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Establishment of Screening Procedures For Genetic Disorders and Risk Factors In The South African Caucasian Population

By

ADEBOYE M. ADELEKAN

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ABSTRACT

Familial hypercholesterolaemia (FH), Hereditary Haemochromatosis (HH), and Methylenetetrahydrofolate reductase (MTHFR) deficiency are genetic disorders linked with cardiovascular disease and organ failure. These mutations have been reported to have a high prevalence and these disorders are often under diagnosed. In order to implement population-screening strategies, it is necessary to establish the prevalence of these genetic disorders. The aim of this study is therefore to:

- Establish rapid screening methods for these genetic disorders;
- Determine the sensitivity, stability and reproducibility of the methods; and
- Determine or confirm the prevalence of the genetic disorders in South African Caucasians.

Three hundred and sixty-three young healthy white male adults were recruited for the study. The methods that were established and used for the screening of these disorders were based on the polymerase chain reaction (PCR). For HH and MTHFR, the products of PCR were determined or detected by restriction fragment length polymorphism (RFLP) and for FH allele specific PCR was used. Sensitivity, stability and reproducibility of PCR reaction master mix were determined after period of 1 week, and after 1 and 2 months storage at -70°C.

Methodologies were chosen or adapted so that PCR master mixes aliquots could be stored at -70°C for over 2 months and still be stable and as sensitive as freshly prepared ones. The possibility of being able to store the PCR master mix improved the turn around time. The method is simple, direct and sensitive and the products are distinct.

Of the 359 and 349 subjects screened for MTHFR and HH respectively, 1 in 12 was homozygote for the mutation for MTHFR, C677T while for HH, 1 in 142 and 1 in 63 were homozygote for C282Y and H63D respectively. Compound heterozygosity for HH, C282Y and H63D was observed to be 1 in 29. The prevalence of FH in 339 subjects studied was found to be 1 in 84.

These results confirm the high prevalence reported for these disorders, which emphasizes the need to screen for the mutations within the population studied. As the penetrance of these mutations has not been established, screening should be limited to patients with indicative biochemical findings of these disorders. The family members of the subjects with these disorders should also be screened and all positive individuals should be appropriately monitored and treated.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	degree centigrade
μ	micro: 10^{-6}
μg	microgram
A	adenine (in DNA sequence, indicating exon sequence)
apo-B	apolipoprotein-B
ARMS	amplification refractory mutation system
bp	base pair
BSA	bovine serum albumin
C	cytosine (in DNA sequence, indicating exon sequence)
CAD	coronary artery disease
CBS	cystathionine β- synthase
CHD	coronary heart disease
CVD	cardiovascular disease
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide
DTT	dithiothreitol: threo-1,4-dimercapto-2,3-butanediol; $C_4H_{10}O_2S_2$
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediamine tetra-acetic acid: $C_{10}H_{16}N_2O_8$
EtBr	ethidium bromide: $C_{21}H_{20}BrN_3$
FDB	familial defective apolipoprotein B-100
FH	familial hypercholesterolaemia
g	gram
G	guanine (in DNA sequence, indicating exon sequence)
HCl	hydrochloric acid
Hcy	plasma homocysteine
HFE	haemochromatosis gene
HFH	homozygous familial hypercholesterolaemia
HH	hereditary haemochromatosis
Hinf 1	restriction endonuclease isolated from *Haemophilus influenzae*, with recognition site 5'-...G↓A...-3'
HLA	human leukocyte antigen
HMG-coA	3-hydroxy-3- methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
kb	kilo base pair
KCl	potasium chloride
LDL	low density lipoprotein
LDL-R	low density lipoprotein receptor
Ltd.	limited

m	milli: 10^{-3}
M	molar
Mb	mega base pair
Mbo 1	restriction endonuclease isolated from an *E. coli* strain that carries the cloned Mbo 1 gene from *Moraxella bovis*, with recognition site 5'-…↓GTAC…-3'
Me_4NCl	tetramethylammonium chloride
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
MTHFR	methylene tetrahydrofolate reductase
NaCl	sodium chloride
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ρmol	picomole
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
Rsa 1	restriction endonuclease isolated from *Rhodopseudomonas sphaeroides*, with recognition site 5'-…GT↓AC…-3'
sec	seconds
T	thymine (in DNA sequence, indicating exon sequence)
Taq DNA Polymerase	deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase from *Thermus aquaticus*
TBE	89.15 mM Tris [pH 8.0], 88.95 mM boric acid, 2.498mM Na_2EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine: $C_6H_{16}N_2$
tHcy	total plasma homocysteine
T_m	melting temperature
Tris	Tris: tris (hydroxymethyl) aminomethan: 2-Amino-2-(hydroxymethyl)-1,3- propanediol: $C_4H_{11}NO_3$
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrchloride: $C_4H_{11}NO_3.H_2O$
Triton X-100	Triton X-100: octylphenolpoly(ethylene- glycolether)$_n$: $C_{34}H_{62}O_{11}$, for n = 10
TS	transferrin saturation
U	unit
UK	United Kingdom
USA	United States of America
V	voltage
ρ	pico: 10^{-12}
Φ	phi
↓	indicates restriction enzyme cutting site

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CHAPTER ONE

1.INTRODUCTION

Molecular diagnosis is a rapidly growing area of diagnosis within virology, microbiology and other areas in pathology. In human genetics it involves the identification of specific mutations that are either risk factors or give rise to specific disorders. Methodologies are based on the identification of alterations in DNA or RNA sequences that are associated with disease. Traditionally, testing involved the measurement of a biochemical parameter such as a metabolite, protein or enzyme levels associated with a specific disorder. Molecular diagnostics involves the detection of the disease causing mutation/s. Not only can the patient presenting clinically with a specific disorder be tested, but family members that are asymptomatic and who are at risk can also be screened. This allows early diagnosis, together with counseling and the early implementation of the treatment strategies, prognosis is greatly improved with some individuals never presenting with clinical symptoms.

The establishment of a genetic diagnostic service requires optimization of screening methods to ensure rapid, cost effective testing, specific for the population for which a service is being provided. Three common disorders that are of late onset and treatable and common within the South African Caucasian population are Hereditary Haemochromatosis (HH), Familial hypercholesterolemia (FH) and hyperhomocyst(e)inemia (Hcy) due to the Methylene-tetrahydrofolate reductase

(MTHFR) C677T mutation. Each disorder represents certain aspects of genetic testing that needs to be taken into consideration when implementing a diagnostic service. The The methodologies used as well as the population specific factors for each of these disorders need to be taken into consideration when diagnostic services are implemented. The latter includes the prevalence of a disorder, gene frequencies, penetration of the mutation, founder effects and compound heterozygosity.

The objectives of this study are to establish rapid screening methods for common genetic disorders that have a high prevalence within the South African population and where early diagnosis and proactive treatment strategies will significantly improve prognosis. Methodologies will be optimized for rapid screening of the MTHFR, (C677T); HH, (C282Y and H63D); and the FH, (D206E) mutations. To ensure that screening and subsequent patient testing is cost effective, parameters such as reagent utilization, the long-term stability of the reagent solutions and test reproducibility will be optimised. The allele and the gene frequency for each mutation has been determined and statistically evaluated. The possibilities of founder effects have been investigated. Although studies have been undertaken to determine the frequencies of these mutations within different South African population groups, no study has been undertaken to determine the mutation frequencies within the same young population group that is yet to develop symptoms associated with these disorders. Information obtained from this study will aid the development of more effective screening strategies. Early diagnosis together with implementation of effective treatment regimes will either prevent or delay the onset of disease. Individuals that are found to be positive for any of these disorders are offered counseling and family screening.

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This will enable the establishment of criteria for family screening, which will impact on the type of counseling services, offered.

Molecular diagnostics is an additional diagnostic tool to assist the pathologist to confirm diagnosis, investigate families of affected patients and support biochemical testing. A patient usually presents with clinical symptoms that indicates the presence of a specific disorder. While the measurement of either raised or reduced biochemical parameter will verify diagnosis, genetic screening will indicate the possible genetic origin of the disorder.

The use of genetic testing as a primary test for the identification of a disorder is of limited use as there is little or no information regarding the penetrance of the mutations. Penetrance is the frequency, expressed as a fraction or percentage of individuals who are phenotypically affected, among persons of an appropriate genotype (i.e. homozygous or hemizygous for recessive, heterozygotes or homozygous for dominants). The little or no information on reliable estimate of the overall clinical pentrance of mutations linked with disease has limited the application and use of population screening[1]. The development of techniques for population screening and information obtained from such screening studies allows the development of testing protocols that are cost effective and population specific.

1.1 The Polymerase Chain Reaction

The development of polymerase chain reaction (PCR) by Kary B. Mullis in 1985, for which he was awarded the Nobel Prize in 1993, has lead to rapid developments in

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molecular diagnostics[2]. PCR is an enzymatic method for producing an extremely large number of copies of a specific DNA sequence by a process called amplification. PCR, by generating identical copies of a target DNA sequence, eventually makes enough analyzable, accessible genetic material to perform tests to determine its identity[2-6]. For successful PCR amplification the following reagents are required, DNA, primer, DNA polymerase, $MgCl_2$, and dNTPs. For detection of a specific mutation all parameters such as annealing temperatures and reagent concentrations need to be optimized. Some of the important factors that affect successful amplification will be discussed. PCR has moved molecular diagnostics from a research environment to the clinical laboratory. Once procedures are optimized and appropriate quantity/quality of genomic DNA is isolated, the methodologies can be automated for rapid diagnosis.

1.1.2 Genomic DNA

For successful amplification of a DNA region using PCR a purified sample of genomic DNA is a required. Although, DNA from any human tissue can be used, the DNA is usually isolated from blood and more specifically from the lymphocyte population. This requires the collection of an uncoagulated blood by an established phlembotomy service within a clinical laboratory. DNA is either isolated from whole blood, the buffy coat or the isolated lymphocytes. All DNA isolation procedures involve the following basic steps (i) cell lysis (ii) solublization of protein and DNA and (iii) selective isolation of DNA from all other cellular components. The most frequently used methods are phenol/chloroform extraction, selective precipitation or selective binding to a membrane, column or particulate matrix. The method of choice should fulfill the following requirements; the isolation of intact pure DNA, limited

exposure to chemical and biological hazards, rapid, with high throughput capacity and high reproducibility[7-9].

1.1.3 Primer Design

Primers are oligonucleotides of 18-24 nucleotides in length that are complementary to a specific DNA sequence. A pre-requirement for successful PCR amplification is that the sequence of the DNA to be amplified is known. This information is either obtained by sequencing the DNA or from published sequences. Two primers that serve to define the region to be amplified are needed

For Restriction Fragment Length Polymorphism (RFLP) PCR methodologies the primers are designed upstream or downstream to the site of the mutation. Alternatively a primer can be designed to recognize the site of the mutation by either amplifying the region of normal or the mutant allele (allele specific methodologies). A second primer complementary to a second sequence at least 80 bp away is used to ensure on amplification a fragment that is large enough to resolve on polyacrylamide gel electrophoresis.

Other factors that are taken into consideration in the design of primers are that the GC content must be between 40 and 60% with a balanced distribution of G/C and A/T rich regions. The primers must contain no internal secondary structure and should not be complementary to each other at the 3' ends to avoid primer-dimer formation. Primers that are used for the amplification of a specific region of DNA should only bind at specific complementary sites and at the optimal annealing temperature

produce a single specific PCR product. The primer/template ratio strongly influences the specificity of the PCR and should be optimized empirically. Too little template may cause the primers not to be able to find their complementary sequences; too many templates may lead to an increase in mispriming events[2, 9].

1.1.4. Taq DNA Polymerase

The primary requirements for the polymerase enzymes used in a PCR reaction are good activity at temperatures of about 75°C and ability to retain the activity after prolonged incubation at the high temperatures used for template denaturation. The polymerase that is most often used in PCR is Taq DNA polymerase isolated from the thermophilic eubacterium *Thermus aquaticus BM*, a strain that lacks Taq I restriction endonuclease. Taq polymerase has 3' to 5' exonuclease activity. The optimal Taq DNA polymerase enzyme activity is between 0.5 and 2.5 units. Increased enzyme concentration sometimes lead to decreased specificity[9].

1.1.5 $MgCl_2$, dNTP's and other Additives

The concentration of Mg^{2+} influences Taq DNA polymerase activity. It increases the T_m of double stranded DNA and forms soluble complexes with the dNTPs to produce the actual substrate that the polymerase recognizes. The concentrations of free Mg^{2+} depend on the concentration of compounds that bind the ion, including dNTPs, free pyrophosphate and EDTA. The concentration of magnesium in the PCR reaction is critical to the success of the reaction. The final magnesium concentration in a reaction

mixture may be optimized by the user according to individual requirements and is usually between 1.0 and 3.5mM. A higher Mg^{2+} increases the PCR yield but decreases the specificity of the reaction (increases the incidence of primer dimers); lower Mg^{2+} concentrations increases the specificity but reduces the yield.

To improve yield and specificity of difficult targets in PCR amplifications, researchers often include enhancing agents in the reaction. The two PCR enhancing agents that deserve particular attention are betaine (N, N, N-trimethylglycine) and dimethyl sulphoxide (DMSO). DMSO is probably the most commonly used enhancing agent and is frequently included as part of a standard optimization of PCR amplifications. Betaine is another agent that has been used successfully for increasing yield and specificity of PCR products. Both of these agents facilitate strand separation; DMSO disrupts base pairing whereas betaine, an iso-stabilizing agent, equalizes the contribution of GC- and AT-bases pairing to the stability of the DNA duplex[10].

1.1.6 PCR Amplification

PCR is the method used selectively for amplifications of a specific target DNA sequence or sequences within a heterogeneous collection of DNA sequences such as total genomic DNA.The primers added to the denatured genomic DNA, will bind specifically to complementary DNA sequences immediately flanking the desired target region. Primers are designed so that, in the presence of a suitable heat-stable Taq DNA polymerase and DNA precursor (the four deoxynucloside triphosphates, dATP, dCTP, dGTP and dTTP), the synthesis of new DNA strands can be initiated.

PCR is a chain reaction because the newly synthesized DNA strands will act as templates for further DNA synthesis in subsequence cycles. After about 30 cycles of DNA synthesis, the products of the PCR will include, in addition to the starting DNA, about 10^5copies of the specific target sequence, an amount which is easily visualized as a discrete band of a specific size when submitted to agarose gel electrophoresis. A heat-stable Taq DNA polymerase is used because the reaction involves sequential cycle composed of three steps: Denaturation-typical at about 93-95°C for human genomic DNA; Reannealing-at temperatures usually from about 50-70°C, depending on the T_m of the expected duplex (annealing temperature is typically about 5°C below the calculated T_m); DNA synthesis-typical of about 70-75°C[2-6, 9]

1.1.7 PCR Techniques

1.1.7.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction enzymes recognize specific sites of different length and base composition. The typical restriction site is an exact palindrome of 4-8bp with an axis of rotational symmetry. The number and the size of fragments generated by a restriction enzyme depend on the frequency of occurrence of the restriction enzyme site in the DNA to be cut. In RFLP a restriction enzyme is used that recognizes the sequence where the point mutation has occurred. If the substitution has occurred the restriction enzyme will recognize this sequence, bind and cut the site resulting in two fragments usually of different size that can be resolved by gel electrophoresis. If the mutation is absent the DNA fragment is not cut. Often if no restriction enzyme is found that recognizes the mutation site, then an enzyme is chosen that will recognize the normal sequence. Subsequently, the reverse is observed: the DNA fragments without the mutation is cut

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and the fragment with the mutation is not. In addition the restriction enzyme will recognize more than one digestion site in a DNA fragment and more complex digestion patterns are observed following electrophoresis. If a restriction enzyme is not found that recognizes either the normal or mutant sequences an allele specific PCR procedure is usually used.

1.1.7.2 Allele Specific PCR

Oligonucleotide primers can be designed so as to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest. This is the PCR equivalent of the allele-specific hybridization, which is possible with allele specific oligonucleotide (ASO) probes. In the case of allele-specific hybridization, alternative ASO probes are designed to have difference in a central segment of the sequence (to maximize thermodynamic instability of mismatched duplexes). However, in the case of allele-specific PCR, ASO primers are designed to differ at the nucleotide that occurs at the extreme 3' terminus. This is so because the DNA synthesis step in a PCR reaction is crucially dependent on correct base pairing at the 3' end. This method can be used to type specific allele at a polymorphic locus, but has found particular use as a method for detecting a specific pathogenic mutation, the so called amplification refractory mutation system (ARMS; Newton et al., 1989)[11]. If hetero- or homozygosity needs to be determined, both the normal and mutant alleles must be detected. This will need two separate PCR mixtures in two separate tubes.

