Analysis of Haemochromatosis Mutations in the North West Population

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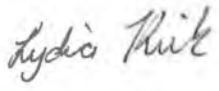
DECLARATION

Declaration

This thesis has not previously been submitted to this, or any other college. With acknowledged exception, it is entirely my own work.

Lydia Kirk



ABSTRACT

Abstract

Background: Hereditary haemochromatosis is a heritable disorder caused by an inborn error in the metabolism of iron. It results in over absorption of iron by the body, which can manifest clinically as fatigue, arthritis, diabetes and cardiovascular problems. The highest prevalence for the genetic mutations that cause hereditary haemochromatosis can be found in the Irish population. Individuals with diabetes may also have haemochromatosis (and vice versa), due to the bi-directional relationship between iron metabolism and glucose metabolism.

Objectives: To determine the incidence of the three haemochromatosis mutations C282Y, H63D & S65C, in a population from the North West of Ireland and to investigate whether there is an increased frequency of these three mutations in a diabetic population from the same region.

Method: DNA was extracted from 500 whole blood samples (250 diabetic samples and 250 'control' samples) using a Wizard™ kit. PCR was conducted utilising specific primers for each mutation and in accordance with a set protocol. Following amplification, PCR product was subjected to restriction endonuclease digestion, where different restriction enzymes (Rsa I, Nde II & Hinf I) were employed to determine the HFE genotype status of samples.

Results: The incidence of C282Y homozygosity (1/83) and C282Y heterozygosity (1/6) in the 'control' group was similar to those reported for the general Irish population (1/83 and 1/5, respectively). Incidences of H63D homozygotes and H63D heterozygotes or 'carriers' in the diabetic population were greater than that of the 'control' population. A significant finding of this study was that of an incidence of 1/32 S65C carriers in the control population. This is, to our knowledge, the highest incidence of the genotype reported to date in the general Irish population. Statistical analysis showed that there was no significant differences between the HFE genotype frequencies in the Diabetic and Control Populations.

Conclusion: Results of the study concord with published literature in terms of C282Y homozygosity and C282Y heterozygosity in the general Irish population. An increased frequency of the H63D mutation in diabetic individuals was also found but was not statistically significant. The biochemical effect of the H63D mutation is still unknown. The significance of such a high incidence of S65C carriers in the 'control' population warrants further investigation.

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1.0 LITERATURE REVIEW

Literature Review

1.1 Introduction

Haemochromatosis is a common heritable disorder which causes the body to absorb excess iron from the diet. Excessive absorption of iron results from disturbances of iron metabolism, the mechanisms of which remain poorly understood. When body iron stores surpass a threshold level, iron cannot be sufficiently excreted and the excess is deposited and builds up, with toxic consequences, in tissues of major organs such as the liver, heart, pancreas, endocrine organs, skin and joints. When organs are overburdened, their ability to function properly is affected and disease often ensues. This is the case with haemochromatosis, where accumulation of iron in tissues of primary organs has been associated with the development of diseases such as diabetes mellitus, cardiomyopathies, impotence, arthritis and skin pigmentation.

1.2 Haemochromatosis: A Historical Review

The first description of haemochromatosis as a clinical or pathological entity was attributed to Trousseau in 1865, who used terms like "pigment cirrhosis" and "bronze diabetes", to describe the condition later termed as "haemochromatosis", by von Recklinghausen in 1889. In later years, Troisier (1871) and Hanot and Chauffard (1882) showed agreement with the proposal that a distinct connection between iron overload and diabetes exists (Barton & Edwards, 2000).

The theory of haemochromatosis as a heritable disorder was initially put forward by Sheldon, a British physician in 1927. Sheldon later published a book entitled "Haemochromatosis", in which the following descriptional monograph of haemochromatosis was contained (1935):

' The most reasonable explanation of haemochromatosis is that it should be classed as an inborn error of metabolism which has an overwhelming incidence in males, and which at times actually has a familial incidence'.

However, it should be noted that not everyone of the time shared the opinion of Sheldon, in that haemochromatosis can be an inherited disorder. In 1964 MacDonald postulated that haemochromatosis and iron overload were consequences of alcoholism and other nutritional factors.

Simon et al., (1976) further hypothesized on the genetic nature of the disorder and reported a strong association between the gene(s) responsible for haemochromatosis and those of the Major Histocompatability Complex (MHC). A later study (Feder et al., 1996) reconfirmed support for the role of a novel MHC-like gene in the development of haemochromatosis.

During the 1980's, several studies were conducted on the relationship of haemochromatosis to; arthritis (Mathews & Williams, 1987), hepatic fibrosis (Bassett et al., 1986), cardiovascular problems (Olson et al., 1987; Fitchett et al., 1980), and on the linkage of haemochromatosis to HLA-H (Bassett et al., 1981). Concurrently, further understanding of the phenotypic expression of haemochromatosis was also achieved.

The quest to understand the biochemical and molecular mechanisms in the body underlying iron absorption (Halberg et al., 1997; Wood & Han, 1998; Fleming et al., 1999; McKie et al., 2001), metabolism (Abboud & Haile, 2000; Eisenstein & Blemings, 1998), and transport (Gunshin et al., 1997; Vulpe et al., 1999; McKie et al., 2000a), dominated research in the last fifteen years.

Sixty years after its inception, in 1996, Sheldon's observation was finally confirmed by a research team at Mercator Genetics, with their discovery of a novel gene called HFE, found to be responsible for hereditary haemochromatosis. Over twenty years had passed between localization of the haemochromatosis gene on chromosome 6p (Simon et al., 1975), and its molecular identification in 1996. The limited number and availability of genetic markers, comparable levels of linkage disequilibrium between markers and the low number of recombinants provided by familial studies, are factors suggested to account for this twenty year delay or search phase.

In the year 2000, expert review of the disorder by groups at the EASL (European Association for the Study of the Liver) International Consensus Conference on Haemochromatosis, resulted in the definition of haemochromatosis as; 'an inherited disorder resulting from an inborn error of iron metabolism which leads to progressive iron loading of parenchymal cells in the liver, pancreas and heart'. (Adams et al., 2000).

A guideline on genetic haemochromatosis was compiled in 2000 by Dr. James Dooley and Professor Mark Worwood on behalf of the Clinical Task Force of the British Committee for Standards in Haematology. The authors conducted a Medline search of world literature to review

existing guidelines. The findings were presented in an open forum at the British Society for Haematology meeting in April 1999. In these guidelines genetic haemochromatosis refers mainly to the accumulation of iron in the body due to the inheritance of mutations in the HFE gene on both copies of chromosome 6. This leads to excessive absorption of iron from food.

1.3 Types of Haemochromatosis

Iron overload is associated with mutations in multiple genes. It is therefore important to note the Online Mendelian Inheritance in Man (OMIM) database classification system that follows;

1.3.1 Type 1 or 'hereditary haemochromatosis' or 'HFE-related hereditary haemochromatosis': is an autosomal recessive condition caused by mutations in the HFE gene (chromosome 6p21.3). Postulated functions of this gene are; interaction with transferrin receptor 1, probably facilitating uptake of transferrin-bound iron and possibly the modulation of hepcidin expression. The major mutations of HFE are C282Y and H63D. Lesser mutations are S65C, C282S, [5569G-A, an intronic polymorphism], VAL53MET, VAL59MET, GLN127HIS, ARG330MET, ILE105THR, GLY93ARG. To date, there have been 37 allelic variants of the HFE gene reported (Pointon et al., 2000). The OMIM number that defines type 1 or hereditary haemochromatosis is 235 200.

1.3.2 Type 2 or 'juvenile haemochromatosis': is a rare, autosomal recessive condition consisting of two different subtypes. The gene implicated in type 2, subtype A is hemojuvelin (HJV) on chromosome 1q21, whose function is not yet confirmed but is thought to play a role in the modulation of hepcidin expression. The gene implicated in type 2, subtype B is HAMP on chromosome 19q13, which is thought to be involved in the down-regulation of iron release by enterocytes, macrophages or placental cells. The OMIM number for type 2A is 602 390 and for type 2B is 606 464.

1.3.3 Type 3 haemochromatosis or 'TFR2-related hereditary haemochromatosis': is an autosomal recessive condition caused by mutations in TFR2, a gene which codes for a transferrin receptor isoform and is located on chromosome 7q22. It is thought that this gene is possibly involved in the uptake of iron by hepatocytes. The OMIM number for type 3 is 604 250.

1.3.4 Type 4 haemochromatosis or 'Ferroportin-related iron overload': is an autosomal dominant condition associated with mutations in the ferroportin 1 gene (also called IREG1 and MTP10) on chromosome 2q32, which codes for a molecule involved in intestinal iron transport (McKusick, OMIM). It's OMIM number is 606 069.

1.3.5 Type 5 haemochromatosis: is an autosomal dominant condition which is caused by a mutation in the H-subunit of the gene for ferritin (Bomford, 2002).

Pietrangelo (2004) concluded that hereditary haemochromatosis should be defined as a unique clinical / pathophysiological entity that can be provoked by pathogenic mutations of one of the four known haemochomatosis genes, HFE, TfR2, HJV and HAMP.

To ensure clarity, all future references to haemochromatosis and / or iron overload in this review / text refers to Type 1 or hereditary haemochromatosis, unless otherwise stated.

1.4 Genetics of Haemochromatosis

1.4.1 Molecular Basis of Haemochromatosis

Haemochromatosis is an inherited disease. Initial studies by Simon et al., (1976) on the linkage between a haemochromatosis locus and the major histocompatability complex, and on the presence of an ancestral hapiotype (combination of genotypes on the same chromosome that tend to be inherited), were of importance in the search for the haemochromatosis gene (Simon et al., 1980). In 1996, the gene responsible for hereditary haemochromatosis was isolated (Feder et al., 1996). Using linkage disequilibrium and full hapiotype analysis, they identified a 250-kilobase region more than 3 megabases telomeric of the MHC. It was within this region that they identified a gene closely linked to the MHC class I family, which they termed HLA-H, that was responsible for hereditary haemochromatosis. The gene was later renamed HFE by the Nomenclature Committee of the Genome Database and can be found on the short arm of chromosome 6 of the human karyotype.

Genetic disorders can be of three main types; chromosomal, single gene (monogenic), or multifactorial (polygenic) diseases. Hereditary haemochromatosis was originally considered a monogenic disorder, in that it is caused by mutations in one or both alleles of a single gene, and

involves changes at the nucleotide level that disrupt the genes normal function. However, with the identification of similar phenotypes associated with mutations in at least four iron-metabolism genes, the condition is more multifactorial in nature than initially realized.

Hereditary haemochromatosis is most commonly the consequence of missense mutations of the HFE gene. The first mutation at the 845 nucleotide locus causes substitution of tyrosine for cysteine in amino acid 282 and is designated as the C282Y (Cys282Tyr) mutation. The second mutation is designated as H63D (His63Asp), as it occurs at the 187 nucleotide locus and causes substitution of aspartic acid for histidine in codon 63. Hereditary haemochromatosis is considered an autosomal recessive disorder, because both copies of the HFE gene (which are located on the two homologues of chromosome 6) need to have a particular mutation in order for the disorder to present itself. If an individual has only one HFE-mutated chromosome they are known as a heterozygote and the remaining non-mutated copy of the HFE is usually sufficient to prevent the onset of symptoms. If a particular mutation (either C282Y or H63D) is present on both chromosomes, an individual is said to be homozygous for that mutation, and there is a strong likelihood that C282Y homozygotes will develop the phenotypic expression of haemochromatosis. When the C282Y mutation is present on one chromosome and the H63D mutation is present on the other, individuals are called compound heterozygotes, and there is a small possibility that some of these individuals may also develop hereditary haemochromatosis. Figure 1.0 shows the different HFE genotypes and inheritance possibilities (Burke et al., 2001). Although C282Y and H63D are the major haemochromatosis mutations, two other mutations S65C and C282S may also contribute towards abnormal iron metabolism. The S65C mutation appears to be in a region implicated in binding the transferrin receptor to the HFE protein, and like the H63D mutation is associated with a milder phenotype than that of the C282Y mutation (ARUP Laboratories, 2002). The S65C mutation may be inherited in the compound heterozygous state with either C282Y or H63D, and is the most clinically significant of the lesser known HFE gene mutations, arising in 2-3% of Caucasians (Mura et al., 1999; Wallace et al., 2002). The C282S mutation is associated with severe iron overload, when in combination with H63D, but because both of the aforementioned mutations have a relatively low frequency, it has been difficult to obtain robust data pertaining to them (Rosmorduc et al., 2000).

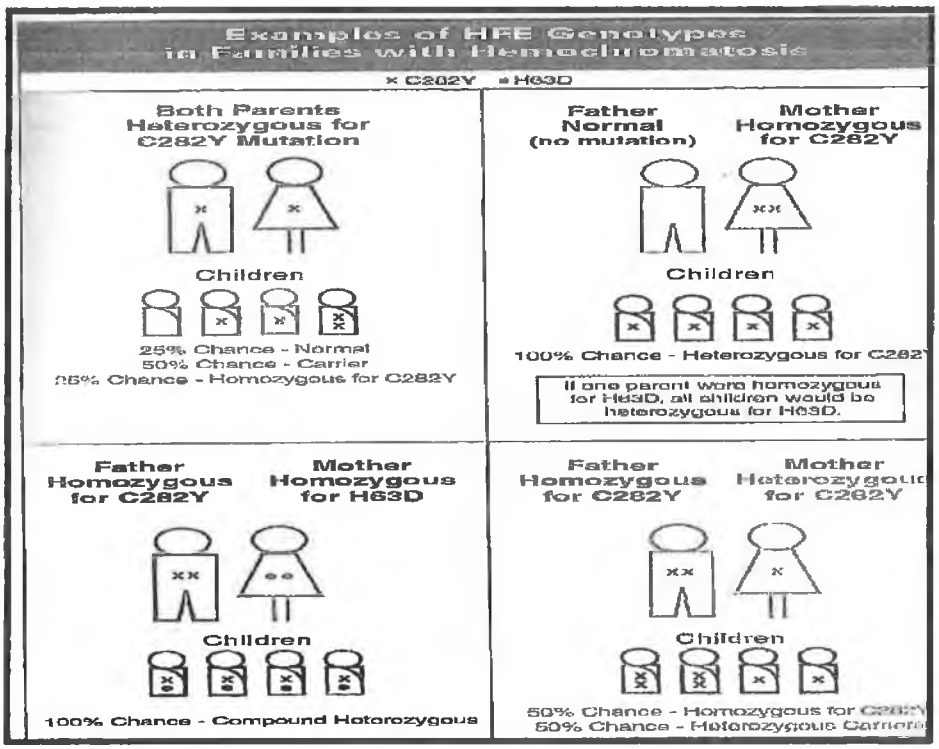


Figure 1.0: Examples of HFE Genotypes in families with Haemochromatosis [Source: Burke, Phatak & Weinberg, 2001]. Permission requested from publisher.

The exact function of the HFE protein in iron metabolism is currently unknown, but it is thought that it may act as a receptor for an iron-binding ligand on the basal cell surface. This activity could function as a negative feedback control so limiting iron absorption, and therefore an abnormality of this protein could result in loss of this negative feedback effect, resulting in excessive iron absorption. Other suggested functions are; signal transduction of body iron stores to the mucosal cell and interaction with immune system components that may influence iron metabolism, however these two functions are still purely speculative (Robson et al., 1997).

The HFE protein has an ancestral peptide-binding groove that is too narrow for antigen presentation (Lebron et al., 1998), so newly synthesized HFE protein binds to beta$_2$-microglobulin facilitating its interaction with the major receptor for transferrin (transferrin receptor 1 or TfR1) (Feder et al., 1998; Gross et al., 1998). The C282Y mutation (but not the H63D mutation) of HFE prevents formation of a disulphide bridge in the protein product, which in turn, prevents it from binding with beta$_2$-microglobulin and reaching the cell surface (Feder et al., 1997). Some investigators have suggested that the C282Y mutation may result in lower levels of the HFE-transferrin receptor complex at the cell surface, which would decrease the uptake of transferrin-

bound iron and so lower a cells iron status. Enterocytes may not be able to distinguish this low iron state from anemia and would therefore stimulate iron absorption, eventually leading to iron overload (Waheed et al., 2002; Wood, 2002). Low iron status stimulates increased expression of transporters, and to support this, haemochromatosis patients have been shown to over-express the iron importer DCT1 (Zoller et al., 1999) and the iron exporter IREG1 (McKie et al., 2000b). The presence of the C282Y mutation in HFE prevents its interaction with TfR1 and leads to iron-deficient crypt cells and in turn, iron-deficient daughter cells. These cells are programmed to react to iron starvation by hyperactively and persistently absorbing iron from the intestinal lumen and transferring almost all of it into the bloodstream, irrespective of the fact that erythropoietic needs may be fully satisfied. This theory is known as the 'crypt-programming model' and is a recently postulated pathogenic model of HFE-related hereditary haemochromatosis (Pietrangelo, 2004).

1.4.2 Origins, Population Genetics, Gene Frequency & Prevalence

Haemochromatosis has a distinctive geographical distribution that reflects the movements of people of northwestern Europe during historic times. This distribution led to the thinking that haemochromatosis is the result of a relatively recent mutation (one that has occurred in hundreds of generations) in a Celtic / North West European population. For a gene to become so prevalent in a population, it must have conferred a selective advantage or survival benefit to the populations which they affected e.g. resistance to dietary iron deficiency or certain infectious diseases. Because haemochromatosis is rarely associated with disability and death during reproductive years, it is unlikely that the disorder would confer a selective disadvantage. It is thought that populations of northwestern Europe that show a high prevalence of haemochromatosis, must have experienced an earlier period where human dietary iron intake was very limited or been areas where populations were repeatedly subjected to blood letting, for example, during war (Fairbanks, 2000). Both of these reasons help explain why the ability to absorb iron at an accelerated rate would confer a selective advantage to a population. Countries with the highest gene frequency for the C282Y mutation include Ireland, Great Britain, The Netherlands, Denmark, Germany, Iceland and Norway and are shown in Figure 1.1.

Figure 1.1: Estimated haemochromatosis gene frequency in Europe and the Mediterranean littoral (principally reflects the distribution and allele frequency of the C282Y mutation of the HFE gene).

[Source: Fairbanks, VF. "Haemochromatosis: population genetics", in Barton & Edwards, Hemochromatosis: genetics, pathophysiology, diagnosis and treatment. Cambridge University Press 2000; pp 43]. Permission requested from publishers.

The prevalence of the major haemochromatosis mutation C282Y in European populations is estimated at 0.4% for the homozygous state and 9.2% for the heterozygous state (Hanson et al., 2001). Studies on people of mixed European descent conducted by Beutler et al., (1996) showed that 83% of patients with hereditary haemochromatosis were homozygous for the C282Y missense mutation. Familial studies conducted by Jawinska et al., (1996) and Jouannolle et al., (1996) supported these findings with even higher mutational frequencies of 91% and 100%, respectively, being observed. The highest allele frequency (in a control population) of 14% for the C282Y mutation has been reported in Ireland (Ryan et al., 1998). In this study thirty-one out of 109 randomly selected people were heterozygous for the C282Y mutation. This allelic frequency of 14% represents a heterozygote or 'carrier' frequency of approximately 1 in 4, and shows agreement with studies by Merryweather-Clarke et al., (1997a) and Edwards et al., (1988) in which a heterozygote frequency of 1 in 5 for the C282Y mutation was observed in Irish people. The carrier frequency in the Irish population is generally accepted to be in the range of 1 in 8 to 1 in 10 people, so the findings by Ryan et al., (1998) of 1 in 5 is extremely high.

1.4.3 Phenotype vs. Genotype

The degree of iron overload and the related clinical complications can vary widely in haemochromatosis with both acquired (environmental) and genetic factors influencing phenotypic expression of the disease. A large proportion of people who may be homozygous for the haemochromatosis gene remain undiagnosed because they are either presymptomatic or they present with one or more of the many symptoms/manifestations of iron overload that may also be ascribed to other, more common conditions. Individuals with hereditary haemochromatosis are generally asymptomatic until toxic concentrations of iron have accumulated within the body, a process which usually takes several decades (Burke et al., 2001). Once this stage has been reached, clinical features such as bronzed skin, joint pain and swelling, diabetes, hypothyroidism and cardiomyopathies can present themselves. By this point, the concentration of iron stored in many body organs is much higher than the normal levels found, e.g. iron levels in the liver and pancreas may be up to 50 to 100 times their normal level. This surplus iron is mainly in the form of hemosiderin and can be visualised in tissues when stained with potassium ferrocyanide / Perls stain (see Figure 1.2).

