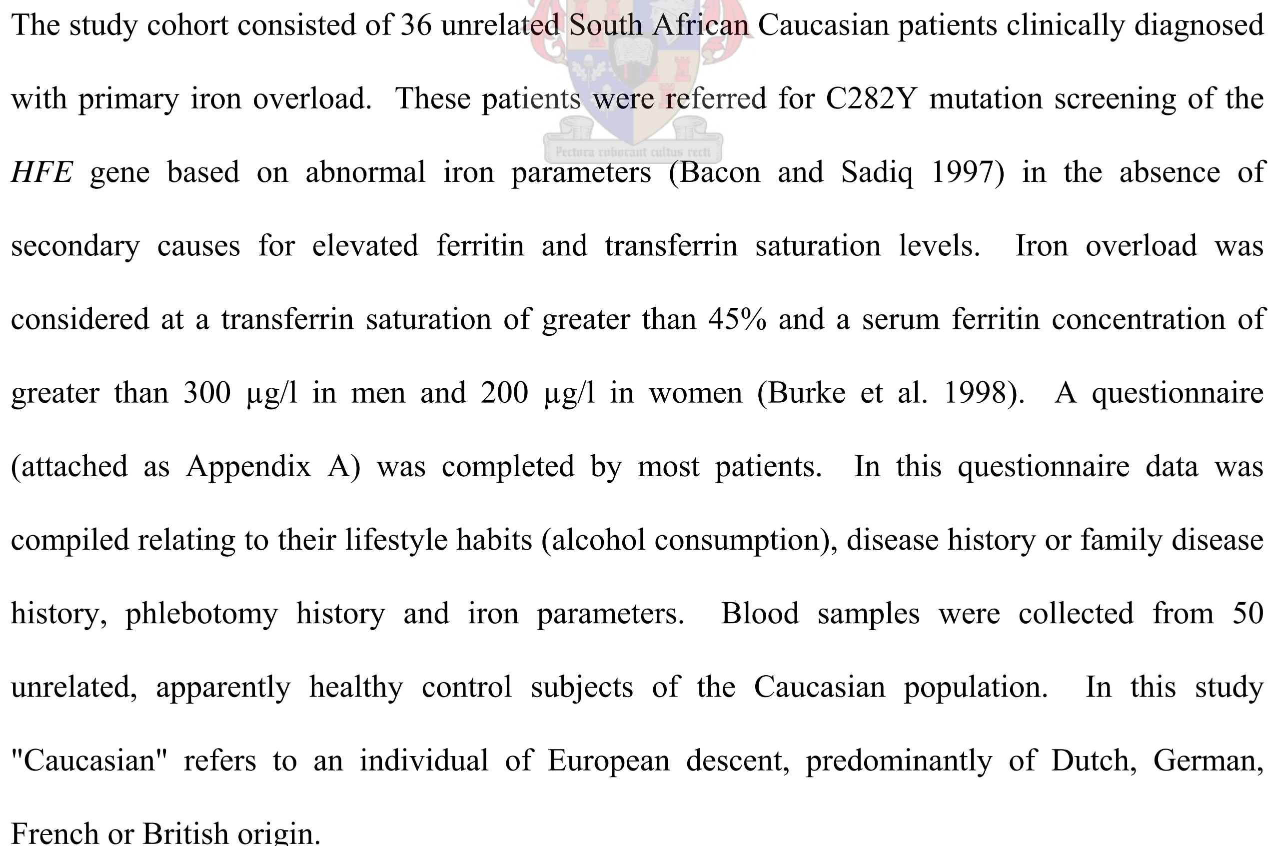
iii. Statistical analysis was performed to establish whether a significant difference could be found between the patient and control groups in relation to each of the variants detected in this study.



CHAPTER TWO: DETAILED EXPERIMENTAL PROCEDURES

Ethical approval for this study was obtained from the Ethics Review Committee of the University of Stellenbosch (reference number: N04/08/123). Prior to sampling written informed consent was obtained from all study participants.

2.1. SUBJECTS



The study cohort consisted of 36 unrelated South African Caucasian patients clinically diagnosed with primary iron overload. These patients were referred for C282Y mutation screening of the *HFE* gene based on abnormal iron parameters (Bacon and Sadiq 1997) in the absence of secondary causes for elevated ferritin and transferrin saturation levels. Iron overload was considered at a transferrin saturation of greater than 45% and a serum ferritin concentration of greater than 300 µg/l in men and 200 µg/l in women (Burke et al. 1998). A questionnaire (attached as Appendix A) was completed by most patients. In this questionnaire data was compiled relating to their lifestyle habits (alcohol consumption), disease history or family disease history, phlebotomy history and iron parameters. Blood samples were collected from 50 unrelated, apparently healthy control subjects of the Caucasian population. In this study "Caucasian" refers to an individual of European descent, predominantly of Dutch, German, French or British origin.

2.2. DNA EXTRACTION

Genomic DNA was extracted from whole blood using a modification of the technique by Miller et al. (1988). Whole blood samples were preserved in ethylenediamine tetra-acetic acid (EDTA, $C_{10}H_{16}N_2O_8$) tubes. A volume of 40 ml cold lysis buffer [155 mM ammonium chloride (NH_4Cl), 10 mM potassium hydrogen carbonate ($KHCO_3$), 0.1 mM EDTA, pH 7.4] was added to 10 ml whole blood in a 50 ml polypropylene tube (Falcon). The solution was placed on ice for 15 minutes and mixed with moderate inversion every 5 minutes to ensure complete lysis of the red blood cells. This was followed by centrifugation of the solution at 250 × g for 20 minutes. The supernatant was discarded and the pellet washed twice with 10 ml phosphate buffered saline solution (PBS). A centrifugation step was performed at 250 × g for 20 minutes and the supernatant discarded. The pellet was re-suspended in 3 ml nuclear lysis buffer [10 mM Tris (hydroxylmethyl) aminomethane (Tris-HCl $(CH_2OH)_3CNH_2$-Cl), 400 mM NaCl, 2 mM EDTA, pH 8.2], 1% (w/v) sodium dodecyl sulphate (SDS) and 1.5 mg/ml proteinase K. This solution was incubated overnight in a water bath at 55˚C.

Subsequent to overnight incubation, 1 ml of 6 M NaCl was added to the solution and vigorously shaken for 1 minute. This was followed by centrifugation at 950 × g for 15 minutes and transfer of the supernatant to a clean 50 ml Falcon tube. The solution was vigorously shaken for 15 seconds, centrifuged at 950 × g for 15 minutes and again transferred to a clean 50 ml Falcon tube. Two times the volume ice-cold ± 99% (v/v) ethanol (EtOH) was added and the solution was allowed to stand until the formation of a spool. The precipitated DNA was transferred to a 1.5 ml tube (Eppendorf) containing 1 ml 70% (v/v) EtOH and centrifuged at 16 000 × g for 30 minutes.

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Following centrifugation the EtOH was discarded and the pellet allowed to air dry at room temperature. The pellet was dissolved overnight in 500 µl sterile SABAX water (ddH_2O) at room temperature and the DNA stored at 4˚C. DNA concentration and purity was measured by using the NanoDrop® ND-1000 spectrophotometer system (NanoDrop Technologies) according to the manufacturer's instructions.

2.3. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

2.3.1. Oligonucleotide Primers

Oligonucleotide primers were designed to screen the coding exonic regions of the genes using Primer3 (Rozen and Skaletsky 2000). The reference sequences for each of the genes were accessed *via* the GenAtlas and Ensembl genome browsers: *HFE* (NM_000410); *HMOX1* (NM_002133); *SLC40A1* (NM_014585); *CYBRD1* (NM_024843); *HAMP* (NM_021175) and *HJV* (ENSG00000168509). The BLAST (Basic Local Alignment Search Tool) software was employed to verify the specificity of all oligonucleotide primer sets.

2.3.2. PCR reaction parameters and programmes

The oligonucleotide primers used for PCR amplification are listed in Table 2.1. PCR amplification of the various amplicons was performed in a Perkin Elmer GeneAmp® PCR 2700 system (Applied Biosystems) in 25 µl reactions. The reaction mixture consisted of 20 ng genomic DNA, 1× Taq buffer with $(NH_4)_2SO_4$ (Fermentas), 0.2 mM of each dNTP (dATP, dTTP,

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dGTP, dCTP) (Fermentas), 1.5 or 2 mM $MgCl_2$ (Fermentas) (as specified for each exon in Table 2.1), 10 pmol of each primer and 0.5 U Taq polymerase (Fermentas). Three different PCR programmes designated as programmes A, B and C (see Table 2.1) were used for amplification of the various exons for each gene. The PCR conditions of programme A were as follows: an initial denaturation step at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at T_A (as specified for each exon in Table 2.1) for 45 seconds, an extension step at 72ºC for 30 seconds, and a final extension step at 72ºC for 10 minutes. PCR amplification conditions for programme B were as follows: an initial denaturation step at 95ºC for 5 minutes, 35 cycles of denaturation at 95ºC for 1 minute, annealing at T_A (as specified for each exon in Table 2.1) for 2 minutes, and a final extension step at 72ºC for 10 minutes. The PCR amplification conditions for programme C were as follows: an initial denaturation step at 94ºC for 5 minutes, 35 cycles of denaturation at 94ºC for 30 seconds, annealing at T_A (as specified for each exon in Table 2.1) for 30 seconds, an extensioC step at 72ºC for 30 seconds, and a final extension step at 72ºC for 10 minutes.