1.1.7.3 Multiplex PCR

To save time and reagents PCR reactions can be multiplexed by using more than one set of primers in one reaction. This type of PCR requires extensive optimization of annealing conditions for maximal amplification efficiency of the different primer-template systems.

1.1.8 Detection of PCR Products

The RFLP digestion products and the Allele Specific PCR products are usually separated according to size by either polyacrylamide or agarose gel electrophoresis. The RFLP digestion produce different banding pattern following gel electrophoresis and these are used to identify whether an individual is homozygous normal, heterozygous or homozygous mutant for a specific mutation. In Allele Specific PCR the presence of an amplified product indicates the presence of a specific allele. Individuals can be identified as homozygous normal, heterozygous or homozygous for the mutation according to the presence or the absence of a specific product. Another technique often used for the sizing and detection of DNA fragments is polyacrylamide capillary gel electrophoresis[4, 5, 9, 11].

1.2 PCR and the Detection of Point Mutations Associated with Disease

The development of PCR based diagnostic methodologies has enabled the screening of large populations for mutations that are associated with genetic disorders.

1.2.1 Population Screening

Population screening follows naturally from the ability to test directly for the presence of a mutation. DNA tests are rather different from many other screening tests because they may be used for both screening and diagnostic purposes at the same time. However, proposals to introduce any population-screening test still need to satisfy the same criteria, regardless of the technology used. The requirement for population screening include: (a) a positive result must lead to some useful action, (b) the whole program must be socially and ethically acceptable, (c) the test must have high sensitivity and specificity, and (d) the benefit of the program must outweigh its costs[1].

1.2.2 Founder Effect

Several well-known genetic phenomena are caused by populations starting at or proceeding through small numbers. When a population is initiated by a small, and therefore genetically unrepresentative, sample of the parent population, the genetic drift observed in the sub-population is referred to as a founder effect. A classical

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human example is the high prevalence of FH among South African Caucasian population.

1.2.3 Statistical Evaluation of Genetic Data

The equilibrium of alleles tested in the population was analyzed using the Hardy-Weinberg principle. The Hardy- Weinberg principle states that with random mating, expected genotypic proportions for an autosomal, diploid locus can be calculated in term of allelic frequencies by a binomial or multinomial function: $(p+q)^2 = p^2 + 2pq + q^2$. The Hardy-Weinberg model was formulated for autosomal loci in a sexually reproducing diploid species and makes the following assumptions: mating is random among phenotypes; generations do not overlap; population sizes are very large to genotypic proportions and do not vary from random drift; there is no migration; there is no mutation; there is no natural selection favoring some genotypes; and allele frequencies are the same in both sexes. If these assumptions hold, Hardy-Weinberg proportions are reached in a single generation. The population will remain indefinitely in these proportions until some event, such as random genetic drift or natural selection changes allelic frequencies[12]. These statistical methodologies will be used to determine the distribution of the methylenetetrahydrofolate reductase C677T mutation, the C282Y and H63D mutations associated with hereditary haemochromatosis and the D206E mutation found in some patients with familial hypercholesterolaemia.

1.3 Homocysteine Metabolism and Methylenetetrahydrofolate Reductase.

1.3.1 Homocysteine Metabolism

Homocysteine is an amino acid intermediate formed during the metabolism of methionine, an essential amino acid derived from dietary protein. It is metabolized by one of two pathways: remethylation and transsulfuration. In the remethylation cycle, homocysteine is salvaged by acquiring a methyl group in a reaction catalyzed by the vitamin B_{12}-dependant enzyme methionine synthase. The donor in this reaction is N^5-methyltetrahydrofolate, and the enzyme N^5, N^{10}-methylenetetrahydrofolate reductase functions as a catalyst in the remethylation cycle. Under conditions in which excess methionine is present or cysteine synthesis is required, homocysteine enters the transsulfuration pathway. In the transsufuration pathway, homocysteine condenses with serine to form cystathionine in a reaction catalysed by the vitamin B_6-dependant rate-limiting enzyme cystathionine β-synthase. Cystathionine is subsequently hydrolyzed to form cysteine, which may in turn be incorporated into glutathione or further metabolized to sulfate and excreted in the urine. Homocysteine does not normally accumulate in plasma because it is very unstable in aqueous solution, and undergoes oxidation to homocystine. Total homocysteine concentrations (fasting and post methionine loading) are measured in EDTA plasma by high-performance liquid chromatography (HPLC) and fluorescence detection. The concentration of homocysteine in blood is normally less than 15μmol/L while in normal urine, the concentration is below the detection limit of most laboratory methods. This back

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ground information on homocysteine metabolism is intended to help to understand how homocysteine elevation occurs in people with defect in its metabolic pathway.

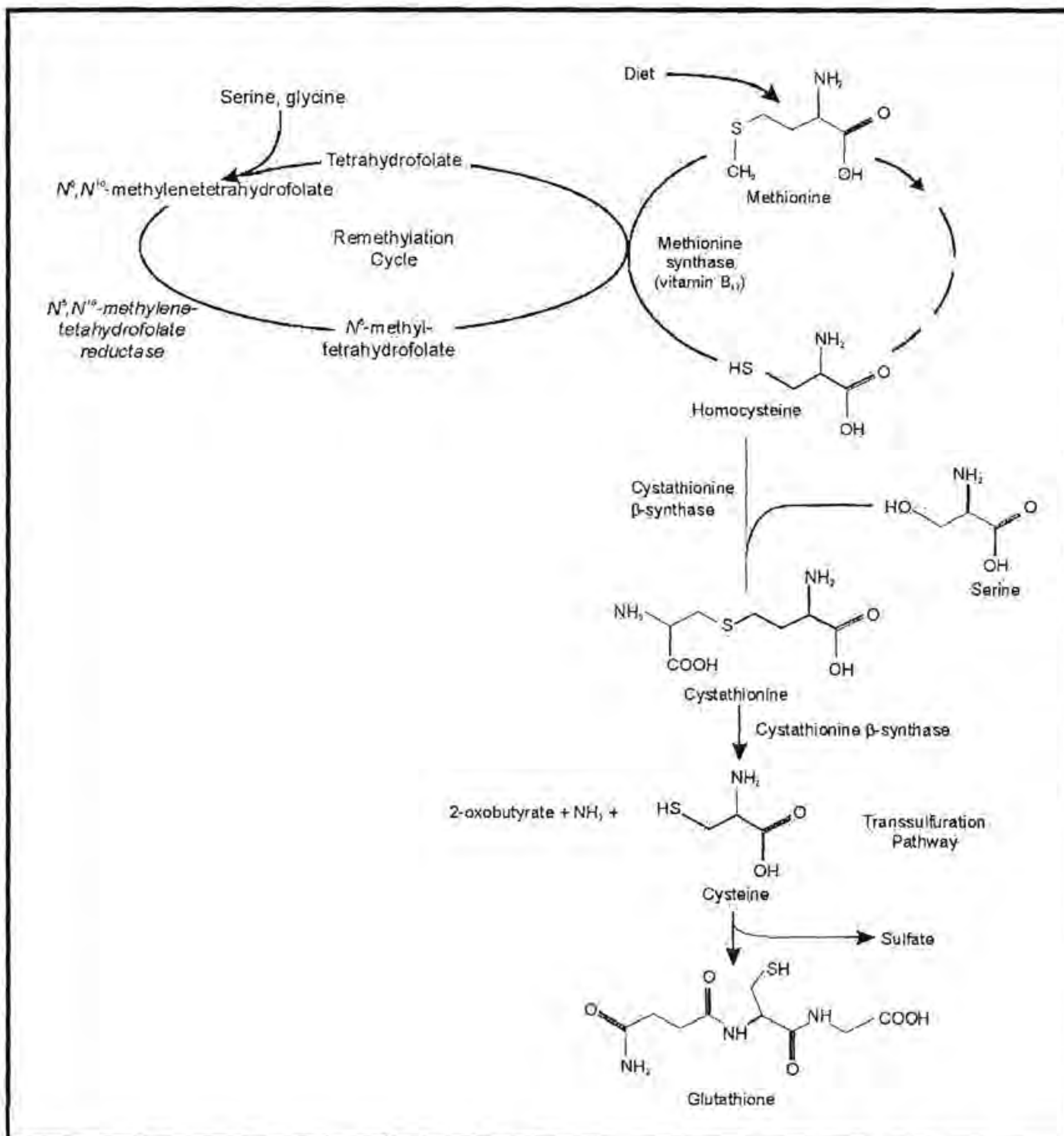

Figure 1: Metabolism of Homocyst(e)ine[13].

Homocystinuria has been reported[14-15] and hyperhomocystinemia is recognized as an independent predictor of arteriosclerotic disease, including stroke, myocardial infarction, and peripheral vascular disease. Homocystinuria can arise as a result of folate deficiency or due to defects in the enzymes involved in folate metabolism. Homocysteinura is linked to a deficiency of cystathionine β-synthase and defects in the N^5-methyltetrahydrofolate-dependent methylation of homocysteine[16-19]. Cardiovascular disease in South African Caucasian population is high and the identification of mutations in the folate pathway can assist in the identification of risk factors for the disease in the population.

1.3.2 The Genetics of Hyperhomocysteinemia

Genetic aberrations in the Cystathionine β-Synthase (CBS) and Methylenetetrahydrofolate Reductase (MTHFR) genes may account for reduced enzyme activities and elevated plasma homocysteine levels.

1.3.2.1 Cystathionine β-synthase (CBS) Deficiency

Cystathionine β-synthase (CBS) is the most common cause of homocystinuria[14, 20]. The deficient activity of this enzyme blocks the formation of cystathionine, with the result that homocysteine and its precursors accumulate, and cysteine and cystine decrease in body fluids [21]. Patients with reduce enzyme activity will respond biochemically and clinically to large doses of pyridoxine, the cofactor required to

activate the enzyme. Those with nul mutation, 0% activity should be treated with a diet low in methionine and supplemented with cystine.

The T833C mutation has been observed in patients with homozygous CBS deficiency but not in cardiovascular disease patients. Hence, the homozygosity for CBS deficiency was concluded not to be involved in premature cardiovascular disease[14].

1.3.4 5, 10-Methylene-tetrahydrofolate Reductase (MTHFR) deficiency

5, 10-Methylene-tetrahydrofolate reductase (MTHFR) deficiency was attributed to the presence of a point mutation (C677T) in the coding region for the MTHFR binding site, leading to the substitution of valine for alanine[21-22]. MTHFR deficiency is rare. When it occurs, death in infancy or childhood is probable; surviving adults are mentally retarded with cerebral atrophy. Treatment consists of administration of folate[22]. Fourteen rare mutations in MTHFR gene have been described and are associated with enzymatic deficiency and homocystinuria[21].

The C677T mutation is one of the more common mutations and is associated with decreased enzyme activity and contributes substantially to the variation in total plasma homocysteine (tHcy) concentration, which predisposes to mild hyperhomocysteinemia. This is even more pronounced in presence of a low folate status [16-17, 21-22]. It has been associated with three-fold increase in risk for premature cardiovascular disease. The genetic-nutrient interaction effect is believed to increase the risk for neural tube defects[18-19] and vascular disease. Homozygotes for A1298C

mutation has been associated with decreased MTHFR activity while the effect T1317C mutation on the enzyme activity is silent[23]. Neither homozygous nor heterozygous A1298C genotype has been associated with higher plasma tHcy concentration, although double heterozygosity C677T and A1298C were associated with reduced MTHFR activity similar to those observed in homozygous C677T allele.

1.3.5 The Prevalence of MTHFR Mutations

C677T has high prevalence in people of Caucasian descent while A1298C was found to have almost equal prevalence in both Caucasian and Asian populations. T1317C mutation is of African origin and is common in individuals of African –American decent[14]. The prevalence of C677T, A1298C and T1317C mutations were studied in healthy adult populations of Caucasian Canadians (n = 197), Asian Canadians (n = 51) and African- Americans (n = 51). The C677T was found in 13% African-American, 30% Asians and 34% Caucasians; A1298C in 20% Asian, 30% Caucasians, 16% African-Americans; and T1317C was found once out of 494 (0.2%) chromosomes in Caucasians and Asian and 37% African-Americans. In the South African Caucasian population, C677T gene and carrier frequency has been reported as 0.22 and 0.34 respectively[24]. For A1298C, gene and carrier frequency is 0.27 and 0.39 while for T1317C the gene and carrier frequency is 0.01 and 0.02[24].

The MTHFR C677T mutation is common in Caucasian populations of Canada and United Kingdom and is believed to increase the risk for neural tube defects and vascular disease. Cardiovascular disease in South Africa is one of the highest in the world. Besides, the high prevalence of FH in South Africa, there could be other risk

factors which also predispose this population to cardiovascular disease. The Methylenetetrahydrofolate reductase (MTHFR) mutation has been identified as a risk factor for cardiovascular disease[14-15, 25-26]. The highest prevalence of this mutation has been found within the Dutch and United Kingdom populations, which are the common ancestors of the South African Caucasian population. A high incidence of MTHFR mutations in the Canadian population reflects the European origin of these mutations. The prevalence of this mutation within the South African population has only been determined within a small population group[14, 24]. A larger study will determine whether the prevelance of the C677T MTHFR mutation is as high as in the Dutch, United Kingdom and Canadian populations. Furthermore, information obtained regarding the frequency of this mutation will provide important information that will contribute to the assessment of patients at risk for developing cardiovascular disease.

The methodology employed for the detection of this polymorphism is a simple restriction length polymorphism (RFLP), polymerase chain reaction (PCR) and is an ideal test system to establish criteria for the standardization of other PCR methodologies.

1.4 Hereditary Haemochromatosis

Hereditary hemochromatosis is an autosomal recessive genetic disease associated with an iron- storage disorder in which an inappropriate increase in intestinal iron absorption result in deposition of iron with eventual tissue damage and functional impairment of especially the liver, pancreas, heart and pituitary[27].

1.4.1 Clinical Effects of Iron Accumulation

Recklinghausen who coined the terms "hemosiderin" for the iron containing compound and heamochromatosis for the clinical disease, first recognized the association of cirrhosis with heavy hepatic deposition of iron-containing pigment in 1889[1]. Sheldon not only provided a full description of the disorder, but also suggested that the disease be inherited. In 1975 Saddi and Feingold made a firm proposal for recessive mode of transmission. The discovery of the association between haemochromatosis and certain HLA antigens by Simon *et al*[1]. made genetic investigation of the disorder possible. Iron over load is classified as primary or secondary, depending on the underlying mechanism. Primary iron overload results from abnormally increased absorption of dietary iron in the small intestine. Secondary overload results mainly from iron accumulated as a consequence of ineffective erythropoiesis, multiple blood transfusions, or prolonged excessive intake of dietary iron.

Primary iron overload associated with Hereditary Haemochromatosis results in excess iron absorbed being deposited in the parenchymal cells of the liver, heart, joints, pancreas and subsequently causing fibrosis and deterioration, resulting in organ failure and ultimately, chronic diseases. The body has no mechanism for excreting iron absorbed from the diet except through incidental losses. Thus, the level of body iron is regulated only through absorption from food. The amount of iron absorbed from diet is influenced by the following factors: the amount of iron stored in the body, the rate and effectiveness of erythropoiesis (functional iron), the amount and chemical form of dietary iron, and the presence of absorption enhancers and inhibitors in the

diet[1, 27]. Persons with normal hemoglobin levels and iron stores (as reflected by serum ferritin value) absorb just enough to meet their daily needs and to balance losses (1mg/day). In contrast, persons with HH continue to absorb high amount of dietary iron even when the body already has enough or too much iron. When total body iron exceed storage capacity (5 to 10 times the normal quantity), tissue and organ damage begins. At this stage, iron overload has occurred. This condition, if left untreated, can result in fatigue, arthralgia and arthritis, diabetes, liver disease, coronary heart disease, hyperpigmentation, endocrine disorders and premature death[1, 27].

The clinical manifestation often appears in people from age 40 and above but sometime at age 20 (juvenile HH)[27]. Sometimes people who are homozygous for the disease mutations never present with clinical signs of the disease[38]. The development of the clinical manifestations of iron overload may be influenced by genetic and environmental factors, including menstruation, diet and blood donation. The early signs of progressive iron overload are asymptomatic elevation of liver enzyme levels, particularly, alanine aminotransferase and aspartate aminotransferase, which later may be accompanied by recurrent right sided abdominal pain and hepatomegaly. Arthropathy is also common and occasional episodes of inflammatory arthritis occur, some of which are caused by deposits of calcium pyrophosphate dihydrate. Other early signs and symptoms include impotence, amenorrhea, irritability, depression, fatigue, gray or bronze skin pigmentation, diabetes mellitus, hypopituitarism, hypogonadism, cardiomyopathy and joint deformity[1, 27].