Taking into account the existence of the highly prevalent C282Y mutation, it is difficult to explain the phenotypic variability of haemochromatosis (Piperno et al., 1996). For example, the iron overload phenotype associated with mutations in the gene which encodes transferrin receptor 2 (TfR2), appears to be very similar, to that of the classic HFE-related haemochromatosis, although very few cases have been described to date (Camaschella et al., 2000; Mattman et al., 2002; Girelli et al., 2002; Hattori et al., 2003). Both of these forms of the disease are representative of adult-onset hereditary haemochromatosis and are characterized by gradual iron loading, with a relatively late onset of parenchymal iron deposition and hepatic organ damage (Pietrangelo, 1998). A study by Lainé et al., (2005) reported that excess body mass is commonly associated with the lack of phenotypic expression in detected C282Y homozygotes. This should therefore be kept in mind with respect to the design and cost-effectiveness of phenotypic screening programs for haemochromatosis. A review article by Whittington & Kowdley (2002) reported that phenotypic expression of hereditary haemochromatosis appears to be different in different ethnic populations. The HealthIron study aims to examine environmental and genetic modifiers of HFE gene expression, and as such, should shed some light on the factors that modify the clinical severity of hereditary haemochromatosis (Gertig et al., 2005).

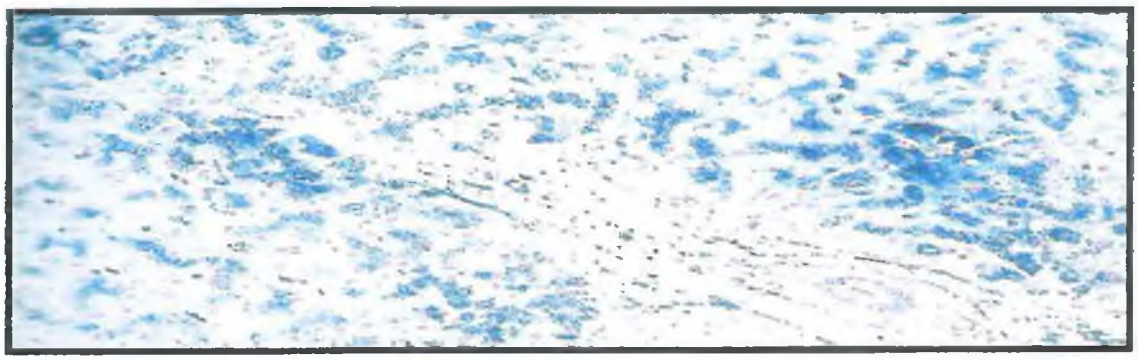


Figure 1.2: Hepatic micrograph showing iron accumulation (blue) in liver cells (Haemochromatosis) using Perls stain. [Source: Burkitt et al. Wheater's Basic Histopathology, a colour atlas and text, 3rd edition (1996), published by Churchill Livingstone. Standard Edition ISBN 0-443-05088-0]. Permission requested from publishers.

1.4.4 C282Y Homozygosity

Individuals who are diagnosed as homozygous for the C282Y mutation and have hereditary haemochromatosis, generally have iron stores that are greatly elevated from 'normal' levels. There have been relatively few large studies investigating iron status in C282Y homozygotes from the general population (Jackson et al., 2001; Burt et al., 1998; Beutler et al., 2000) that have included random samples. The study by Jackson et al., (2001) of HFE mutations, iron deficiency and overload in 10,500 blood donors, showed that 1 in 147 (72) were homozygous for the C282Y mutation. Studies by Beutler et al., (2000) and Burt et al., (1998) demonstrated that both transferrin saturation levels and serum ferritin concentration was considerably higher in C282Y homozygotes than in their wild-type counterparts. A study by Olynyk et al., (1999), showed that a high proportion of C282Y homozygotes had increased hepatic iron levels.

The penetrance of homozygosity for HFE C282Y is low, ranging between 1% and 4%. It remains unclear as to why only few patients homozygous for the C282Y mutation develop the full-blown form of the disease whilst others do not. The variation in penetrance may be due to epigenetic mechanisms like the presence of retrotransposons influencing expression nearby, environmental factors (such as alcoholism) and genetic modifiers such as mutations in other genes involved in iron metabolism (Beutler et al., 2003). However, a study by Lee et al., (2002) suggested that mutations in the genes coding for transferrin, ferroportin, TfR1, TfR2, haem-oxygenase 1 and 2, NRAMP1, NRAMP2, IRP2, haptoglobin, hepcidin and caeruloplasmin did not contribute to manifestation of the disease in patients homozygous for C282Y. Phatak et al., (2005), showed that disease expression varied considerably in C282Y homozygotes and that the observed variation is not explained by variations in dietary iron, fibre, ascorbic acid or ethanol intake. The

authors suggest that other unknown environmental variables or alternatively, modifier genes, may play a role in modulating disease expression in these patients. Candidate genes that could have implications for the clinical penetrance of C282Y, are the genes coding for hephaestin, duodenal cytochrome b and those of the heavy and light chains of ferritin (Bomford, 2002), as well as the gene that causes juvenile haemochromatosis (Roetto et al., 1999). There is no doubt that homozygosity for C282Y is a required cause of clinical haemochromatosis but other causal factors must also play a role in order to account for the incomplete penetrance of the disorder (Beutler et al., 2003).

A study in 2004 (Livesey et al.,) found that the frequency of the 16189 variant of mitochondrial DNA, is elevated in individuals with haemochromatosis who are homozygous for the C282Y allele, compared with population controls and with C282Y homozygotes who are asymptomatic. Homozygosity for the C282Y mutation appears to be the prevailing cause of clinically overt hereditary haemochromatosis (Milman et al., 2003). It is important to emphasize that homozygosity for C282Y does not confer a diagnosis of haemochromatosis but rather indicates a tendency towards iron overload (Whittington & Kowdley, 2002). Non-expression is common, especially in women (Crawford et al., 1998). A study in 2002 by van Aken et al., showed that no increase in mortality and morbidity was evident for carriers of the C282Y mutation. It is noteworthy that a study by Rossi et al., (2004) found that none of the C282Y homozygotes or compound heterozygotes expressed the phenotype of iron-overload disease.

1.4.5 C282Y Heterozygosity

Most studies report elevated transferrin saturation levels in C282Y heterozygotes when compared to their wild-type counterparts (Burt et al., 1998; Beutler et al., 2000; Datz et al., 1998). A study by Distante et al., (1999) supports this statement by reporting that 11% of heterozygotes had elevated transferrin saturation compared with 3% of wild-type individuals. Serum ferritin concentrations did not appear to differ significantly (Burt et al., 1998; Datz et al., 1998; Rossi et al., 2001). To date, there is little data on the prevalence of increased hepatic iron levels in C282Y heterozygotes taken from the general population.

A study of 1784 Danes suggested that C282Y heterozygosity may be associated with shorter life expectancy, particularly in women (Banthum et al., 2001). One of the main results of a study by Chambers et al., (2003) was a falling prevalence of C282Y heterozygosity with increasing age.

Both of the aforementioned findings are not consistent with those of Beutler et al., (2002 (a), 2002 (b)) and Waalen & Beutler (2004).

1.4.6 C282Y/H63D Compound Heterozygosity

Indices for the iron status of C282Y/H63D compound heterozygotes appear to lie between those of C282Y homozygotes and heterozygotes. Beutler et al., (2000) and Rossi et al., (2001) reported higher transferrin saturation levels in both male and female compound heterozygotes than in their wild-type counterparts. Serum ferritin levels were reported to be higher in men than in women also (Beutler et al., 2000). Compound heterozygotes are at increased risk of clinical haemochromatosis. Numerous studies have also pointed towards an increased risk of liver disease in compound heterozygotes. Rossi et al., (2001) expected a prevalence of around 2% in the general population of Australia, yet a study by Bacon et al., (1999a) reported that out of 132 patients with liver disease, 6% were compound heterozygotes. In 2000, Willis et al., demonstrated that individuals with cirrhosis had a prevalence rate of 3% for C282Y/H63D compound heterozygosity compared to a prevalence rate of 0.9% (for the same mutationai status), in the general population. Results from a study by Aguilar-Martinez et al., (2005) highlights that females who are compound heterozygotes for C282Y and H63D, are also at risk of developing clinical pictures of iron overload as severe as C282Y homozygous females.

1.4.7 H63D mutations

The H63D mutation (and that of the S65C), are associated with a milder phenotype than that of C282Y. H63D has an allele frequency of approximately 16% in the general American population (Arup Laboratories, 2002). A study by Gochee et al., (2002), reported elevated transferrin saturation levels in individuals with H63D mutations alone (i.e. no C282Y mutation). There was no significant differences in serum ferritin levels, although 9% of male H63D homozygotes and 3% of H63D heterozygotes had increased transferrin saturation and serum ferritin levels compared with 0.7% of the wild-type individuals. H63D is the major HFE mutation found in the Thai population with an average allele frequency of 3% (Viprakasit et al., 2004). The H63D mutation is now widely accepted to have few if any clinical sequelae (Galhenage et al., 2004; Burke et al., 2000; Gochee et al., 2002).

1.4.8 S65C mutations

In recent years, a new HFE amino acid variant known as S65C has been reported. This variant results in a single base substitution at nucleotide 193 in exon 2 and has been implicated, through association, to be involved in the pathogenesis of haemochromatosis (Mura et al., 1999; Barton et al., 1999). A study was conducted by Arya et al., (1999) to evaluate the association of S65C with iron overload that is unexplained by C282Y. Results showed that the S65C variant is not associated with increased transferrin saturation in voluntary blood donors and did not support the hypothesis that the S65C allele is associated with a genetic predisposition to iron overload. Wallace et al., (2002) investigated the haemochromatosis phenotype of compound heterozygotes for C282Y and S65C. They found that some C282Y/S65C compound heterozygotes had elevated serum iron indices and iron overload. The penetrance of this genotype is low and it is thought that other genetic and environmental factors may influence the expression of iron loading. Screening for the S65C mutation may be useful in people with iron overload who are neither C282Y homozygotes or C282Y/H63D compound heterozygotes. An allele frequency of 0.013% for S65C was reported by Willis et al., (2003), and was similar to that reported by Simonsen et al., (0.015%) in 1999. This suggests that the frequency of S65C homozygosity is about one in 5000, and that of C282Y/S65C compound heterozygosity is about one in 1400. The S65C mutation has a European allele frequency of 0.1-2% (Mura et al., 1999; Arya et al., 1999; Holmstrom et al., 2002; Koefoed et al., 2002; Candore et al., 2002; Couto et al., 2003; Willis et al., 2003; Salvioni et al., 2003; Pietrapertosa et al., 2003).

1.5 Penetrance

Clinical penetrance may be defined as the extent to which the mutations predict disease. In a review by Worwood (2005) it is stated that prior to the discovery of the HFE gene it was assumed that every person found homozygous for haemochromatosis would eventually accumulate enough iron to cause tissue damage ultimately resulting in morbidity. A recent study has suggested that the clinical penetrance of the C282Y mutation may be as low as 1%, however, this figure may be underestimated because the studied population was not representative of the population as a whole, symptom definition was poor (therefore it may not be reflective of the true health status of C282Y homozygotes in general), and because the sample size may have been inadequate. It should be noted, however, that the frequency of lethargy, arthralgia and diabetes was the same in both the study group and the controls. There was also a small but significant

increase in the percentage of individuals with either raised transaminase activity or fibrosis/cirrhosis in the C282Y homozygous group (Beutler et al., 2002a). A large study in Norway showed iron accumulation in C282Y homozygotes but little morbidity (Asberg et al., 2002). A study by Jackson et al., in 2001 identified 72 C282Y homozygotes out of a sample population of 10,500 blood donors. Most of the men and 45% of the women had elevated transferrin saturation levels. Sixty-three of the C282Y homozygotes that were interviewed did not show any physical signs of iron overload or were aware of relatives with haemochromatosis. Worwood (2005) states that despite much debate about ascertainment bias in family and population surveys, it is becoming clear that most men homozygous for C282Y will have raised transferrin saturation before the age of thirty, a proportion will have raised serum ferritin levels, but only a minority will eventually develop fibrosis and liver cirrhosis. Only around 50% of C282Y homozygous women will have raised transferrin saturation. Iron accumulation and tissue damage will usually, but not always, occur at a slower rate.

An article by Födinger and Sunder-Plassmann (2003) stated that almost all large-scale studies to date, have focused on the clinical penetrance of HFE C282Y homozygosity. Very few studies have investigated the clinical penetrance of other HFE mutations like H63D and S65C, and compound heterozygosity for C282Y and H63D.

1.6 Iron Metabolism in Haemochromatosis

The pathophysiology of hereditary haemochromatosis lies in a defective gene that controls the intestinal mucosal barrier to iron absorption. Lifelong absorption of dietary iron results in excessive body iron loading and haemochromatosis develops as a consequence. Although an essential element in many metabolic and enzymatic body processes (e.g. as an electron carrier), iron may also be extremely toxic due to lack of specific excretion pathways for the metal *in vivo*. Except for natural blood loss, maintenance of body iron levels relies on regulation of absorption alone. Intestinal absorption of iron that has entered the body via the gastrointestinal tract, can be split into three components; mucosal uptake by the enterocyte following iron presentation to the cells, intracellular enterocyte transport and storage, and extra-enterocyte transfer (Abdou, 2002; Fletcher & Halliday, 2002). Some regulation may occur during each phase. For absorption to occur, elemental iron must first be bound to oxygen and exposed to acidic conditions in the stomach, where it becomes ferrous iron and is absorbed in the duodenum. Absorbed iron is

captured by villi, finger-like projections, on the surface of the intestinal wall and so enters the bloodstream, where it is met by transferrin, a transport protein molecule.

Under normal conditions, each transferrin molecule can bind two atoms of Fe^{3+} and transports it to all tissues, vital organs and bone marrow, where it plays a vital role in metabolism, synthesis of DNA and erythropoiesis (Burke et al., 2001). Figure 1.3 shows the cycling of transferrin across the cell membrane. Current research has shown that caeruloplasmin and divalent metal transport ions (DMTI) are also involved with transferrin and the transport of iron in the blood. Transferrin is normally saturated 25 to 35 percent with iron, but when excess iron is present in the blood, transferrin becomes oversaturated and its ability to bind iron tightly, decreases. Transferrin saturation is a measurement of the amount of iron that transferrin can bind and transport and therefore reflects the changeability of iron from its storage form to its utilization. This measurement is the most reliable measure of the key process underlying haemochromatosis, as it shows the increased saturation of the body's TIBC (total iron binding capacity) in relation to serum iron levels (*Haemochromatosis (Inherited)* by The Haemochromatosis Society Australia Inc., 2000). The role of transferrin and its significance in transcellular transfer has been scrutinized. Studies have shown that hypotransferrinaemic mice and humans can develop iron overload due to enhanced iron absorption despite low serum transferrin concentrations, and hypoxaemic animals with enhanced iron absorption do not have increased mucosal cell transferrin, in spite of having increased plasma transferrin concentrations (Simpson et al., 1991; Osterloh et al., 1987). Additional studies in man indicate that transferrin may not play a controlling role in transcellular iron transport, as no significant difference was found in duodenal mucosal cell transferrin concentrations between iron-deficient patients and normal subjects (Whittaker et al., 1989). Mucosal cell transferrin concentration is not correlated with body iron stores or with non-haem or haem iron absorption. The concentration of transferrin within mucosal cells is probably achieved as a consequence of the cell's iron requirements during cellular growth and development.

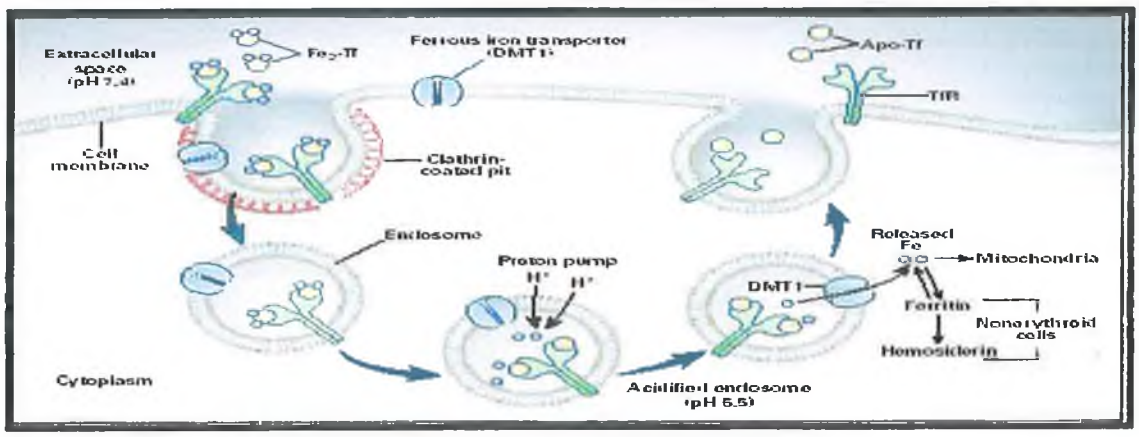


Figure 1.3: The Transferrin Cycle:

Iron-laden transferrin (Fe_2-Tf) binds to transferrin receptors (TfR) on the surface of erythroid precursors. These complexes localize to clathrin-coated pits, which invaginate to form specialized endosomes. A proton pump decreases the pH within the endosomes, leading to conformational changes in proteins that result in the release of iron from transferrin. The iron transporter DMT1 moves iron across the endosomal membrane, to enter the cytoplasm. Meanwhile, transferrin (Apo-Tf) and transferrin receptor are recycled to the cell surface, where each can be used for further cycles of iron binding and iron uptake. In erythroid cells, most iron moves into mitochondria, where it is incorporated into protoporphyrin to make heme. In nonerythroid cells, iron is stored as ferritin and hemosiderin.

[Source: Andrews NC. Disorders of Iron Metabolism, Review Article, Medical Progress, *The New England Journal of Medicine* 1999, pp 1987]. Permission requested from publishers.

Absorbed iron that is not needed for metabolism or production of DNA and erythrocytes may be stored within hepatocytes as ferritin. This large protein molecule can hold up to 4500 atoms of iron and acts as a containment reservoir for the metal when present in plentiful supply, or when free iron has the potential to damage ones health. When stored iron is in its soluble, mobile, diffuse form it is known as ferritin. The insoluble form is called hemosiderin and may be found as aggregated deposits throughout the tissues of the body. In cells, ferritin concentration reflects its repository function of sequestering excess cellular iron that has not been absorbed into the circulation. Free or unbound iron has toxic potential within the body through, amongst other things, its elicitation of free radical activity. Ferritin sequesters iron within the cell and prevents it from catalysing potentially cytotoxic free-radical generating reactions. Excessive absorption and deposition of iron within organs induces an inflammatory reaction, which in turn can induce organ fibrosis. In iron overload, plasma transferrin concentration decreases (transferrin saturation increases), while plasma ferritin is greatly elevated; consequently these measurements serve as important indicators of disease status. A review on the evaluation of iron overload by Jensen in 2004 highlighted that although the determination of serum transferrin saturation is commonly

used as an indication of iron status, and is therefore useful in screening for hereditary haemochromatosis, its diagnostic predictive value is higher than that of the serum ferritin test (Cook et al., 1976; Worwood et al., 1997). Serum ferritin, although easy to perform frequently, exhibits high variability, but to date, no alternative serum/plasma marker provides a better indicator of total body iron. A study, published in 2003, by Guillygomarc'h et al., concluded that although circadian variations in transferrin saturation levels exist, the test may be performed at any time during the day. O'Hara et al., (2003) concluded that in the absence of a national genetic screening programme for haemochromatosis in Ireland, transferrin saturation could be used as part of a health-screening panel, to detect individuals prior to the occurrence of organ damage.