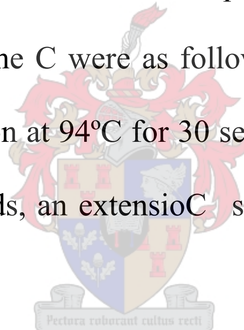

Table 2.1. Intronic oligonucleotide primers designed for PCR amplification

Gene	Exon	Primer Name	Sequence (5'-3')	T_M (ºC)	T_A (ºC)	PCR product size (bp)	$MgCl_2$ (mM)	PCR amplification programme
HFE	1	HFE-1Fa	TTACTGGGCATCTCCTGAGC	63	57	256	1.5	A
		HFE -1Ra	CTAGTTTCGATTTTTCCACCCC	61				
	2	HFE -2FAb	ACATGGTTAAGGCCTGTTGC	60	55	298	1.5	A
		HFE -2RAa	TACCCTTGCTGTGGTTGTGTGA	60				
		HFE -2FBa	TGACCAGCTGTTCGTGTTCT	60	55	257	1.5	A
		HFE -2RBb	CAGCTGTTTCCTTCAAGATGCA	60				
	3	HFE -3FAa	CTTGGGGATGGTGGAAATAG	60	57	279	1.5	A
		HFE -3RAa	CTCCAGGTAGGCCCTGTTCT	65				
		HFE -3FBa	CGAGGGCTACTGGAAGTACG	65	57	280	1.5	A
		HFE -3RBa	CTGCAACCTCCTCCACTCTG	65				
	4	HFE-4Fb	TGGCAAGGGTAAACAGATCC	60	57	396	1.5	A
		HFE-4Rb	TACCTCCTCAGGCACTCCTC	64				
	5	HFE -5Fa	GAGAGCCAGGAGCTGAGAAA	63	55	297	1.5	A
		HFE -5Rb	CAGAGGTACTAAGAGACTTC	58				
	6	HFE -6Fb	TAGTGCCCAGGTCTAAATTG	58	55	202	1.5	A
		HFE -6Rb	TGAGTCTCTAGTTTTCTCTCC	59				

TM = 2(nA+nT) + 4(nG+nC), Thein and Wallace (1986); Abbreviations: ºC = degrees Celsius; bp = base pairs; F = forward primer; HFE = high-iron gene; mM = millimolar; R = reverse primer; TA = annealing temperature; TM = melting temperature

References: [a]Primers designed by VR Human, [b]Primer sequences supplied by Prof C. Camaschella (Zaahl 2003)

Table 2.1. Intronic oligonucleotide primers designed for PCR amplification (Continued)

Gene	Exon	Primer Name	Sequence (5'-3')	T_M (ºC)	T_A (ºC)	PCR product size (bp)	$MgCl_2$ (mM)	PCR amplification programme
HMOX1	**1**	HMOX1-1F[c]	CCGCCGAGCATAAATGTG	60	60	300	1.5	B
		HMOX1-1R[c]	GCACAGGCAGGATCAGAAC	62				
	2	HMOX1-2F[d]	CAGCCAGCTTTGTGTTCACC	72	60	236	1.5	B
		HMOX1-2R[d]	AACCACTGGTCTGAGCCTTG	70				
	3	HMOX1-3FA[d]	TAGTGGACGGGACGGACAGA	74	60	187	1.5	B
		HMOX1-3RA[d]	CCTTGCGGTGCAGCTCTTCT	76				
		HMOX1-3FB[d]	TGAGCGCAACAAGGAGAGCC	76	60	314	1.5	B
		HMOX1-3RB[d]	GGAAGGTGAAGAAGGCCAGG	74				
		HMOX1-3FC[d]	GCCTGGCCTTCTTCACCTTC	74	60	276	1.5	B
		HMOX1-3RC[d]	TGGCAGTGCTGGAACTCTGG	75				
	4	HMOX1-4F[d]	GGACCTGGTAGCATCTCTCA	67	60	314	1.5	B
		HMOX1-4R[d]	GCGAGAACCTGTCCTTACAG	68				
	5	HMOX1-5FA[d]	CCACCTGTTAATGACCTTGC	68	60	343	1.5	B
		HMOX1-5RA[d]	GAAGATGCCATAGGCTCCTT	69				
		HMOX1-5FB[d]	GGAAGGAGCCTATGGCATCT	71	60	537	1.5	B
		HMOX1-5RC[d]	CTCCTACCGAGCACGCAAGA	74				
		HMOX1-5FC[d]	CACTCTGTTCCTGGCTCAGC	71	60	254	1.5	B
		HMOX1-5RC[d]	CTCCTACCGAGCACGCAAGA	74				

T_M = 2(nA+nT) + 4(nG+nC), Thein and Wallace (1986); Abbreviations: ºC = degrees Celsius; bp = base pairs; F =forward primer; *HMOX1* = haem oxygenase 1 gene; mM = millimolar; R = reverse primer; T_A = annealing temperature; T_M = melting temperature

References: [c]This study, [d]Primers designed by H. Waso

Table 2.1. Intronic oligonucleotide primers designed for PCR amplification (Continued)

Gene	Exon	Primer Name	Sequence (5’-3’)	T_M (ºC)	T_A (ºC)	PCR product size (bp)	$MgCl_2$ (mM)	PCR amplification programme
SLC40A1	1	SLC40A1-1FA[e]	CCAGTCGGAGGTCGCAGG	67	55	318	1.5	A
		SLC40A1-1RA[e]	CAGGAGTGCAAGGAACTGG	62				
		SLC40A1-1FB[e]	CCAAAGTCGTCGTTGTAGTC	60	55	276	1.5	A
		SLC40A1-1RB[e]	TTCCTCCAGAACTCGTGTAG	60				
	2	SLC40A1-2F[f]	TGGATAAGCATTCTGCCCTC	60	55	275	1.5	A
		SLC40A1-2R[f]	AAAGCATGTGTACTTGGATG	56				
	3	SLC40A1-3F[e]	GATAAGGAAGCAACTTCCTG	58	55	339	1.5	A
		SLC40A1-3R[e]	CCTGGTTGTTTCTCTCCTAG	60				
	4	SLC40A1-4F[f]	GGATAAGAACAGTCTCACTG	58	55	243	1.5	A
		SLC40A1-4R[f]	TTCATCCTTTACCACTACCAG	60				
	5	SLC40A1-5F[f]	TTAAACTGCCTTGTTTAGTG	54	55	278	1.5	A
		SLC40A1-5R[f]	GCCTCATTTATCACCACCG	58				
	6	SLC40A1-6F[e]	TTGTGTAAATGGGCAGTCTC	58	55	368	1.5	A
		SLC40A1-6R[e]	CATTTAAGGTCTGAACATGAG	57				
	7	SLC40A1-7FA[e]	GCTTTTATTTCTACATGTCC	54	55	352	1.5	A
		SLC40A1-7RA[e]	CCAGTTATAGCTGATGCTC	58				
		SLC40A1-7FB[e]	GGGTACGCCTACACTCAG	62	55	298	1.5	A
		SLC40A1-7RB[e]	CAGTTGTAATTTCAGGTATC	54				
		SLC40A1-7FC[e]	GAAGATATCCGATCAAGGTTC	59	55	259	1.5	A
		SLC40A1-7RC[e]	TTAATGGATTCTCTGAACCTAC	57				
	8	SLC40A1-8FA[e]	TTGAAATGTATGCCTGTAAAC	55	55	343	1.5	A
		SLC40A1-8RA[e]	TTCCTTCCTAACTTCTTTTGC	57				
		SLC40A1-8FB[e]	CCGATTTGCCCAAAATACTC	58	55	297	1.5	A
		SLC40A1-8RB[e]	TTTCCATGCCTCAACATAAGG	59				
		SLC40A1-8FC[e]	GTTTTTACCACAGCTGTGCC	60	55	359	1.5	A
		SLC40A1-8RC[e]	GTCTTCATACTTGAAGAATTTG	55				

T_M = 2(nA+nT) + 4(nG+nC), Thein and Wallace (1986); Abbreviations: ºC = degrees Celsius; bp = base pairs; F = forward primer; mM = millimolar; R = reverse primer; *SLC40A1* = solute carrier family 40 (iron-regulated transporter) member 1 gene; T_A = annealing temperature; T_M = melting temperature

References: [e]Primers designed by LM Bloem, [f]Njajou et al. 2001

Table 2.1. Intronic oligonucleotide primers designed for PCR amplification (Continued)

Gene	Exon	Primer Name	Sequence (5'-3')	T_M (ºC)	T_A (ºC)	PCR product size (bp)	$MgCl_2$ (mM)	PCR amplification programme
CYBRD1[g]	**1**	CYBRD1-1F CYBRD1-1R	GAGACAGCCCCAAGAAGTCG TTCACGGAGGACCCTCTGCC	65 67	60	378	2.0	C
	2	CYBRD1-2F CYBRD1-2R	CCAGTGTGTCAAACTGTTC CATTTACAGTCTGAATTG	58 54	51	346	2.0	C
	3	CYBRD1-3F CYBRD1-3R	TTGTCATACACATATTGC CATTTTCCCAGTGAACAAGTA	53 57	51	318	2.0	C
	4	CYBRD1-4FA CYBRD1-4RA	GCATGTTGCTGTATCATCCTGT AGAGTAGGCTGGCATGGAAC	61 63	57	254	2.0	C
		CYBRD1-4FB CYBRD1-4RB	AAATGGAGGCACTGAACAGG AGGAGAAGCAAAACTGTAGAGC	60 61	57	217	2.0	C
HAMP[h]	**1**	HAMP-1F[i] HAMP -1R[i]	GAGACAGCCCCAAGAAGTCG TTCACGGAGGACCCTCTGCC	71 60	60	262	1.5	B
	2	HAMP -2F HAMP -2R	CCAGTGTGTCAAACTGTTC CATTTACAGTCTGAATTG	60 60	55	235	1.5	B
	3	HAMP -3F HAMP -3R	TTGTCATACACATATTGC CATTTTCCCAGTGAACAAGTA	60 60	55	272	1.5	B

T_M = 2(nA+nT) + 4(nG+nC), Thein and Wallace (1986); Abbreviations: ºC = degrees Celsius; bp = base pairs; *CYBRD1* = cytochrome b reductase gene; F = forward primer; *HAMP* = hepcidin antimicrobial peptide gene; mM =millimolar; R = reverse primer; T_A = annealing temperature; T_M = melting temperature

References: [g]This study, [h]Primers designed by VR Human, [i]Primer sequences supplied by Dr A. Merryweather-Clarke (Zaahl 2003)