1.4.2 Diagnosis of Hereditary Haemochromatosis

1.4.2.1 Transferrin Saturation and Serum Ferritin Levels

For over 10 years, laboratory tests for assessing iron burden (transferrin saturation and serum ferritin levels) have been widely used in population screening, in conjunction with diagnostic protocols aimed at differentiating HH from other acquired and inherited causes of iron overload[29].

Ferritin is an iron protein complex formed by the union of ferric ions with apo-ferritin. It regulates iron storage and transport from the intestinal lumen to plasma. Serum ferritin level >200µg/L for pre-menopausal females, and >300µg/L for males and post-menopausal females are suggestive of HH[27; 30]. Transferrin is a non-heme β_1-globulin of the plasma, acting, as an iron transporting protein, and transferrin saturation (TS) is the amount of transferrin in percentage that is bound to iron. TS ≥50% for pre-menopausal females and ≥60% for males and post-menopausal females are suggestive for HH.

1.4.2.2 The Genetics of Hereditary Haemochromatosis

In 1996, a candidate gene for hereditary hemochromatosis, originally termed 'HLA-H', but more correctly 'HFE (Haemochromatosis gene)' [31-32], that is located on chromosome 6 (6p22.1)[33] has been identified on which two mutations found, cysteine to tyrosine at amino acid 282 (C282Y) and histidine to aspartate at amino acid 63(H63D), were associated with HH[33-37]. The protein product of the HFE gene is a trans-membrane glycoprotein, termed HFE, which modulates iron uptake. Mutations

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in the HFE protein compromise its function and produce disease symptoms. Other mutations such as S65C are also associated with HH. However, these mutations are rare.

1.4.3 The Prevalence of Hereditary Haemochromatosis Mutations

In the United Kingdom, over 90% of Haemochromatosis patients are homozygous for the C282Y mutation[37] whereas only 1 in 200-control individuals was homozygous for the mutation[38]. The H63D mutation alone is not associated with iron overload, showing a frequency of 2% in a patient and 16% in a control population. However, subjects who have one copy of each mutation, termed compound heterozygous, are at risk of iron overload and may develop clinical hemochromatosis[39]. Possible founder effects have been described for HH[40] within the South African population. The presence of a founder effect within the South African population needs to be verified and the prevalence of compound heterozygosity still needs to be determined. In this study, the possibility of a founder effect as well as the prevalence of compound heterozygosity will be investigated by using techniques that will allow rapid population screening.

1.5 Familial Hypercholesterolaemia

1.5.1 Clinical Effects of Increased Cholesterol Levels

Familial hypercholesterolaemia (FH) is characterized clinically by increased plasma low density lipoprotein (LDL) cholesterol concentration, cholesterol deposition in

skin, tendons, and in arteries. Hypercholesterolemia is present at birth in most FH patients and persists throughout life. In heterozygotes, xanthomas appear towards the end of the second decade and clinical manifestations of atherosclerotic disease appear during the fourth decade[41-42]. In homozygotes, the unique yellow-orange cutaneous xanthomas develop by the age of 4years, if they are not already present at birth[43]. An affected individual suffer from atherosclerosis and premature coronary heart disease (CHD), which may manifest in childhood and progress to early death[44] . Death from myocardial infarction invariably occurs in homozygotes before the end of the third decade[45-46].

1.5.2 The Metabolism of Cholesterol

The LDL receptor is a membrane protein of 839 amino acid that is responsible for cholesterol uptake into cell via receptor mediated endocytosis of cholesterol rich lipoproteins secreted by the liver. The receptor binds one of two ligands, apo-B or apo-E to perform its function, and is recycled for further use. Mutations at the LDL-receptor gene affect either the number and or function of the LDL- receptor protein. This has two different effects on the endogenous lipoprotein pathway that cause hypercholesterolaemia. Firstly, the rate of LDL removal is decreased and secondly, the production of LDL from intermediate density lipoprotein (IDL) is increased as a result of impaired IDL clearance from the circulation. An alternative genetic disorder that cause pathological decreased LDL clearance is familial defective apolipoprotein-B (apo-B) which functions as a ligand of the LDL receptor. However this disorder will not be discussed, as it is rare in the South African population.

The significance of LDL-R is evident in patients with genetic disorder FH and their defects have been described. It may be deficient or it may be present and bind LDL normally, but unable to internalize the LDL particles. In subjects who are homozygote for the gene defect, functional receptors are few or absent. Heterozygote subjects have approximately half the normal number of LDL receptor (LDL-R). The inability of the LDL moiety to deliver cholesterol to the cell of FH patient result in increased HMG-coA reductase activity and increased cholesterol synthesis. Reduction in the removal of LDL from plasma causes elevated plasma levels of LDL- cholesterol, increased uptake of LDL by macrophage and smooth muscle cells, cholesterol deposits in arterial walls and consequently, premature atherosclerosis[41].

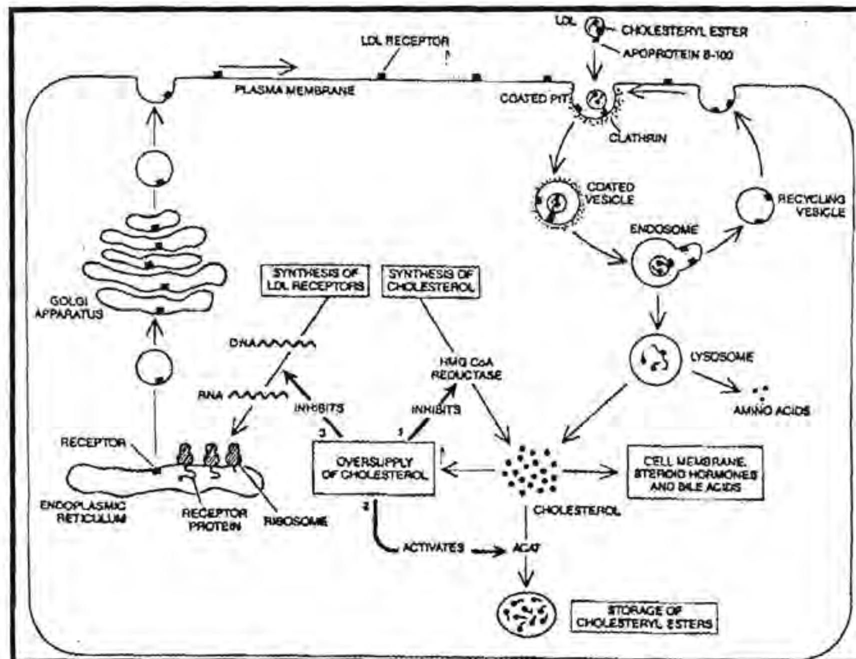

Figure 1.2: Pathway of LDL metabolism[41]. LDL, low density lipoproteins; LDL-R, low density lipoprotein receptor; ACAT, acyl-CoA cholesterol acyltransferase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase. Because of the presence of apolipoprotein B-100 on its surface, the LDL particle is recognized by a specific receptor in a coated pit and taken into the cell in a coated vesicle (top right). Coated vesicle fused together to form an endosome. The acidic environment of the endosome causes the LDL particle to dissociate from the receptors, which return to the cell surface. The LDL particles are taken to a lysosome, where apolipoprotein B-100 is broken down into amino acids and cholesterol ester is converted to free cholesterol for cellular needs. The cellular cholesterol level is self-regulated. Oversupply of cholesterol will lead to (1) decreased rate of cholesterol synthesis by inhibiting HMG-CoA reductase, (2) increased storage of cholesteryl ester by activating ACAT, and (3) inhibition of manufacture of new LDL receptors by suppressing the transcrition of the receptor gene into mRNA[30].

1.5.3 Diagnosis of Hypercholesterolaemia

1.5.3.1 LDL Cholesterol and Hypercholesterolaemia

FH is diagnosed on typical trait: elevation of LDL cholesterol up to twice the normal level, presence of cutaneous and tendon xanthomata, and a family history of premature coronary artery disease. Features may manifest at a very young age in children inheriting two mutant genes, but are usually not apparent in heterozygotes until adulthood[47].

The mean plasma LDL cholesterol in children and adult heterozygotes is usually two to three times that of normal people of similar age, whereas the mean plasma LDL cholesterol of homozygotes is four to six times that of normal subjects.
Once identified, the hyperlipidemia of these carriers is responsive to treatment by diet and drugs. Children who inherited two defective alleles of the LDL receptor (R) usually have little response to treatment by diet and drug. Many suffer a major coronary event in the first or second decade of life. Life expectancy can be extended by appropriate treatment, which may include gene therapy.

1.5.3.2 The Genetics of Hypercholesterolaemia

Five classes of mutations at the LDL receptor locus have been identified on the basis of the phenotypic behaviour of the mutant protein. Up to date more than 400 mutations of this gene have been characterized worldwide and a database of 400 mutations is available on the website http://www.ucl.ac.uk/fh. Within a geographically or culturally isolated population, or where a large proportion of people are related by decent because of migration (founder effect), there may be a single or a couple of mutations causing FH in many of these patients.

1.5.3.3 Genetic Testing for Hypercholesterolaemia

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder, expressed in either heterozygous or homozygous mode. It is caused by primary genetic defect in the plasma low-density lipoprotein-receptor (LDL-R) due to mutations in the gene encoding for it[48]. The LDL-R is a membrane protein of 839 amino acids that is

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responsible for LDL uptake into cells via receptor bindings of one of the two ligands, apo-B or apo-E, to perform its function and is recycled for further use. The LDL-R gene is located on the short arm of chromosome 19, spans 45kb and comprises of 18 exons and 17 introns. Five classes of mutations at the LDL-R locus have been identified on the basis of the phenotypic behavior of the mutant protein. The autosomal dominant nature of the disease implies that only one copy of the defective gene (in the case LDL-R gene) is sufficient to induce the onset of the disease[49].

FH is genetically heterogeneous and large number of mutations including both gross rearrangements and point mutations have been reported in various countries and ethnic groups. The gross rearrangement of the LDL-R gene includes deletion of various sizes, but only one insertion has been reported so far[50]. The defect as discovered in patients with FH includes reduced LDL binding because of defective or absent LDL receptors. In another variant of the disorder, the person may make defective LDL receptors that bind LDL normally but that cannot efficiently internalize the LDL particles[48]. Heterozygous FH is one of the most common genetic metabolic disorders, with an incidence of 1 in 500 in the United States and most European populations, while a founder's effect increased the frequency among Afrikaners in South Africa to about 1 in 80[41, 51-53]. The prevalence of homozygous FH is about one in million people[41, 53].

The development of DNA based methods for the direct detection of specific LDL-R gene mutations enabled the establish a molecular diagnostic service for FH. This specialized service is of particular relevance and was applied in the Afrikaner population of South Africa, where a founder gene effect increased the prevalence of

FH to about 5-10 times than that found in most population groups. Three point mutations, FH Afrikaner-1 (D206E), -2 (V408M), and -3 (D154N) in the LDL-R gene were shown to account for approximately 90% of the defect in South African Afrikaner. The D206E, V508M and D154N mutations were present in 70%, 20%, and 10% respectively of the alleles that were studied so far. FH may also result from other different genetic defect in the LDL-R gene. The same clinical characteristics can be seen in individuals with a defect in apolipoprotein B (apo-B) which is formed as the ligand of the LDL-R. This defect is called familial defective apo-B (FDB) and is rare the South African population.

Heterozygous FH in many patients can be the diagnosis on the basis of increased plasma cholesterol. However, it has been shown that there is an overlap between affected and normal individuals, even when measuring the LDL receptor function on patients' monocytes. By contrast, a genetic approach gives an unequivocal result, which is very advantageous especially when diagnosing children and relatives of a FH patient. In South Africa (SA), 90% of the homozygous FH patients can be diagnosed by screening for these specific mutations while the other 10% of the patients with elevated cholesterol values can be screened for the apo-B mutation or unknown mutation by using single-strand conformation polymorphism and heteroduplex analysis[54-57].

Patients often present with moderately raised cholesterol levels. Referrals to the Molecular Diagnostic Laboratory of the Department of Chemical Pathology, University of Pretoria, indicates that either this mutation is absent in the population for which the service is provided or that these patients are not sent for genetic testing,

often with dire consequences. An objective of this study is to determine the prevalence of the D206E within a young normal male population in order to define diagnostic screening criteria.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 MATERIALS

All primers used in this study were purchased from Whitehead Scientific, Cape Town, South Africa, and were synthesized by Integrated DNA Technologies, Coralville, USA. All other PCR reagents, restriction enzymes and chemicals used for electrophoresis were from Promega Corporation and supplied by Whitehead Scientific, Cape Town, South Africa. PCR reactions were carried out using a Thermal Cycling System from Hybaid Limited, Teddington, Middlesex, UK, supplied by the Scientific Group, Cape Town, South Africa. PCR tubes and tips were manufactured in USA by Quality Scientific Plastics and supplied by Sterilab Services, Johannesburg, South Africa. The QIAamp DNA Blood Mini Kit used was manufactured by Qiagen and supplied by Southern Cross Biotechnology, Cape Town, South Africa.

All water used in this study was double distilled, deionized with a Continental Water System and sterilized by filtration through a Millex 0.22 µm filter. All glassware was sterilized at 140°C for 20 min in a Speedy Autoclave HL-341.

2.2 SUBJECTS

Blood samples with EDTA as anti-coagulant were collected from each of the 363 young adult healthy Caucasian male recruited. The blood samples were collected in 5ml tubes and were stored in freezer at –20°C.

2.3 THE ISOLATION OF DNA

2.3.1 Procedure for the Isolation of DNA from Whole Blood Samples

For the isolation of DNA from the frozen blood samples, the tubes were removed from the freezer, placed on the bench top and thawed to room temperature. Genomic DNA was isolated from thawed EDTA blood specimens using QIAamp DNA Blood Mini Kit.

The procedure is as follows: 20µl volume of the QIAGEN Protease solution was pipetted into the bottom of 1.5ml microcentrifuge tube. A 200µl volume of the thawed blood sample was added followed by 200µl Buffer AL. The sample was mixed by pulse-vortexing for 15 seconds. The sample was incubated at 56°C for 10 minutes in a water-bath. Following incubation, the sample was centrifuged at 6 000 x g for 30 seconds in a Hereus Biofuge 15 Centrifuge to remove droplets from the inside of the lid. The tube was opened and 200µl ethanol was added to the sample, the sample was mixed again by pulse-vortexing for 15 seconds and the centrifugation step was repeated.

The lysed blood sample was carefully applied with a pipette to the QIAamp spin column ensuring that the rim remained dry. The cap was closed, and the microfuge tube with the spin column was centrifuge at 6 000 x g for 1 min. The spin column was placed into a clean 2ml collection tube, and the tube containing the filtrate was discarded. The QIAamp spin columns were opened carefully and 500µl of Buffer AW1 were added without wetting the rim. The cap was closed and the sample was

centrifuged at 6 000 x g for 1min. The spin column was removed, placed into a clean 2ml-collection tube, and the collection tube containing the filtrate was discarded. The spin column was carefully opened and 500µl Buffer AW2 was added without wetting the rim. The cap was closed and the sample was centrifuged at 20 000 x g for 3min. The spin column was removed from the collection tube, dried with a paper towel to reduce possible buffer carryover. The collection tube was replaced with a new tube and centrifuged at 8 000 x g for 1 min.

The spin column was placed in a clean 1.5ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The column was carefully opened and 200µl Buffer AE was added. The spin column was incubated at room temperature for 1 min, then centrifuge at 6 000 x g for 1 min to extract the DNA. The tube containing the DNA sample was closed, labeled and stored at 4°C.

2.3.2 Determination of DNA Concentration and Quality

A pooled blood sample was prepared from seven different individuals. The blood was stored at –20°C and DNA was isolated as described in Section 2.3.1.

2.3.2.1 Electrophoretic Analysis of Isolated DNA

The DNA isolated were separated by agarose gel electrophoresis. The 1% agarose gel was prepared in 1x TBE buffer (0.089M Tris, 0.079M Boric acid, 0.002M EDTA at pH 8.3) containing 0.01% ethidium bromide. Electrophoresis was carried out using a Hoefer Submarine Gel Electrophoresis System coupled to a Pharmacia PS 3000 DC

power supply at 140V for 1 hour. The gel was then visualized by ultraviolet radiation and photographed using UVIdoc Gel Documentation System manifactured by UVItec Limited, St John's Innovation Centre Cowley Road, Cambridge, UK and supplied by Whitehead Scientific, Cape Town, South Africa. The DNA samples were diluted 1:1 with loading buffer (60% sucrose in 1x TBE buffer containing 0.001% bromophenol blue). A DNA stock solution of 100 ng/ml Calf Thymus DNA (Boehringer Mannheim supplied by Whitehead Scientific, Cape Town, South Africa) was prepared. A concentration series representing 50-1000ng/μl was loaded onto the agarose gel.