Whilst for many years it had been suggested that both total body iron levels and the rate of erythropoiesis regulated iron absorption (Bothwell et al., 1958), it is only recently that a number of other factors have been proposed that may influence iron absorption. These molecules, called iron stores regulators and erythroid regulators, are thought to play an important role in regulatory aspects of the absorption pathway (Finch, 1994; Sayers et al., 1994).

1.7 Diagnostic techniques for iron overload

Diagnosis of hereditary haemochromatosis is generally by way of molecular testing. Haemochromatosis mutation detection can supplement biochemical measurements like transferrin saturation and serum ferritin, to confirm a diagnosis in symptomatic individuals and to detect sub-clinical cases. Molecular analysis detects people at risk of developing haemochromatosis independent of environmental factors, and consists of testing for the presence of the two major mutations C282Y and H63D. Most assay systems are PCR-based, meaning that specific gene regions are amplified and the products analysed.

The Polymerase Chain Reaction (PCR) is a technique for selective amplification of a part of DNA from the total genome. This technique makes amplification of large quantities of DNA from a DNA template, relatively simple and rapid. A task which prior to PCR, was only possible by labour-intensive cloning.

The process of PCR faithfully replicates a defined segment of DNA millions of times and is dependent on the enzyme *Taq* polymerase. There are three main steps that make up the amplification procedure, and these steps are repeated typically 25 – 35 times. The first step is

denaturation, a process which involves the separation of the two strands of the DNA double helix. Each strand can then be used as a template for the synthesis of a new strand. Denaturation occurs by subjecting the DNA to high temperature. The second step involves the annealing of DNA primers. Primers are short, synthetic pieces of DNA that match the two end points of a defined segment of DNA and bind on to them by complementary base pairing. They mark the starting point of the synthesis of the new DNA and prime the amplification reaction from their 3' OH end. The final or third step is extension. Here, single nucleotide bases are inserted to form the new DNA strands. It is in this way that the new strand is manufactured with a sequence complementary to the original template. At the end of the first cycle of PCR the DNA segment of interest has been duplicated. This cycle of three steps is repeated up to 35 times, with each subsequent step doubling the number of copies of DNA (See Figure 1.4). The main components of polymerase chain reaction are: oligonucleotides, buffers, *Taq* DNA polymerase, deoxyribonucleotide triphosphates, primers and target sequence / DNA (Innis et al., 1990).

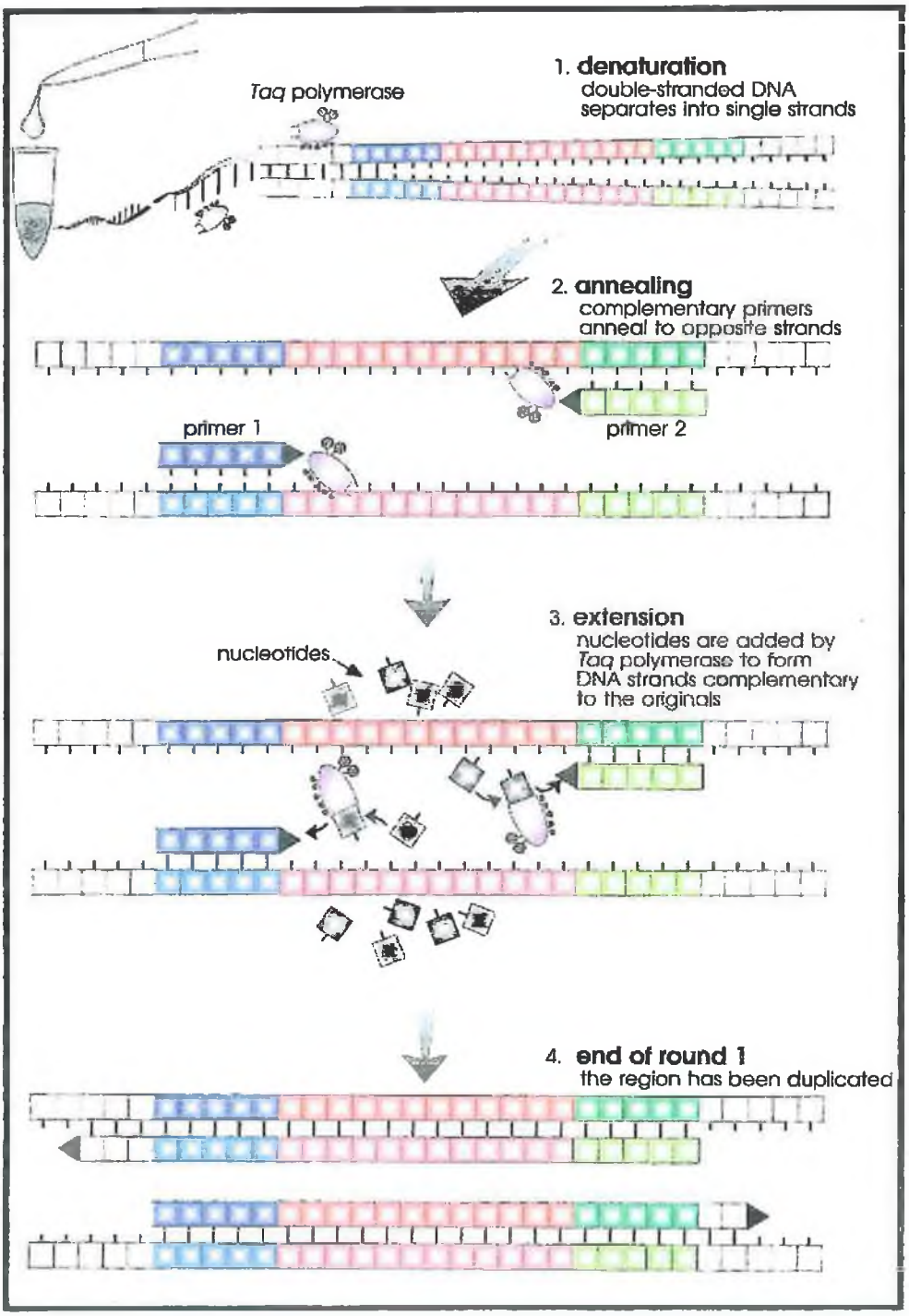


Figure 1.4: Polymerase Chain Reaction (round 1 amplification). [Source: Rudin, N. and Inman, K. An Introduction to Forensic DNA Analysis, 2nd edition, 2001, published by CRC Press, USA]. Permission to use requested from publishers.

PCR product can be then be subjected to further analysis, for example to determine the presence or absence of genetic mutations.

The most common method to differentiate between alleles is restriction fragment length polymorphism (RFLP) analysis. This method employs restriction enzymes to 'cut' alleles that contain certain mutations (Lyon & Frank, 2001). Variations of this analysis include multiplex PCR-SSCP (Polymerase Chain Reaction with Sequence Specific Primers), a simple and rapid technique that permits simultaneous detection of C282Y, H63D and S65C mutations (Simonsen et al., 1999; Guttridge et al., 1998) and MS-PCR, a method specific enough to distinguish between different genotypes of C282Y (Merryweather-Clarke et al., 1997b). Other methods include the oligonucleotide ligation assay (Feder et al., 1996) and allele-specific oligonucleotide hybridisation (Beutler et al., 1996). Both of the aforementioned methods have disadvantages in that they require either sophisticated automated technology or the use of radioisotopes. The advantages of using heteroduplex technology to genetically screen for haemochromatosis has been described by Jackson et al., (1997). This method involves the construction of a heteroduplex generator, by site-directed mutagenesis, which is subsequently used in PCR followed by capillary electrophoresis, for the detection of haemochromatosis mutations. Advantages to this type of analysis are that it permits rapid processing of large sample numbers and has higher specificity than RFLP analysis. A study by Gómez-Llorente et al., (2004) presented the first application of a multiplex multicolour assay for the simultaneous detection of the three most frequent HFE mutations; C282Y, H63D and S65C. The method is based on single base extension followed by competitive allele-specific PCR, using fluorescent detection and capillary electrophoresis. This method will facilitate automated genotyping for routine molecular diagnostics and large-scale genetic studies. Results from a study by Rossi et al., (1999) indicated that, overall, genotyping for the C282Y mutation is a useful test for the diagnosis of genetic haemochromatosis in clinical practice.

In this study, PCR for detection of the C282Y haemochromatosis mutation was conducted with the forward C282Y primer used by Feder et al., (1996) but a different reverse primer was used. The base sequence of the reverse primer that was used by Feder et al., (1996) was: C282YR: 5'CTC AGG CAC TCC TCA ACC-3'. This primer was discarded for use in this study (as it may cause overestimation of C282Y homozygosity) and an alternative one used by Jeffrey et al., (1999) was employed. Both the H63D and the S65C haemochromatosis mutations were detected using the forward and reverse primers employed by Merryweather et al., (1997a). The same set of primers could be used to detect both mutations as they are contained in the same region / exon of the HFE gene that is amplified by the aforementioned primers (see Figure 1.5).

Nucleotide no. 4652

↓

gttcacact ctctgcacta cctcttcatg ggtgcctcag agcaggacct tggtctttcc ttgtttgaag ctttgggcta cgtggatgac cagctgttcg tgttctatga tcatgagagt cgccgtgtga agccccgaac tccatgggtt tccagtagaa tttcaagcca gatgtggctg cagctgagcc agagtctgaa agggtgggat cacatgttca ctgttgactt ctggactatt atggaaaatc acaaccacag caag

↑

Nucleotide no. 4915

Figure 1.5: Exon 2 of the HFE gene and location of the H63D and S65C mutations (in bold) [Source: adapted from results of GenBank search on NCBI, http://www.ncbi.nlm.nih.gov].

Restriction endonuclease digestion in conjunction with electrophoresis allows simple determination of sample mutational status.

Electrophoresis through agarose (or polyacrylamide) gels is the standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid and capable of resolving fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. The location of DNA within the gel can be determined directly by the use of ethidium bromide staining detected under UV light.

Agarose gels have lower resolution than their polyacrylamide counterparts, but a greater range of separation. DNAs from 200bp to nearly 50kb in length can be separated on agarose gels of different concentrations (Sambrook et al., 1989).

Agarose, which is a neutral polysaccharide extracted from seaweed, is a linear polymer whose basic structure is:

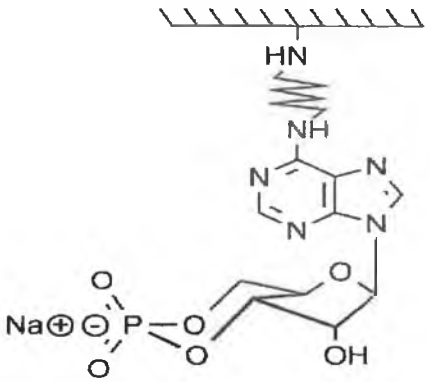


Figure 1.6: Chemical structure of agarose [Source: http://www.biolog.de/a036.html].

Special types of low melting temperature agarose can be used to analyze very small fragments of DNA (10-500bp) and have a greater resolving power than gels made with normal agarose.

Agarose gels are cast by melting an amount of agarose in a volume of buffer until a clear, transparent solution is achieved. This solution is then poured into a mold and allowed to harden or polymerize. On hardening, the agarose forms a matrix, the density of which being determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The rate of DNA migration is determined by the:

- molecular size of the DNA (larger molecules migrate more slowly because of greater frictional drag and because they pass through gel pores less efficiently than smaller molecules).
- agarose concentration (gels of different agarose concentrations can separate linear DNA of different sizes).
- conformation of the DNA (different conformations of DNAs of the same molecular weight migrate through agarose gels at different rates)
- applied voltage (migration rate of linear DNA fragments is proportional to the voltage applied. For maximum resolution of DNA fragments greater than 2kb in size, gels should be run at no more than 5 V/cm.
- direction of the electric field
- base composition and temperature
- Presence of intercalating dyes (ethidium bromide is a dye which intercalates between stacked base pairs, extending the length of DNA molecules and increases their rigidity).
- Composition of the electrophoresis buffer (DNA electrophoretic mobility is affected by the composition and ionic strength of the buffer. If there are no ions, electrical conductance is minimal and DNA migration is very slow) (Sambrook et al., 1989).

Liver biopsy was once considered essential by many authorities, when the diagnosis of haemochromatosis was suspected due to either clinical or biochemical abnormalities (Sallie et al., 1991; Powell, 1996). The procedure for hepatic biopsy is invasive and may not be advisable in reluctant or older patients, or those with other complicating medical conditions. Alternative

methods that may be of use in the diagnostic evaluation of severe hepatic iron deposition, are computed tomography (CT) scanning and magnetic resonance imaging (MRI), but both techniques are quite expensive (Bonkovsky et al., 1990; Chezmar et al., 1990).

1.8 Screening

By definition, a screening test is one administered on a single occasion in seemingly normal individuals to allow detection of a disease at a time when instigation of appropriate treatment will substantially improve outcomes (Prorok, 1992). Haemochromatosis represents the current model for genetic screening for disease predisposition followed by preventative therapy. A study by Niselle et al., in 2004, investigated the implementation of a workplace-based genetic screening programme for haemochromatosis. Results of the investigation showed that widespread screening for hereditary haemochromatosis is readily accepted in the workplace and a "one-to-many" education programme is effective. It was also found that the level of participation varied greatly and that the advertising and logistics of the screening programme should be adapted to reflect this and suit the specific features of each workplace.

A review on hereditary haemochromatosis by Limdi & Crampton (2004) reiterates that the condition meets a number of WHO criteria for population screening (Wilson & Junger, 1968), such as a latent period, availability of a screening test, and safe, cost-effective treatment. Despite this, there is still much debate as to whether population screening for the haemochromatosis mutations is worthwhile. A review by the European & UK Haemochromatosis Consortia (2000) highlighted that although there are many epidemiological arguments in favour of developing screening programmes for haemochromatosis, the condition of hereditary haemochromatosis does not yet fulfill the criteria of Wilson & Junger (1968) for implementation of a screening programme.

Serum transferrin saturation (preferably in the fasting state to help eliminate confounding factors such as iron fortified preparations or oral contraceptives) is the best initial screening test for haemochromatosis (Edwards & Kushner, 1993; George et al., 1995). Although population screening studies have traditionally used a transferrin saturation threshold of > 45-75 %, it is likely that a value of 55 % gives the best positive predictive value. Following determination of transferrin saturation, serum ferritin is usually measured and it is only if concentrations > 300 µg/l that genotyping is recommended (Dooley & Worwood, 2000). Results from the HEIRS

(Hemochromatosis and Iron Overload Screening) study, presented by Adams et al., (2005(b)), have suggested that the unbound iron binding capacity (UIBC) be used as an alternative biochemical screening test to transferrin saturation to identify individuals with C282Y-linked haemochromatosis. An advantage of population screening is that it would permit early identification of those at risk from iron overload before clinical complications of the disorder occur. Disadvantages may be its cost-effectiveness and potential implications for negative bias in health insurance and employment (Cifuentes Henderson et al., 2002).

The optimal time for screening is between the ages of 18 and 30, a time when haemochromatosis is evident from biochemical tests, but before serious organ damage has occurred (Bacon et al., 1999b). Screening of children is currently not recommended (Adams et al., 2000).

A recent study by Patch et al., (2005) looked at factors affecting the uptake of two different strategies for screening, i.e. phenotypic versus genotypic. Results showed that the uptake of screening with a genotypic strategy was not inferior to that in a phenotypic strategy. A study by Gason et al., (2004) on the novel concept of genetic susceptibility screening in schools, indicated that it could be a public health success, provided community support was obtained. Allen et al., (2005) have presented data that demonstrates that population-based screening, prior to the onset of symptoms, is both practicable and acceptable to the community. Of the 11,307 individuals they screened, genotyping was completed on 11,287 individuals. Out of the 51 C282Y homozygotes identified, 46 have taken steps to treat or prevent iron overload.

Population genetic screening programmes will remain a contentious issue until it can be demonstrated that the benefits outweigh any potential harm or costs (Gertig et al., 2003; Yapp et al., 2001), and optimal screening strategies are designed to meet various medical, ethical, legal and social issues, whilst identifying 'at risk' individuals (Adams 2005a; Olynyk et al., 2005; Delatycki et al., 2005; Powell, 2005). The role of HFE genotyping in asymptomatic populations has yet to be defined (The European & UK Haemochromatosis Consortia, 2000). Figure 1.7 is a typical algorithm for the evaluation of hereditary haemochromatosis.

Cadet et al., (2003) recommend that patients, presenting to hospital clinics with haemochromatosis associated conditions, should be screened biochemically for iron overload. They also state that patients at greatest risk of having undiagnosed haemochromatosis are those

presenting with unstable diabetes, or fatigue and/or arthralgia in the absence of any other explanation.

The HEIRS study aims to evaluate the prevalence, genetic and environmental determinants, and potential clinical, personal and societal impact of hemochromatosis and iron overload in a multiethnic, primary care-based sample of 100,000 adults over a five-year period. Information from the study will inform policy regarding the feasibility, optimal approach and potential health benefits and risks of primary care-based screening for hemochromatosis and iron overload (McLaren et al., 2003).

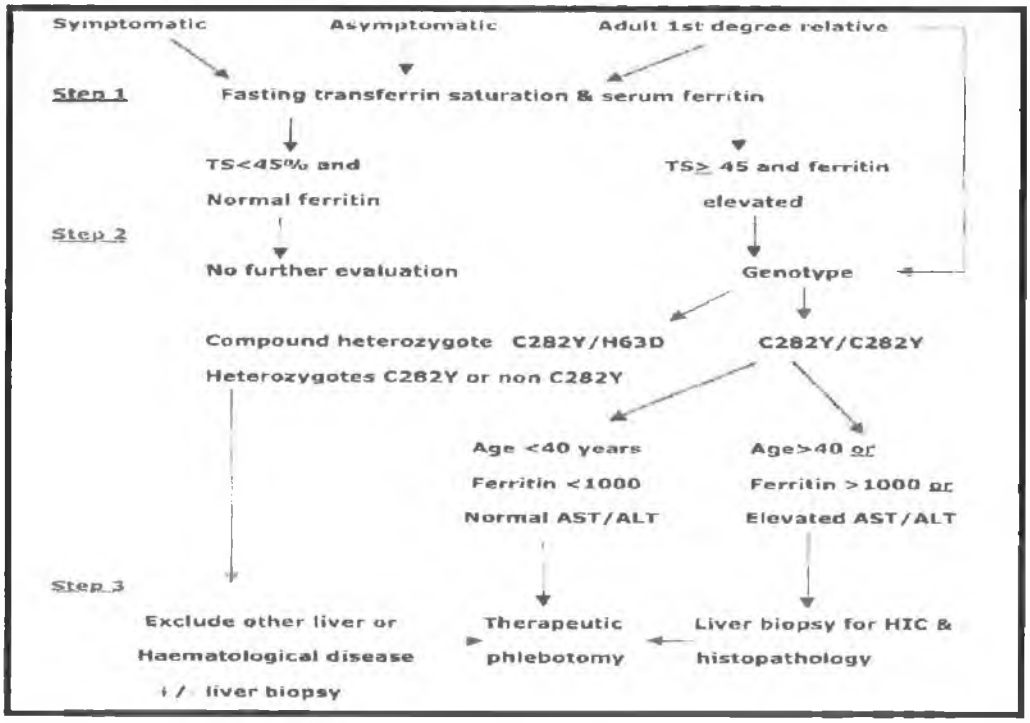


Figure 1.7: AASLD (American Association for the Study of Liver Disease) algorithm for the evaluation of hereditary haemochromatosis. From Tavill AS. Diagnosis and management of haemochromatosis AASLD practice guidelines. Hepatology;33:1321-8. Copyright © 2001, Wiley-Liss, Inc. Reproduced with permission of John Wiley & Sons, Inc.

Imperatore et al., (2004) listed the generally accepted criteria for an effective population-based screening test. The criteria were as follows; the disorder screened for must be well-defined and represent an important health problem whose natural history is understood. The prevalence must be known and the disorder common enough that population screening is cost-effective. Suitable screening tests with known performances must be available, and for individuals identified as screen positive, there must be an adequate and acceptable protocol for diagnosis and effective

treatment. There should be adequate facilities available to support screening, diagnosis and treatment and costs must have been examined and found reasonable. Above all the approach must be considered ethical and must be acceptable to both health care providers and consumers.