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Table 2.1. Intronic oligonucleotide primers designed for PCR amplification (Continued)

Gene	Exon	Primer Name	Sequence (5'-3')	T_M (ºC)	T_A (ºC)	PCR product size (bp)	$MgCl_2$ (mM)	PCR amplification programme
HJV[j]	**1**	HJV-1F HJV-1R	CTGTCCAGTACTCTGGCCAG GAGAGATCCCAGCATTTGGA	65 60	57	313	2.0	C
	2	HJV-2F HJV-2R	CACTCCACATTATCCTTACC ATGCCCACCCCTACATAGC	58 62	55	284	2.0	C
	3	HJV-3FA HJV-3RA	ACACTCCGATAGAGCAGAGG TCTTCGATGCCATGTACCG	63 60	57	298	2.0	C
		HJV-3FB HJV-3RB	TAGAGGTGGGGGTTCATCAG CGGCCTTCATAGTCACAAGG	63 63	57	300	2.0	C
		HJV-3FC HJV-3RC	GACCTGATGATCCAGCACAA TGGCTTGGACAAAGAGGAAG	60 60	55	287	2.0	C
		HJV-3FD HJV-3RD	CCGGACCCTTGTGACTATGA GTGCCCGTGGAAGAATCTC	63 62	57	279	2.0	C
	4	HJV-4FA HJV-4RA	TCAAGGATTGAGGGCCATAG TGGATCTCCACATGGTTCC	60 60	55	300	2.0	C
		HJV-4FB HJV-4RB	GGTGGATAATCTTCCTGTAGC CGACGATTGCGCTCTGAT	61 59	55	288	2.0	C
		HJV-4FC HJV-4RC	GCTCTCCTTCTCCATCAAGG CTGAGCTGCCACGGTAAAGT	63 63	57	256	2.0	C
		HJV-4FD HJV-4RD	GGGCTTCCAGTGGAAGATGC CCCCTTACTGAATGCAAAGC	65 60	57	238	2.0	C
		HJV-4FE HJV-4RE	CATCTCTTCCCCTCAGATGC GATCCGGAATGCAGTAACCT	63 60	57	300	2.0	C
		HJV-4FF HJV-4RF	AAGCAGGGCCTAGGAGACAC TGCTTTCAGCTCTTGCCTCT	65 60	57	283	2.0	C
		HJV-4FG HJV-4RG	CTGCATTCCGGATCTCTGTG TTTTGAATCAAGAAAGCAGAACA	63 56	55	291	2.0	C

T_M = 2(nA+nT) + 4(nG+nC), Thein and Wallace (1986); Abbreviations: ºC= degrees Celsius; bp = base pairs; F = forward primer; *HJV* = hemojuvelin gene; mM = millimolar; R = reverse primer; T_A = annealing temperature; T_M = melting temperature

References: [j]This study

2.4. AGAROSE GEL ELECTROPHORESIS

All PCR products were resolved on a 2% (w/v) agarose gel [gel mix consisting of: 4g agarose in 200 ml 1 × TBE (90 mM Tris-HCl, 90 mM boric acid (H_3BO_3) and 1 mM EDTA, pH 8.0)] to test for successful amplification. To allow for visualisation of the PCR products, 0.01% (v/v) ethidium bromide (EtBr) was added to the gel mix. A total volume of 10 µl was loaded onto a gel consisting of an equal volume (5 µl) Cresol Red loading buffer [2 mg/ml Cresol Red solution in 35% (w/v) sucrose solution] and PCR product. To ascertain the amplification of the correct PCR product a molecular size marker (Fermentas, 100 bp O' GeneRuler^{TM}) was loaded along with the PCR products on the agarose gel. The PCR products were electrophoresed at 120V for 1 hour in 1 × TBE buffer and visualised by ultraviolet light transillumination.

2.5. HETERODUPLEX SINGLE STRAND CONFORMATION POLYMORPHISM (HEX-SSCP) ANALYSIS

The successfully amplified PCR products were subjected to HEX-SSCP analysis (Kotze et al.1995) performed on a 30 cm vertical gel apparatus (Hoefer SE 660 Series Electrophoresis Units, Hoefer Inc). Twelve percent polyacrylamide (PAA) gels supplemented with urea [gel consisting of 7.5% (w/v) urea, 1.5 × TBE (135 mM Tris-HCl, 135 mM boric acid and 2 mM EDTA, pH 8.0), 12% PAA (w/v) (1%C of a 40% stock) [99 acrylamide (AA): 1 bisacrylamide (BAA)], 0.1% ammonium persulphate (APS) and 0.01% N,N,N'N'-tetramethylethylene diamine (TEMED)] was prepared on which PCR fragments were electrophoresed. The gel mixture was

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poured between two glass plates and allowed to polymerise. The assembled gel plates with attached upper chamber were lowered into the lower chamber of the Hoefer electrophoresis tank filled with 1 × TBE. The upper chamber was filled with 1.5 × TBE buffer [135 mM Tris-HCl, 135 mM boric acid and 2 mM EDTA, pH 8.0]. Before loading of the samples 15µl bromophenol blue loading buffer [95% (v/v) formamide ($HCONH_2$, de-ionized), 20 mM EDTA, 0.05% (w/v) xylene cyanol ($C_{25}H_{27}N_2NaO_6S_2$) and 0.05% (w/v) bromophenol blue ($C_{19}H_{10}Br_4O_5S$)] was added to 20 µl of the PCR products. The prepared samples were heat denatured at 95ºC for 5 minutes and immediately placed on ice. A volume of 15µl of the denatured PCR product was loaded onto the gel. Gels were electrophoresed at 350 V for 16 hours at 4°C and subsequently stained using a standard silver staining technique (Beidler et al. 1982).

2.5.1. Silver staining of Polyacrylamide Gels

Following electrophoresis the PAA gels were dismantled and covered with fixing solution [10% (v/v) EtOH, 0.5% (v/v) acetic acid (CH_3COOH), stored at room temperature] and allowed to shake on an Orbital Shaker (Stovall Life Science, Inc) for 10 minutes. The fixing solution was discarded and the gels rinsed twice for 1 minute with dH_2O. Following rinsing, the dH_2O was discarded and the gels covered with staining solution [0.1% (w/v) silver nitrate ($AgNO_3$)]. Gels were again allowed to shake on an Orbital Shaker for approximately 10 minutes. The staining solution was discarded, and the gels rinsed for 5 seconds with dH_2O. The dH_2O was discarded and the gels were allowed to agitate in fresh developing solution [1.5% (w/v) sodium hydroxide (NaOH), 0.4% (v/v) formaldehyde (HCHO) (added a few minutes before use)] on an Orbital Shaker. Developing was continued until visibility of clear DNA bands. The developing solution

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was discarded and the gels rinsed thoroughly with distilled water. The stained gels were then stored and sealed between two transparencies as a preservation measure.

2.6. RESTRICTION ENZYME ANALYSIS

The presence of the *HFE* exon 2, IVS2+4T→C and *CYBRD1* exon 4, S266N variants was confirmed by restriction digestion with enzymes, *RsaI* (Fermentas) and *TspRI* (New England BioLabs), respectively. In the case of IVS2+4T→C, a total reaction mixture of 20 µl was prepared, which consisted of 10 µl PCR product, 1× Tango™ Buffer [33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] and 2 U *RsaI*. The samples were digested in a waterbath for 16 hours at 37ºC according to the manufacturer's instructions. For screening of S266N, a total reaction mixture of 20 µl was prepared which consisted of 10 µl PCR product, 1× NEBuffer 4 [50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9], 100 µg/ml BSA and 10 U *TspRI*. This was followed by incubation of the samples in a waterbath for 16 hours at 65ºC. A total volume of 25 µl comprising, 20 µl of the digested product and 5 µl Cresol Red loading buffer was resolved on a 2% agarose gel for 1 ½ hours at 90 V.

2.7. SEMI-AUTOMATED DNA SEQUENCING

PCR products demonstrating aberrant HEX-SSCP patterns upon analysis were subjected to semi-automated DNA sequencing. The amplified samples were bi-directionally sequenced using the same primers as for PCR amplification (see Table 2.1).

2.7.1. Purification of PCR products

The PCR products were purified with a Wizard® SV Gel and PCR Clean-Up kit (Promega) according to the manufacturer's protocol. An equal volume of membrane binding solution [4 500 mM guanidine isothiocyanate, 500 mM potassium acetate ($KC_2H_30_2$, pH 8.0)] was added to 20 µl of the PCR product. The prepared PCR product was transferred to the SV minicolumn assembly and incubated for 1 minute at room temperature. This was followed by centrifugation at 16 000 × g for 1 minute. The liquid in the collection tube was subsequently discarded and the membrane column washed by adding 700 µl membrane wash solution [10 mM potassium acetate (pH 5.0), 80% (v/v) EtOH, 0.0167 mM EDTA (pH8.0), diluted with 75 ml 95% (v/v) EtOH] to the SV minicolumn. The SV minicolumn assembly was centrifuged for 1 minute at 16 000 × g and the liquid discarded following centrifugation. Washing of the membrane column was repeated by adding 500 µl of the membrane wash solution to the SV minicolumn and centrifugation for 5 minutes at 16 000 × g. The liquid was discarded from the collection tube and the column assembly centrifuged for 1 minute at 16 000 × g to allow evaporation of any residual EtOH. The SV minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and the PCR product eluted with 50 µl nuclease-free water. This was incubated at room temperature for 1 minute and centrifuged for 1 minute at 16 000 × g. Purified PCR products were stored at 4ºC. To ascertain the concentration of the PCR products, 5 µl was resolved on a 2% agarose gel for 1 hour at 120 V.