2.3.2.2 Spectrometric Analysis of Isolated DNA

The absorbency at 260 and 280 nm was measured using a UV/VIS Lambda2 Spectrophotometer (supplied by Perkin Elmer, Johannesburg, South Africa). A 20μl aliquot of DNA diluted 1:100 with 1xTBE buffer, was measured against 1xTBE buffer. The concentration of the sample was determined from the 260 nm absorbance reading and the purity from the 260/280 nm ratio.

2.4 SCREENING FOR THE MTHFR C677T MUTATION

2.4.1 Restriction Fragment Length Polymorphism (RFLP) Analysis of MTHFR, C677T mutation

The presence of the C677T polymorphism was detected by PCR amplification of the region containing the mutation. The PCR products were digested with the restriction enzyme, Hinf 1 that identifies the presence of the single nucleotide substitution, C→T

at nucleotide 677. The digested products were separated by polyacrylamide gel electrophoresis and from the different banding patterns three different genotypes could be identified, homozygous normal (HN, -/-), C677T heterozygous (H, +/-) and C677T homozygous mutant (HM, +/+).

2.4.1.1 PCR Amplification of MTHFR 198bp Fragment

PCR amplification was carried out in total volume of 15µl consisting of 2.5µl volume of genomic DNA and 12.5µl PCR reaction mix. The primer stock solution was prepared to a final concentration of 100 ρmol/ml in water. Volumes of 12.5µl were aliquoted and stored at –20 °C.

The primer sequences were obtained from Goyette P *et al.* 1995[58] is as follows:

MTHFR Forward 5'TGA AGG AGA AGG TGT CTG CGG GA-3'

MTHFR Reverse 5'-AGG ACG GTG CGG TGA GAG TG-3'

The dNTP solution was prepared by mixing 10µl of each 10mM dATP, dGTP, dCTP, and dTTP together and preparing a final volume of 100µl and concentration of 1mM by adding 60µl H_2O. Aliquots of 10µl were prepared and stored at –20°C. All other reagents were stored at –20°C and thawed to room temperature.

For a PCR amplification of 10 tubes the following mixture of all PCR reagents was prepared and reagents were added in the following order: 92.6µl water, 15µl of 10 x PCR buffer, 10.5µl of 25 mM $MgCl_2$, 3µl of 40mM dNTP, 1.5µl of the two 100ρmol/ml primers and 0.9µl of 5U/µl Taq DNA polymerase into 1.5 ml

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microcentrifuge tube. A volume of 12.5µl of the PCR mixture was aliquoted into ten 600µl PCR microcentrifuge tubes. A 2.5µl volume of genomic DNA which had been mixed by gentle vortexing was added to the tubes. The caps of the tubes were closed, the contents were mixed by vortex and the samples were centrifuged for 30 seconds at 6000 x g.

The final PCR reaction consisted of 200µM each of dATP, dGTP, dCTP, and dTTP, 10x PCR buffer [10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton X-100], 1.75 mM $MgCl_2$, 0.45U Taq DNA polymerase, and 6 pmol/µl primers.

A PCR tube containing all PCR components except DNA was included as a negative control. Additional two tubes containing positive controls were also included. These contained all PCR components and DNA either from an individual that had previously tested C677T homozygote (positive) and or homozygote normal (negative) for the C677T mutation. These controls were included in each batch of samples that were subjected to PCR amplification and restriction enzyme digestion.

The samples were placed in a Hybaid Touchdown Thermocycler and subjected to the following cycling conditions. One cycle of denaturation (94°C for 10 minutes), 30 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 30 seconds) and extension (72°C for 30 seconds). A final extension step was performed at 72°C for 7 minutes. No oil overlay was used during amplification.

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2.4.1.2 Polyacrylamide Gel Electrophoresis (PAGE) of PCR Amplification Products

The presence of a 198bp PCR product was determined by polyacrylamide gel electrophoresis (PAGE). A 5µl aliquot of loading buffer [60% sucrose in 10x TBE (0.89M Tris, 0.79M Boric Acid, 0.02M EDTA made up to 500ml with ddH_2O) buffer and 0.01% Bromophenol Blue] was mixed together with 5µl of PCR product. The samples were mixed, centrifuged and were loaded onto a polyacrylamide (10% T, 2.9%C) gel and separated at room temperature on a Hoefer Mighty Small Electrophoresis System in 1xTBE buffer at 140V for 45 minutes.

Polyacrylamide gels were prepared by adding 3.3ml of 30% polyacrylamide (0.957g acrylamide and 0.033g bisacrylamide in 3.3ml warm water), 1ml (10 x TBE) to 5.6ml distilled water in a small beaker. A 40µl aliquot of a 20% ammoniumpersulphate solution and 40µl TEMED was added and the solution was mixed well. Gels with well size of 64 mm^3 were prepared using the Hoeffer Mighty Small SE 245 dual gel caster. The gels were allowed to polymerized for 10 minutes at room temperature. The combs were removed the wells rinsed with 1xTBE, removed from the casting apparatus and placed onto the Hoeffer Mighty Small Unit. The samples were loaded and the gels were subjected to an electric field using a Pharmacia Biotech Power Pack set at 140V, 42mA and 6W. The gels were removed from between the glass plates placed in staining solution (0.01% Ethidium Bromide (EtBr) in H_2O) for 5 minutes. The bands were visualized with ultra violet light in a UVIdoc Gel Documentation System and photographed with a Mitsubishi P_{91} video copy processor.

A molecular weight size markers (ΦX174 DNA/Hinf 1) (24-726bp) was included in each electrophoretic run. The molecular markers was prepared by digesting 1μg ΦX174 DNA with 10U Hinf 1 in 10x Buffer B [6mM Tris-HCl, 6mM $MgCl_2$, 50mM NaCl, 1mM dithiothreitol (DTT), (pH 7.5 at 37°C)] and made to a final volume of 100μl with ddH_2O at 37°C overnight.

2.4.1.3 Preparation of Master Mix and Optimization of Storage Parameters

Twelve and half micro-liters, aliquots of the PCR reaction mix were prepared as described in Section 2.4.1.1 and stored at −70°C. Genomic DNA was added, the sample was vortexed and centrifuge at 6000 x g for 30 seconds as described in Section 2.4.1.1. The stability of the PCR reaction mix was evaluated by polyacrylamide gel electrophoresis (Section 2.4.1.2), 1 week, and 1 and 2 months after storage. This was determined by visual evaluation of the band on the gel. Specific attention was given to factors such as band intensity, the presence of smears and primer dimers.

2.4.1.4 Restriction Enzyme Digestion

Fifteen micro-liters volume of the PCR product was digested with 5U Hinfl restriction enzymes in 2μg bovine serum albumin (BSA), 10x Buffer B made up to final volume of 20μl with ddH_2O and incubated at 37°C for 12 hours in a Merck D-64271 Incubator. The digested products and size markers (ΦX174 DNA/Hinf 1) were

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separated by electrophoresis at 140V for 60 minutes on a polyacrylamide (10%T, 2.9%C) gel and visualized as described in Section 2.4.1.2.

2.4.1.5 Identification of the C677T MTHFR Genotypes

Hinf1 digestion recognizes the presence of the C→T substitution. Hinf1 digests alleles containing this mutation into two fragments of 175 and 23 bp (Figure 2). The 23 bp band is eluted from the 10% PAGE gel and therefore Homozygote normal (HN, -/-) subjects were identified by a single band of 198bp, C677T heterozygous (H, +/-) by 198bp and 175bp bands and homozygote mutant (HM, +/+) with a single band of 175bp.

MTHFR-C677T

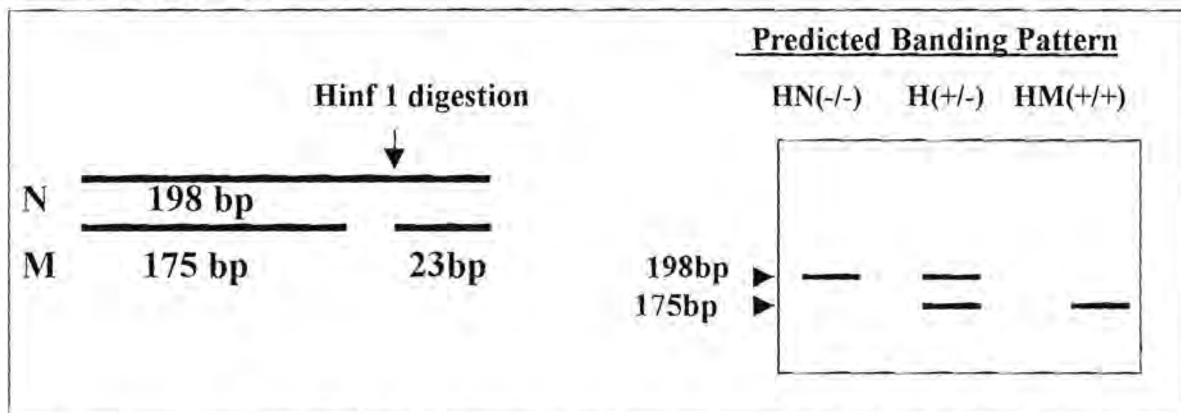

Figure 2.1: Schematic representation of restriction enzyme (Hinf 1) digestion of the normal (N) and mutant (M) MTHFR alleles. The predicted gel electrophoresis banding patterns of the digested MTHFR PCR products: (i) Homozygote Normal (HN, -/-); (ii) Heterozygote (H, +/-); and (iii) Homozygote Mutant (HM, +/+) for the C677T mutation.

2.4.1.6 Population Screening and Statistical Evaluation of the MTHFR C677T genotypes

The number of individuals that were homozygote normal, heterozyogote and homozygote was determined and tabulated. Each individual was screened in duplicate. If for a specific individual, there was a discrepancy in the genotype, the original blood sample was thawed and a new sample of genomic DNA was prepared and the entire procedure was repeated. The carrier frequency for each genotype on the basis of the observed genotype distribution was calculated. The expected occurrence rates of the different genotypes within each group was calculated according to the Hardy-Weinberg principle and compared with the observed occurrence rates by the Chi-square test.

2.5 SCREENING FOR THE HAEMOCHROMATOSIS C282Y AND H63D MUTATIONS

2.5.1 Restriction Fragment Length Polymorphism (RFLP) Analysis of the Heamochromatosis C282Y and the H63D mutations.

The presence of the C282Y and H63D mutations associated with Hereditary Haemochromatosis was determined using separated PCR reactions for each mutation. PCR amplification of the regions containing the mutations resulted in a single product of 343bp and 294 bp for the C282Y and H63D mutations respectively.

The restriction enzyme, Rsa 1 recognizes a restriction site in both the normal and mutant C282Y alleles and with digestion two fragments of 230 and 140 bp form. The

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presence of the C282Y mutation results in the recognition of a second digestion site within the 140-bp fragment, which is digested into a 111 and 27 bp fragment.

In contrast, for the H63D mutation the presence of the mutation results in the loss of a restriction site. The restriction enzyme, Mbo 1 recognizes a digestion site in both the normal and mutant allele. A second restriction site is recognized in the normal allele and is absent in the mutant allele.

2.5.1.1 PCR Amplification of C282Y and H63D Fragment

The PCR reaction for the C282Y and H63D mutation was carried out in separate tubes and only relevant primers were added to the appropriate reaction tubes. The PCR amplification for each mutation was carried out in a total volume of 25µl consisting of 5µl volume of genomic DNA and 20µl PCR reaction mix. The primer stock solution was prepared to a final volume of 100 ρmol/ml in water. Volumes of 20µl were aliquoted and stored at –20 °C. The dNTP solution was prepared as described in Section 2.4.1.1. All other reagents were stored at –20°C and thawed to room temperature.

The primer sequences were from Merryweather-Clarke *et al.* 1997[38] and is as follows for the C282Y mutation

C282Y Forward 5'-CAA GTG CCT CCT TTG GTG AAG GTG ACA CAT-3' and

C282Y Reverse 5'-CTC AGG CAC TCC TCT CAA CC-3'.

The primer sequences for the H63D mutation was

H63D Forward 5'-ACA TGG TTA AGG CCT GTT GC-3' and

H63D Reverse 5'-CTT GCT GTG GTT GTG ATT TTC C-3'.

For a PCR amplification of 10 tubes, the following mixture of all PCR reagents were prepared and reagents were added in the following order: 148μl water, 25μl of 10x PCR buffer, 15μl of 25mM $MgCl_2$, 5μl of 40mM dNTP, 3μl of each 100ρmol/μl primer and 1μl of 5U/μl Taq DNA polymerase. The final PCR reaction mix consists of 200μM of each of dATP, dGTP, dCTP, and dTTP, 10x PCR buffer [10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton X-100], 1.5mM $MgCl_2$, 0.5U Taq DNA polymerase and 12ρmol/μl of each primer.

A PCR tube containing all PCR components except DNA was included as a negative control. Additional two tubes, which include one positive patient control and one negative patient control, were also included. The positive patient control contains all PCR components and DNA either from an individual that had previously tested C282Y or H63D homozygote. The negative patient control was an individual that previously tested negative for C282Y and H63D. These controls were included in each batch of samples that were subjected to PCR amplification, restriction enzyme digestion, gel electrophoresis and genotype determination.

PCR tubes containing primers for the C282Y and the H63D mutation were placed in the same block and were subject to the following PCR cycling conditions. The PCR cycling conditions were one cycle of denaturation (94°C for 5 minutes), 30 cycles of denaturation (94°C for 1 minute), annealing (58°C for 1 minute) and extension (72°C for 1 minute).

2.5.1.2 Polyacrylamide Electrophoresis of PCR Amplification Products

To determine the presence of a PCR product, 5µl of loading buffer was mixed with 5µl PCR product. The samples were mixed, centrifuged and the samples were loaded onto a polyacrylamide (10% T, 2.9%C) gel and separated at room temperature on a Hoefer Mighty Small Electrophoresis System in 1x TBE buffer at 140V for 60 minutes. The gels were removed from between the glass plates, stained with a EtBr solution, visualized with ultra violet light and photographed in a UVIdoc Gel Documentation System. A molecular weight size marker (ΦX174 DNA/Hinf 1) (24-726bp) was included in each electrophoretic run.

2.5.2 Preparation of Master Mix and Optimization of Storage Parameters

Twenty micro-liters, aliquots of the PCR reaction mix were prepared as described in Section 2.5.1.1 and stored at –70°C. The stability of the PCR reaction mix for the amplification of the C282Y and H63D mutations was evaluated by polyacrylamide gel electrophoresis (Section 2.5.1.2), 1 week, and 1 and 2 months after storage. This was determined by visual evaluation of the band quality and position (343 bp for C282Y and 294 bp for H63D). Specific attention was given to factors such as band intensity, and the presence of smears and primer dimers.

2.5.3 Restriction Enzyme Digestion and Genotype Analysis

Twenty-five µl volume of PCR product, was digested with restriction enzyme solution containing 3U Rsa1 for C282Y or Mbo1 for H63D in 10x Buffer C [10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$ and 1mM DTT (pH 7.9 at 37°C)] made up to final volume of 30µl with ddH_2O and incubated in Merck D-64271 Incubator for 16 hours at 37°C. The digested products were separated and visualized as described in Section 2.4.1.2

The smaller band of 29 bp eluted from the gel. Individuals that were homozygote normal (HN, -/-) were identified by the presence of two bands of 203 and 140bp. Heteozygotes (H, +/-) presented with three bands on gel electrophoresis, 203, 140 and 111bp while individuals C282Y homozygote mutant (HM, +/+) presented with two bands of 203 and 111bp.

HH-C282Y

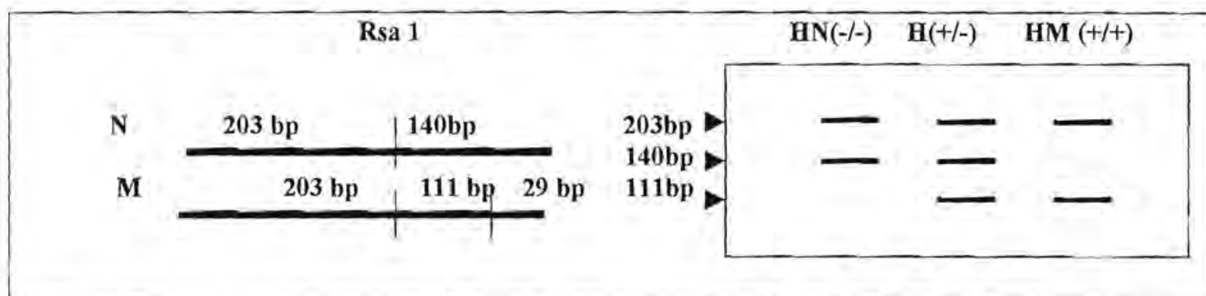

Figure 2.2: Schematic representation of restriction enzyme (Rsa 1) digestion of the normal (N) and mutant (M) C282Y alleles. The predicted gel electrophoresis banding patterns of digested C282Y PCR products: (i) Homozygote normal (HN, -/-); (ii) Heterozygote (H, +/-); and (iii) homozygote mutant (HM, +/+) for the Hereditary Haemochromatosis C282Y mutation.