1.9 Therapy

1.9.1 Phlebotomy

Therapeutic phlebotomy was the first successful treatment described for iron overload (Davis & Arrowsmith, 1952) and is still the preferred treatment for the condition today (Powell et al., 1994; Witte et al., 1996). For subjects who have already accumulated iron, the usual treatment is phlebotomy carried out at weekly intervals. A record should be kept of the volume or weight of blood venesected so that it is possible to calculate the amount of iron removed by treatment. Guidelines on genetic haemochromatosis compiled on behalf of the British Committee for Standards in Haematology recommend the following treatment regime:

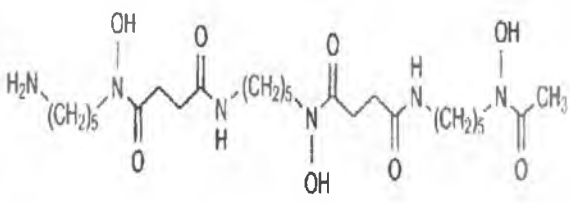
' Venesect 450-500 ml blood once weekly until serum ferritin concentration is below 20 µg/l and transferrin saturation s under 16%. Haemoglobin levels should be monitored weekly and the rate of venesection reduced in the event of anaemia developing. Serum ferritin should be monitored on a monthly basis and transferrin saturation measured as the ferritin concentration decreases below 50 µg/l. Calculation of the amount of iron removed by therapy can be achieved by weighing the blood bag before and after phlebotomy (density of blood is 1.05 g/ml) and assuming that 450 ml blood (Hb concentration = 13.5g/dl) contains 200 mg of iron. If daily iron absorption of 3 mg is allowed for, the assumption is that 25 weekly venesections will remove 4.5 g of iron ' (Dooley & Worwood, 2000).

Iron depletion by phlebotomy usually alleviates malaise, weakness and ease of fatigue, symptoms not attributable to specific organ dysfunction, but which affect approximately 80% of patients with iron overload (Powell et al., 1994; Witte et al., 1996). Therapeutic phlebotomy can help resolve many liver-associated abnormalities. Cardiomyopathies and arrhythmias are also alleviated by the aggressive removal of stored iron (Cutler et al., 1980; Candell-Riera et al., 1983; Rivers et al., 1987). Patients with haemochromatosis, who do not have hepatic cirrhosis, diabetes mellitus or cardiomyopathy and who undergo and maintain depletion of iron stores by phlebotomy, can expect to have a normal life expectancy. For patients who have these

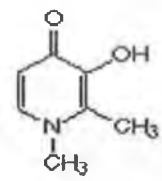
aforementioned complications, therapeutic phlebotomy often improves the quality of life and may increase longevity (Niederau et al., 1985 & 1996; Powell 1986; Adams et al., 1991(b)). Cirrhosis of the liver rarely resolves with venesection therapy (Knauer et al., 1965; Weintraub et al., 1966; Powell & Kerr, 1970). De Valk et al., in 2000 stated that phlebotomy treated haemochromatosis homozygotes maintain a high and potentially harmful non-transferrin-bound iron (NTBI). Haemochromatosis heterozygotes have a higher serum NTBI than normal controls. The reported increased risk of cardiovascular events in heterozygous haemochromatosis may be explained by NTBI-catalyzed LDL peroxidation (De Valk et al., 2000).

1.9.2 Chelation Therapy

Although phlebotomy is well established for the treatment of haemochromatosis, it requires an intact haemopoietic system to maintain its efficacy. Therefore, it is unsuitable for patients with genetic and other chronic anaemias who require blood transfusions for their survival, and the only available method of protection against consequences of transfusional iron overload for these individuals, is chelation therapy (Hershko et al., 2000). Chelation therapy can be used to achieve safe tissue iron concentrations by promoting negative iron balance. The therapy can also serve to detoxify excess iron in the body. Chelatable iron is that which comes from the continuous catabolism of red cell haemoglobin as well as iron obtained from the breakdown of ferritin within lysosomes. Chelation of iron can be achieved by either subcutaneous infusion of deferoxamine or oral therapy with deferiprone, see Figure 1.8 (Porter, 2001; Nielsen et al., 2003). The dose regime for both forms of chelation therapy involves a balance between the risks of excess iron and those of excess chelator. Lower doses, given more continuously are generally more efficient and less toxic than larger, more intermittent doses of chelator (Porter, 1997). The efficiency of chelation regimes is low, with 90% of subcutaneous deferoxamine (Porter et al., 2000) and 96% of deferiprone (Al-Refaie et al., 1995) being excreted without binding iron.



Deferoxamine B



Deferiprone

Figure 1.8 : Iron Chelators [Source: http://www.medicinescomplete.com; http://www.lipomed.com]

1.10 Social & Ethical Issues

Genetic testing for haemochromatosis raises concerns about stigmatisation and discrimination which in turn raises concerns about possible breaches of privacy and confidentiality. Diagnosis of, or a predisposition to, the condition may lead to possible loss of insurance coverage and employment, both of which have been reported by Alper et al., (1994). Such instances of discrimination in health insurance and employment should be prohibited by law, but as yet have not been defined with enough clarity to afford legal protection to those concerned. Once a person has been diagnosed with haemochromatosis it is necessary to explain that other family members may also be at risk. Genetic testing and counselling should be offered to all first-degree relatives of the proband. It is important to note that some but not all insurance companies take the view that haemochromatosis, when properly diagnosed and managed, does not justify refusal of insurance cover or increased premiums.

1.11 Relationship to other disorders

There may be a high frequency of heterozygosity for haemochromatosis in patients with porphyria cutanea tarda (PCT), sideroblastic anaemia and hereditary spherocytosis with iron overload, amongst other conditions (Simon, 1985).

A study by Elder & Worwood (1998), showed a high frequency of HLA-A3 in patients with PCT, which is suggestive of the presence of a haemochromatosis mutation also. Although patients with PCT exhibit some iron accumulation, very few of them have an iron overload which would be considered diagnostic for haemochromatosis. Roberts et al., (1997a, 1997b), also showed that PCT patients demonstrated a high frequency of HLA-A3 in addition to a high frequency of D6S265-1, D6S105-8 and D6S1260-4, all of which are allelic markers associated with the ancestral haplotype for haemochromatosis. A study by Sampietro et al., (1998) showed findings opposite to that of these two studies, in that PCT patients did not show an increased frequency of the C282Y mutation. The study did, however, demonstrate a significant increase in the frequency of the H63D mutation when compared to the control population or other patients suffering with viral hepatitis. The relationship between PCT and haemochromatosis can be summarized by stating that; haemochromatosis *per se* does not precipitate PCT (Brissot et al., 1978) and inheritance of the haemochromatosis gene is an important susceptibility factor for sporadic PCT,

but the coexistence of the two conditions needs further investigation (Roberts et al., 1997a, 1997b).

A review by George et al., (1999) provides evidence that mild to moderate iron loading due to either homozygosity or heterozygosity for the haemochromatosis mutations, acts as a hepatotoxin by aggravating liver damage from other causes of liver disease, like non-alcoholic steatohepatitis, chronic hepatitis C, PCT and possibly primary liver cell cancer.

Co-inheritance of heterozygosity or homozygosity for haemochromatosis with that for beta-thalassaemia, may increase the rate at which iron overload occurs (Rees et al., 1997). Incidences have been reported where patients with sideroblastic anaemias have expressed HFE mutations (Yaouanq et al., 1997; Cotter et al., 1999). Results from a study by Jazayeri et al., (2003), suggested that the mutations C282Y and H63D are more frequent in Iranian beta-thalassemia minor patients than in the normal population but cause no significant changes in serum ferritin level.

In Wilson's disease, genetic mutations cause excess copper to accumulate in the liver or brain and because there is increasing evidence for an interaction between copper and iron metabolism, it is reasonable to assess iron indices in Wilson's disease sufferers in order to detect iron overload (Erhardt et al., 2002).

it has been suggested that if iron plays an important role in the pathogenesis of atherosclerosis and ischaemic heart disease, subjects homozygous or heterozygous for haemochromatosis might be expected to experience high rates of the disease (Burt et al., 1993; Sempos & Looker, 1999). However, systemic reviews do not support the existence of strongly positive or strongly negative epidemiological associations between iron status and cardiovascular disease (Kelly, 2002). Worwood (2002) stated that heterozygosity for either C282Y or H63D does not appear to be a risk factor for common conditions like diabetes, liver and cardiovascular disease. Hetet et al., (2001) reviewed three association studies between the haemochromatosis gene and the risk of cardiovascular disease. None provided consistent evidence supporting the hypothesis that HFE mutations are associated with an increased risk of cardiovascular disease and with the development of arteriosclerosis. A case-control study by Claeys et al., (2002) also reported no association between genetic haemochromatosis and myocardial infarction. No evidence to support an association between haemochromatosis and atherogenesis was found, when a study

into the prevalence of haemochromatosis in premature atherosclerotic vascular disease was conducted by Franco et al., in 1998.

Iron is potentially carcinogenic as it has a catalytic effect on the formation of hydroxyl radicals (initiation), it is an essential nutrient for tumour cells (promotion) and it has the potential to suppress the activity of immune cells. Based on epidemiological surveys, the possibility of a positive association between measures of iron status and cancer, in the general population, has been proposed (Knekt et al., 1994; Stevens et al., 1988 & 1994). The evidence relating iron to cancer and cardiovascular disease risk is inconsistent and equivocal for the general population (Kelly, 2002).

1.12 Summary of mutational incidence (worldwide)

The figures presented below (Figures 1.9 to 1.14) summarise the incidence of HFE mutational genotypes in 25 clinically diagnosed populations versus 64 general populations. In clinically diagnosed proband populations (those with haemochromatosis), the commonest mutational genotype was C282Y homozygosity followed by C282Y/H63D compound heterozygosity (see Figure 1.9). The reverse was the case, when general (control) populations were screened (see Figure 1.7). The Reference Code for the information compiled below is as follows:

*	= Rivard et al., (2000).
#	= http://www.cdc.gov/genomics/hugenet/reviews/tables/HFE_Tables.htm
~	= Milman et al., (2003).
^	= Imperatore et al., (2003)
$	= Guix et al., (2002)
+	= Beutler et al., (2002a)
£	= Jackson et al., (2001)
>	= Deugnier et al., (2002)
<	= Cadet et al., (2003)
!	= Rossi et al., (2004)

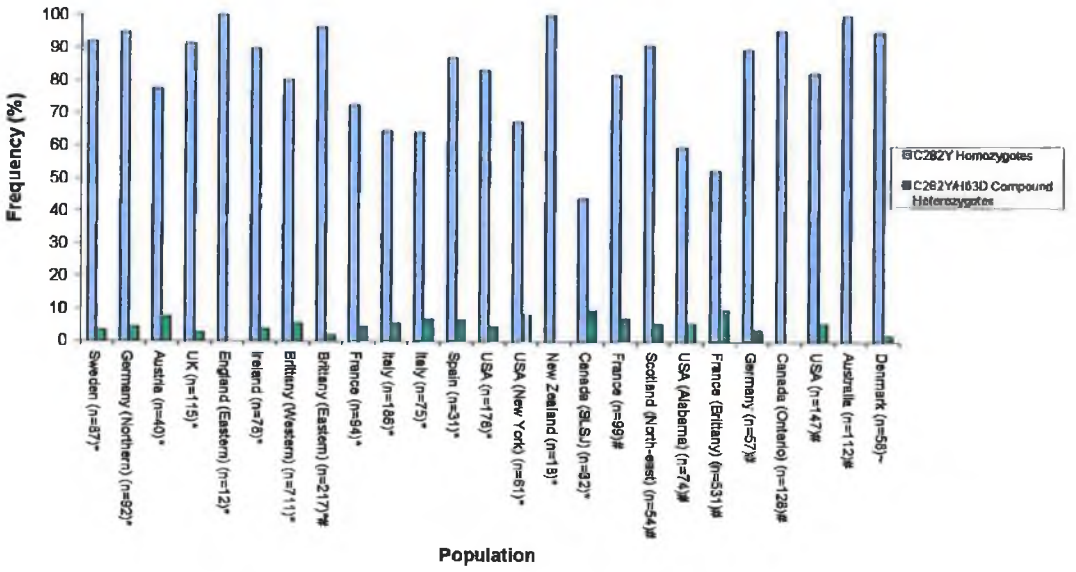


Figure 1.9: Incidence of C282Y homozygosity & C282Y/H63D compound heterozygosity in clinically diagnosed proband populations.

Of the data presented for 25 countries/regions, the frequency of (a) C282Y homozygosity in clinically diagnosed proband populations, ranged from 43.8% in Canada's Saguenay-Lac-Saint-Jean (SLSJ) region to 100% in each of Australia, New Zealand and England (Eastern). The frequency in Ireland was reported at 89.7%. (b) C282Y/H63D compound heterozygosity in clinically diagnosed proband populations, ranged from 0% in England (Eastern), New Zealand, Canada (Ontario) and Australia, to 9.6% in France (Brittany). The frequency in Ireland was reported at 3.8% (see Figure 1.9).

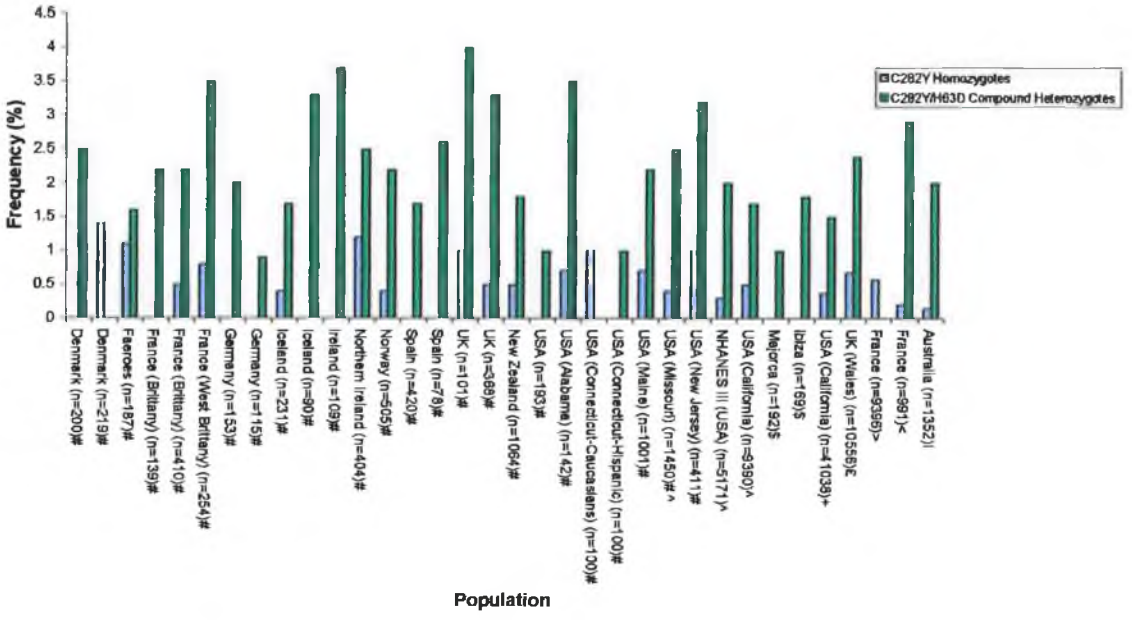


Figure 1.10: Incidence of C282Y homozygosity & C282Y/H63D compound heterozygosity in general populations.

Of the data presented for 25 countries/regions: the frequencies of C282Y homozygosity in general populations, ranged from 0% in 12 out of 25 countries/regions to 1.4% in Denmark (n=219), the frequency in Ireland was reported at 0%, and C282Y/H63D compound heterozygosity in general populations, ranged from 0% in Denmark (n=219), USA (Connecticut-Caucasians) and France (n=9396), to 4% in the UK (n=101). The frequency of C282Y/H63D compound heterozygosity in Ireland and Northern Ireland was reported at 3.7% and 2.5%, respectively (see Figure 1.10).

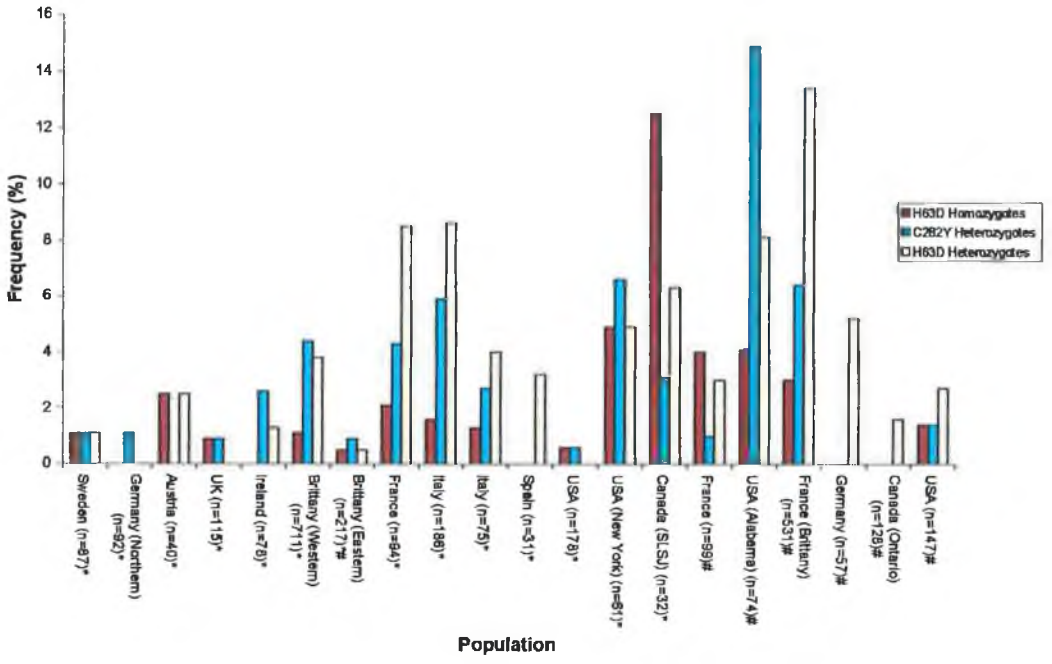


Figure 1.11: Incidence of H63D homozygosity, C282Y heterozygosity & H63D heterozygosity in clinically diagnosed proband populations.

Of the data presented for 20 countries/regions: the frequencies of H63D homozygosity in clinically diagnosed proband populations, ranged from 0% in 5 out of 20 countries/regions to 12.5% in Canada (SLSJ), and the frequency in Ireland was reported at 0%. The frequencies of C282Y heterozygosity in clinically diagnosed proband populations, ranged from 0% in 4 out of 20 countries/regions to 14.9% in USA (Alabama), and the frequency in Ireland was reported at 2.6%. The frequencies of H63D heterozygosity in clinically diagnosed proband populations, ranged from 0% in 3 out of 20 countries/regions to 13.4% in France (Brittany), and the frequency in Ireland was reported at 1.3% (see Figure 1.11).