2.7.2. Cycle Sequencing reaction and programme

The cycle sequencing reaction was performed in 7 µl reactions consisting of: 9.9 ng PCR product, 1 µl termination ready reaction mix [BigDye® Terminator v3.1 Cycle Sequencing Kit,

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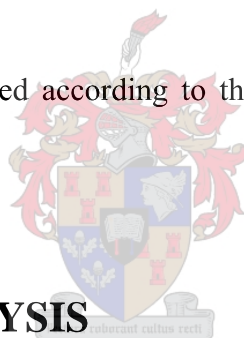
Applied Biosystems] and 3.3 pmol of each primer. Cycle sequencing reactions were performed on a Perkin Elmer GeneAmp® PCR 2700 system (Applied Biosystems). The reaction programme used was as follows: an initial denaturation step at 96ºC for 10 seconds, 25 cycles of denaturation at 96ºC for 10 seconds, annealing at 55ºC for 10 seconds and an extension step at 60ºC for 4 minutes. Samples were sequenced on an ABI PRISM 3130Xl genetic analyzer (Applied Biosystems). Following electrophoresis, sequence electropherograms were analysed using Chromas 2.13 and the BioEdit Sequence Alignment Editor (Hall 1999).

2.8. NUCLEOTIDE NUMBERING

All sequence variations were numbered according to the nomenclature system set out by den Dunnen and Antonarakis (2001).



2.9. STATISTICAL ANALYSIS

Allele and genotype frequencies were calculated by allele counting using Microsoft® Excel 2000. The control population group was tested for Hardy-Weinberg equilibrium for each of the variants identified. Hardy-Weinberg was accepted at a probability (*P*) value greater than 0.05. For those variants not in Hardy-Weinberg equilibrium no further statistical calculations were carried out. The Fisher's exact test and/or chi-squared (χ^2) analysis were used to estimate whether allele and genotype frequencies observed between the patient and control groups were statistically significant. The StatSoft® STATISTICA version 6 and the Epi Info™ version 3.3.2 computer software programmes were used to calculate these values. A *P* value less than 0.05 was regarded

as significant. Analysis of variants within the same gene and those in different genes were performed by comparing the frequency of carriers for two gene variants in the patient and control group (Butt et al. 2003). Microsoft® Access 2000 was used to generate both observed and expected values for combinations of alleles between two variants. Any statistical significant differences ($P<0.05$) between variants were regarded as potential association between alleles within the same gene or between two different genes.

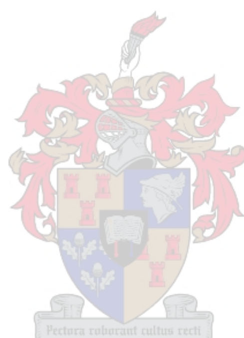


Table 2.2. List of generally used chemicals/reagents and their suppliers

Chemical/Reagent	**Supplier**
Acetic acid	Associated Chemical Enterprises
Acrylamide (AA)	Fluka
Agarose	BIO BASIC
Ammonium chloride (NH_4Cl)	Merck
Ammonium persulphate (APS)	Associated Chemical Enterprises
Bisacrylamide	Sigma
Boric acid	KIMIX
Bromophenol blue	Merck
Cresol red	Merck
Ethanol (EtOH)	KIMIX
Ethidium bromide (EtBr)	Fluka
Ethylenediamine tetra-acetic acid (EDTA)	Merck
Formaldehyde (HCHO)	Associated Chemical Enterprises
Formamide ($HCONH_2$, de-ionised)	Merck
PBS pellets	Roche Diagnostics
Potassium hydrogen carbonate ($KHCO_3$)	Merck
Proteinase K	Roche Diagnostics
SABAX water (ddH_2O)	Adcock Ingram
Silver nitrate ($AgNO_3$)	Merck
Sodium chloride (NaCl)	Sigma

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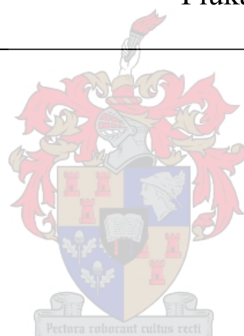
Table 2.2. List of generally used chemicals/reagents and their suppliers (Continued)

Chemical/Reagent	**Supplier**
Sodium dodecyl sulphate (SDS)	Merck
Sodium hydroxide (NaOH)	Merck
Sucrose	Associated Chemical Enterprises
N,N,N'N'-tetramethylethylene diamine (TEMED)	Fluka
Tris (hydroxylmethyl) aminomethane (Tris-HCl)	Fluka
Urea	Sigma
Xylene cyanol	Fluka



CHAPTER THREE

INVESTIGATION OF IRON-REGULATING GENES IN SOUTH AFRICAN PRIMARY IRON OVERLOAD PATIENTS: A PILOT STUDY

ABSTRACT

Hereditary haemochromatosis (HH), a heterogeneous disease of iron metabolism has a carrier frequency of approximately one in six, for the common C282Y mutation, in the South African Caucasian population. Although the homozygous C282Y mutation has been shown to be the cause of primary iron overload in most cases, increasing evidence exists highlighting the possible role of modifier genes in expression of this genotype. We investigated the role of six genes involved in iron regulation, transport and storage, including *HFE, HMOX1*, *SLC40A1*, *CYBRD1*, *HAMP* and *HJV* in a cohort of 36 patients referred for *HFE* mutation screening, who tested either negative or displayed heterozygosity for C282Y. Mutation screening of these genes were performed by PCR amplification and heteroduplex single strand conformation polymorphism (HEX-SSCP) analysis. Semi-automated DNA sequencing revealed 16 previously described and two novel variants. The novel variant, -89C→T was identified in the 5' untranslated region of the *CYBRD1* gene, and S333, a silent mutation in exon 4 of the *HJV* gene. These variants were

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absent from the population-matched controls screened and could explain the non-*HFE* iron overload presented by 14% of patients.

INTRODUCTION

Hereditary haemochromatosis (HH), an autosomal recessive disease, occurs predominantly in populations of northern European descent, affecting an estimated one in 200 to 300 individuals (Simon et al. 1977; Adams et al. 2000; Camaschella et al. 2000). The disease is characterized by excessive absorption of iron from the gut, leading to progressive iron accumulation and damage in organs such as the liver, heart and pancreas if left untreated (Bothwell et al. 1995).

The majority of HH patients are either homozygous for the C282Y mutation in the *HFE* gene or compound heterozygous for the C282Y/H63D mutations (Feder et al. 1996). Homozygosity for the C282Y mutation accounts for more than 80% of HH cases in individuals of European descent (Worwood et al. 1997). In Asian, Australasian, Amerindian and African populations C282Y occurs rarely or not at all (Beckman et al. 1997; Chang et al. 1997; Merryweather-Clarke et al. 1997; Agostinho et al. 1999; Rochette et al. 1999; Sohda et al. 1999; Barut et al. 2003; Zorai et al. 2003; Karimi et al. 2004; Kotze et al. 2004; Sassi et al. 2004; Leone et al. 2005). Other mutations in the *HFE* gene have been identified as a cause of the disease phenotype in approximately 2-10% of cases (reviewed by Pointon et al. 2000).

Several non-HFE related forms of HH have been identified, but are rare (Pietrangelo 2004). These include types 2, 3 and 4 haemochromatosis. Type 2 haemochromatosis, also referred to as

juvenile haemochromatosis or HFE2 has two genetic forms, subtype 2A associated with mutations in the hemojuvelin (*HJV*) gene (Roetto et al. 1999; Papanikolaou et al. 2004), and subtype 2B associated with mutations in the hepcidin antimicrobial peptide (*HAMP*) gene (Roetto et al. 2003). Type 3 haemochromatosis, or HFE3, results from mutations in the transferrin receptor 2 gene (*TFR2*) (Camaschella et al. 2000), and type 4 haemochromatosis, or HFE4, from mutations in the solute carrier family 40 (iron-regulated transporter) gene (*SLC40A1*).

Increasingly, novel mutations are identified in a number of genes implicated in iron homeostasis and varying forms of HH, which explains iron overload in cases not presenting with the typical C282Y homozygous status (Beutler 2005). Numerous studies have been conducted to investigate the role of modifier genes in the varying phenotypic expression observed in HH (Biasiotto et al 2003; Merryweather-Clarke et al. 2003; Biasiotto et al. 2004; Jacolot et al. 2004; Le Gac et al. 2004; Pietrangelo et al. 2005). Digenic inheritance of mutations in both the *HFE* and *HAMP* genes has been reported, where the simultaneous presence of *HFE* (C282Y) and *HAMP* (ATGG deletion in exon 2) heterozygous mutations was shown to be a determinant for the severity of the disease (Merryweather-Clarke et al. 2003).

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In this study genes involved in iron regulation (*HFE*, *HAMP* and *HJV*) or iron transport and storage (*HMOX1, SLC40A1 and CYBRD1*) were investigated in patients presenting with primary iron overload who were not homozygous for the C282Y mutation in the South African Caucasian population.

MATERIALS AND METHODS

Refer to chapter two of this thesis for the detailed experimental procedures employed in this study.

RESULTS

The allelic frequency distributions of the variants identified in both the patient and control groups are displayed in Table 3.1. Hardy-Weinberg equilibrium was demonstrated for all of the variants in the control group, with the exception of IVS4+48G→A in the *HFE* gene. As a result of this deviation no further statistical analysis of this variant could be performed between the patient and control groups. The electropherograms and results from HEX-SSCP analysis of the novel variants identified are illustrated in Figures 3.1 and 3.2.