For the H63D mutation the smaller band of 57bp is eluted from the gel. The H36D, homozygote normal (HN, -/-) individuals were identified by two bands of 138 and 99bp. Heterozygotes (H, +/-) presented with three bands of 237, 138 and 99bp and homozygote mutants (HM, +/+) with one band of 237bp

HH-H63D

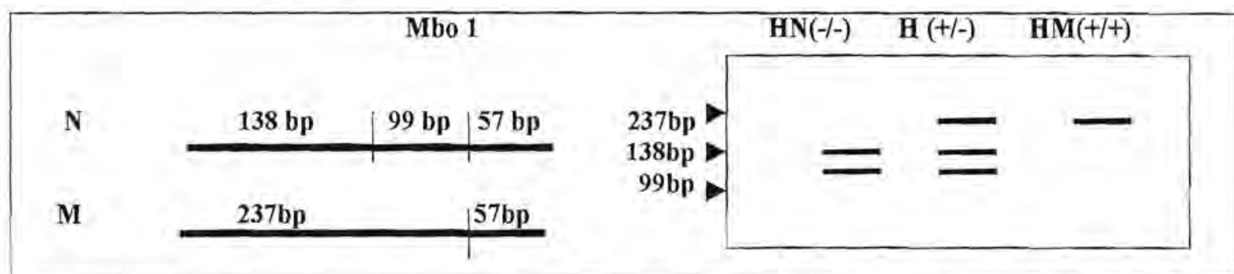

Figure 2.3: Schematic representation of restriction enzyme (Mbo 1) digestion of the normal (N) and mutant (M) H63D alleles. The predicted gel electrophoresis banding patterns of digested PCR products: (i) Homozygote Normal (HN, -/-); (ii) Heterozygote (H, +/-); and (iii) homzygote mutant (HM, +/+) for the hereditary haemochromatosis H63D allele.

2.5.4 Population Screening and Statistical Evaluation

The number of individuals that were homozygote normal, heterozyogote and homozygote (positive) was determined for both the C282Y and H63D mutations and tabulated. Each individual was screened in duplicate. If there was a discrepancy in the genotype, the original blood sample was thawed and a new sample of genomic DNA was prepared and the entire procedure was repeated. The carrier frequency was for each genotype on the basis of the observed genotype distribution was calculated. The expected occurrence rates of the different genotypes within each group was calculated

according to the Hardy-Weinberg principle and compared with the observed occurrence rates by the Chi-square test.

As compound heterozygosity is also associated with disease the frequency of compound heterozygotes C282Y/H63D was determined, tabulated and of the observed genotype distribution was calculated and compared to the observed frequency by the Chi-square test.

2.6 FAMILIAL HYPERCHOLESTEROLAEMIA

2.6.1 Methodologies for the identification of the Familial Hypercholestrolaemia Mutations

The presence of the D206E mutation was initially detected by a multiplex allele specific PCR methodology, which detects the presence of FH_1, D206E and FH_2, V408M mutations. This method could not distinguish between individuals that are heterozygote and homozyote for either the D206E or the V408M mutations. For the purpose of this study where only frequency of the D206E mutation needed to be determined an allele specific methodology was developed that could identify the normal and mutant alleles and it was now possible to distinguish between individuals that are normal, heterozygote or homozygote for the D206E mutation.

2.6.1.1 Multiplex Allele Specific PCR Detection of the D206E and V408E Familial Hypercholesterolaemia Alleles.

The final PCR amplification volume was 50μl consisting of 15μl volume genomic DNA and 35μl PCR reaction mix. The PCR reaction mix consist of 200μM of each of dATP, dGTP, dCTP, and dTTP, 10x PCR buffer [10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton X-100], 1.5mM $MgCl_2$, 3.2mM Me_4NCl, 15% Glycerol 2U Taq DNA polymerase, 0.5ρmol/ml COMM 1, 0.8ρmol/ml COMM 2, 1ρmol/ml ARMS1, 0.5ρmol/ml ARM2, and 2ρmol/ml COMM3 primers. The primer sequences were from Kotze *et al.* 1995[55] as follows:

COMM 1: 5'-CGA GGC CTC CTG CCC GGT GCT CAC C-3'

COMM 2: 5'-GCT CAC CTG CAG ATC ATT CTC TGG G-3'

COMM 3: 5'-GGG ACC CAG GG ACAG GTG ATA GGA C-3'

ARMS 1: 5'-CCC GCC CAT ACC GCA GTT TTC CTC C-3'

ARMS 2: 5'-AGC CTC ATC CCCAAC CTG AGG ACC A-3'

For PCR amplification of 10 tubes the following mixture of all PCR reagent was prepared and reagents were added in the following order: 46μl double distilled water, 150μl of 50% glycerol, 20μl of 80mM Me_4NCl, 50μl of 10x buffer, 30μl of 25mM $MgCl_2$, 10μl of 40mM dNTP, 10μl of 25ρmol COMM1, 10μl of 40ρmol COMM2, 10μl of 50ρmol ARMS1 and 10μl of 100ρmol COMM3 and 4μl of 5U/μl Taq DNA polymerase into 1,5 ml sterile centrifuge tube. A 35μl aliquot of the PCR mixture was pipetted into ten 600μl PCR microcentrifuge tubes. A 15μl volume of the genomic DNA which had been mixed by gentle vortexing was added to the tubes. The caps of

the tubes were closed, the contents were mixed with vortex, centrifuged for 1 minute at 6000 x g and were then placed in a Hybaid Touchdown Thermocycler.

A PCR tube containing all PCR components except DNA was included as a negative control. Additional two tubes, which include one positive patient control and one negative patient control, were also included. The positive patient control contains all PCR components and DNA either from an individual that had previously tested heterozygote for the D206E or the V408M mutations respectively. The negative patient control was an individual that previously tested negative for both mutations. These controls were included in each batch of samples that were subjected to PCR amplification, restriction enzyme digestion, and gel electrophoresis in genotype determination.

2.6.1.2 Allele Specific Amplification of D206E Fragment

The final PCR amplification volume was 25μl consisting of 2μl genomic DNA and 23μl PCR reaction mix. Two different PCR reaction mixes were used, both containing a common primer COMM1 and either a primer for the normal (FH1-N) or the mutant (FH1-M) allele.

For the amplification of 10 tubes the following mixture of all PCR reagents was prepared and reagents were added in the following order for both tubes labeled M and N: 179μl double distilled water, 25μl of 10x PCR buffer, 15μl of 25mM $MgCl_2$, 5μl of 40mM dNTP, 2.5μl of 20pmol/μl FH_1-C, 1μl of 5U/μl of Taq DNA polymerse, 2.5μl of 20pmol/μl of primer FH_1-M into tube M and 2.5μl of 20pmol/μl FH_1-N into

tube labeled N. A volume of 23µl of the PCR mixtures were aliquoted into ten 600µl PCR microcentrifuge tubes. A N and M series of tubes containing 23 µl PCR mix was prepared and 2µl volume genomic DNA were added to both a N and M tube.

The final PCR reaction consist of 200µM of each of dATP, dGTP, dCTP, and dTTP, 10x PCR buffer [10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton X-100], 1.5mM $MgCl_2$, 0.5U Taq DNA polymerase and 2ρmole of each primer.

The primer sequences were from Kotze e*t al.* 1995 [55] as follows:

FH_1-C:	5'-CGA GGC CTC CTG CCC GGT GCT CAC C-3',
FH1-M:	5'-CCC GCC CAT ACC GCA GTT TTC CTC C-3' and
FH1-N:	5'-CCC GCC CAT ACC GCA GTT TTC CTC-T3'.

A negative control (all reagents except DNA), and patient negative controls (Homozygote, Normal (HN, -/-)) and a positive controls (Heterozygote, (H, +/-)) were included in each PCR amplification batch.

For both the Multiplex Allele Specific PCR and the Allele Specific PCR the PCR cycling conditions were one cycle of denaturation (94°C for 5 minutes), 15 cycles of denaturation (94°C for 1 minute), annealing (67°C for 1 minute) and extension (72°C for 2 minute), and 20 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute) and extension (72°C for 2 minute).

2.6.2 Preparation of Master Mix and Optimization of Storage Parameters for the (a) Multiplex Allele Specific PCR of D206E and V408M and (b) Allele Specific PCR for the D206E FH Mutations.

Thirty-five micro-liters (Section 2.6.1.1) and 23μl (Section 2.6.1.2), aliquots of the PCR reaction mixes were stored at –70°C. The stability of the PCR reaction mix for both methods, Section 2.6.1.1.and 2.6.1.2 was evaluated by polyacrylamide gel electrophoresis, 1 week, and 1 and 2 months after storage. This was determined by visual evaluation of the band quality and position. Specific attention was given to factors such as band intensity, and the presence of smears and primer dimers.

2.6.3. Polyacrylamide Electrophoresis and Genotype Analysis for the FH Mutations

Equal volumes of PCR product and loading buffer were mixed together, centrifuged and loaded onto a polyacrylamide (10% T, 2.9%C) gel and separated at room temperature on a Hoefer Mighty Small Electrophoresis System in 1x TBE buffer at 140V for 45 minutes. For the detection of D206E mutation (Section 2.6.1.2) samples were loaded in the order of M and N for each sample of genomic DNA that was amplified. The gels were removed from between the glass plates and stained in EtBr solution for 5 minutes. The band/s were visualized with ultra violet light and photographed as described in Section 2.4.1.2.

2.6.3.1 Genotype Analysis of FH Alleles (Multiplex Allele Specific PCR of D206E and V408M FH Mutations).

The presence of a 330 bp indicated successful amplification of all sample. Individuals that are either heterozygote, or homozygote for the D206E mutation are identified by the presence of a band of 285bp. Individuals either heterozygote or homozygote for the V408M mutation are identified by the presence of a 100bp band.

FH-D206E and V408M

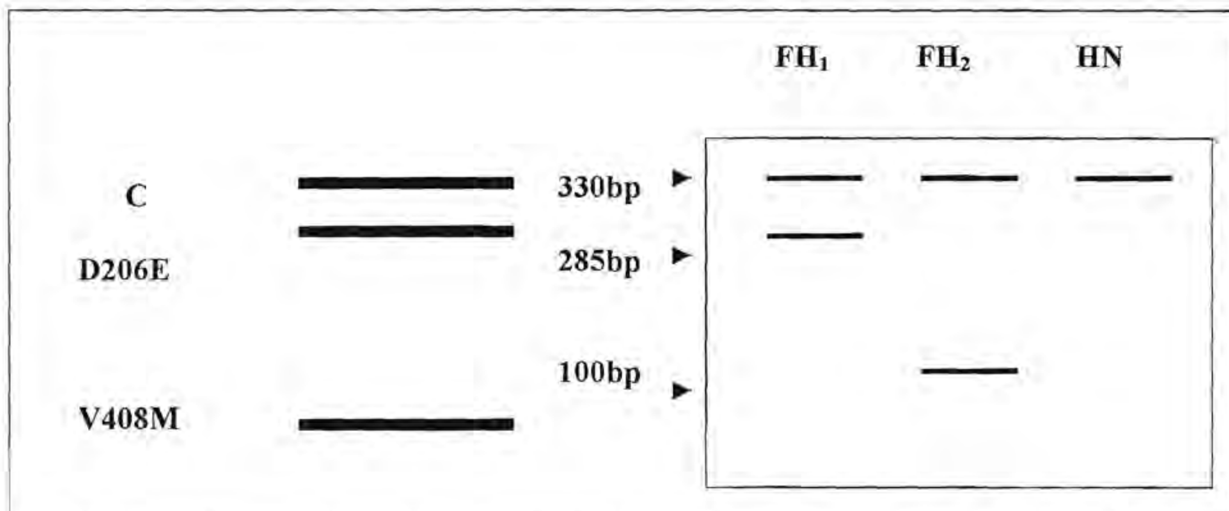

Figure 2.4: Schematic representation of FH alleles in multiplex allele specific PCR of FH_1 (D206E) and FH_2 (V408M) mutations. C = control band, D206E, FH_1, either homozygote or heterozygote and V408M, FH_2 either heterozygote or homozygote. Predicted gel electrophoresis banding patterns for FH_1, either heterozygote or homozygote, FH_2 either heterozygote or homozygote. HN homozygote normal for both mutations.

2.6.3.2 Genotype Analysis of FH alleles (Allele Specific PCR for the D206E FH Mutation).

For each individual analysis of the N and M lanes are used to identify the genotype of an individual. Individuals that are homozygote normal for the D206E mutation present with a single band in N and no band in M, heterozygote for D206E presents with a band in each lane and homozygote, a single band in M lane only

FH-D206

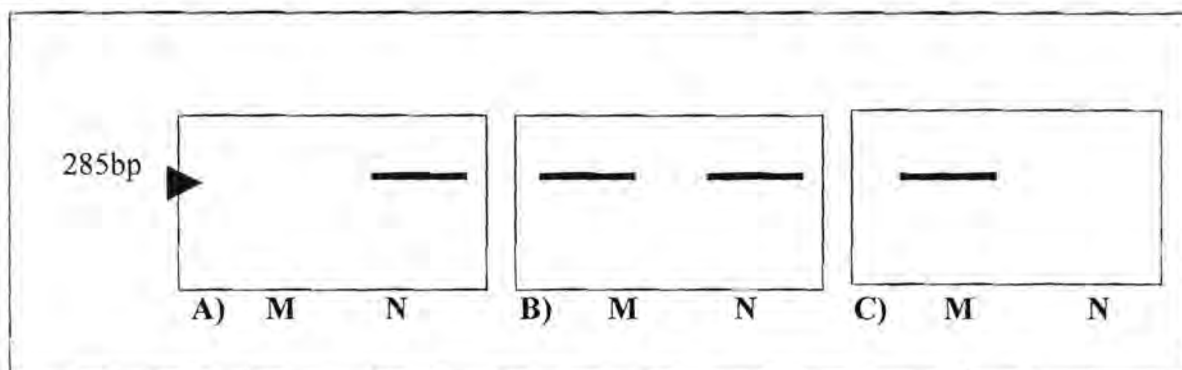

Figure 2.5: Schematic representation of the predicated banding patterns for the allele specific PCR for the detection of the D206E mutation. A) Homozygote Normal (HN, -/-), B) Heterozygote (H, +/-) and C) Homozygote Mutant (HM, +/+)

2.6.4 Population Screening and Statistical Evaluation of the FH D206E Genotypes

The number of individuals that were homozygote normal, heterozyogote and homozygote (positive) was determined and tabulated. Each individual was screened in duplicate. If for a specific individual there was a discrepancy in the genotype, the original blood sample was thawed and a new sample of genomic DNA was prepared and the entire procedure was repeated. The carrier frequency was for each genotype

on the basis of the observed genotype distribution was calculated. The expected occurrence rates of the different genotypes within each group was calculated according to the Hardy-Weinberg principle and compared with the observed occurrence rates by the Chi-square test.

The carrier and gene frequency for each genotype was determined on the basis of the observed genotype distribution. The expected occurrence rate of the different D206E genotype in each group was calculated according to the Hardy-Weinberg P_1 principle and compared with the observed occurrence rates by the Chi-square test.

CHAPTER THREE

3. RESULTS

3.1 ISOLATION OF GENOMIC DNA

A single sample of whole blood with anticoagulant was collected from a group of 359 young white male adults. The tubes of blood were stored at -20°C. Prior to DNA isolation the blood samples were thawed, mixed well and an aliquot was used for DNA isolation. The blood samples were then placed back into the freezer and stored again at -20°C for further DNA isolation when required.

Blood samples from 7 individuals were pooled to determine the average concentration of the DNA and the quality of the isolated DNA. A sample of pooled blood was prepared and the DNA was isolated using the QIAamp DNA Mini Kit. The isolated DNA was loaded onto a 1% agarose gel together with a DNA concentration series of 200-1000 ng DNA (Figure 3.1, Lane 1-5). Following electrophoresis and EtBr staining the isolated DNA had migrated to the same position as the DNA of the standard series. It was a single well defined band without streaking, indicating the absence of DNA fragmentation (Figure 3.1, Lane 6-10). From the concentration series the DNA concentration was determined as 200 ng with a final concentration of 40 ng/µl.