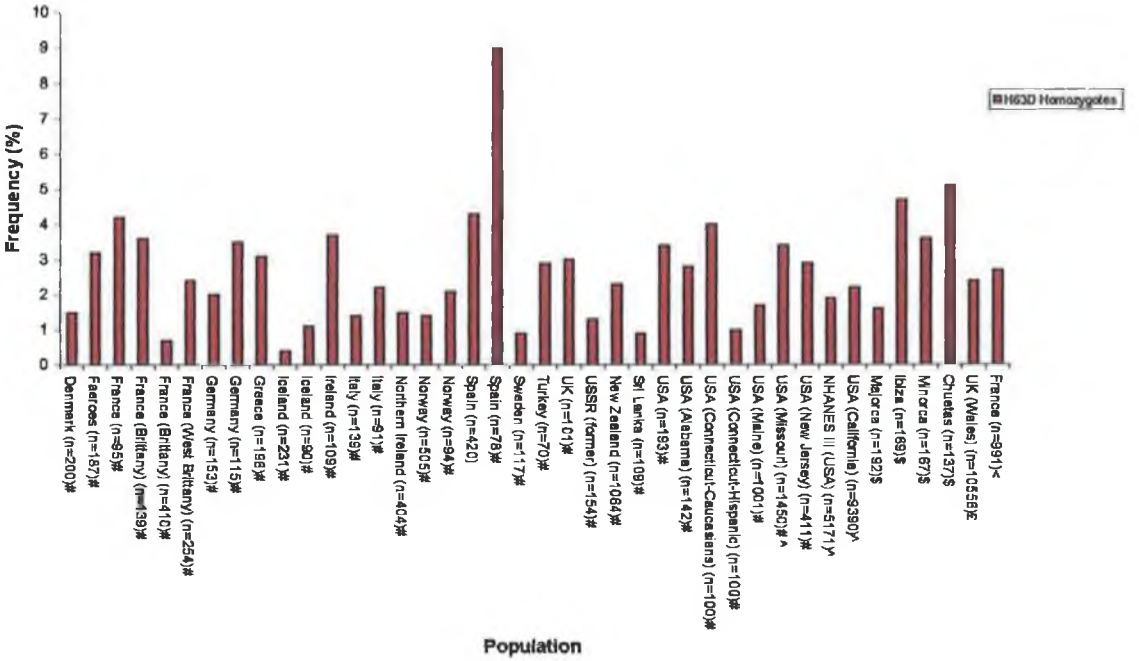


Figure 1.12: Incidence of H63D homozygosity in general populations.

Of the data presented for 40 countries/regions, the frequency of H63D homozygosity in general populations, ranged from 0.4% in Iceland (n=231) to 9% in Spain (n=78). The frequency in Ireland was reported at 3.7% (see Figure 1.12).

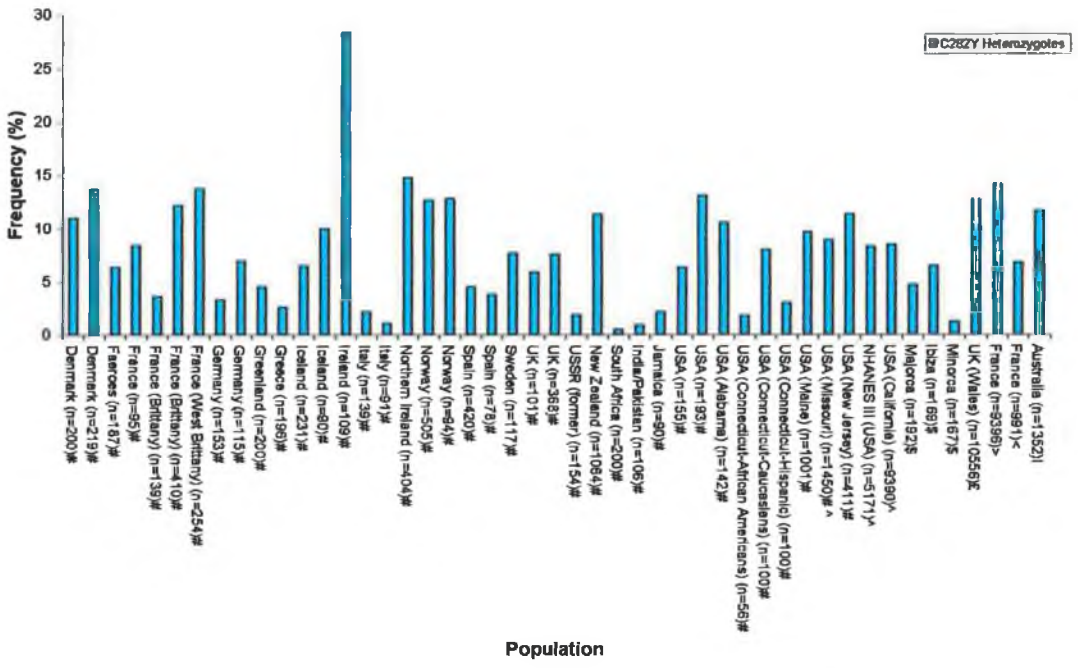


Figure 1.13: Incidence of C282Y heterozygosity in general populations.

Of the data presented for 47 countries/regions, the frequency of C282Y heterozygosity in general populations, ranged from 0.5% in South Africa to 28.4% in Ireland (see Figure 1.13).

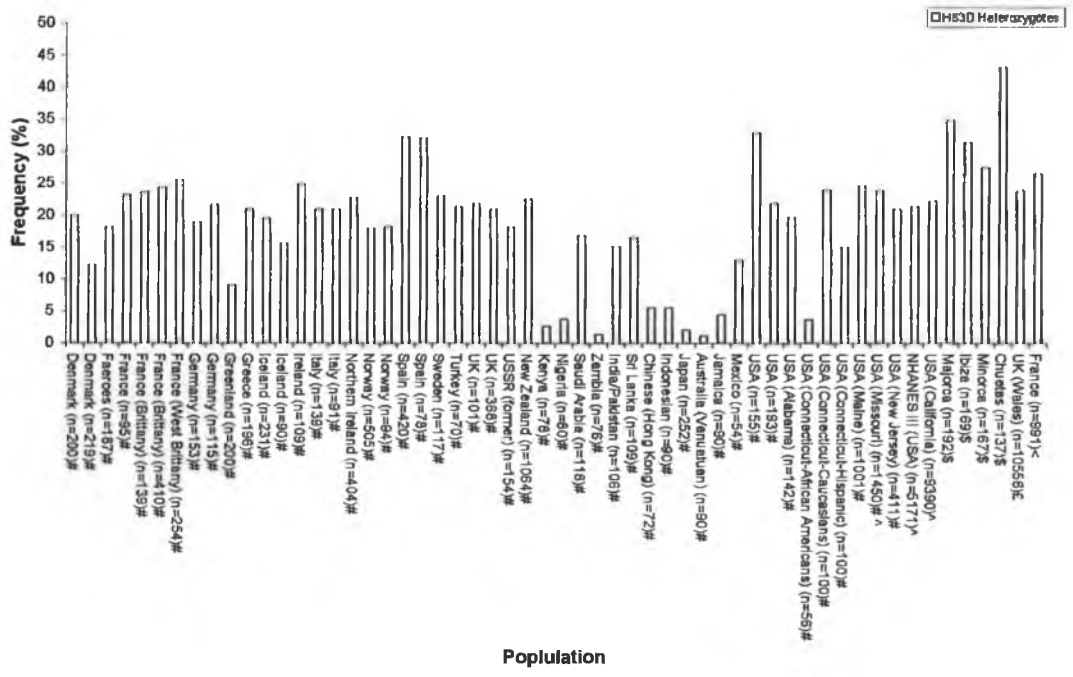


Figure 1.14: Incidence of H63D heterozygosity in general populations.

Of the data presented for 56 countries/regions, the frequency of H63D heterozygosity in general populations, ranged from 1.1 % in Australia (Vanuatuan) to 43.1% in the Chuetas (Majorcan jew) population. The frequency in Ireland was reported at 24.8% (see Figure 1.14). It should be noted, however, that sample population sizes differed in each country/region presented in Figures 1.9 to 1.14.

As discussed previously, results from studies of HFE mutations in North African populations have revealed that the C282Y mutation is extremely rare. Sassi et al., (2004) reported allele frequencies for C282Y and H63D in a Tunisian population to be 0.09% and 15.17%, respectively. Similar allele frequencies for C282Y and H63D (13.2% and 0.9%) were reported in individuals with a North African ancestry, but who were living in Southern France (Aguilar-Martinez et al., 2001).

A study by Guix et al., (2000) reported a C282Y mutation frequency in Majorcans of 2.62 ± 0.8%. Another study Guix et al., (2002) on the prevalence of C282Y and H63D mutations in the Balearic Islands showed the populations were not homogeneous. The C282Y chromosomal frequency was very low in Minorca (1.2%) when compared to the other islands of Majorca (4.7%) and Ibiza

(6.5%). The heterozygote frequency of the H63D mutation in all three islands was similar and quite high (27.5%, 34.9% and 31.4%, respectively).

The frequency of the haemochromatosis C282Y mutation was reported to be 2.59 ± 1.25% in Hungarians, and 1.07 ± 1.20% in Romanies, living in eastern Hungary (Szakony et al., 1999). Miedzybrodzka et al., (1999) reported high allele frequencies for C282Y (8%) and H63D (15.7%) in North-East Scotland. In Sweden, a study by Cardoso et al., (1998) showed the chromosomal frequency for C282Y in the control population was 3.8%, and for the H63D mutation was 12.4%. An allele frequency of 4.8% for C282Y was found in control subjects from Northern Germany (Nielsen et al., 1998). In comparison to these results, Murphy et al., (1998) estimated allele frequencies of 9.9% and 14.10% for the C282Y and H63D mutations, respectively in an Irish population.

The first global population analysis of 2978 people showed a prevalence for C282Y of 1.9% and for H63D of 8.1% (Merryweather-Clarke et al., 1997a). A high C282Y allele frequency of 9.44% has been reported for a population from western Brittany (Jezequel et al., 1998). A review by Mortimore et al., (1999) details that the C282Y mutation is virtually absent in African, Asian and indigenous Australasian populations and has a very low frequency (generally below 1.4%), on the Indian subcontinent and in indigenous Americans. The highest C282Y allele frequency was found in Irish samples and was approximately 10% (Merryweather-Clarke et al., 1997a).

The H63D mutation is frequently observed in European populations at frequencies ranging anywhere between 6.7 and 30.4% (Merryweather-Clarke et al., 1997a). The same mutation was also detected at high frequencies (6.5-9.2%), in North Africans (Roth et al., 1997), Mexicans and Asian Indians. Low frequencies for H63D (between 0.6 and 2.8%) were observed in the following populations; Sub-Saharans, Jamaicans, Vancouver Island Indians, Hong Kong Chinese, Indonesians and Vanuatuans. There was a complete absence of the H63D mutation in the following populations; Senegalese, Taiwanese, Australian Aboriginals and indigenous people form Papua New Guinea and Colombia (Merryweather-Clarke et al., 1997a). Another study by Cullen et al., (1998) reported findings of very low mutational frequencies for C282Y (< 1.07%) in Australian Aboriginal, Melanesian and Polynesian populations. It also reported an absence of the C282Y mutation in Chinese, Javanese, Micronesian and Nepalese individuals.

1.13 Diabetes: A brief history & Classification

The first description of diabetes is usually credited to Arataeus of Cappadocia in Asia Minor in the first century AD, who was also responsible for giving the disease its name. However, the greatest figure in the history of diabetes in the first half of the nineteenth century was undoubtedly Claude Bernard, who discovered that the liver stored glycogen and secreted a sugary substance into the biood. In 1879, Von Mering, a German physician discovered that removing the pancreas caused diabetes. This showed that the pancreas contained an antidiabetic substance later known as insulin. Insulin was first crystallized in 1926 by J.J. Abel (Alberti et al., 1997).

Classification of Diabetes

Diabetes mellitus is a syndrome or a collection of disorders that have hyperglycaemia and glucose intolerance as their hallmarks. The contemporary classification of diabetes was developed by the National Diabetes Data Group of the National Institutes of Health, USA (1979) and later endorsed by the World Health Organization (WHO) Expert Committee on Diabetes (1980), and the WHO Study Group on Diabetes Mellitus (1985). These groups recognized two main types of diabetes, insulin dependent diabetes mellitus (IDDM, type I diabetes) and non-insulin dependent diabetes mellitus (NIDDM, type II diabetes).

Criteria for the diagnosis of Diabetes Mellitus is as follows;

(1) Classic symptoms of diabetes and plasma glucose concentration ≥ 11.1 mmol/L (200 mg/dL) regardless of the time of the preceeding meal
(2) Fasting (no calorific intake for at least 8 hours) plasma glucose level of ≥ 7 mmol/L (126 mg/dL)
(3) 2-hour postioad plasma glucose concentration of ≥ 11.1 mmol/L during the oral glucose tolerance test (200 mg/dL) (American Diabetes Association, 1997)

Diabetes occurs either because of a lack of insulin or because of the presence of factors that oppose the action of insulin. Diabetes is diagnosed by way of a glucose tolerance test. The two most common forms of diabetes are discussed below.

Type I Diabetes (Insulin Dependent Diabetes Mellitus/ IDDM):

This form of diabetes is generally characterized by abrupt onset of severe symptoms and dependence on exogenous insulin to sustain life. Type I diabetes is due to the destruction of β-

cells in the pancreatic islets of Langerhans, which in turn results in loss of insulin production. It is generally thought that a combination of environmental and genetic factors cause an autoimmune attack on the β-cells. The highest incidence of type I diabetes is in Scandinavia, of medium incidence in Europe and the USA and lowest in Oriental or tropical regions (Diabetes Epidemiology research International Group (1990). It is now apparent that type I diabetes is a chronic autoimmune disorder that develops gradually over time, with suggestions that both MHC Class II genes and T lymphocytes are important in the pathogenesis of islet B-cell destruction (Mordes et al., 1987; Rossini et al., 1984). The main defect in type I diabetes appears to be related to the activation of T lymphocytes which then mediates the destruction of the islets. T-cell activation through gene rearrangement may be a close step to the development of type I diabetes in an individual who is genetically predisposed to the disease, and environmental factors may act by triggering or selecting the appropriate T-cell receptor rearrangement (Hoover & Capra, 1987; Ito et al., 1988; Millward et al., 1987). Environmental agents could play several roles in type I diabetes in that they may function as initiating factors i.e. factors which begin or continue the aetiologic processes, which eventually terminate in type I diabetes. Alternatively, they may act as factors that convert pre-clinical diabetes into clinical disease (Bosi et al., 1987; Gamble, 1980).

Type II Diabetes (Non-Insulin Dependent Diabetes Mellitus / NIDDM / Adult-onset diabetes):
Type II diabetes constitutes approximately 90% of all cases of diabetes. People with type II diabetes are not dependent on exogenous insulin for the prevention of ketonuria but they may require insulin for correction of fasting hyperglycemia. The underlying mechanism of this form of diabetes is due either a diminished insulin secretion or increased hepatic glucose output. As many as 98% of cases of Type II diabetes are 'idiopathic', meaning no specific causative defect of the condition has yet been identified. Type II diabetes has a strong genetic basis as evidenced by its frequent familial pattern of occurrence, high prevalence in certain ethnic groups and genetic admixture studies (Kaprio et al., 1992; Newman et al., 1987), however the gene(s) causing most cases of Type II diabetes remain obscure. It is thought that the causes of Type II diabetes lie in environmental and lifestyle factors superimposed on genetic susceptibility (Harris and Zimmet, 1997).

Figure 1.15 demonstrates the role of insulin in development of diabetes. The figure shows that if the body is functioning normally, it is insulin that permits glucose entry to cells where it is subsequently used to provide energy. In the case of Type I diabetes, no insulin is being produced by the body and glucose is therefore not able to enter cells. With Type II diabetes,

insulin is being produced by the body but in insufficient quantities to allow adequate glucose entry to the cells.

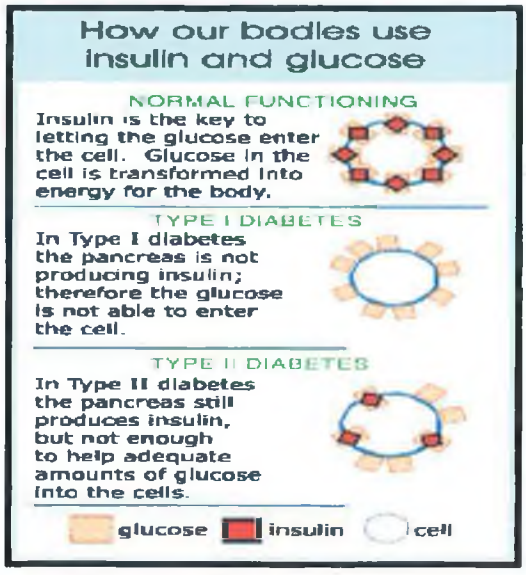


Figure 1.15: Type I & Type II Diabetes Mellitus [Source: http://www.diabetes-and-diabetics.com] Accessed on 25/05/2005.

1.14 Genetics of Diabetes

The main information used to support the hypothesis that diabetes mellitus is a hereditary disorder is its familial incidence. Diabetic individuals have an increased family history of the disease with the frequency of diabetics having positive family histories ranging from 25 to 50%. The relative frequency of a positive family history in non-diabetics is less than 15% (Rimoin & Schimke, 1971). Studies on twins have also confirmed the importance of genetic factors in the aetiology of diabetes. Most investigators have reported a concordance rate of between 45 and 96% for monozygotic twins (i.e. twins that share all genes), and 3-37% for dizygotic twins (twins that share half of their genes), when clinical diabetes was used as the criterion. The theory of genetic heterogeneity proposed in 1967 by Rimoin, was backed up by the following indirect evidence;

- the existence of distinct genetic disorders that have glucose intolerance as one of their features (Rotter et al., 1992).
- genetic heterogeneity exhibited in diabetic animal models

- the ethnic variability in prevalence rate and clinical features
- the clinical variability between individuals e.g. thin, ketosis-prone insulin-dependent juvenile diabetes vs. obese, non ketotic, insulin-resistant, adult-onset diabetes
- physiologic variability i.e. in the plasma insulin levels of individuals.

Direct evidence for genetic heterogeneity came from clinical genetic studies, the results of which suggested that insulin-dependent and non-insulin dependent patients differed genetically within families (Rotter et al., 1978a; Simpson, 1976).

Clinical and genetic evidence from twin and familial studies eventually led to the separation of type I and type II diabetes. Physiologic studies further supported this separation and immunologic studies highlighted the importance of immune mechanisms in the aetiology of Type I diabetes, but not Type II diabetes. Finally, the major argument for aetiologic differences between the two types of diabetes is the consistent association of Type I diabetes with HLA antigens, with approximately 95% of Type I diabetes patients having DR3 or DR4 or both (Nerup et al., 1977; Maclaren et al., 1988; Platz et al., 1981; Rotter et al., 1983; Wolf et al., 1983).

1.14.1 Genetics of Type I Diabetes Mellitus

There is no consensus as to which gene or combination of genes in the major histocompatability complex is responsible for HLA-related susceptibility to diabetes, or other genes outside the HLA region that are involved. Difficulties in analyzing the genetics of Type I diabetes include the reduced penetrance of the disorder, the confounding effect of linkage and association and the heterogeneity within the disorder (Rotter & Rimoin, 1978b). Genetic determinants are important risk factors for Type I diabetes, especially certain histocompatability antigens (HLA) located on chromosome 6. Studies have shown that in Europe and North America around 95% of young Type I diabetes patients have either HLA DR3 or HLA DR4 or both, compared to only 20% of the general population (Nerup, 1976; Cudworth & Woodrow, 1976). Other MHC genes like DPA1 and DPB1 may act as candidate susceptibility determinants for type I diabetes. A positive association has been found between a DPB1 polymorphism and diabetes in a European population but not in other races (Eastel et al., 1990). Susceptibility to Type I diabetes has been associated with the C4A3 allele of the C4 complement locus and an increase in the frequency of C4AQ0/C4BQ0 (null) alleles (Thomsen et al., 1988; White, 1989). It has even been suggested that single gene copies of the C4 genes were a common feature of diabetes-associated

haplotypes (Serguado et al., 1992). Other non-HLA genes that are candidates for susceptibility to type I diabetes include the insulin gene and T-cell receptor genes. Results from comparative mapping studies using animal models of Type I diabetes, propose that a gene residing on chromosome 11q23 might cause susceptibility to type I diabetes in women. This gene was designated as human IDDM2 by the Eleventh International Workshop on Human Gene Mapping (Junien & van Heyningen, 1991). Support has come from animal models of diabetes, for the importance of non-HLA region genes in susceptibility to Type I diabetes. The availability of large numbers of multiple-case pedigrees (Bain et al., 1990; Lernmark et al., 1990) allowed type I diabetes to be one of the first complex disorders subjected to a systematic search for susceptibility loci, using PCR or automated DNA technology. A landmark study by Morton et al., in 1983 confirmed the dominance of MHC-encoded susceptibility (called IDDM1) to type I diabetes. Linkages of Type I diabetes mellitus to chromosomes 11q and 6q were confirmed by replication studies (Hashimoto et al., 1994; Field et al., 1994; Pociot & Nerup, 1993; Barbosa et al., 1982).