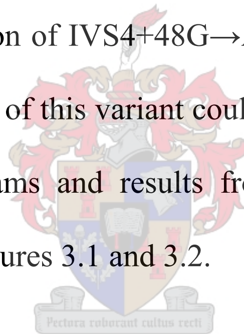

Table 3.1. The allelic distribution and *P*-values obtained with the Fisher's exact test of the variants detected in the HH patient and control groups

Gene	Exon/Intron	Variant	Allele frequencies: Patient Group 2n=72	Allele frequencies: Control Group 2n=100	*P*-values	References for variants
HFE	2	IVS2+4T→C[a]	0.32	0.34	0.45	de Villiers et. al. 1999
	2	H63D[b]	0.11	0.14	0.38	Feder et al. 1996
		S65C[d]	-	0.04	-	Henz et al. 1997
	3	Q127H[b, c]	0.01	-	-	de Villiers et al. 1999a
	4	C282Y[a]	0.18	0.12	0.19	Feder et al. 1996
	4	IVS4-44T→C[b]	0.08	0.04	0.19	Beutler and West 1997
		IVS4+48G→A[b]	0.25	0.46	-	Totaro et al. 1997
	5	IVS5-47G→A[a]	0.35	0.48	0.06	Beutler and West 1997
HMOX1	4	IVS5+51delTGGCTGTCTGACT[a]	0.07	0.01	0.05	Lee et al. 2002
SLC40A1	4	I109 (321C→T)[b,c]	0.03	-	-	Zaahl et al. 2004
	6	V221 (663T→C)[a]	0.57	*0.66* (2n = 96)	0.16	Devalia et al. 2002
CYBRD1	5'UTR	-89C→T[b, c]	0.01	-	-	This study
	1	IVS1-4C→G[a]	0.21	*0.18* (2n = 94)	0.40	Zaahl et al. 2004
	2	IVS2+8T→C[a]	0.78	*0.85* 2n = 94)	0.16	Zaahl et al. 2004
	4	S266N[a]	0.68	0.72	0.35	McKie et al. 2001
HJV		S264 (792G→C)	-	0.01	-	Lee et al. 2004
	4	A310G (929C→G)	-	0.01	-	Lee et al. 2004
		S333 (1001G→A)[c]	0.07	-	-	This study

[a]Denotes the variant in both the homozygous and heterozygous state, [b] Denotes the variant in only the heterozygous state, [c]Denotes the variant only in the patient group, [d]Denotes the variant only in the control group, Abbreviation: *P* = probability value; 5'UTR = 5' untranslated region

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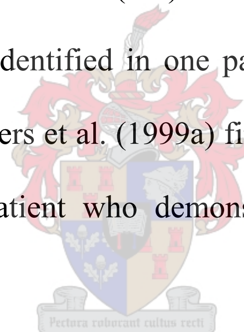
HFE gene

Eight sequence changes, including four exonic and four intronic, were detected in the *HFE* gene. All of these have previously been characterized. The C282Y heterozygous mutation was present in 13 of the 36 (37%) patients in this study. Of these patients, two also had the H63D mutation. The remaining 23 (64%) patients, tested negative for the C282Y mutation. In six (17%) of these C282Y negative patients, H63D was present. In the control group, ten of the 50 (20%) individuals were identified as heterozygous and only one homozygous for C282Y. Compound heterozygosity for C282Y/H63D was identified in three of the controls. The heterozygous state of the H63D mutation was present in 11 of the 39 (28%), C282Y negative control individuals. Heterozygosity for S65C was identified in four (8%) controls. This mutation was absent from the patient group. Variant Q127H was identified in one patient who tested negative for both the C282Y and H63D mutations. de Villiers et al. (1999a) first described this mutation in a severely affected variegate porphyria (VP) patient who demonstrated compound heterozygosity with H63D.



The presence of IVS2+4T→C was confirmed by digestion of the amplicons with *RsaI* (Figure 3.2). Restriction enzyme digestion of the amplicons yielded 44 bp, 181 bp and 275 bp fragments in homozygous individuals, and, 44 bp and 181 bp fragments in heterozygous individuals. The allele frequency of this variant was determined to be 0.32 in the 36 HH patients, compared with 0.34 in the control group. IVS4-44T→C was present in both the patient, and control groups, with six of the 36 HH patients and four of the 50 control patients being heterozygous. The IVS4+48G→A variant, shown to be in linkage disequilibrium with C282Y (Jeffrey et al. 1999), was detected in 18 of the 36 (50%) HH patients. Furthermore, in comparison to the patient group

this variant was detected with a significantly higher frequency in the control group (50% vs 92%). The IVS4+48G→A variant, however, deviated from the Hardy-Weinberg law. Variant IVS5-47G→A, was detected in the homozygous state in four (11%) patients, while 17 (47%) of patients were heterozygous. The frequency of this variant in the control group was 13 (26%) in the homozygous state and 22 (44%) in the heterozygous state. Out of the total of the 36 HH patients, only one was identified without any of these *HFE* variants. The calculation of the comparisons of all allele distributions in the *HFE* gene did not prove statistically significant.

***HMOX1* gene**

Mutation analysis of the five exons comprising *HMOX1* identified the common IVS5+51delTGGCTGTCTGACT variant in intron 5 of the gene. Only three of the 36 patients (8%) had the IVS5+51delTGGCTGTCTGACT variant, two in the heterozygous and one in the homozygous state. This deletion was identified in the heterozygous state in only one control individual. A marginal statistically significant difference, $P<0.05$, was observed for this variant when comparing the patient and control groups.

***SLC40A1* gene**

Only two variants were identified in the *SLC40A1* gene, I109 in exon 4 and V221 in exon 6. I109 was identified in two patients, but was absent from the population-matched controls. The V221 variant was common in both the patient and control groups, with an allele frequency of 0.57 and 0.66 respectively.

***CYBRD1* gene**

Upon analysis of the *CYBRD1* gene a novel -89C→T variant, resulting in a C to T transition in the 5' untranslated region at nucleotide position -89 from the start codon was detected. Figure 3.1 displays the electropherogram and results from HEX-SSCP analysis for this variant. It was identified in a single patient and was absent from the population-matched control group. The IVS1-4C→G variant was identified in 14 of the 36 patients; 13 (36%) of these patients were heterozygous and one (3%) homozygous. This variant was identified in the heterozygous state with a similar frequency in the control group, 17 of 50 (34%). Homozygosity for IVS1-4C→G was not observed in the control group. The IVS2+8T→C variant was present with almost equally high frequencies in both the patient and controls, with allele frequencies of 0.78 and 0.85 respectively. In the patient group, 21 of the 36 (58%) of the patients were homozygous and 12 of the 36 (33%) were heterozygous. Homozygosity for IVS2+8T→C was identified in 35 of the 50 (70%) and heterozygosity in 10 of the 50 (20%) controls.

Digestion with the *TspRI* restriction enzyme confirmed the presence of S266N in exon 4 (Figure 3.3). The digestion product sizes for the wild-type are 14 bp, 75 bp and 228 bp; the homozygous digestion products are 14 bp, 75 bp, 203 bp and 228 bp, while the heterozygous product sizes are 14 bp and 203 bp. The variant was detected in the homozygous state in 15 of the 36 (42%) patients and 26 of the 50 (52%) controls. Heterozygosity for S266N was shown to be at 53% in the patient group, while in the control group a frequency of 40% was observed. Only two individuals lacked the S266N mutation in the patient group, while it was absent in four of the control individuals. Only one individual did not have any of the three variants identified in the *CYBRD1* gene.

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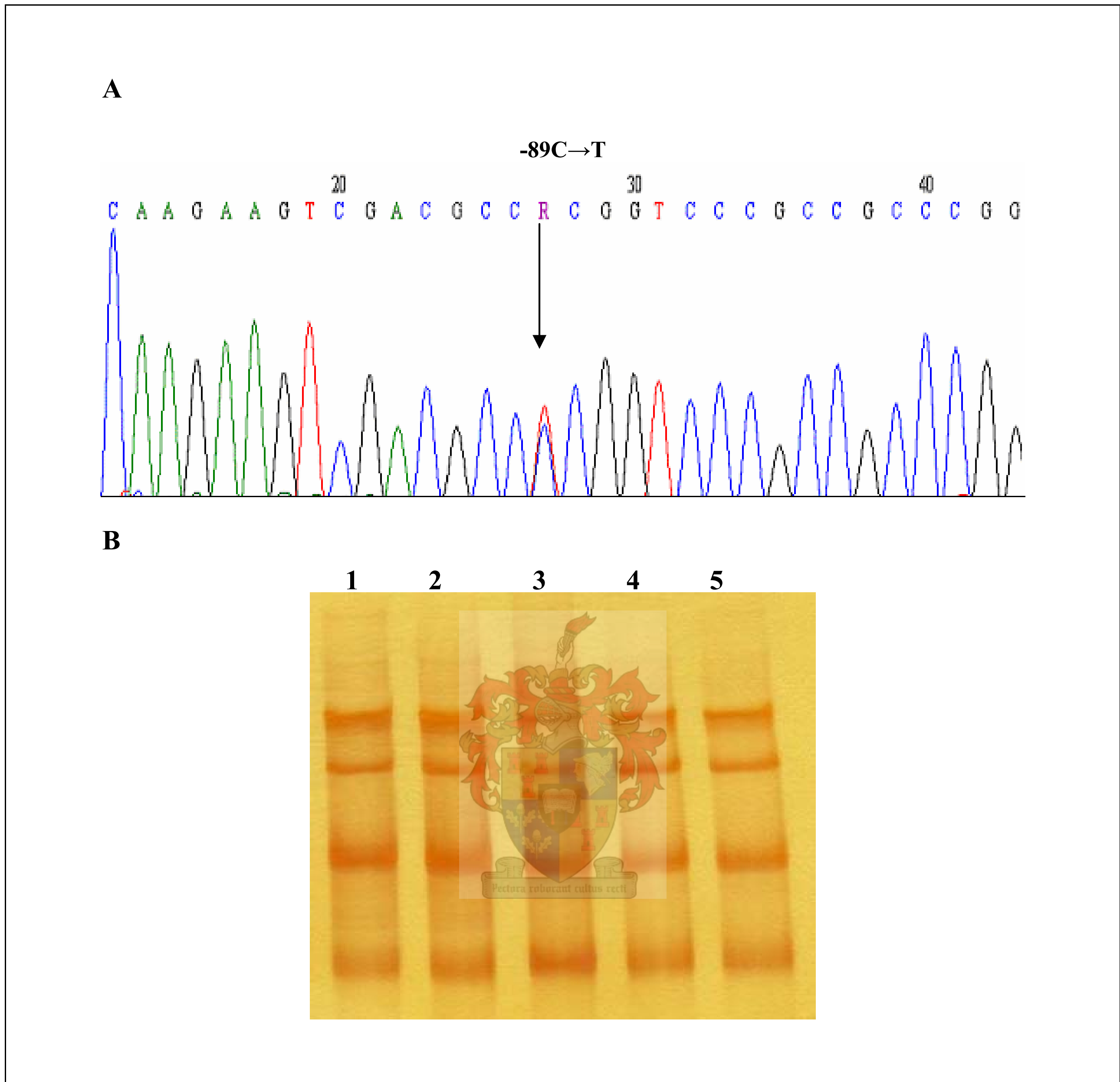


Figure 3.1. (A) Electropherogram of a novel variant identified in the promoter region of the *CYBRD1* gene. The arrow indicates the point of variation. **(B)** A 12% (v/v) polyacrylamide gel (1%C, supplemented with 7.5% urea) visualized with a standard silver staining technique. Lanes 1, 2, 4 and 5 contain PCR-amplified DNA of normal control patients, while lane 3 contains an individual heterozygous for the novel -89C→T variant.