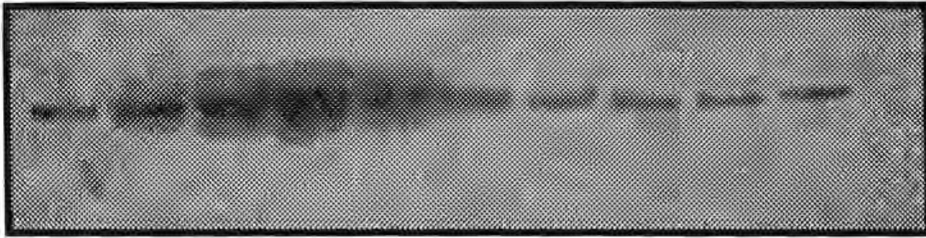

Figure 3.1: Agarose gel electrophoresic analysis of genomic DNA isolated from whole blood. Lanes 1-5 DNA standard concentration series of 200, 400, 600, 800 and 1000 ng. Lanes 6-10, genomic DNA of 5μl (40ng/μl)

The purity of the DNA was determined by measuring the absorbance at 260 and 280 nm and calculating the ratio of 260 /280 nm. This ratio was 1.85, which is within the range 1.8 –2.0 required for DNA that is used for molecular diagnostics[8]. The DNA concentration of each isolated DNA sample was not determined. The assumption was made that the DNA quality and concentration is comparable to that found for the pooled DNA sample.

3.2. SCREENING FOR THE MTHFR C677T MUTATION

3.2.1.Restriction Fragment Length Polymorphism (RFLP) Analysis of C677T mutation

PCR amplification the region containing the C677T mutation resulted in a single specific product of 198 bp without primer dimers or any non-specific product (Figure 3.2). The inclusion of a negative control that contains all components of the PCR except genomic DNA was used to exclude the presence of contamination by endogenous DNA (Figure 3.2, Lane 6). The final PCR reaction volume was 15μl and successful amplification was achieved with 100ng/ml DNA. This is sufficient for successful restriction enzyme digestion and genotype analysis (Figure 3.3).

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A ΦX174 DNA/Hinf1 molecular weight size markers was prepared by digesting the ΦX174 DNA plasmid overnight with Hinf1. This produced a ΦX174 DNA/Hinf1 molecular weight size marker series (24 to 726bp) that was comparable with commercially available products such as (ΦX174 DNA/Hinf1 Dephosphorylated markers, produced by Promega and supplied by Whitehead Scientific) with a significant savings in cost.

Figure 3.2: MTHFR C677T, PCR product was separated by 10% PAGE, stained with EtBr and visualised with UV. Lane 1-5, the 198-bp PCR product, Lane 6 Negative control (no genomic DNA) and Lane MW, ΦX174 DNA/Hinf1 molecular weights size markers.

The 198bp PCR product was digested overnight with Hinf1 and the digestion products were separated by polyacrylamide gel electrophoresis. Staining with EtBr and UV visualisation resulted in the identification of the different genotypes. The absence of the C677T mutation resulted in a PCR product without a digestion site for Hinf1 and these individuals were identified as homozygote normal (Figure 3.3, Lane 5). The presence of one allele with the C677T mutation and one normal allele in an individual that is heterozygote is observed following electrophoresis as two bands of 198 and 175bp (Figure 3.3, Lane 3 and 4). In an individual that is homozygote, both alleles are affected and a single band of 175 bp is observed (Figure 3.3, Lane 2). The small fragment of 23 bp that formed as a result of digestion of the 198 bp fragment is eluted together with the primers from the gel.

A positive patient control, an individual that was homozygote for C677T was not only used to identify this genotype but was also used to indicate successful digestion. Digestion was complete if only a band of 175 bp was observed with total absence of a band at 198 bp as seen, when Figure 3.3, Lane 2 is compared with Figure 3.2, Lane 5.

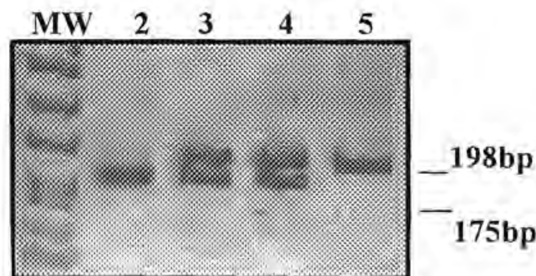

Figure 3.3: MTHFR genotype analysis. Lane MW: ΦX174 DNA/Hinf1 molecular weight size markers; Lane 2: Homozygote Mutant (HM, +/+); Lane 3 and 4: Heterozygote (H, +/-) and Lane 5: Homozygote Normal (HN) for the C677T mutation.

To enable the rapid and cost effective screening of a large population the PCR reaction volume was decreased. Furthermore to make this screening strategy less time consuming it was necessary to determine whether 12.5µl aliquots of the PCR reaction mixture could be stored at -70°C for an extended time interval. This would mean that the required number of tubes could be removed from the freezer, thawed, the genomic DNA added, samples mixed and amplified. Aliquots of the PCR mixture was prepared, stored at -70°C, after 1 week, and after 1 and 2 months the tubes of PCR mix were thawed and DNA was added. The samples were mixed well and loaded into the PCR apparatus and amplified. The samples were then digested with Hinf 1, separated by gel electrophoresis, stained and the banding patterns were evaluated. The presence of primer dimers and the formation of non-specific product were evaluated. No primer dimers or non-specific product was observed. Only the results are shown

for samples evaluated using freshly prepared PCR mix (Figure 3.3) and that which had been stored for 1 week and 2 months (Figure 3.4A and B). No differences were observed in the results obtained when using freshly prepared PCR mix or PCR reaction mix stored for 2 months. Frozen PCR reaction mixes were used to screen a male Caucasian population in duplicate for the C677T mutation.

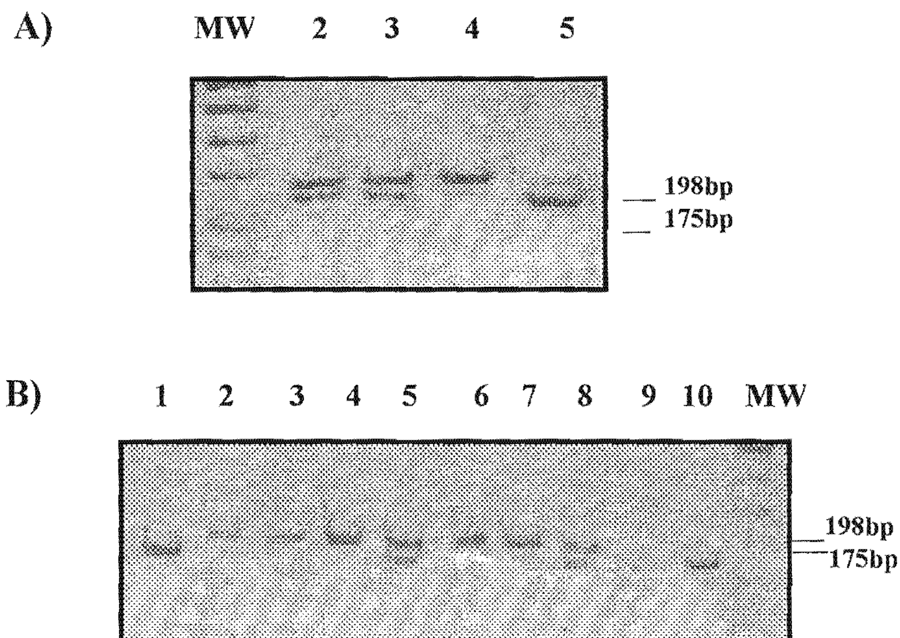

Figure 3.4: MTHFR genotype analysis after storage of the PCR mix at -70°C for 1 week (A) and 2 months (B). Lane MW, ΦX174 DNA/Hinf1 molecular weight sizes markers. For (A) Lane 2, 3 and (B) Lane 5 and 8: Heterozygote (HN, +/-); (A) Lane 4 and (B) Lane 2, 3, 4, 6 and 7: Homozygote Normal (HN, -/-) and (A) Lane 5 and (B) Lane 1 and 10: Homozygote Mutant (HM, +/+) for the C677T mutation.

3.2.2 Population Screening of a Caucasian Male Population for the C677T, MTHFR Mutation

3.2.2.1 Genotype and Allele Frequency Analysis

A population of 359 Caucasian males were screened for C677T mutation. All screening was in duplicate and results were tabulated and are presented in Table 1.1. In this population group, 186 were normal homozygotes, 134 were heterozygotes and 39 homozygotes for the C677T mutation. A carrier frequency was found to be 0.416 with a gene frequency of 0.295. The population was in Hardy Weinberg equilibrium. Data was evaluated using the Chi-square goodness of fit and a X^2 of 3.98 was obtained.

Table 1.1: C677T Distribution, Gene and Allele Frequencies in a Male Caucasian Population (n=359)

C677T			
Genotype	-/-	+/-	+/+
Observed	**186**	**134**	**39**
Expected	**178.4**	**149.3**	**31.2**

Carrier frequency = 0.416
Gene frequency = 0.295

Table 1.2 Chi-square for Goodness of Fit for the C677T Mutation

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2/E$
-/-	186	178.4	7.6	57.76	0.32
+/-	134	149.0	-15	255	1.71
+/+	39	31.2	7.8	60.8	1.95
Total	359	358.6			X^2=3.98

$X^2_{2df} = 3.98$
P ($X^2_{0.99}$) = 10.6, difference is not significant

3. 3 SCREENING FOR THE HAEMOCHROMATOSIS C282Y AND THE H63D MUTATIONS

3.3.1 RFLP Analysis of the Haemochromatosis C282Y and H63D Mutations

3.3.1.1 PCR Amplification of Regions Containing the C282Y and the H63D Mutations

The region of the DNA containing H63D and the C282Y mutations was amplified as described in Section 2.5.1.1. The amplification product was a single band of 294 bp for the H63D (Figure 3.5a) and 343 bp for the C282Y (Figure 3.5b) regions.

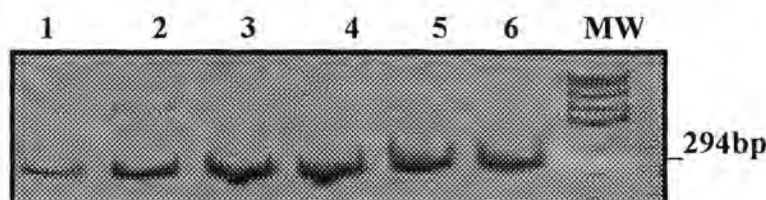

Figure 3.5a: HH, H63D PCR product was separated by 10% PAGE, stained with EtBr and visualised with UV. Lane 1-6 the 294 bp PCR product and Lane MW, ΦX174 DNA/Hinf1 molecular weight size markers.

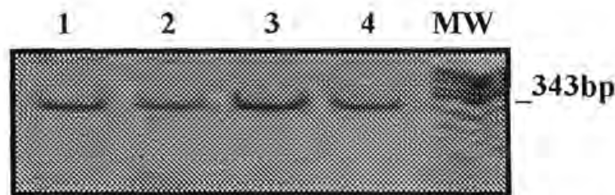

Figure 3.5b: HH, C282Y PCR product was separated by 10% PAGE, stained with EtBr and visualised with UV. Lane 1-4 the 343 bp PCR product and Lane MW, ΦX174 DNA/Hinf1 molecular weight size markers.

3.3.1.2 Restriction Enzyme Digestion, Polyacrylamide Gel Electrophoresis and Genotype Analysis

Rsa 1 and Mbo 1 restriction enzyme digestion was used to identify the presence of the C282Y (Figure 3.6a) and H63D (Figure 3.6b) mutations respectively. Digestion of the C282Y, 343 bp product resulted in the different banding pattern for each genotype. For the normal allele a single restriction site is identified and this results in two products of 203 and 140 bp (Figure 3.6a, Lane 1, 2, 4 and 5). If the C282Y mutation is present the smaller fragment of 140 bp is further digested into a 111 and 29 bp fragment. Only the 111 bp fragment is seen following gel electrophoresis (Figure 3.6a, Lane 3 and 6). Individuals that are C282Y homozygote are identified by a 203 and 111 bp bands (Figure 3.6a, Lane 6). Heterozygosity for this mutation results in a banding pattern that is a combination of homzygote normal and mutant (Figure 3.6a, Lane 3). A negative control consisting of PCR reaction mix without genomic DNA was included to exclude presence of contamination and ΦX174 DNA/Hinf1 molecular weight size markers was used to determine the size of product formed in base pairs.

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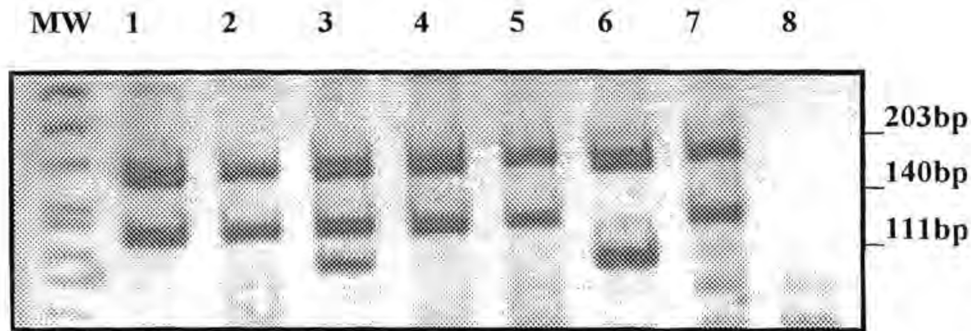

Figure 3.6a: HH, C282Y genotype analysis. MW, molecular mass standards; ΦX174 DNA/Hinf1 molecular weight size markers; Lane 1, 2, 4, 5, and 7:Homozygote Normal (HN, -/-); Lane 3: Heterozygote (H, +/-); Lane 6: Homozygote Mutant (+/+); Lane 8: Negative control (no genomic DNA).

For the normal H63D allele the restriction enzyme Mbo1 recognises two digestion sites and the 294 bp product is digested into a 237 and 57 bp fragment. The smaller fragment is eluted from the gel. The 237 bp fragment is further digested into a 138 and a 99 bp fragment (Figure 3.6b, Lane 3, 4 and 6). If the H63D mutation is present there is loss of this restriction site. If the H63D mutation is present the 237 bp fragment remains undigested and an individual that is homozygote for the H63D mutation has a single band following electrophoresis of 237 bp (Figure 3.6b, Lane 2). Heterozygosity is a combination of these two patterns (Figure 3.6b, Lane 5). Compound heterozygosity is diagnosed if an individual has a heterozygote banding pattern for the C282Y (Figure 3.6a, Lane 3) and the H63D (Figure 3.6b, Lane 5) mutation.

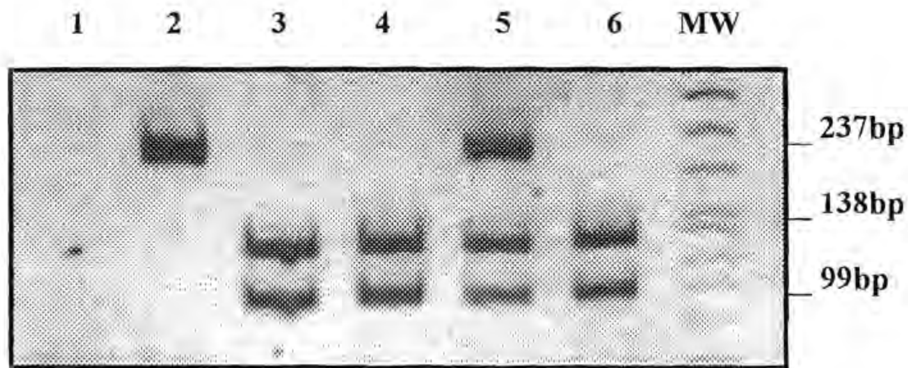

Figure 3.6b: HH, H63D genotype analysis. Lane 1: Negative control (no genomic DNA); Lane 2: Homozygote Mutant (HM, +/+); Lane 3, 4, and 6, Homozygote Normal (HN, -/-); Lane 5: Heterozygote (HN, +/-) for the H63D mutation. Lane MW: ΦX174 DNA/Hinf1 molecular weight size markers.

3.3.1.3 Stability of PCR Reaction Mix

As with the MTHFR C677T mutation the PCR reaction mix for the C282Y and the H63D mutations were stored at -70°C. Aliquots were evaluated after 1 week, and after 1 and 2 months of storage. For both the C282Y and H63D the stored aliquots of reaction mix could be used for the successful amplification and restriction enzyme digestion of the C282Y (Figure 3.7A and B) and the H63D (Figure 3.8A and B) alleles. These stored PCR reaction mixes were used for population screening.