1.14.2 Genetics of Type II Diabetes Mellitus

Evidence of a genetic contribution to Type II diabetes mellitus has come from animal studies which suggest that diabetes can be modified by genes other than those directly responsible for obesity and/or diabetes in mice (Coleman, 1982; Coleman & Hummel, 1973; Zhang et al., 1994). Several studies have shown that in populations with a high prevalence of Type II diabetes, the distribution of glucose tolerance may be bimodal. This means that fasting glucose levels appear to be distributed around two distinct mean values. Interpretation of this suggests that there is a major gene which influences glucose tolerance (Elston et al., 1974; Raper et al., 1984; Rushforth et al., 1971; Zimmet & Whitehouse, 1978). However, it should be noted that in most populations, blood giucose values are unimodally distributed due to their heterogeneous nature. Twin and familial studies have long suggested a strong 'genetic' susceptibility to Type II diabetes. Studies in monozygotic twins demonstrate almost complete concordance for Type II diabetes (Pyke, 1979; Barnett et al., 1981), yet the aggregation of clinical disease or glucose levels in families is not consistent with a single, simple mode of inheritance (Rotter & Rimoin, 1981). The most likely explanation for this would be genetic heterogeneity, although environmental factors are known to be important as well. Several studies have suggested that impaired insulin secretion is an early defect in the development of Type II diabetes (Cerasi & Luft, 1967; O'Rahilly et al., 1986; Polonsky et al., 1988; Mitrakou et al., 1992). Genetic susceptibility appears to be the primary

determinant for development of Type II diabetes and in all probability, at least some forms of Type II diabetes require the presence of one or more gene defect to cause clinical diabetes. Genetic defects could influence any of the many steps in glucose metabolism and these defects, alone or in concert with other defects, could result in Type II diabetes mellitus. This makes studying the genetics of Type II diabetes problematic, as this extensive etiologic heterogeneity underlies the disease (Scheuner et al., 1997).

1.15 Summary of incidence (worldwide)

A large number of studies support the theory that the 'diabetes epidemic' is to continue well into the future (King et al., 1998; Wild et al., 2004; International Diabetes Federation 2003; Lefebvre 2002; Amos et al., 1997; Zimmet et al., 2001; Larkin, 2001). Results of a study by Wild et al., on the global prevalence of diabetes were published in 2004. This study utilized data on diabetes prevalence by age and gender from a limited number of countries, to extrapolate to all World Health Organization member states. The data was subsequently applied to United Nations population estimates, to calculate diabetes prevalence for the year 2000 and to make projections of prevalence for the year 2030. The authors estimated the prevalence of diabetes to be 2.8% in 2000 and 4.4% in 2030. This means, that the overall number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. Given that these figures will continue to rise even if obesity levels associated with diabetes mellitus were to remain constant, it is likely the prevalence of diabetes may be underestimated as obesity is on the increase. In 2003 the International Diabetes Federation (IDF) also released estimates of numbers of people with diabetes for 2003 and forecasts for 2025 of 194 million and 334 million, respectively.

Studies on the Irish population (King et al., 1998) estimated a diabetes prevalence of 1.8% for Ireland in the year 1995 and 2000 which would rise to 2.3% in 2025.

1.16 Haemochromatosis and Diabetes

A large number of mutations have been mapped to chromosome 6 (McKusick et al., OMIM, 2006). Most of these mutations can be found on 6p (the short arm of chromosome 6), which is also the area where the HFE gene is localized. It is presumed that only genes on 6p may be in linkage disequilibrium with HFE, but most interestingly the gene causing insulin dependent / type I diabetes (IDDM1), is in close proximity. The chromosomal location of both disorders is band

p21.3 of chromosome 6 (i.e. 6p21.3), however exact locational status of the IDDM1 gene remains elusive, and the status of other genes associated with type 1 diabetes on the long arm of chromosome 6 (6q) remain provisional.

A conclusive answer as to whether the HFE gene is associated with or linked to other disease causing genes on chromosome 6 cannot be obtained until positional cloning of the candidate genes e.g. IDDM1 is conducted. Even though subjects / individuals with haemochromatosis have not been examined prospectively for other chromosome 6 genetic disorders, no suggestion for an association or linkage of the haemochromatosis allele to any other disorder on chromosome 6, other than that concerning diabetes mellitus, has been reported (Sheldon, 1935; Bassett et al., 1981, 1984; Simon et al., 1977, 1980, 1987; Edwards et al., 1981, 1982, 1988; McKusick et al., OMIM; Saddi et al., 1981; Kravitz et al., 1979; Cartwright et al., 1979; Beaumont et al., 1979; Valberg et al., 1980; Dadone et al., 1982; Lalouel et al., 1985; Milman et al., 1994; Borwein et al., 1983, 1984; Bulaj et al., 1996).

Early studies have reported an almost 80% prevalence of diabetes mellitus in haemochromatosis (Finch & Finch, 1966). Studies conducted later reported the prevalence to be about 50 – 60% (Niederau et al., 1985).

Although diabetes meilitus may be common in people with haemochromatosis (it is the most commonly observed endocrine disorder), it does not affect everyone. However, in late-stage haemochromatosis, iron deposition is often visible in the pancreas which leads invariably to pancreatic fibrosis. The severity and frequency of pancreatic dysfunction depends on the degree of iron overload within the body (Milman, 1991), and particularly the proportion of excess iron deposited in the islets of the pancreas. In 1995, a review by Yaouanq indicated that patients with haemochromatosis, who were diagnosed in the preclinical or early iron-loading phase, exhibited a lower prevalence of diabetes mellitus and less severe diabetes than patients with symptomatic iron overload. The clinical spectrum of diabetes mellitus in haemochromatosis is variable, having no consistent pattern of presentation and can range from latent diabetes to non-insulin dependent diabetes to insulin dependent diabetes. Such a wide spectrum of clinical manifestations favours the view that the severity of pancreatic dysfunction and diabetes may be related to the severity of iron deposition in the pancreatic islet cells, and therefore body iron overload (Milman, 1991). This view is supported by the observation that 40-50% of haemochromatosis patients with diabetes

show improved carbohydrate tolerance following phlebotomy therapy. Insulin resistance is also markedly improved after this therapy (Dymock et al., 1972; Stremmel et al., 1988).

The prevalence of diabetes in people with clinically overt haemochromatosis has been reported as 40-55% (Milman, 1991; Niederau et al., 1985; Yaouanq et al., 1990), whereas it was only 13-17% in people with biochemical evidence of haemochromatosis without clinical signs (Yaouanq et al., 1990; Adams et al., 1991b). Homozygosity for the C282Y mutation occurs with a presumed prevalence of 0.4 - 0.5% and type 1 diabetes mellitus occurs with a similar lifetime cumulative prevalence of approximately 0.4% (Bain et al., 1997). This, combined with the high prevalence of diabetes in patients with haemochromatosis, meant that it seemed reasonable to postulate a genetic linkage between the two conditions (Dymock et al., 1972; Balzercak et al., 1968; Pozza & Ghidoni, 1968; Saddi & Feingold, 1974). Although there is no documented evidence of a linkage between the haemochromatosis locus and the IDDM1 gene, the disorder is strongly associated with HLA-haplotypes known as 'low-risk' markers for type I diabetes, and there is even a suggestion that HLA-A3, B7 may have a protective effect against type I diabetes through its significant association with the aforementioned markers.

The close association between diabetes and haemochromatosis is well established (Fernandez-Real et al., 2002) with hepatic cirrhosis, damage to pancreatic beta-cells and genetic predisposition being the major causal factors of impaired glucose tolerance and diabetes in haemochromatosis (Niederau et al., 1981, 1984, 1985; Saddi & Feingold, 1974; Strohmeyer et al., 1976; Saudek, 1980). Yaouanq's study in 1990 showed that diabetics have twice as much mobilisable iron as non-diabetic patients. The decreased prevalence of haemochromatosis-associated diabetes over the last few decades is mainly due to higher numbers of people being diagnosed in the early stages, when glucose metabolism is still normal or only slightly abnormal. The prevalence of unrecognized haemochromatosis in diabetics has been estimated at 5-8 cases per 1000 in Europe (O'Brien et al., 1990; Czink & Tamas, 1991; Singh et al., 1992) and 9.6 cases per 1000 in Australia (Phelps et al., 1989), therefore it has been recommended that people with diabetes should also be evaluated for the possible presence of haemochromatosis.

Non-insulin dependent diabetes mellitus (NIDDM / Type II diabetes) is a common complication of diseases of iron overload such as haemochromatosis, with 53-80% of individuals with haemochromatosis developing diabetes (Witte et al., 1996). Salonen et al., (1999) conducted a study which showed an association between body stores of iron and the incidence of diabetes.

Their data supported the theory that increased iron stores even in the range not considered to be associated with haemochromatosis, contribute towards development of Type II diabetes.

A study by Tuomainen et al., (1997) proposed that three mechanisms may associate between an elevated iron status and an increased risk of type II diabetes; (a) the effect of iron on insulin synthesis and excretion and the pancreatic cells, (b) the oxidation of free fatty acids by free radicals leading to decreased utilization of glucose by muscle tissue, which in turn leads to increased insulin resistance and (c) accumulation of iron in the liver, which interferes with its capacity to remove insulin from the blood.

In 1999, a large study was conducted by Ford & Cogswell, to investigate whether non-haemochromatotic individuals with elevated iron levels had a higher risk of type II diabetes. It reported an increased risk of newly diagnosed diabetes in both sexes, with elevated serum ferritin concentrations. However, the cross-sectional nature of this study, compounded by the fact that both risk factor and outcome were measured at the same time point, means it is impossible to determine whether it is the elevated serum ferritin (an acute phase protein) causing the diabetes, or diabetes-induced inflammation causing elevated serum ferritin.

Niederau et al., (1985) reported that patients with hereditary haemochromatosis are at increased risk of type II diabetes and are seven times more likely to die from diabetes than the general population. However, there is conflicting evidence as to whether there is an increased risk of type II diabetes in C282Y heterozygotes (Salonen et al., 2000). A study by Jiang et al., (2004) showed that higher iron stores (reflected by an increased ferritin concentration and a lower ratio of transferring receptors to ferritin), are associated with an increased risk of type II diabetes in healthy women independent of known diabetes risk factors. A study in 2001 evaluated a possible association between HFE mutations and either chronic liver disease or type II diabetes. Three hundred and twenty seven individuals were tested for the C282Y, H63D and S65C mutations of haemochromatosis. One hundred individuals from this tested population had type II diabetes. Genotypic analysis of this group (n = 100), showed that 3% were homozygous for H63D, 27% were heterozygous for H63D, 1% were heterozygous for S65C and the remaining 69% had none of the three haemochromatosis mutations tested for (Campo et al., 2001).

Prior to the era of HFE gene testing, the most convincing data in support of screening a diabetic population for haemochromatosis was published by Phelps et al., (1989). The biochemical based

screening strategy employed in their study, revealed a prevalence rate for hereditary haemochromatosis of 9.6 per 1000 in diabetic individuals. A similar but separate study conducted in the U.K reported a prevalence rate of 0.5% (5 per 1000), in diabetics tested (George et al., 1995). Subsequent to the availability of HFE testing numerous case-control studies have investigated the prevalence of HFE mutations among diabetic patients, compared to gender- and age matched non-diabetic controls (Dubois-Laforgue et al., 1998; Frayling et al., 1998; Sampson et al., 2000; Braun et al., 1998; Fernandez-Real et al., 1999 and Florkowski et al., 1999). The Spanish study (Fernandez-Real et al., 1999), found a significantly increased prevalence of the H63D allele in diabetics and serum ferritin levels were also significantly higher in type II diabetics that carried HFE mutations. A study by Kwan et al., (1998) found that there was an increased prevalence of the C282Y mutation in type II diabetics compared to individuals with type I diabetes (reported incidence rates of 21.9% versus 11.7%). In 2001, Moczulski et al., conducted a study on the role of C282Y and H63D mutations in the development of type II diabetes and diabetic nephropathy. Their results showed a significantly increased frequency of the C282Y mutation in diabetics when compared to the controls. They also observed an increased frequency of the H63D mutation in diabetics with microalbuminuria, which suggests that this mutation may play a role in susceptibility to diabetic nephropathy. Ellervik et al., (2001) concluded from a recent, large retrospective study in Denmark, that all individuals with late-onset type I diabetes should be screened for hereditary haemochromatosis.

Serum ferritin levels are increased in individuals with type II diabetes (in the absence of a reciprocal decrease of soluble transferrin receptor levels), thereby suggesting that the elevated ferritin levels seen in diabetics are a result of an inflammatory mechanism rather than due to iron overload (Hernandez et al., 2004).

Some 'typical' symptoms of haemochromatosis like diabetes and arthritis may be no more common in C282Y homozygotes than in matched controls (Adams, 2005c).

Tables 1.0 & 1.1 below, show the incidence of HFE mutations in both diabetic populations and in populations without diabetes (controls), and conversely, the prevalence of diabetes mellitus in haemochromatotic populations. Figure 1.16 illustrates the survival rates of individuals with each permutation of the two conditions i.e. haemochromatosis and diabetes mellitus.

Table 1.0: Screening studies of HFE mutations in diabetics compared with controls [Source: DuBois & Knowdley, Alimentary Pharmacology & Therapeutics 2004;20(1):1-14]

Author	Year	Sample size (N)	Population	HFE mutations
Sampson et al.,	2000	- 220 normal subjects - 220 type II diabetes mellitus (DM2)	England	No difference
Fernandez-Real et al.,	1999	- 108 normal subjects - 170 DM2	Spain	No difference in C282Y; increased frequency of H63D allele in DM2 (22.64 ± 4.4 % versus 15.7 ± 0.9 %)
DuBois-Laforgue et al.,	1998	- 87 normal subjects - 184 DM2	France	No difference
Frayling et al.,	1998	- 215 normal subjects - 238 DM2	England	No difference
Kwan et al.,	1998	- 103 type I diabetes mellitus (DM1) - 105 DM2	Canada	Increased C282Y frequency in DM2: (21.9% DM2, 11.7% DM1)
Florkowski et al.,	1999	- 1064 normal subjects - 230 DM2	New Zealand	No difference
Braun et al.,	1998	- 355 normal subjects - 401 DM2	Germany	No difference

Table 1.0 shows that only two out of seven studies reported a difference in the incidence of HFE mutations in individuals with diabetes compared to that of control individuals. One study reported an increased H63D allele frequency in individuals with type II diabetes mellitus (Fernandez-Real et al., 1999). Other workers have reported an increased C282Y frequency in individuals with type II diabetes mellitus (Kwan et al., 1998).

Table 1.1: Prevalence of diabetes mellitus in primary haemochromatosis [Source: Strohmeyer & Niederau. Diabetes mellitus and haemochromatosis in Barton & Edwards Haemochromatosis: Genetics, pathophysiology, diagnosis and treatment. Cambridge University Press 2000]. * Indicates review from previous literature.

Reference	Year	Number of patients (n)	Prevalence of diabetes (% of patients)
Sheldon	1935	311*	82
Butt & Wilder	1938	30	87
Finch & Finch	1966	707*	78
Wöhler	1964	1500*	76
Dymock & Williams	1971	115	63
Stocks & Powell	1973	33	63
Saddi & Feingold	1974	96	69
Strohmeyer et al.,	1976	40	70
Niederau et al.,	1981	74	62
Niederau et al.,	1985	163	55
Yaouanq et al.,	1990 & 1995	474	40
Niederau et al.,	1996	251	48
Adams et al.,	1991a	85	21

Table 1.1 shows that between 21 and 87% of individuals with haemochromatosis have diabetes mellitus also.

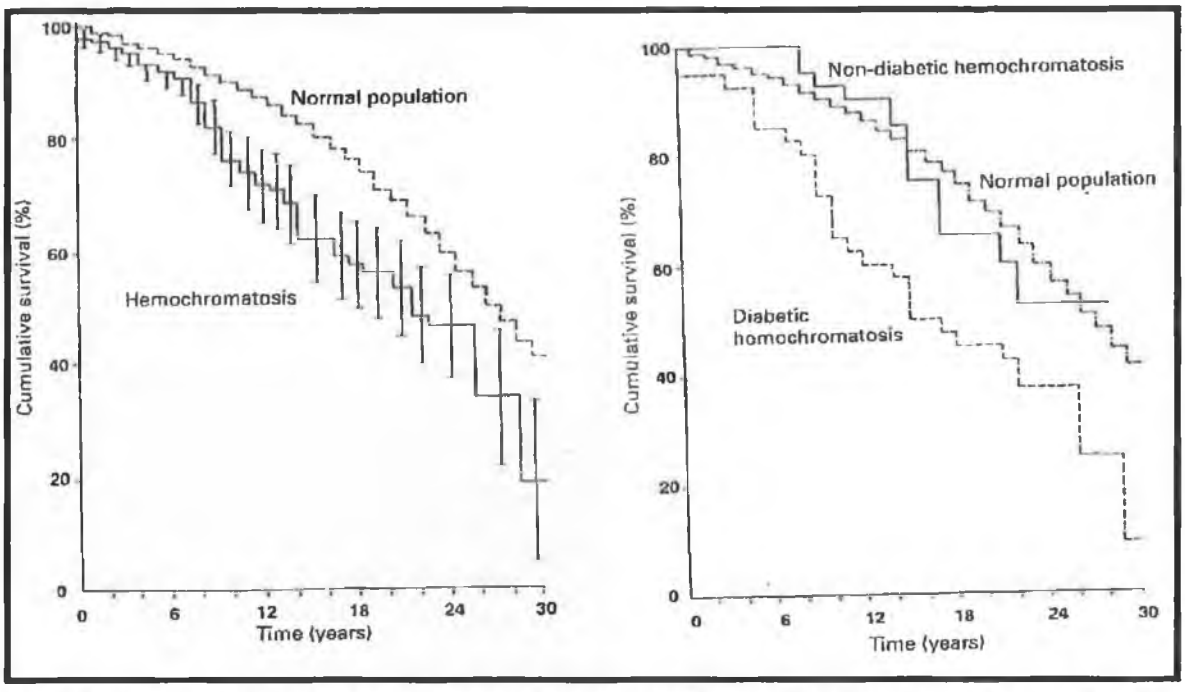


Figure 1.16: Cumulative survival rates of patients with haemochromatosis, haemochromatosis with and without diabetes mellitus and the normal population i.e. individuals that do not suffer with haemochromatosis or diabetes or both. [Adapted from Niederau et al. Long-term survival in patients with hereditary hemochromatosis. Gastroenterology 1996;110:1107-19. by Niederau & Strohmeyer. Survival in hemochromatosis in Barton & Edwards Hemochromatosis: genetics, pathophysiology, diagnosis and treatment. Cambridge University Press 2000].

Figure 1.16 shows that patients with haemochromatosis have cumulative survival rates lower than those of the normal population. It also shows that non-diabetic haemochromatosis -affected individuals have higher cumulative survival rates than those of diabetic haemochromatosis – affected individuals (over a 30 year period).

Although the pancreas is a site of heavy iron deposition in haemochromatosis, the diabetes that occurs in haemochromatosis is the result of inherited genetic factors and cirrhosis as well as islet cell dysfunction due to iron overload (Stocks & Powell, 1973). A review by George & Powell (1997) states that there is a definite trend towards increased haemochromatosis rates in diabetic populations. Prevalence rates between 1 in 100 and 1 in 200 have been confirmed by liver biopsy (George et al., 1995) but proving statistical significance between the two conditions is difficult.

1.17 Information on incidence of haemochromatosis and diabetes in Ireland

Ireland has a reported C282Y homozygote frequency of 1 in 83 (Ryan et al., 2000, Gleeson et al., 2004).

Although the biochemical expression of the C282Y mutation is high in individuals with hereditary haemochromatosis, the clinical expression has not been fully investigated. A study by Gleeson et al., (2004), was conducted to determine the clinical, biochemical and histological penetrance of the C282Y homozygous mutation in Irish individuals originally identified by family screening. Results showed that 43.4% of males and 23.3% of females had elevated transferrin saturation and ferritin levels. Approximately one third (32.3%) of male subjects also exhibited abnormal liver enzyme levels and diabetes was noted in 2.8% of males.