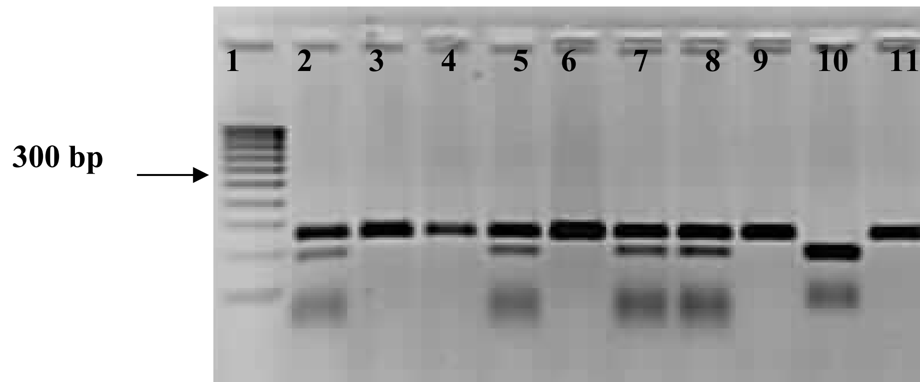


Figure 3.2. A 2% (w/v) agarose gel of the IVS2+4T→C variant (*RsaI* restriction digest). Lane 1 contains a 100 bp ladder loaded as a molecular size marker. Lanes 2, 5, 7 and 8 contain the homozygous digestion products of 44 bp, 181 bp and 275 bp, while lanes 3, 4, 6, 9 and 11 contain the wild-type digestion products and lane 10 contains the heterozygous digestion product of 44 bp and 181 bp.

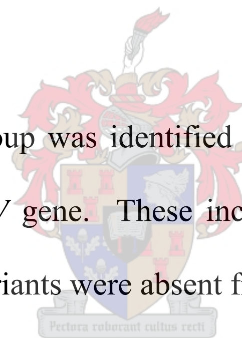
Figure 3.3. A 2% (w/v) agarose gel of the S266N variant (*TspRI* restriction digest). Lane 1 contains a 100 bp ladder loaded as a molecular size marker. Lanes 2, 3, 5, 7 and 8 contain the wild-type digestion products of 14 bp,75 bp and 228 bp, while lanes 4 and 9 contain the heterozygous digestion products of 14 bp and 203 bp, and lanes 6, 10 and 11 contain the homozygous digestion products of 14 bp, 75 bp, 203 bp and 228 bp.

***HAMP* gene**

HEX-SSCP analysis of the *HAMP* gene revealed no variation in exons 1 to 3. This observation might be underscored by the fact that mutations in this gene, related to HFE1 are rare.

***HJV* gene**

Mutation analysis of exon 4 of the *HJV* gene produced abberant banding patterns in three patients. Subsequent semi-automated DNA sequencing analysis revealed a novel S333 silent mutation. These results are shown in Figure 3.4. Screening of the control individuals confirmed this variant to be absent from this group. Both the *HFE,* H63D and C282Y mutations were absent in these individuals.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A single individual in the control group was identified with compound heterozygosity for two known variants in exon 4 of the *HJV* gene. These include the silent mutation, S264 and the missense mutation, A310G. These variants were absent from the patient group.

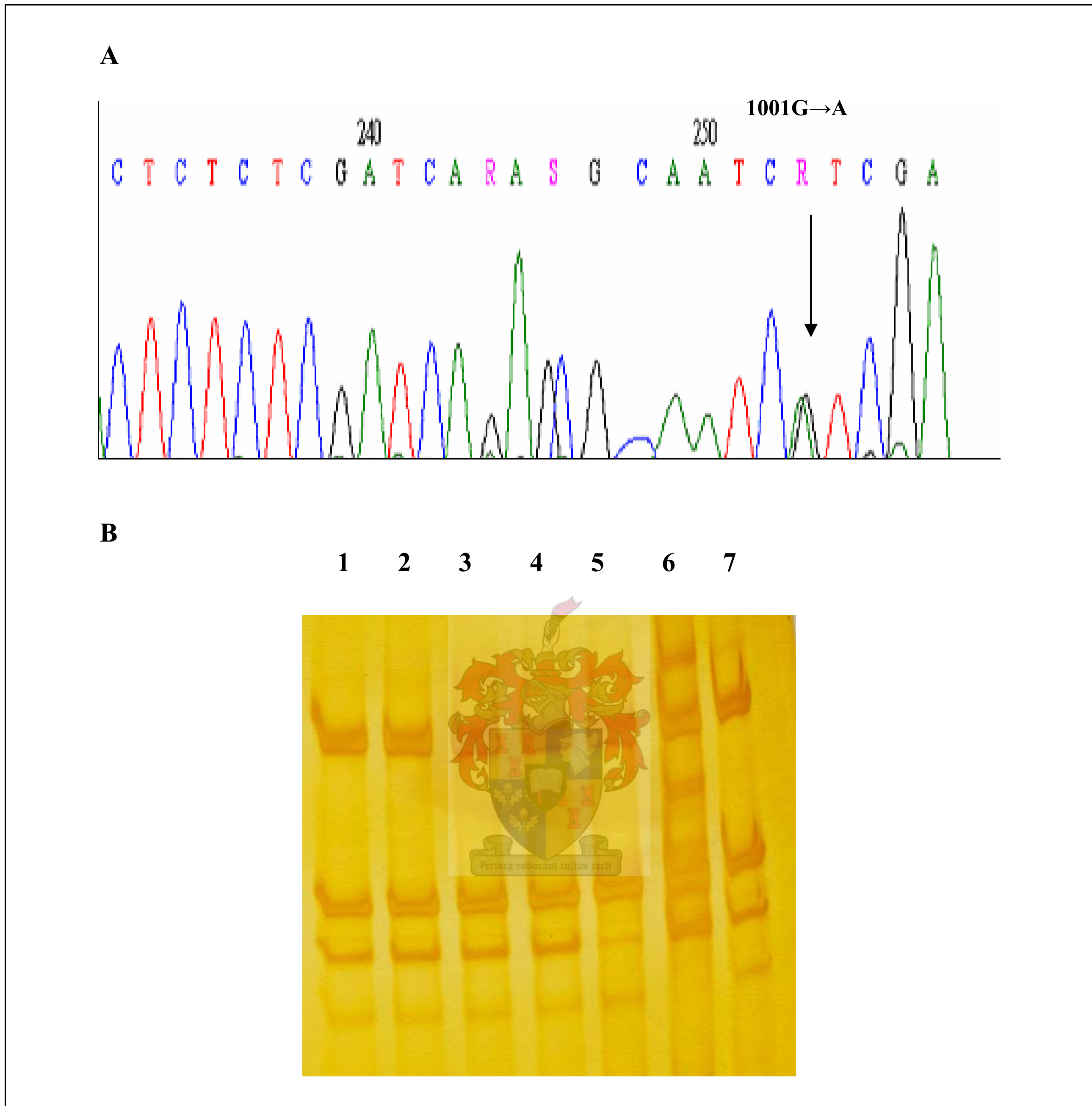


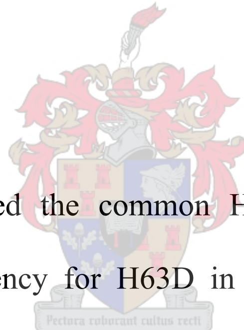
Figure 3.4. (A) Electropherogram of a novel variant identified in exon 4B of the *HJV* gene. The arrow indicates the variant. **(B)** A 12% (v/v) polyacrylamide gel (1%C, supplemented with 7.5% urea) visualized with a standard silver staining technique. Lanes 1, 2, 3, 4, 5 and 7 contain PCR-amplified DNA of control individuals, while lane 6 contains an individual heterozygous for the novel S333 variant.

DISCUSSION

The importance of modifier genes on HH expression and their involvement in the varying penetrance observed for this disease has been highlighted by a number of studies (Merryweather-Clarke et al. 2003; Biasiotto et al. 2004; Zaahl et al. 2004). Although homozygosity for the C282Y mutation is present in the majority of Caucasian HH patients, evidence exists which indicate that it is insufficient to result in the disease phenotype. A study was conducted by Zaahl et al. (2004), where the potential involvement of the *HFE*, *SLC40A1*, *HAMP* and *CYBRD1* genes was investigated in South African Caucasian, and Black primary iron overload patients, with either a negative or heterozygous C282Y status. This study provided a possible explanation for iron overload in 11% of their Caucasian patients. Even though non-HFE related forms of haemochromatosis are uncommon, primary iron overload in many South African patients remains unresolved. In this study we screened six iron related genes to determine whether variants in these genes play a role in augmenting primary iron overload in a group of C282Y negative and heterozygous patients.

Mutation analysis of the *HFE*, *HMOX1, SLC40A1*, *CYBRD1* and *HJV* genes revealed sequence changes in the 5' untranslated, exonic and intronic regions. Only two of these variants were identified as novel, whereas the majority have previously been documented. Most of these variants are classified as single nucleotide polymorphisms (SNPs). SNPs are single base substitutions that occur with a frequency of more than 1% in a population (Chanock 2003). The human genome contains an estimated 15 million SNPs; between 50 000 and 250 000 of these may alter gene expression or function (Chanock 2001, Chanock 2003).