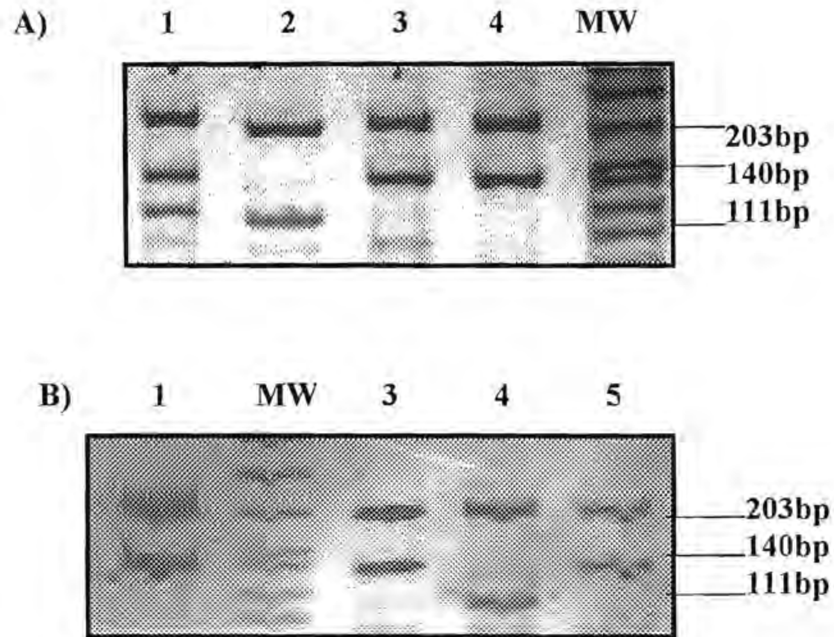

Figure 3.7: HH, C282Y genotype analysis after storage of the PCR mix at -70°C for 1 week (A) and 2 months (B). For (A) Lane 3, 4 and (B) Lane 1, 3: Homozygote normal (-/-); (A) Lane 1: Heterozygote (-/+) and (A) Lane 2 and (B) Lane 4: Homozygote (+/+); Lane MW, ΦX174 DNA/Hinf1 molecular weight size markers.

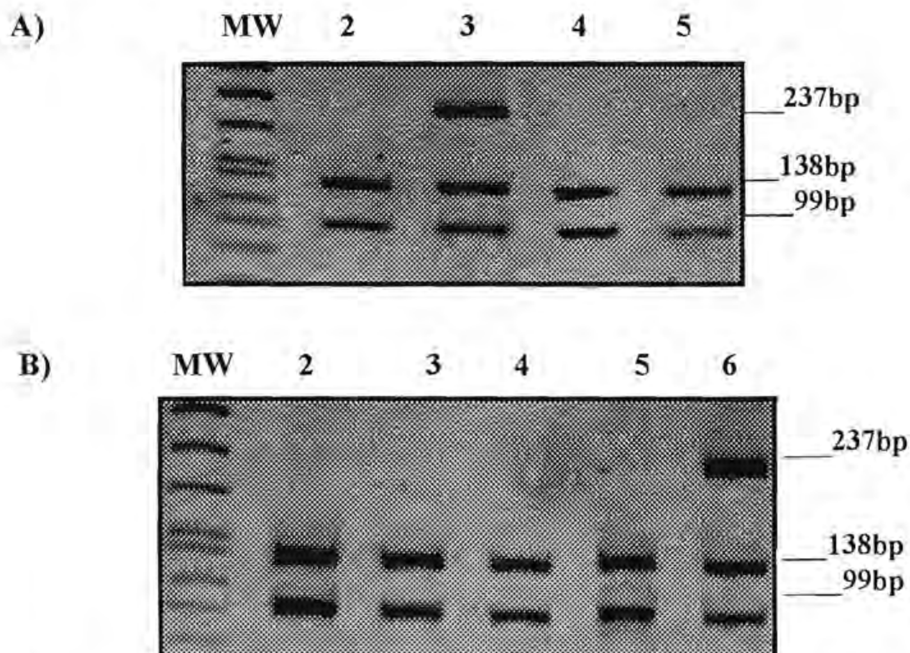

Figure 3.8: HH, H63D genotype analysis after storage of PCR mix at -70°C for 1 week and 2 months. For (A) Lane 2, 4 and 5 and (B) Lane 2-5: Homozygote normal (-/-); and (A) Lane 3 and (B) Lane 6: Heterozygote (-/+). Lane MW: ΦX174 DNA/Hinf1 molecular weight size markers.

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3.3.2.1 Genotype and Allele Frequency Analysis for the C282Y and H63D Haemochromatosis Mutations

A population of 349 male Caucasians was screened in duplicate for the C282Y and H63D mutations (Table 2.1). In the population, 293 were found to be homozygote normal, 53 heterozygote and 3 homozygote mutant for the C282Y mutation. In the same population 269 were homozygote normal, 72 heterozygote and 8 homozygote mutant for H63D mutation. The carrier frequency was 0.153 and 0.220 for the C282Y and H63D mutations respectively. The gene frequency for the C282Y and the H63D mutation was 0.084 and 0.126 respectively. The population group was in Hardy Weinberg equilibrium with a X^2 of 0.128 and 1.41 for the C282Y and the H63D mutations respectively.

Table 2.1: Distribution of the C282Y and H63D Mutations in a Male Caucasians Population

	C282Y			H63D		
Genotype	-/-	+/-	+/+	-/-	+/-	+/+
Observed	**293**	**53**	**3**	**269**	**72**	**8**
Expected	**292.1**	**53.6**	**2.46**	**266.6**	**76.78**	**5.54**
Gene frequency	**0.084**			**0.126**		
Carrier frequency	**0.153**			**0.220**		
Homozygosity	**1 in 142**			**1 in 63**		

Table 2.2 Chi-square for Goodness of Fit for the C282Y Mutation

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2/E$
-/-	293	292.1	0.9	0.81	0.002
+/-	53	53.6	-0.6	0.36	0.007
+/+	3	2.46	0.54	0.29	0.119
Total	349	348.16			X^2=0.128

$X^2_{2df} = 0.128$
P ($X^2_{0.99}$) = 10.6, difference is not significant

Table 2.3 Chi-square for Goodness of Fit for the H63D Mutation

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2/E$
-/-	269	266.6	2.4	5.76	0.02
+/-	72	76.78	-4.78	22.84	0.30
+/+	8	5.54	2.46	6.05	1.09
Total	349	348.92			X^2=1.41

$X^2_{2df} = 1.41$
P ($X^2_{0.99}$) = 10.6, difference is not significant

3.3.2.2 Genotype and Frequency for C282Y and H63D Compound Heterozygosity.

Compound heterozygosity for C282Y and H63D is also associated with disease and the frequency of both alleles in the individual was determined. In the population screened, 12 subjects were compound heterozygote and two subjects were H63D homozygote and C282Y heterozygote. A carrier frequency of 0.153 and 0.220 was found for the C282Y and the H63D mutations respectively. For both mutations together the population was in Hardy Weinberg Equilibrium (Table 3.1) and the frequency for compound heterozygosity is 1 in 29.

Table 3.1: Combined Frequencies of the C282Y and the H63D Mutations in a Male Caucasian Population

Genotype C282Y/H63D	Observed (O)	Expected (E)
-/-; -/-	227	223.2
-/-; +/-	60	64.3
-/-; +/+	6	4.6
+/-; -/-	39	41.1
+/-; +/-	12	11.8
+/-/+/+	2	0.9
+/+; -/-	3	2
+/+; +/-	0	0.6
+/+; +/+	0	0.04

Table 3.2.Chi -square for Goodness of Fit for the C282Y and H63D Mutations

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2/E$
-/-; -/-	227	223.2	3.8	14.44	0.065
-/-; +/-	60	64.3	-4.3	18.5	0.29
-/-; +/+	6	4.6	1.4	1.96	0.426
+/-; -/-	39	41.1	-2.1	4.41	0.107
+/-; +/-	12	11.8	0.2	0.04	0.003
+/-/+/+	2	0.9	1.1	1.21	1.344
+/+; -/-	3	2	1	1	0.5
+/+; +/-	0	0.6	-0.6	0.36	0.6
+/+; +/+	0	0.04	-0.04	0.0016	0.04
					X^2= 3.375

Df =(r-1) x (c-1) = 8 x 1 =8, X^2= 3.38

3.4 SCREENING FOR THE D206E AND/OR THE V408M MUTATIONS ASSSOCIATED WITH FAMILIAL HYPERCHOLESTEROLAEMIA

3.4.1. PCR Amplification of D206E and the V408M FH Mutations with Multiplex Allele Specific PCR

The Multiplex Allele specific PCR method of Kotze *et al.* was is used to detect the mutant alleles V408M and D206E associated with FH_2 and FH_1 respectively[49, 55]. The presence of the 330 bp product indicates that amplification was successful (Figure 3.9). The absence of any other band means that the individual is homozygote normal for both the D206E and the V408E mutations. A band of 285 bp means that this individual has at least one copy of the D206E allele. However with this method it is not possible to determine whether the individual is heterozygote or homozygote for the E206E mutation. Likewise a band of 100 bp indicates than an individual is either homozygote or heterozygote for the V408M mutation and further analysis is necessary to determine the specific genotype of the patient.

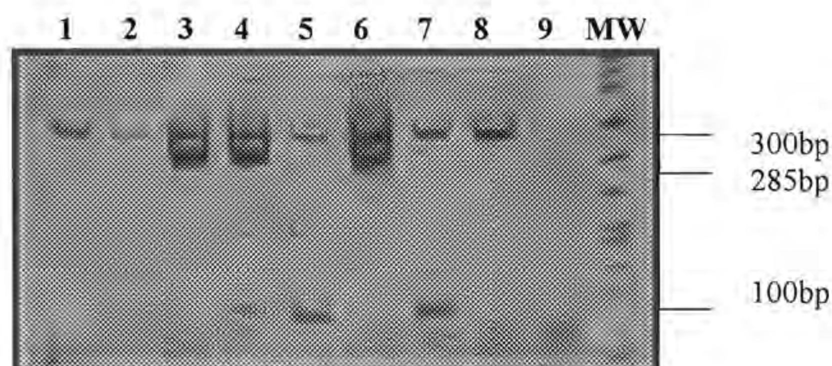

Figure 3.9: Multiplex allele specific PCR for the D206E, FH_1, and the V408M, FH_2 mutations. The amplified PCR products were separated by 10% PAGE. Lane 1, 2 and 8: Homozygote normal; Lane 3, and 6: FH_1 either heterozygote or homozygote for D206E, Lane 4: FH_1 and FH_2 either heterozygote or homozygote, Lane 5 and 7: FH_2: either heterozygoye or homozygote V408M, Lane 9: Negative Control (no genomic DNA) and Lane MW: ΦX174 DNA/Hinf1 molecular weight size markers.

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3.4.1.1 Allele Specific Amplification of the D206E Allele

The region of the LDL-R gene containing the D206E mutation was amplified as described in Section 2.6.2. The primers for this allele specific method were the same used in the multiplex allele specific PCR described in Section 3.3.1 except that a second primer that recognised the normal allele was used. Using a primer set that could distinguish between the normal and the mutant allele, it was possible to identify an individual as either heterozygote or homozygote normal for the mutation (Figure 3.10, Lanes 1(M and N) and 2 (M and N)). A single band of 285bp was produced and no non-specific product was present (Figure 3.9). Although two PCR reactions are required for each patient the reaction volume was reduced from 50 µl to 20 µl. The amount of DNA required was reduced from 15 µl (600 ng) to 2 µl (80 ng) for each PCR reaction. Inclusion of negative control (no genomic DNA) excluded presence of contamination and ΦX174 DNA/Hinfl molecular weight size markers was included to determine the size of the amplified products in bp.

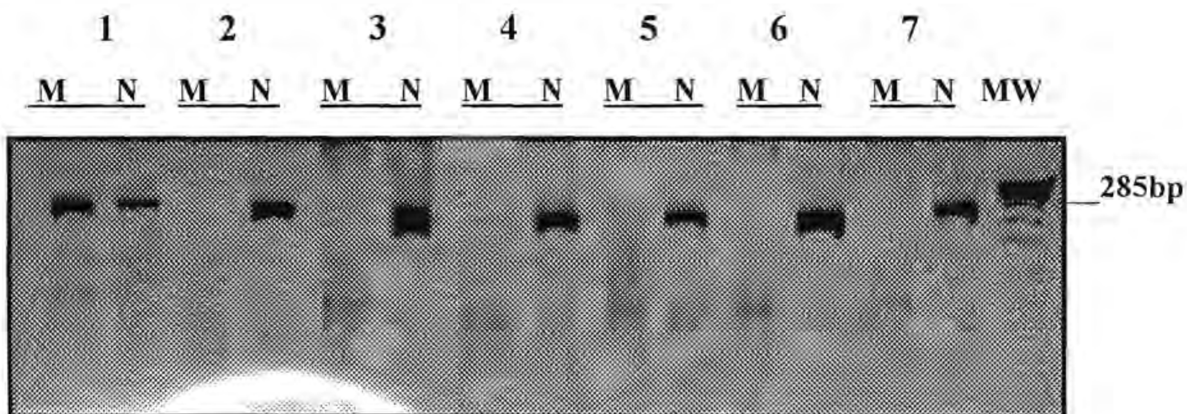

Figure 3.10: D206E allele specific PCR to identify the FH_1 genotype. The PCR amplification products were separated by 10% PAGE. The M lanes represent the mutant D206E allele and the N lanes the normal allele. Lane 1: Heterozygote; Lane 2-7, Homozygote Normal and Lane MW: ΦX174 DNA/Hinfl molecular weight size markers.

3.4.1.2 Stability of PCR Reaction Mix

The PCR reaction mix for 3.3.1.1 and 3.3.1.2, stored at -70°C were evaluated after 1 week and after 1 and 2 months. Storage of the PCR reaction mix for the multiplex allele specific method at -70°C resulted in no amplified product as shown in Figure 3.11. Only streaks of nucleotide material that stained with EtBr were observed following gel electrophoresis.

The allele specific PCR reaction mix could be stored at -70°C, thawed and used for the successful amplification of the D206E allele (Figure 3.12). The allele specific methodology was used for the screening of the male Caucasian population for the D206E mutation.

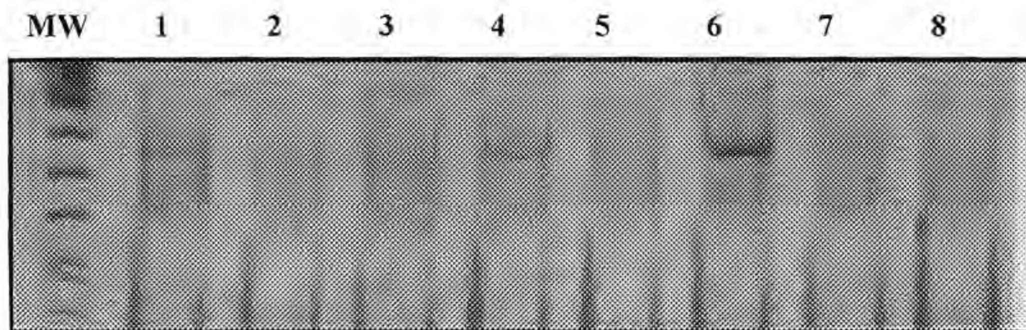

Figure 3.11: Gel electrophoresis of the multiplex allele specific PCR amplification product for the D206E, FH_1, and the V408M, FH_2 mutations using a PCR reaction mix stored for 1 week at -70°C. MW, 1: ΦX174 DNA/Hinf1 molecular weight size markers; Lane 2-5: Homozygote normal; Lane 6: FH_1 either heterozygote or homozygote D206E, Lane 7 either heterozygote ot homozygote for the V408M, FH_2 mutation.

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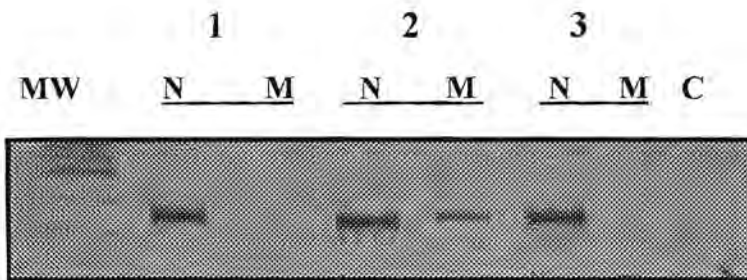

Figure 3.12: Allele specific PCR amplification for the FH_1, D206E genotype separated by 10% PAGE prepared using PCR mix stored for 1 month at -70°C. The N lanes represents the normal allele and the M lanes the mutant allele. Lane 1 (N and M) and 3 (N and M) are homozygote normal, Lane 2 (N and M) Heterozygote. Negative control (C) (containing no genomic DNA) and Lane MW: ΦX174 DNA/HinfI molecular weight size markers.

3.4.2.1 Genotype and Allele Frequency Analysis for the D206E Mutation

A population of 339 Caucasian males were screened for the D206E mutation. In this population group, 335 were found to be homozygous normal, 4 were heterozygous and there was no homozygote for the mutation (Table 4.1). A gene frequency of 0.006 was observed and a frequency for heterozygosity was 0.012. The population was in Hardy Weinberg equilibrium with a X^2 of 0.01 and the difference is not significant.