Research into haemochromatosis by Kelleher et al., (2004) stated that genetic variability (i.e. polymorphisms), within the DMT 1 gene, are not responsible for phenotype variability in hereditary haemochromatosis.

A study by Ryan et al., (1998) showed that thirty-one out of 109 randomly sampled individuals from a hospital were heterozygous for the C282Y mutation when tested. This correlates to a C282Y allele frequency of 14% and a heterozygote or 'carrier' frequency of approximately 1 in 4. This is the highest allele frequency reported in a control population and supports the theory of a Celtic origin for the genetic condition. The prevalence of C282Y was assessed in a cohort of thirty haemochromatosis patients and 404 normal controls. Ninety per cent of the haemochromatosis patients were homozygous for C282Y and there was an increased frequency of the C282Y mutation in the normal population (17.3%), which was also reflected in the higher frequency of C282Y homozygotes (1.24%), (Murphy et al., 1998).

Merryweather-Clarke et al., (1997(a)) found the highest global prevalence of the C282Y allele in 45 Irish individuals (10%). They also found a high H63D prevalence of 30.4% in 28 Basque individuals.

A study on the genetics and pathophysiology of hereditary haemochromatosis in Ireland was completed by Valerie Byrnes in 2002. The study analysed a random sample of neonates (n=1000) from the Irish population, for genotypic and phenotypic frequencies of hereditary

haemochromatosis. Genotypic results for 800 neonates reported C282Y and H63D allele frequencies of 10.7% and 15.1%, respectively. The allele frequency of approximately 11% is the highest globally reported result to date. The study also reported that 1% of the sample population had the genetic susceptibility to develop the phenotype of hereditary haemochromatosis. If this figure is extrapolated to represent the Irish population in general, an estimate of 1 in every 83 Irish people is genetically susceptible to developing iron overload. The high C282Y allele frequency in the Irish population and its close linkage to hereditary haemochromatosis indicate that C282Y genotyping is the preferred screening strategy for the disease in Ireland (Byrnes et al., 2001).

Taking the following factors into account; (a) that Ireland has the highest C282Y allele frequency reported worldwide, (b) that there is repeated suggestion of an association of the haemochromatosis allele and diabetes and (c) that the prevalence of diabetes in individuals with haemochromatosis is high, it is reasonable to propose an investigation into the potential interrelatedness of the two conditions be conducted.

1.18 Research Question / Objectives of the Study

(1) To determine the incidence of the C282Y, H63D & S65C haemochromatosis mutations in a population from the North West of Ireland.

(2) To determine if there is an increased incidence of these mutations in a diabetic population from the same region.

2.0 MATERIALS & METHODS

2.1 Materials

DNA Purification

Wizard™ Genomic DNA Purification Kit:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Cat. # A1120.

Eppendorf Safe-Lock Microtubes, 1.5ml capacity, PCR clean:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: Z60,631-6 (1000 each).

PCR

Human Genomic DNA:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Cat.# G3041.
This DNA is sourced from multiple anonymous donors. The genomic DNA is purified by the method described by Sambrook *et al.*, (1989). Greater than 90% of the DNA is longer than 50kb in size as measured by pulsed-field gel electrophoresis. The DNA is suitable for Southern Blot hybridisations, genomic analyses (including PCR), and genomic library construction. Concentration: 173 µg/ml.

Human Placental DNA Ultrapure:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: D 4642.
42 % GC; Tm 87°C, purified by equilibrium buoyant density ultracentrifugation in caesium chloride, dialysed against a solution of 1mM sodium chloride, 1mM EDTA and 1mM Trizma HCl, pH 7.5 and lyophilised at a concentration of approximately 2 units per ml. Approximately 20 A_{260} units per mg DNA. % GC is the percentage of G/C base pairs. Tm is the temperature at the midpoint of the thermal denaturation profile. The DNA quality and integrity are evaluated using pulsed field gel electrophoresis and visualisation after ethidium bromide staining. The DNA is free of RNA and contaminants that might interfere with restriction endonuclease digestion. Mol wt. 10 MDa (= 16 kb).

Mineral Oil for molecular biology:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: M 5904.
Suitable for overlaying aqueous reactions and centrifuge gradients, free from DNase, RNase, protease, density of 0.84 g/ml.

ReadyMix™ Taq PCR Reaction Mix without $MgCl_2$ kit:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: P4475.
ReadyMix Taq PCR Reaction Mix is a prepared solution containing everything needed for a PCR reaction except the specific primers and template. The mix includes Taq DNA Polymerase, 99% pure deoxynucleotides and buffer in a 2x optimized reaction concentrate. For reaction set-up, add the ReadyMix (25 µl) to the primers, template and water (total volume 50 µl). Using ReadyMix Taq PCR Reaction Mix reduces pipetting steps and risk of contamination. This saves time and reduces errors while still providing the great performance of Sigma's Taq Polymerase.

- Amplifies targets up to 7 kb in length
- A separate vial of 25 mM $MgCl_2$ is provided for optimization with the ReadyMix Taq PCR Reaction Mix without $MgCl_2$.

- ReadyMix Taq PCR Reaction Mix offers the same performance as Taq DNA Polymerase in a more convenient design.

Supplied with a vial of PCR grade water for dilution. Unit definition: one unit incorporates 10 nmol of total dNTP's into acid-precipitable DNA in 30 min. at 74°C, Concentration is 1.5 units/reaction (50 µl reaction volume). The kit contains:

- PCR Ready-Mix solution, Product No. P 0351, contains 20mM Tris-HCl (pH 8.3), 100mM KCl, 0.4mM dNTP mix (dATP, dCTP, dGTP, dTTP), stabilizers, 60 units Taq Polymerase/ml. Provided as 20 reactions, 5 x 20 reactions.
- 25mM Magnesium Chloride, Product No. M 8787, provided as 1 vial
- Sterile Water, Product No. W 1754, provided as a 1.5ml vial.

PCR Master Mix:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Cat. # M7502.
PCR Master Mix includes Nuclease-Free Water and PCR Master Mix, 2X. PCR Master Mix is a premixed, ready-to-use solution containing Taq DNA Polymerase, dNTP's, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. PCR Master Mix, 2X contains 50 units/ml of Taq DNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$.

Primers:

The following primers were manufactured by TAGN Ltd., Unit 11b Station Approach, NE11 OZF, Gateshead, U.K.

C282YF: 5'-TGG CAA GGG TAA ACA GAT CC-3'
C282YR: 5'-TAC CTC CTC AGG CAC TCC TC-3'
H63DF: 5'-ACA TGG TTA AGG CCT GTT GC-3'
H63DR: 5'-CTT GCT GTG GTT GTG ATT TTC C-3'

See Appendix 1 for Certificate of Analysis.

Thermo Hybaid Omne E thermal cycler:
Hybaid Ltd., 111-113 Waldegrave Road, Teddington, Middlesex TW II 8LL, United Kingdom.

Restriction Endonuclease Digestion

Nde II, restriction endonuclease:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Product Code: R7291.
Source: *Neisseria dentrificans,*
Recognition sequence: 5'...▼ GATC...3'
3'...CTAG▲ ...5'

Rsa I, restriction endonuclease:

Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Product Code: R6371.
Source: *Rhodopseudomonas sphaeroides*,
Recognition sequence: 5'...GT▼ AC...3'
3'...CA▲ TG...5'

Hinf I, restriction endonuclease:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Product Code: R6201.
Source: *Haemophilus influenzae Rf (1)*
Recognition sequence: 5'...G▼ ANT C...3'
3'...C TNA▲ G...5'

PCR tubes, 0.2ml thin-walled tubes with flat caps, RNase, DNase, DNA and pyrogen free. Molecular Bioproducts, San Diego, California, USA. Product Code: 3412.

Controls

Positive control samples for C282Y homozygosity and C282Y/H63D compound heterozygosity were supplied by the Haematology Department of Sligo General Hospital. Positive control samples for S65C heterozygosity and S65C/H63D compound heterozygosity were supplied by Dr. Sharon Barrett (Centre for Liver Disease, 55 Eccles Street, Dublin 7).

Agarose Gel Electrophoresis

Water:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: W 4502.
Molecular Biology reagent, CAS # 7732-18-5, EC no. 231-791-2, 0.2 µm filtered, DNase and RNase free.

TBE Buffer, 10X (Tris-borate-EDTA):
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Cat. #H5241.
TBE buffer, 10X (pH 8.3), is used for polyacrylamide and agarose gel electrophoresis and has been optimized for use in DNA applications. Composition: 890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA.

Agarose:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: A 9539.
Molecular Biology reagent, CAS # 9012-36-6, EC no. 232-731-8. Agarose is ideal for routine analysis of nucleic acids by gel electrophoresis or blotting (Northern or Southern) and is also suitable for protein applications such as Ouchterlony and radial immunodiffusion (RID), has low ethidium bromide and SYBR Green background staining, ≤ 10 % water, ≤ 1.0 % ash, DNase and RNase free, EEO of 0.09-0.13 and a gel point of 36°C (± 1.5°C),

50 bp DNA Step Ladder:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Product Code: G4521.
The 50 bp DNA Step Ladder consists of 16 DNA fragments ranging from 50 bp to 800 bp in exactly 50 bp increments. The 800 bp band appears 2-3 times more intense than all other bands, which are of approximately equal intensity. Concentration: 0.34 µg/µl.

Lambda DNA Hind III digest buffered aqueous solution (for molecular biology):
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: D 9780.
Concentration: 334µg/ml, Contains 8 fragments, 125-23,130 bp, solution in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA,

Blue/Orange Loading Dye, 6X:
Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA. Product Code: G1881.
Blue/Orange Loading Dye, 6X, is a marker dye containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0). It is provided in a premixed, ready-to-use form. The dye is used for loading DNA samples into gel electrophoresis wells and tracking migration during electrophoresis. In a 0.5-1.4% agarose gel in 0.5X TBE, xylene cyanol FF migrates at approximately 4kb, bromophenol blue at approximately 300 bp and orange G at approximately 50 bp.

Ethidium bromide:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: E 8751.
(2, 7-Diamino-10-ethyl-9-phenylphenanthridinium bromide; EtBr; 3, 8-Diamino-5-ethyl-6-phenylphenanthridinium bromide; Homidium bromide, [1230-45-8], $C_{21}H_{20}N_3Br$, FW = 394.3), Ethidium bromide (EtBr) is a nucleic acid intercalating agent and frameshift mutagen. It can also be used in conjunction with acridine orange to differentiate between viable, apoptotic and necrotic cells. It is the most commonly used nucleic acid stain for PAGE or agarose gel electrophoresis. EtBr intercalates double-stranded DNA and RNA. The fluorescence of EtBr increases 21-fold upon binding to double-stranded RNA and 25-fold on binding double-stranded DNA so that destaining the background is not necessary with a low stain concentration (10 µg/ml). EtBr has been used in a number of fluorimetric assays for nucleic acids. It has been shown to bind to single-stranded DNA and triple-stranded DNA. For staining a gel after electrophoresis, dilute a sample of stock solution to 0.5 µg/ml with water and incubate the gel for 15-30 min. Destaining is usually not needed but can be carried out in water for 15 min if decreased background is necessary. The DNA bands can then be detected on a UV light box (254 nm wavelength). EtBr can also be incorporated into the gel and running buffer at 0.5 µg/ml and visualised immediately after electrophoresis.

Electrophoresis Power Supply, EPS-250 Series II:
C.B.S. Scientific Co. Inc., Del Mar, California, USA.

Horizontal Electrophoresis unit (Maxi):
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: Z33,882-6.

Comb, 28 Sample wells, 1.0mm, for 20cm tray:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: Z33,934-2.

Syngene DigiGenius Documentation System:
Syngene, Beacon House, Nuffield Road, Cambridge C4 ITF, United Kingdom.

Consumables

Pipette Tips (for Hamilton SoftGrip pipettes, precision pipette tips):
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland.

- 1ml volume, Product Code: P6733 (1000 each),
- 200µl volume, Product Code: P5983 (1000 each),
- 300µl volume, Product Code: P6358 (1000 each),
- 10µl volume, Product Code: P5608 (1000 each),
- 10µl volume, filter tips, Product Code: P5858 (1pkg)

Decontamination / Contamination Prevention

DNA Away:
Molecular Bioproducts, San Diego, California, USA. Product Code: 7010

Ethidium bromide extraction:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: Z361569-2 EA.
An extractor device manufactured by Schleicher & Schuell as a way to safely remove ethidium bromide from nucleic acid staining buffer waste for easy disposal. It consists of two layers of specially formulated activated carbon filters sealed into a polypropylene housing. Using a simple vacuum filtration procedure, >99% of ethidium bromide is removed from up to 10 liters of staining solution through adsorption to the activated carbon matrix. The decontaminated filtrate can then be safely disposed of down the drain.

Marigold™ Suretouch vinyl gloves:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland. Product Code: Z30,163-9
Small, powder-free with low chloride, sulphur and sodium ion levels, they provide minimum particulate contamination with good sensitivity, grip and tear resistance.

Instrumentation / Apparatus

Hamilton SoftGrip Pipettes:
Sigma-Aldrich Ireland Ltd., Airton Road, Tallaght, Dublin 24, Ireland.

Micro-Centrifuge
Sanyo MSE Micro Centaur, Sanyo Biomedical (previously Sanyo Gallenkamp), 1062 Thorndale Avenue, Bensenville, Illinois 60106, USA.

Incubators (37°C and 65°C)
Memmert Model 600, Memmert GmbH + Co. KG-91107 Schwabach, Germany.

Methods

2.2 Study Design

In the initial stages of study design, a statistician was consulted to determine the appropriate population sample size. Using the sample size formula for a single proportion or percentage, which allows for 80% power and a 5% two-sided significance, and an increase / decrease of 100% from the prevalence documented for the general Irish population (Kelleher & Crowe, 2001), N was calculated to be 1530 individuals / subjects (N=765, for each of the 'control' and diabetic groups). In actuality, the population sample size employed in the study, was N=500. The main reasons for deviating from the calculated N value, were limitations on the time period for analysis, available personnel and equipment constraints and lack of willing participants. The original study design was aimed at genotypic analysis for two haemochromatosis mutations, C282Y and H63D, but plans for analysis of a third mutation (S65C) were incorporated at a later date. Randomisation of study subject's for both populations, was achieved as a result of both the volunteer nature for study participation and the method of sample selection.

2.3 Ethics

Prior to study initiation, full ethical approval for the study was obtained from the Ethics Committee of Sligo General Hospital by submission of the requisite application (see Appendix A).

2.4 Research Subject Recruitment

Initial recruitment of research subjects (diabetic population) took place in the diabetic clinic of the Out Patient department of Sligo General Hospital and participation was on a volunteer basis. Study participation was by way of informed, documented consent (following review of an information sheet about the study by individuals, and by their subsequent signature of consent forms – See Appendix A). The following inclusion criteria for subjects was also applied; aged greater than 20 years, residence in the North West region of Ireland, and of a Celtic/North European ancestry or origin. Patients who had recent excessive blood loss or blood transfusion, were pregnant, aged less than 20 years or of a non-Celtic/North European ancestry were excluded from the study. Due to project time constraints and the limited availability of suitable collection personnel, only twenty six diabetic subjects were recruited in this manner (n=26), it was therefore decided that the only way to increase the sample size of the test population to

significant levels was to include 224 blood samples sent to the laboratory (for routine HbA1c testing) from patients with diabetes mellitus.

'Control' subject samples were obtained by selecting (N= 250), randomly from blood samples entering the aforementioned laboratories for routine haematological testing. All details regarding samples obtained in this manner were anonymous and were blind to the tester, save for their hospital number (blinded to the researcher), which ensured duplication of samples did not occur during analysis.

2.5 Blood / Serum Samples

Peripheral blood samples were collected by qualified hospital personnel in accordance/compliance with routine clinical procedures in place at Sligo General Hospital. The blood was collected into EDTA-coated tubes during routine attendance of research subjects at Out Patient departments in Sligo General Hospital. All blood samples underwent routine processing procedures on arrival at Sligo General Hospital Laboratories. Whole blood samples, which were to be used in the study, were subsequently located and stored frozen at -20°C in a designated area, prior to future genotypic analysis. The 250 whole blood samples collected from each of the diabetic and 'control' populations were numbered sequentially from 1 to 250 and C1 to C250, respectively. The purpose of this was to permit identification of samples in the two populations.

2.6 TBE Buffer Preparation

Tris-Borate EDTA (TBE) Buffer at a 1X concentration and with an ethidium bromide concentration of 0.5 µg/ml was used in this study: (a) as a running buffer for gel electrophoresis and (b) for gel preparation. The buffer was supplied from the manufacturer as a 10X concentrate and a 1:10 dilution was therefore necessary before usage. Two litres of buffer was prepared freshly at various intervals, as required during analysis. This was performed by adding 200 mls of 10X TBE concentrate and 100 µl of ethidium bromide (stock solution concentration 1 mg/ml) to 1799.9 mls of molecular grade water in a 2000 ml volumetric flask. A homogenous solution was then ensured by inverting the flask at least 30 times to mix the contents thoroughly. By incorporating ethidium bromide at this stage it eliminated the need for staining and destaining of gels after electrophoresis. TBE Buffer was subsequently stored at room temperature.

2.7 *Agarose Gel Preparation*

Agarose gels of two different concentrations were used in this study and were prepared in the following manner:

- 1% Agarose Gels: were prepared by the dissolution of 3.5 grams of molecular grade agarose in 350 millilitres of 1X TBE buffer (see Section 2.5) in a clean beaker. Heat in the form of a hotplate and continuous stirring was utilized to ensure a homogenous solution. The agarose gel was ready to pour when it went completely clear in colour. 1% agarose gels were used in Quantitation of DNA Yield post DNA purification procedure.

- 2% Agarose Gels: were prepared by the dissolution of 7 grams of molecular grade agarose in 350 millilitres of 1X TBE buffer (see Section 2.5) in the same manner as that employed for the preparation of 1% gels (see above). 2% agarose gels were used in PCR Optimisation procedures and Restriction Enzyme Digestion Analysis.

2.8 *Molecular Weight Marker Preparation*

Two molecular weight markers were employed in this study to help identify restriction fragments of varying sizes during mutational analysis. The markers were a 50 bp DNA Step Ladder (Promega) and a Lambda Hind III digest. Before use the markers were prepared in the following way:

(a) DNA Step Ladder: 5 µl of a 50 bp DNA Step Ladder was added to 1 µl of 6X gel loading dye (as per manufacturers instructions) and was mixed by gentle pipetting. 2 µl of the resultant mixture was added to each of two wells of the gel at varying positions to function as a molecular weight marker.

(b) Lambda Hind III Digest: 5 µl of Lambda Hind III Digest was added to 5 µl of molecular grade water and 2 µl of 6X gel loading dye. The solution was mixed by gentle pipetting and 5 µl of the resultant mixture was added to each of two wells of the gel at varying positions to function as a molecular weight marker.

2.9 *Primer Stock Solution & Working Solution Preparation*

Primers were supplied from the manufacturers in a lyophilized form. Prior to usage all four primers (a forward and reverse primer for each of C282Y and H63D), were made into 1 µg/µl stock solutions in the following way:

C282Y Forward Primer (C282YF): 315.6 µg* was dissolved in 315.6 µl of molecular grade water.

C282Y Reverse Primer (C282YR): 348.6 µg* was dissolved in 348.6 µl of molecular grade water.

H63D Forward Primer (H63DF): 258 µg* was dissolved in 258 µl of molecular grade water.

H63D Reverse Primer (H63DR): 309.5 µg* was dissolved in 309.5 µl of molecular grade water.

All solutions were mixed thoroughly to ensure homogeneity.

* = quantity of lyophilized primer supplied by the manufacturer

For PCR, primer working solutions of a concentration of 5 picomoles/µl were required. These working stock solutions of each primer were prepared in the following way at various intervals when required during analysis (calculations were based on primer molecular weight).

C282YF: 20 µl of C282YF stock solution (1µg/µl) was added to 596 µl of molecular grade water.

C282YR: 20 µl of C282YR stock solution (1µg/µl) was added to 618 µl of molecular grade water.

H63DF: 20 µl of H63DF stock solution (1µg/µl) was added to 598 µl of molecular grade water.