All variants, but two, for which restriction enzyme digestion was preferred, were initially identified using HEX-SSCP analysis and confirmed with semi-automated DNA sequencing. Kotze et al. (1995) reported SSCP analysis to be more effective when combined with heteroduplex analysis. SSCP analysis is ideal for amplicons between 130 to 250 bp and its sensitivity has been shown to be between 70% and 100% for fragments in this size range (Ellison et al. 1993; Hayashi and Yandell 1993; Sheffield et al. 1993). Due to its cost-effectiveness and simplicity it was favoured in this study because of the number of genes screened. Semi-automated DNA sequencing has been proclaimed the “gold standard” for detection of both known and unknown sequence specific nucleotide variations (Kristensen et al. 2001). However, because of the high costs related to this method it could not exclusively be employed for mutation analysis.



Screening of the *HFE* gene revealed the common H63D, S65C and C282Y amino acid substitutions. The population frequency for H63D in Europeans, available on the HapMap database was shown to be, 0.129 (refSNP ID: rs1799945) for the mutant G-allele. The most recent study on *HFE* in South African Caucasian primary iron overload patients reported an allele frequency of 0.17 (Zaahl et al. 2004). Potekhina et al. (2005) observed an allele frequency of 0.256 in the Russian population. We demonstrated an allele frequency of 0.11, consistent with results from the first two documented studies. In our control group the frequency for H63D was slightly higher in comparison to the patient group (0.14 *vs* 0.11), whereas Zaahl et al. (2004), showed the patient and control frequencies to be 0.17 *vs* 0.15 respectively. These results confirm that heterozygosity for H63D alone cannot confer an iron overload status. The S65C mutation has been implicated in a mild form of iron overload. This mutation was absent from our patient

group. Zaahl et al. (2004) identified S65C in their patient group only, with an allele frequency similar to that found in our control group, i.e. 0.03 *vs* 0.04. Both H63D and S65C involve amino acids that have remained evolutionarily conserved in humans, mice and rats (de Villiers et al. 1999a). The allele frequency of C282Y was 0.18 in the patient group in comparison with a slighter lower, 0.12 in the control group. The HapMap database reports a frequency of 0.083 (refSNP ID: rs1800562) for the C282Y mutant A-allele in one European population. Although H63D and many other variants in this study reported with a slightly higher frequency in the control group, this observation is likely due to chance, as our control samples were randomly selected from the general Caucasian population.

Mutation Q127H, identified in exon 3 of the *HFE* gene, was present in a single patient. This mutation is situated in the α2 chain of the HFE protein (de Villiers et al. 1999a). The C282Y mutation has been shown to disrupt a key disulphide bond in the α3 extracellular loop of the HFE protein, thus Q127H could have a similar effect. The mutation is an A to C transversion at nucleotide position 317. It causes the substitution of a glutamine, a polar, uncharged amino acid with histidine, a weakly positively charged amino acid. No other published results, except for the de Villiers et al. (1999a) article could be found regarding Q127H or its effect. We generated predictive results for the effect of the Q127H variant by using the ESEfinder (ESE-exonic splice element) programme. A SF2/ASF binding site was created in the presence of the mutated C-allele, which might result in generating aberrant mRNAs that are either unstable or code for defective protein isoforms. de Villiers et al. (1999a) suggested the role of variants in the *HFE* gene in conferring porphyria-like symptoms in patient referrals lacking VP-related mutations. The patient in which this variant was identified in our study displayed clinical symptoms of

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portal hypertension, decompensated liver illness and liver cirrhosis; which are all symptoms related to both VP and HH. When looking at the iron parameters obtained for this individual, we observed a serum ferritin level of 1451 µg/l out of a reference range of 18.7 to 323 µg/l, which is extremely high; the fourth highest in our patient group for that matter. The heterozygous Q127H mutation could thus most likely account for the iron overload presented by this individual.

Approximately 15% of point mutations within introns affecting human disease cause mRNA splicing (Krawczak et al. 1992). None of the *HFE* intronic variants identified, i.e. IVS2+4T→C, IVS4-44T→C, IVS4+48G→A and IVS5-47G→A has been reported to affect splicing of the gene, and thus can be excluded as causative factors for iron overload. A study by Christiansen et al. (1999), verified that IVS2+4T→C has no effect on the splicing of the *HFE* mRNA using reverse transcription-PCR analysis. The allele frequencies of the intronic variants were as follows: for IVS2+4T→C (0.32 *vs* 0.34), IVS4-44T→C, (0.08 *vs* 0.04), IVS4+48G→A (0.25 *vs* 0.46) and IVS5-47G→A (0.35 *vs* 0.48) in the patients and controls respectively. Once again, the control group displayed a slightly higher frequency for these variants, except for IVS4-44T→C, confirming them as frequent polymorphisms. The observation that the control population deviated from Hardy-Weinberg equilibrium for the IVS4+48G→A variant underlines the need to expand both the patient and control group.

The only variant identified in the *HMOX1* gene, was IVS5+51delTGGCTGTCTGACT, which causes a 13 base pair deletion starting at position 51 at the splice acceptor site of intron 5. No significant differences could be deduced between the patients and controls (three patients' *vs* one control).

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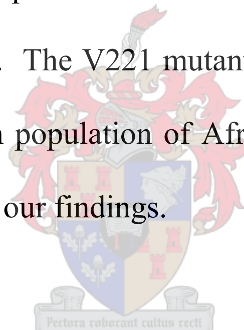
Although silent mutations represent neutral polymorphisms, the potential role of the *SLC40A1* I109 variant in two isolated cases cannot completely be eliminated. HapMap results for I109 in a European population illustrated the mutant T-allele to be absent in Europeans, whereas in an African American group the allele frequency was shown to be 0.065 (refSNP ID: rs11568345). The allele frequency of I109 in our group was 0.03, and Zaahl et al. (2004) reported an allele frequency of 0.008 for this variant. Interesting enough, the patient with the Q127H mutation, also has the I109 heterozygous mutation. A potential interaction between the Q127H and I109 variants can only be verified through further studies. However, we predict that Q127H is more likely to explain iron overload in this individual than I109. V221 in exon 6 of the *SLC40A1* gene was identified with similar high frequencies in both the patient and control groups, thus representing a frequent polymorphism. The V221 mutant C-allele frequency was 0.457 (refSNP ID: ss48422628) in a North American population of African Americans and Caucasians on the HapMap database, corresponding with our findings.



We identified a 5’ untranslated, one exonic and two intronic variants in the *CYBRD1* gene. These include a novel -89C→T variant, S266N, IVS1-4C→G and IVS2+8T→C. Non-significance *P*-values were obtained for all of these variants, except for -89C→T. The -89C→T variant results in a C to T transition at nucleotide position 81 in the 5’ untranslated region of the *CYBRD1* gene. It was identified in only one patient and was absent from the control group. The UTRscan programme was used to search for functional regulatory elements within the region of the -89C→T variant (Pesole and Liuni 1999). This programme sifts through a collection of functional sequence patterns situated in 5' or 3' UTR sequences. No matches were found within the region of the -89C→T variant with any of the functional patterns defined on this site. The promoter

regions of genes initiate transcription and contain a number of essential *cis*-acting elements in the genome which are responsible for regulating gene expression (Hoogendoorn et al. 2003). A third of promoter variants have been found to modify gene expression by 50% or more, suggesting these regions to be an essential source of functionally relevant polymorphisms. Due to the fact that -89C→T is situated within the regulatory region of the *CYBRD1* gene functional studies are needed in order to further characterize it in a larger patient group. The original study by Zaahl et al. (2004) proposed the potential of IVS1-4C→G in affecting splicing of *CYBRD1* mRNA. The results from our study confirm this variant as a common polymorphism, as it was highly prevalent in both our patient and control groups. Only one homozygous patient for IVS1-4C→G was identified. It would be interesting to investigate whether homozygosity for this variant contributes an iron overload phenotype. The allele frequency of the IVS2+8T→C mutant C-allele was 0.792 in one European group on the HapMap database (rs7586144). The mutant A-allele of S266N in a European population was at 0.729 (refSNP ID: rs10455) corresponding to a frequency of 0.68 in our patient group. Both IVS2+8T→C and S266N are frequent polymorphisms.

HJV exonic mutations have been identified in the homozygous or compound heterozygous state in cases of JH or HFE1; these mutations, however, are rare. A study by Le Gac et al. (2004) demonstrated exactly how rare mutations in the *HJV* gene are. They identified only two missense mutations, in three out of a total of 333 French control subjects. The mutation frequency of the *HJV* gene was shown to be at 0.0045 in this study. Another study of a Caucasian and African American cohort in the general Alabama population identified only one *HJV* mutation in a total of 240 Caucasian subjects (Barton et al. 2004). None of the African American subjects were

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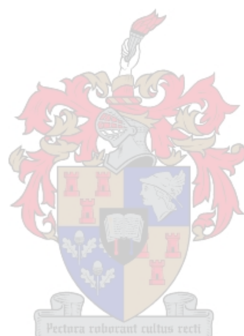
shown to harbour any *HJV* mutations. We identified a novel S333 variant in exon 4, resulting in a G to A transition at nucleotide 1001 which does not change the amino acid. An interesting observation from results obtained in the *HFE* gene of this study in these patients, showed variant IVS4-44T→C to be absent from two of these patients and present in one, in the heterozygous state. The two patients lacking this variant were shown to be heterozygous for IVS4+48G→A, while this variant was absent from the patient with IVS4-44T→C. This observation is likely due to chance, as no evidence exists that intronic variants in the *HFE* gene have a functional impact on the gene. In order to fully elucidate the role of this variant as a potential factor in iron overload in these patients further studies are warranted. HapMap results for the A310G (refSNP ID: rs7540883) variant identified in one control individual shows it to be absent in two European cohorts in the HapMap database. The mutant G-allele frequency was found to be 0.043 in the African American population, 0.092 in a Sub-Saharan African cohort and 0.068 in a combined group of Caucasians and African Americans. In summary, results from our study and the aforementioned documented studies confirm that mutations in the *HJV* exonic regions have a low frequency in the general Caucasian population.