Table 4.1: Frequencies of the D206E Mutation in a Male Caucasian Population (n=339).

D206E			
Genotype	-/-	+/-	+/+
Observed	**335**	**4**	**0**
Expected	**334.94**	**4.04**	**0.01**
Gene Frequency = 0.006			
Carrier Frequency = 0.012			
Prevalence of affected individuals = 1 in 84			

Table 4.2 Chi-square for Goodness of Fit for the D206E Mutation

Genotype	Observed (O)	Expected (E)	(O-E)	$(O-E)^2$	$(O-E)^2/E$
-/-	335	334.9	0.1	0.01	0.00003
+/-	4	3.97	0.03	0.009	0.0022
+/+	0	0.01	-0.010	0.0001	0.01
Total	339	337.98			X^2=0.01

X^2_{2df} =0.01
P ($X^2_{0.99}$) = 10.6, difference is not significant

CHAPTER FOUR

4. DISCUSSION

4.1 Introduction

The South African Caucasian population presents with unique genetic founder effects either with increased prevalence of unique mutations or mutations found in many other populations. A founder effect of the population specific mutations have been found for the R95W mutation[1, 40, 59] associated with Porphyria Variegata and all the three mutations: D206E (FH_1), V408M (FH_2) or D154N (FH_3) for Familial Hypercholesterolaema [54-55, 49, 60]. An increased prevalence of the C282Y and the H63D mutations associated with Herediatary Haemochromatosis has also been reported for the South African population[40]. The increase that is observed in this study has been postulated to be due to founder effect[40, 61-62]. In contrast other mutations often associated with Familial Hypercholestrolaemia such as the ApoB100 gene defect (R3500 mutation) is rarely found in the South African population [54,56].

To implement a diagnostic service for many of these mutations within the South African Caucasian population presents with unique challenges, regarding the screening methods used, the prevalence and penetrance of these mutations within the population.

4.1.1 Genomic DNA

To screen for a specific mutation using techniques such as PCR amplification, the quality of DNA used has an impact on the results obtained. Blood specimens sent for mutations screening arrived on irregular basis. Hence, there is need to batch specimen for DNA isolation and mutation screening. Batches of blood specimens were kept in the freezer at -70°C. Before the DNA isolated from the blood in this manner could be used for mutation screening, it was necessary to determine the quality of the DNA isolated. To determine the DNA quality three important factors need to be determined, i.e. the concentration, integrity and purity of the DNA sample. The criteria were determined using a pooled blood sample. DNA was isolated using the QIAamp DNA blood mini kit. This method of isolation was chosen, as it required small volumes of blood.

The concentration of DNA was determined by agarose gel electrophoresis. Using this method it is also possible to determine whether the DNA has become fragmented either physically or by DNase activity during storage or isolation. The DNA was intact and the purity of DNA was determined by measuring the A_{260}/A_{280nm} absorbance ratio. The concentration and purity of a pooled blood sample was determined. The DNA isolation were done using the QIAamp DNA mini kit on pooled blood that have been kept in the freezer at -70°C. The DNA concentration was 40ng/µl and the purity was 1.85, which is of sufficient quantity and quality for PCR amplification.

4.2 Polymerase Chain Reaction (PCR)

Not only must a diagnostic service be appropriate for the population being tested but the procedures used must be simple, reliable and give reproducible results. Genetic testing using PCR technology involves the amplification of the region of DNA containing the mutation. The presence of the mutation is recognized either by using allele specific primers or restriction enzyme digestion that identifies mutation site. The products are separated according to size by gel electrophoresis and from the banding patterns the genotype of an individual can be identified[7, 63]. Based on this technology wide ranges of instruments and technologies (Light cycler, Taq Man104) have been developed for the screening of a wide range of disorders. However, this type of technology is expensive for small laboratories with low throughputs and its application is limited when screening for unique population specific mutations. Therefore it was necessary to make use of robust methods and to devise ways to simplify manual PCR genotyping. This would also enable the rapid screening of large populations to determine the prevalence of specific mutations within the South African Caucasian population. In this study by PCR methodologies where a PCR mixture containing primers, dNTP's, Taq polymerase and PCR buffers could be stored for at least 2 months at -70°C and still be used for successful PCR amplification. The stability of enzyme solutions at -70°C is well documented[64]. The PCR mixtures have been found to be stable for more than 2 months when stored at -70°C. However, it was found that for each PCR amplification reaction- either RFLP or ARMS procedures, aliquotes of PCR reaction mix containing only one set of primers could be used. Multiplex RFPL and ARMS PCR reaction mixtures could not be stored at -70°C for 2 months. Although the storage of PCR solutions is not

recommended[64] it was found that storage of the PCR mixes for all tests used in this study had no affect on the results. This method of storage could be used for both RFLP and allele specific PCR methodologies. The aliqoting of small amounts of PCR mix into tubes and the subsequent storage at -70°C greatly facilitates the testing of a large group of samples. It simplifies the screening procedures by decreasing preparation times and improves turn around times. It could also help to eliminate or to reduce possibility of cross contamination or random error.

In order to prevent the possibility of false negative or false positive results and detect contamination or non-specific bands (primer dimer), four levels of controls were used which include: positive control (DNA specimen positive for mutation), normal control (DNA specimen negative for mutation), negative control (PCR reaction mix without DNA) and molecular weight size markers. The positive or normal control was used to determine that the PCR reaction worked under optimum reaction conditions. Negative controls were used to detect presence of contamination in PCR reaction mixtures and molecular weight size marker to identify the DNA fragment or band needed for diagnosis or detection of mutation. With these, sources of error in the screening processes were eliminated.

4.3 Methylene Tetrahydrofolate Reductase (MTHFR)

Cardiovascular disease is the most common cause of death in males within the South African population[53-54, 65-66]. Although the prevalence of familial hypercholesterolaemia is high[65, 67] other factors such as raised levels of homocysteine are associated with increased risk of coronary heart disease[14-15, 22, 24-25]. Raised

homocysteine levels are due to a nutritional deficiency of folate or a genetic defect in one of the enzymes found in the folate metabolic pathway such as the C833T mutation of cystathione β-synthetase and C677T mutation of methylenetetrahydrofolate reductase[14]. The screening of a population already at high risk for cardiovascular disease for the MTHFR C677T will identify individuals whose possible risk is due to raised homocysteine levels[22]. The gene frequency of C677T mutation within this study population was 0.084, which implies that 1:12 individuals are homozygous for this mutation. Gardemann *et al*[26] reported that individuals homozygous for this C677T mutation with high coronary risk profiles had clearly higher cardiovascular heart disease scores than individuals with heterozygous C677T alleles[25]. Allele frequency of this mutation is 38% for the French Canadian and 5 to 15% for the general Canadian population[13]. In the African American, Asian and Caucasian[23] population it is 13, 30 and 34% respectively and is associated with elevated plasma homocyst(e)ine concentration. The frequency of this polymorphism in the South African population is 8.4%, which is similar to the frequency in the general Canadian population[23].

Individuals that are compound heterozygotes with the MTHFR, C677T and A1298C polymorphism also present with raised homocysteine levels[14]. The gene frequency and carrier frequency of this mutation in the South African population was reported to be 0.22 and 0.34 respectively[23]. Further screening for this mutation should be considered to identify individuals that have raised homocysteine levels due to compound heterozygosity. Furthermore, moderately elevated plasma homocysteine level is associated with parental history of cardiovascular disease (CVD) and that homozygosity for C677T mutation occurs more frequently in FH children with

parental history of CVD than in a group without[22], all patients with a history of premature atherosclerosis should be screened to establish risk factors, including elevated plasma homocysteine. Genetic risk factors in families with a history of CVD should be assessed and appropriate measures should be taken (e.g. determination of folate status and supplementation when inadequate) to prevent onset of disease.

4.4 Hereditary Haemochromatosis

Hereditary Haemochromatosis is another autosomal recessive disorder that has been reported to have a high incidence in the South African population[40]. Previous studies have been done on small population sizes (102 vs 359) and this is the first study that also determines the prevalence of compound heterozygosity within the South African population. The methodology used for the detection of the C282Y and H63D mutation was a modification of the multiplex method described by Merry weather-Clarke *et al*[38]. Storage of PCR reaction mixes containing more than one primer pair at -70°C resulted in poor amplification, possibly due to dimer formation between the different primers. The preparation of PCR reaction mix for each specific mutation and its subsequent storage at -70°C as significantly reduced the time taken for the preparation of the PCR mix and increased between batch reproducibility. Successful amplification of both alleles was achieved even after 2 months storage.

The prevalence of Hereditary Haemochromatosis was 0.05% (1 in 2000) in parts of Finland and nearly 0.5% (1 in 200) in Utah in the USA[1]. Subsequently, the genotype analysis for these patients for the H63D and C282Y mutations revealed that 60 to 100% of patients are C282Y homozygote with a small percentage appearing to be

compound heterozygote (C282Y and H63D), some apparently heterozygous for the C282Y mutation and some other unknown mutations in the gene[1]. An incidence of about 1 in 300 has been reported in Brisbane (Australia), Denmark, Germany, Iceland, part of Sweden and the UK[1, 30]. Within the South African Afrikaner population, the HH gene frequency was reported to be approximately 1 in 100[40]and a carrier frequency of 1 in 5. de Villiers *et al*[40] reported in a group of 22 clinically diagnosed patients that 77% were C282Y homozygote and 14% were C282Y and H63D compound heterozygote. In 9% of this population group the genetic cause of this disorder was unknown. The same group of researchers reported that the allele frequency was 0.09 and 0.12 for the C282Y and the H63D mutation respectively. In this study, within a three times larger group size a similar allele frequency of 0.084 and 0.127 was found for the C282Y and the H63D mutations respectively.

Jackson *et al*[68] studied the prevalence of the C282Y and the H63D mutations in a group of 10 556 Welsh blood donors and reported a prevalence of 0.082 and 0.15 for the C282Y and the H63D alleles respectively. Jackson *et al* reported that within the population studied 0.68% and 2.4% were C282Y homozygote and C282Y/H63D compound heterozygote respectively. In the United States the incidence of C282Y homozygosity is 0.28% and for C282Y/H63D compound heterozygosity 1.97%. However, in this South African Caucasian population, 0.86% and 3.44% prevalence for C282Y homozygote and C282Y/H63D heterozygote was observed. Within the South African population de Villiers *et al*[40] reported that up to 1:115 South African Caucasians of European descent are at risk of being C282Y homozygote. From this study we find that risk for being C282Y homozygote is 1:142 and for C282Y/H63D compound heterozygosity is 1:29.

Jackson *et al*[68] reported that in a population of Welsh blood donors 1 in 7 donors carried the C282Y mutation and 1 in 4 the H63D mutation, which is one of the highest in Europe. Likewise in this study group 1 in 7 individuals were heterozygote for C282Y and 1 in 5 were heterozygote for H63D. From this the general population risk for C282Y homozygosity is 1:200 and for C282Y/H63D it is 1:114. Due to the high prevalence of the C282Y and H63D mutations in the South African population, many parents that are C282Y or H63D homozygote have an increased risk of 1:2 of having offspring that are either C282Y homozygote or C282Y/H63D compound heterozygote, with the general population risk becoming 1:102 for C282Y homozygotes and 1:57 for C282Y/H63D compound heterozygotes. The penetrance of these mutations is age related and gender influenced. The genotype/phenotype correlation and phenotypic expression are more accurately addressed by age and gender. Penetrance of C282Y homozygosity in men >40 years of age is 95% and 80% in men younger than 40 years for iron overload. In women, iron overload is seen in 80% C282Y homozygous older than 40 years and in 39% younger than 40 years. New phenotypic data show a high penetrance of iron overload for C282Y homozygous males (85%) and females (69%) in relatives of haemochromatosis patients[27].

The issue concerning the clinical significance of the DNA mutations since homozygosity for the C282Y defect does not guarantee that an individual will develop HH is still unsolved as many individuals that are homozygous for C282Y never develop the clinical symptoms of HH. Furthermore, cases of patients with clinical conditions of HH and yet negative for the common mutations are also documented[1, 27, 61]. This could be due to lack of precise information on the penetrance of the common mutations. Nevertheless, HH symptoms often begin after the age of 50 years.

Symptoms occur more frequently in males than females at an estimated male to female ratio of 3:1. The decreased incidence of symptoms in female is attributed to the protection effects of menstruation blood loss and pregnancy in premenopausal women. MseI polymorphism identified in intron 4 (IVS4+48G→A) of the HFE gene, within the binding area of the antisense primer used for C282Y mutation screening was associated with the possible cause of overestimation of C282Y homozygote prevalence in HH, due to linkage disequilibrium with the mutation[69]. de Villers and Kotze reported that the presence of MseI polymorphism, could result in the overestimation of C282Y homozygosity within the South African population[69]. However, the proposed overestimation was excluded when the C282Y homozygosity observed was confirmed by re-amplification of the DNA samples, using the new antisenes primer designed by Jeffery et al[70]. In this study, the possibility of overestimation was also considered but was excluded by the confirmation of the C282Y homozygosity by re-amplification of the DNA sample and controls by blinding. The presence of this polymorphism can be confirmed or eliminated by sequencing the DNA of patients, which tested positive for C282Y homozygote.

The high frequency of C282Y and H63D mutations detected in the study population confirmed the high prevalence reported and stresses the need to monitor HH aggressively in South Africans of European descent. This high prevalence observed could be due to founder effect as has been suggested in Caucasian population in UK, Canada and South Africa[40, 61-62]and confirmed in Australia[61].The result of this study will provide a valuable framework for the implementation of a diagnostic service for HH mutation screening. Early detection screening, appropriate monitoring using serum ferritin level, serum transferrin saturation and iron binding capacity

measurements and subsequent treatment with repeated phlebotomy or venesection can prevent disease onset in affected individuals[40] and enhance normal life expectancy[1]. The fact that the majority of cases of haemochromatosis may be prevented by early detection through selective biochemical testing of young adults from 30 years or genetic testing of all young adults, and monitoring those at risk by measuring transferrin saturation at regular intervals (once in 1 or 2 years) can make a significant contribution to the health of the South African population.

4.5 Familial Hypercholesterolemia

A founder effect has also been described for FH within the South African population. Three unique founder mutations namely account for most of the cases of FH found in South Africa. Heterozygous FH is one of the most commonly seen genetic metabolic disorder, with an incidence of 1 in 500 in the United States and most European population while founder's effect increased the frequency among Africaners in South Africa to about 1 in 80[51-53, 41]. The prevalence and distribution observed when 27 unrelated homozygous and 79 unrelated heterozygous FH Afrikaner patients from Transvaal and Cape Province in South Africa were compared was similar with FH_1 (66%), FH_2 (27%), and FH_3 (7%)[56]. As for HH it was necessary to establish criteria for rapid populations screening using a method that could distinguish between individuals that were either homozygous or heterozygous for the mutation. The further requirement was that the method used should be robust and the PCR mix could be stored at -70°C for rapid analysis. The multiplex allele specific PCR method for the detection of FH mutations did not fulfill these requirements, as it was not possible to distinguish between individuals that are heterozygous or homozygous.

FH, especially FH_1 is under-diagnosed and that not even one quarter of patients with the condition is recognized in clinical practice. Therefore it was decided to develop a method specifically to determine the prevalence of FH_1. Using the same primer sequences, a allele specific PCR procedure was developed to identify the normal and mutant alleles. This method could distinguish between individuals that were heterozygote or homozygote for the D206E mutation. Furthermore, this PCR mixes could be stored at -70°C for at least 2 months without a decreased in product formation. A frequency of 1 in 84 individuals in this study group was heterozygous for the E206D FH_1 mutation. Most individuals are not diagnosed until middle age, when atherosclerotic disease is already rampant. With the availability of screening methods needed for early detection of the disease-causing mutation, as well the knowledge and awareness of its prevalence, this disorder can be detected and treated in early stages. It has become necessary to adopt MEDPED (make early diagnosis, prevent early deaths), initiated by late Professor Roger Williams[71-72], whereby people below 30 years should be encourage to go for screening. The screening of the family members of those found with the disease mutations should be made compulsory and the people at risk should be treated[65, 67].

Although, this study confirmed the incidence of a number of risk factors and disorders within the South African population this was the first time that the prevalence of these mutations has been determined in a young unrelated apparently healthy male population. The population group screened is larger than most other population studies undertaken within South Africa, and reveals that within this population group 1 in 20 individuals will develop a genetic disorder either due to HH or FH. It is recommended that voluntary screening for both disorders should be implemented.

Furthermore, valuable information has for the first time been obtained regarding the incidence of compound heterozygosity for HH within the South African population. The screening of the population for C677T provided some information regarding the prevalence of the mutation in the South African population and served as important tool in standardizing storage parameters for all subsequent PCR methodologies. The same method of PCR mix storage has been implemented for routine diagnostics within the molecular diagnostic laboratory and has reduced testing turn around times.

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