Few studies exist that have investigated the risk of common diseases and several predisposing polymorphisms collectively (Weedon et al. 2006). Most studies have shown that polymorphisms on their own have very little effect on increasing the risk for developing a particular disease. A single polymorphism for example will is not very informative about the risks relating to clinical symptoms of a disease, as most diseases are accompanied by an array of symptoms. The lack of well published studies on the effect of multiple alleles on diseases is the result of the fact that not many common risk alleles for any disease have been confirmed with certainty as yet.

The current study is the first, to our knowledge, to investigate the role of the *HMOX1* and *HJV* genes as modifiers of iron overload in the South African population. Although we were unable to identify modifier variants which could potentially explain iron overload in most of the patients, results from this study may indicate that none of the variants identified are responsible for conferring primary iron overload in this specific group. Although most of the variants identified proved to be highly frequent polymorphisms in the general population, this study clearly demonstrates both the complexity and importance of undertaking studies on multiple genes in iron overload. With further identification and screening of potential candidate genes in South African HH individuals with unexplained primary iron overload, the precise role of modifier genes is still an ongoing challenge.



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CHAPTER FOUR: CONCLUSIONS AND FUTURE PROSPECTS

4.1. CONCLUSIONS

Non-HFE related forms of haemochromatosis are uncommon, but have extensively been documented. Roetto et al. (2002) reported mutations in the *HAMP* gene as a cause of juvenile haemochromatosis. Similarly, mutations in the *HJV* gene have been identified, which are also related to this early onset form of haemochromatosis (Papanikolaou et al. 2004). Other forms have been described that are related to the *TFR2* and *SLC40A*1 genes (Camaschella et al. 2000; Montosi et al. 2001; Njajou et al. 2001). Zaahl et al. (2004) suggested the role of *CYBRD1* and *SLC40A1* gene mutations as causative factors of iron overload in South African Caucasian and Black individuals who tested either negative or heterozygous for the *HFE* C282Y mutation. In an attempt to identify mutations that may confer an iron overload status in a group of C282Y heterozygous or negative individuals, we screened the entire coding region of the *HFE*, *HMOX1*, *SLC40A1*, *CYBRD1*, *HAMP* and *HJV* genes.

We demonstrated the potential role of variants, Q127H in the *HFE* gene, I109 in the *SLC40A1* gene, -89C→T in the *CYBRD1* gene and S333 in the *HJV* gene in the iron overload status of approximately 14% of our patients. Cartegni et al. (2002) have identified certain silent mutations that have an effect on the mRNA level by affecting the translated product. Both I109 and S333, although silent mutations in the *SLC40A1* and *HJV* genes, cannot completely be ruled out to have a potential effect on these genes, and hence contribute to an iron overload

phenotype in the patients in which it was identified. The effect of the 5'untranslated variant, -89C→T in the *CYBRD1* gene on mRNA structure and gene expression can only be confirmed through functional studies. It may be possible that this variant is in linkage disequilibrium with a functional regulatory element upstream of the *CYBRD1* gene or with a functional variant in close proximity. In order to fully elucidate the effects of these variants in South African patients with iron overload that cannot be explained by the common C282Y mutation, the study needs to be extended to a larger patient and control group.

The IVS2+4T→C, IVS4-44T→C, IVS4+48G→A and IVS5-47G→A in the *HFE* gene, IVS1-4C→G, IVS2+8T→C and S266N in the *CYBRD1* gene, and the V221 variant in the *SLC40A1* gene, most likely represent frequent polymorphisms in the South African Caucasian population. While most of these variants occur with slightly higher frequencies in the control group, they could potentially provide a protective advantage against iron overload in these individuals, a theory that needs to be confirmed in a larger patient cohort. The lack of mutations in the *HAMP* gene may be attributed to the small cohort size. Mutations in this gene are also usually rare and related to juvenile haemochromatosis.

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Failure to identify potential mutations conferring iron overload in 86% of patients in this study may be underscored by the fact that we failed to screen the entire gene, particularly the promoter regions. An explanation of iron overload in the majority of individuals in this study might therefore lie within the promoter regions of these genes or within other genes not screened in this study. Conversely, when comparing this study to that of Zaahl et al (2004) it is clear that almost similar results were observed for many of the variants identified in the *HFE*, *SLC40A*1 and *CYBRD1* genes. This could imply that the answer of iron overload in South African Caucasian patients might not lie in further investigation of other genes; it might

just lie within those genes for which comparable results were obtained between these two studies. In order to answer these questions, further investigation of the *SLC40A1*, *CYBRD1* and *HJV* genes in the South African Caucasian population is warranted.

Although HEX-SSCP analysis has been proclaimed by many as a highly effective mutation screening technique with a sensitivity of 70%, it may have missed the identification of approximately 30% of variants in this study. The low mutation frequency yielded from this study may also be accounted for by the small patient cohort screened or interactions from other genetic and environmental sources (Bataller et al. 2003; Hellier et al. 2003; Yee 2004). A major problem encountered by many studies with such small cohorts is the possibility of missing the identification of SNPs (Douabin-Gicquel et al. 2001). In larger studies the potential of identifying rare SNPs are usually more likely.

No significant associations between the patient and control groups were observed when gene-gene interactions were investigated. The only explanation for this might be the size of the patient group screened. Genotype-phenotype comparisons were hampered by a lack of clinical data on patients and iron parameters for some and most control individuals. To improve the chance of not missing association between variants, as well as correlations between genotype and phenotype, we suggest that patients be divided into well defined clinical groups with information such as iron parameters and age of onset of disease.

In summary, results from this study sparked the possibility of mutations further upstream of the *CYBRD1* gene, in the promoter regions, in the modification of disease expression in these patients, a potential that must be investigated. Even though, the exact cause of primary iron overload in most of our patient group remains elusive, the Q127H, I109, S333 and -89C→T

variants may provide a possible explanation for iron overload in some patients. This study clearly highlights the need to elucidate the role of modifier genes in HH expression, particularly in the South African population.

4.2. FUTURE PROSPECTS

As a future prospect for the HH project, we aim to expand both our patient and control numbers, as well as include individuals from the South African Black and Coloured (Mixed ancestry) populations. This should yield some interesting and novel results as not much is known about the cause of primary iron overload in these individuals. The patient group will also be extended to include C282Y homozygous individuals with variable clinical expression, as well as family cases. In research studies it is often difficult to obtain sufficient data regarding the iron status of both patients and controls, and obtain clinical information on age-related disease expression in patients. We hope in future with sampling of patients to compile data such as this fully as these prove valuable when undertaking comparisons of gene-gene interactions and genotype-phenotype correlations. It would also be interesting to recruit H63D homozygous patients as not much evidence exists that it alone can promote expression of iron overload.

We also envisage investigating other genes implicated in iron homeostasis including ceruloplasmin, hephaestin and *TFR2*, as well as screen the promoter regions of these genes and those investigated in the present study. With the identification of novel polymorphisms particular in the promoter region of genes, the next and most logical step would be functional studies to confirm the effects of these polymorphisms; something we aim to prioritise with future studies.

4.3. SOUTH AFRICA AND HH

Because the carrier frequency of the C282Y homozygous mutation is so high in the South African Caucasian population in particular, it is vital to promote awareness among patients, their families, the general public and medical professionals treating these patients. The Haemochromatosis Society of South Africa (HSSA) was established for exactly that purpose and is actively busy in promoting research studies, such as this at South African universities. The results from this study, and future studies on HH in the South African population could lead to improved counselling of patients and their families.

CHAPTER FIVE: REFERENCES

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5.2. ELECTRONIC REFERENCES

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APPENDIX A

Patient Information Sheet

Research Project: Analysis of genes implicated in iron regulation in individuals presenting with primary iron overload in the South African population

Name/No……………………………….Date of Birth……………….Gender……….
Telephone number………………………..Length (m)…………..Weight (kg)………..
Address…………………………………………………………………………….
Race/Ethnic background………………………………Home language……………….
Nationality of parents / grandparents…………………………………………………...

Any of the following symptoms:

□ Continuous tiredness	□ Heart disease	□ Weakness
□ Loss of libido	□ Sun induced skin rash	□ Diarrhoea
□ Arthritis / Joint pain	□ Abdominal pain discomfort	□ Constipation
□ Liver disease/ cancer	□ Tendency to low blood pressure	□ High blood pressure
□ Depression	□ Uncommon skin pigmentation	□ Diabetes mellitus

More Information / Other relevant symptoms…………………………………………………..

Any of the symptoms due to medication……………………………………………………..

Participant or any the family members diagnosed with:

	Participant	**Family member**	**Detail (e.g. type)**
1) Iron overload			
2) Iron deficiency			
3) Porphyria			
4) Multiple Sclerosis			
5) Diabetes Mellitus			

6) Chronic Tiredness			
7) Any infections			
8) Cancer			
9) Heart Disease			

Other:

Menstrual or relevant problems……………………………………………………………..

Hysterectomy (age)…………………………………………………………………………….

Blood group…………………………………………………………………………………….

Blood donor……………………….from…………………..

Vegetarian………………………..from…………………..

Diet supplementation with iron……………..mg/day………………..in the past…………..

Vitamin/mineral supplements……………….daily…………………..in the past…………...

No of pregnancies…………………………..Pregnancies terminated……………………

Circumstances that may cause blood loss…………………………………………………...

Smoke/day…………………………(cigarettes/pipe)…………….

Alcohol use/week……………………(beer/home-brewed beer/spirits)…………………….

IRON PARAMETERS

Serum iron	
Transferrin	
Transferrin saturation	
Ferritin	
Liver biopsy result (if available)	
Liver function test	
DNA results (if available)	
Other	