H63DR: 20 µl of H63DR stock solution (1µg/µl) was added to 544 µl of molecular grade water.

All solutions were mixed thoroughly to ensure homogeneity (see Appendix B for Primers Certificate of Analysis).

2.10 DNA Purification

Whole blood samples were thawed completely at room temperature before use. Each tube was inverted gently to ensure thorough mixing of the contents prior to use. DNA was extracted or 'purified' from these whole blood samples using Promega's Wizard™ Genomic DNA Purification Kits (Cat. # A1120) and in accordance with the following procedure from product inserts.

The Wizard™ Genomic DNA Purification Kit is designed to isolate genomic DNA from white blood cells. The procedure is based on a four-step process. The first step is lysis of red blood cells using Cell Lysis Solution (which leaves white blood cells intact). The second step is lysis of the white blood cells and their nuclei, and their solublisation in the Nuclei Lysis Solution. The third step removes cellular proteins by salt precipitation, leaving only high molecular weight genomic DNA in solution. In the final step the genomic DNA is concentrated and desalted by isopropanol precipitation.

According to the manufacturer, the following procedure consistently yields in the range of 5-15 µg of DNA per 300 µl of whole blood. This procedure involves no organic extractions or proteinase digestions. DNA purified using this system is suitable for amplifications, restriction endonuclease digestion and membrane hybridisation. Promega has tested the performance of genomic DNA isolated from fresh whole blood collected in EDTA-, heparin- and citrate-coated tubes and has detected no adverse effects upon subsequent manipulations of the DNA including PCR.

The manufacturer recommends that the DNA isolated from the purification procedure should be quantitated by agarose gel electrophoresis method. This method is more accurate than the spectrophotometric method of DNA quantitation.

Procedure for Genomic DNA Purification from Whole Blood (300 µl sample volume):

1. Add 900 µl of Cell Lysis Solution to a sterile 1.5 ml microcentrifuge tube.

2. Gently rock the tube of blood until thoroughly mixed; then transfer 300 µl of blood to the tube containing the Cell Lysis Solution. Invert the tube 5-6 times to mix.

3. Incubate the mixture for 10 minutes at room temperature (mix once during the incubation) to lyse the red blood cells. Centrifuge for 20 seconds at 13,000-16,000 x *g* at room temperature.

4. Remove and discard as much supernatant as possible without disturbing the visible white pellet. Approximately 10-20 µl of residual liquid will remain in the tube.

   Frozen samples may require repeat of Steps 1-4 until the pellet is white. There may be some loss of DNA from frozen samples.

   Note: Some red blood cells or red cell debris may be visible aiong with the white blood cells. If the pellet appears to contain only red blood cells, add an additional aliquot of 300 µl of Cell Lysis Solution after removing the supernatant above the cell pellet. Then repeat Steps 3-4.

5. Vortex the tube vigorously until the white blood cells are resuspended (10-15 seconds).

   Note: To obtain efficient cell lysis, it is essential that the white blood cells are completely resuspended.

6. Add 300 µl of Nuclei Lysis Solution to the tube containing the resuspended cells. Pipette the solution 5-6 times to lyse the white blood cells. The solution should become very viscous. If clumps of cells are visible after mixing, incubate the solution at 37°C until the clumps are disrupted. If the clumps are still visible after 1 hour, add an additional 100 µl of Nuclei Lysis Solution and repeat the incubation.

7. OPTIONAL STEP: Add 1.5 µl of RNase Solution to the nuclear lysate and mix the sample by inverting the tube 2-5 times. Incubate the mixture at 37°C for 15 minutes. Cool the sample to room temperature before proceeding.

8. Add 100 µl of Protein Precipitation Solution to the nuclear lysate and vortex vigorously for 10-20 seconds. Small protein clumps may be visible after vortexing.

   Note: If it was necessary to use additional Nuclei Lysis Solution in Step 6, add a total of 130 µl of Protein Precipitation Solution.

9. Centrifuge at 13,000-16,000 x *g* for 3 minutes at room temperature. A dark brown pellet should be visible.

10. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube containing 300 µl of room temperature isopropanol.

    Note: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

11. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.

12. Centrifuge for 1 minute at 13,000-16,000 x *g* at room temperature. The DNA will be visible as a small white pellet.

13. Decant the supernatant and add 300 µl of room temperature 70% ethanol to the DNA. Invert the tube gently several times to wash the DNA pellet and the sides of the microcentrifuge tube. Centrifuge for 1 minute at 13,000-16,000 x g at room temperature.

14. Carefully aspirate the ethanol using either a drawn Pasteur pipet or a sequencing pipette tip. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into the pipet. Invert the tube on clean absorbent paper and air-dry the pellet for 10-15 minutes.

15. Add 100 µl* of DNA Rehydration Solution (10mM Tris-HCl/1mM EDTA, pH 7.4) to the tube and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the

solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature (♠ later changed to 50 µl).

16. Store the DNA at 2-8°C (prior to amplification by PCR etc).

Quantitation of the DNA (Yield Gel)

1. Mix 3.5 µl of the DNA solution prepared by the above method with 0.7 µl of gel loading dye and centrifuge at 12,000 x g for 2 seconds in a microcentrifuge. [5:1 ratio for volume of DNA to that of the loading dye].

2. Prepare a 1% agarose gel (see section 2.6).

3. Load the sample into one of the wells. In the adjacent wells, load equal volumes of a series of DNA concentration standards in the range 25-500 ng/µl.

4. Run the gel at 5V/cm. When the bromophenol blue tracking dye has migrated at least 2 cm from the wells, the run can be stopped.

5. Examine the gel on an ultraviolet light transilluminator. Intact DNA will be visible as a band near the wells. A smear extending from the well to the dye front indicates that the DNA has been fragmented. Visualize the gel under UV light and document the results through photography.

6. From the photograph, estimate the quantity of DNA in the test samples by comparison to the DNA concentration standards.

The yield should be in the range of 5-15 µg of DNA per 300 µl of fresh whole blood with an average size of ≥50kb. It should be noted that DNA yield may decrease with prolonged storage and repeated freeze-thaw cycles.

At the end of the purification procedure, sufficient DNA should be obtained for use in the Polymerase Chain Reaction (PCR).

A second method of DNA purification was also tried and is detailed below.

Chelex DNA Extraction from Whole Blood (Walsh et al., 1991).

1. Pipet 1 ml of sterile distilled water into a sterile 1.5-ml microcentrifuge tube. Add 3 µl of whole blood and mix gently.

2. Incubate at room temperature for 15 – 30 minutes. Mix occasionally by inversion or gentle vortexing.

3. Spin in a microcentrifuge for 2 – 3 minutes at 10,000 – 15,000 x g.

4. Carefully remove the supernatant (all but 20 – 30 µl) and discard.

5. Add 5% Chelex to a final volume of 200 µl.

6. Incubate at 56°C for 15 – 30 minutes.

7. Vortex at high speed for 5 – 10 seconds.

8. Incubate in a boiling water bath for 8 minutes.

9. Vortex at high speed for 5 – 10 seconds.

10. Spin in a microcentrifuge for 2 – 3 minutes at 10,000 – 15,000 x g.

11. The sample is now ready for the amplification process. it is recommended that 20 µl of the supernatant be added to the PCR mix.

12. Store the remainder of the sample at 2° - 8°C or frozen. To re-use, repeat Steps 9 – 11.

2.11 PCR

PCR is a method used to amplify a specific DNA sequence in vitro by repeated cycles of synthesis using specific primers and DNA Taq polymerase enzyme. Optimised PCR components and run parameters ensure successful PCR. The DNA product obtained from the optimised PCR process can subsequently be analysed in a variety of different ways, but in this case restriction enzyme digestion was employed for mutational analysis of haemochromatosis for C282Y, H63D and S65C.

2.11.1 Optimisation of PCR

It is necessary to carry out optimization experiments in order to establish optimal conditions for each procedure. The following factors should be investigated during the optimization process because all may influence the specificity, fidelity and yield of the desired product.

(a) Enzyme Concentration

The concentration of *Taq* DNA polymerase required for use in PCR can vary with respect to target templates or primers. Too high a concentration of enzyme and background products may accumulate but with too low a concentration, an insufficient amount of desired product is made.

(b) Deoxynucleotide Triphosphates (dNTP's)

The four dNTP's should be used at equivalent concentrations to minimize misincorporation errors. Low dNTP concentrations minimize mispriming at nontarget sites and reduce the likelihood of extending misincorporated nucleotides (Innis et al., 1988). Working stock solutions of 1 mM for each dNTP are recommended.

(c) Buffer

Buffers generally recommended for PCR are Tris-HCl or Tris-borate based buffers (pH 8.3 - 8.8). Optimal buffer conditions are required to help facilitate primer annealing and to optimize *Taq* DNA polymerase activity.

(d) Magnesium Concentration

The magnesium ion concentration may affect; primer annealing, strand dissociation temperatures of template and PCR product, product specificity, enzyme activity and fidelity and formation of

primer-dimer artifacts. Taq DNA polymerase requires free magnesium on top of the amount generally bound by template DNA, primers and dNTP's.

2.11.2 Optimisation of PCR in terms of $MgCl_2$ Concentration

As initial attempts at PCR were conducted using Readymix™ Taq without $MgCl_2$, it was unnecessary to optimize in terms of enzyme and dNTP concentration or buffer capacity. The product is supplied as a ready-prepared mixture of Tris-HCl, KCl, dNTP's and Taq DNA polymerase, and the product insert recommends a volume of 25 µl of PCR Readymix be used in each PCR reaction. This means that there is a final concentration of 1.5 units of Taq polymerase and 0.2 mM dNTP's per reaction within the optimum pH range.

It was however necessary to optimize in terms of magnesium chloride ($MgCl_2$) , when using the aforementioned Sigma-Aldrich product. The exact protocol employed in this optimization is detailed below.

Procedure for Optimization of Magnesium Chloride ($MgCl_2$) Concentration:

1. PCR tubes were set up according to Table 2.0 below.

2. Varying volumes of $MgCl_2$ were added to each tube to give finai magnesium chloride concentrations of: 0 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM and 4 mM.

Table 2.0: Preparation of Tubes for Optimisation of PCR in terms of Magnesium Chloride Concentration.

	Tube Number								
Tube Contents	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***
DNA Template	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
C282YF* (5 picomoles/µl stock solution)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
C282YR* (5 picomoles/µl stock solution)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
$MgCl_2$ (25 mM stock solution)	0 µl	1 µl	2 µl	3 µl	4 µl	5 µl	6 µl	7 µl	8 µl
Readymix™ Taq polymerase	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
Ultrapure / Molecular biology grade water	14 µl	13 µl	12 µl	11 µl	10 µl	9 µl	8 µl	7 µl	6 µl
Final Volume	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***

* see section 2.9

3. The contents of each of the tubes above were over layed with PCR mineral oil prior to PCR to prevent sample evaporation.

4. All tubes underwent PCR under the following conditions: Initial denaturation at 95°C for 5 minutes → Denaturation at 94°C for 45 seconds → Annealing at 55°C for 45 seconds → Extension at 72°C for 45 seconds (30 cycles).

5. Following PCR, 10 µl of each PCR product was removed to a set of new tubes and 2 µl of 6X gel loading dye was added to each. The solution was then mixed by gentle pipetting.

6. 5 µl of each of the resultant mixtures was then loaded into wells of a 2% agarose gel (see section 2.7).

7. Molecular weight markers were prepared and used as detailed in section 2.8.

8. The agarose gel underwent electrophoresis at a rate of 5V/cm for 3 hours, after which time it was visualized over UV light on a transilluminator. All results were photographed and archived using the Syngene DigiGenius Documentation System.

(e) PCR Run Conditions

(i) Denaturation Time and Temperature: Incomplete denaturation of the target template and/or the PCR product is the most likely cause for failure of a PCR. Incomplete denaturation of DNA, results in the inefficient use of template in the first cycle of PCR and a poor yield of PCR product will be obtained.

It is generally accepted that an initial denaturation step of an incubation at 95°C for 1-3 minutes be performed for templates with GC contents of 50% or less. For GC-rich templates this incubation period should be extended for up to 10 minutes. For the initial denaturation step, *Taq* DNA polymerase should only be added to the initial reaction mixture if the incubation period is no longer than 3 minutes and the temperature is not greater than 95°C otherwise the enzyme's stability decreases (Sambrook et al., 1989).

Because the PCR product synthesized in the first round of amplification is considerably shorter than the template DNA (and is completely denatured under these conditions), a denaturation step of 0.5-2 minutes at 94-95°C is sufficient for PCR (MBI Fermentas, 2002-2003).

2.11.3 Optimisation of PCR in terms of Primer Annealing Temperature

(ii) Primer Annealing: The length of time and temperature required for primer annealing is dependant on the base composition, length and concentration of the amplification primers. Annealing temperature is generally 5°C below the true T_m of the primer-template DNA duplex. The melting temperature (T_m) can be calculated by the following equation (Hybaid Omne E thermal cycler, user instruction manual, 1994):

$$T_m = 2 \times (A + T) + 4 \times (C + G)$$

Where: A is the number of adenine bases in the primer
T is the number of thymine bases in the primer
C is the number of cytosine bases in the primer
G is the number of guanine bases in the primer

Annealing temperatures in the range of 55 to 72°C generally yield the best results. Incubation for 0.5 – 2 minutes is usually sufficient, but if nonspecific PCR products are obtained in addition to the expected product, the annealing temperature may need to be increased.

The following forward (F) and reverse (R) primer pairs were used to detect the two major haemochromatosis mutations C282Y and H63D.

C282YF: 5'-TGG CAA GGG TAA ACA GAT CC-3'
C282YR: 5'-TAC CTC CTC AGG CAC TCC TC-3'
H63DF: 5'-ACA TGG TTA AGG CCT GTT GC-3'
H63DR: 5'-CTT GCT GTG GTT GTG ATT TTC C-3'

Calculation of primer melting temperature was as follows and was based on the equation above;

T_m of C282YF = 2 x (7 + 3) + 4 x (4 + 6) = (2 x 10) + (4 x 10) = 20 + 40 = 60°C
T_m of C282YR = 2 x (3 + 5) + 4 x (10 + 2) = (2 x 8) + (4 x 12) = 16 + 48 = 64°C
T_m of H63DF = 2 x (4 + 6) + 4 x (4 + 6) = (2 x 10) + (4 x 10) = 20 + 40 = 60°C
T_m of H63DR = 2 x (1 + 11) + 4 x (4 + 6) = (2 x 12) + (4 x 10) = 24 + 40 = 64°C

The melting temperatures indicated on the manufacturers Certificate of Analysis for the primers were; 57.3°C for C282YF, 61.4°C for C282YR, 57.3°C for H63DF and 58.4°C for H63DR. By applying the hypothesis that the optimum annealing temperature is 5°C below the melting temperature, it can be said that the optimum annealing temperature for C282YF is somewhere between 52.3°C and 55°C. Using the same principle, the optimum annealing temperature for C282YR is between 56.4°C and 59°C, for H63DF is between 55°C and 55.3°C and for H63DR is between 53.4°C and 59°C. The following temperatures were therefore decided on for optimization work: 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 64°C and 70°C.

Procedure for Optimization of Annealing Temperature:

1. Thirteen tubes were prepared for PCR by adding the following reagents together.

DNA Template / Genomic DNA [50ng/µl]	3.2 µl
C282YF primer♦ [5 pmoles/µl stock]	2.5 µl
C282YR primer♦ [5 pmoles/µl stock]	2.5 µl
PCR Master Mix	25 µl
Molecular Grade Water	16.8 µl
Final Volume	**50 µl**

2. The contents of each of the tubes above were over layed with PCR mineral oil prior to PCR to prevent sample evaporation.

3. One tube was subjected to PCR at each of the 13 annealing temperatures listed above for a cycle time of 45 seconds. All other PCR conditions remained as determined i.e. Initial denaturation at 95°C for 5 minutes → Denaturation at 94°C for 45 seconds → Annealing (at each relevant temperatures above) for 45 seconds → Extension at 72°C for 45 seconds (30 cycles).

4. Following PCR, 10 µl of each PCR product was removed to a new set of tubes and 2 µl of 6X gel loading dye was added to each. The solution was then mixed by gentle pipetting.

5. 5 µl of each of the resultant mixtures was then loaded into wells of a 2% agarose gel (see section 2.7).

6. Molecular weight markers were prepared and used as detailed in section 2.8.

7. The gel then underwent electrophoresis at a rate of 5V/cm for 3 hours, after which time it was visualized over UV light on a transilluminator. All results were photographed and archived using the Syngene DigiGenius Documentation System.

♦ A second set of 13 tubes was prepared using the H63D forward and reverse primers and annealing temperature was optimized by the same method. See section 2.9 for primer solution preparation.

(iii) Cycle Number: The optimum number of cycles to use for PCR depends mainly on the amount of starting/template DNA and on the expected yield of the PCR product. For less than 10 copies of the template, 40 cycles should be performed, but if the initial quantity of template is higher, 25-35 cycles is usually sufficient. Too many cycles can increase the amount and complexity of nonspecific background products, yet too few will give a poor product yield. Based on published literature, a cycle number of 30 was utilized for PCR.

2.11.4 Optimisation of PCR in terms of Primer Concentration

(iv) Primers: Typical primers are 18 to 28 nucleotides long and have a GC content of between 50 and 60%. Primers are used at concentrations between 0.1 and 0.5 µM for optimal results. Higher concentrations may promote accumulation of nonspecific product, mispriming and the probability of primer-dimers occurring (MBI Fermentas, 2002-2003).

Procedure for Optimization of Primer Concentration:

1. From the stock solutions of the C282YF and C282YR primer, working solutions of the following concentrations were prepared: 0.1 µmole/µl, 0.01 µmole/µl, 1 µmole/µl, 4 µmole/µl, 1 nmole/µl, 0.1 nmole/µl, 0.01 nmole/µl, 1 pmole/µl and 5 pmole/µl.

2. Nine tubes were set up for PCR according to Table 2.1. Each of the 9 tubes used primer working solutions of a different concentration (see above). A volume of 2.5 µl of each of the forward and reverse primer was used for each PCR reaction. A different set of tubes was prepared in the same manner for the H63D primer pair.

Table 2.1: Preparation of tubes for Optimisation of PCR in terms of Primer Concentration

	Tube Number								
Tube Contents	***1*** 1 µmole/µl primer working solution	***2*** 0.1 µmole/µl primer working solution	***3*** 0.01 µmole/µl primer working solution	***4*** 1 nmole/µl primer working solution	***5*** 0.1 nmole/µl primer working solution	***6*** 0.01 nmole/µl primer working solution	***7*** 1 pmole/µl primer working solution	***8*** 5 pmole/µl primer working solution	***9*** 4 umole/µl primer working solution
DNA Template	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
C282YF■	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**
C282YR■	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**	**2.5 µl**
$MgCl_2$ (25 mM stock solution)	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl
Readymix™ Taq polymerase	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
Ultrapure / Molecular biology grade water	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl
Final Volume	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***	***50 µl***

■ or H63DF and H63DR primers (see section 2.9 for primer solution preparation)

3. The contents of each of the tubes above were over layered with PCR mineral oil prior to PCR to prevent sample evaporation.

4. All tubes underwent PCR at the following conditions: 95°C for 5 minutes → Denaturation at 94°C for 45 seconds → Annealing at 55°C for 45 seconds → Extension at 72°C for 45 seconds (30 cycles).

5. Following PCR 10 µl of each PCR product was removed to a set of new tubes and 2 µl of 6X gel loading dye was added to each. The solution was then mixed by gentle pipetting.

6. 5 µl of each of the resultant mixtures was then loaded into wells of a 2% agarose gel (see section 2.7).

7. Molecular weight markers were prepared and used as detailed in section 2.8.

8. The gel then underwent electrophoresis at a rate of 5V/cm for 3 hours, after which time it was visualized over UV light on a transilluminator. All results were photographed and archived using the Syngene DigiGenius Documentation System.

In order to ensure PCR was continuously and reproducibly conducted at optimized conditions in terms of enzyme, magnesium chloride and dNTP concentrations, and to minimize possible sources of error, it was decided to utilize Promega's PCR Master Mix for all subsequent PCR work. This product has been optimized for use in routine PCR reactions for amplifying DNA template in the range of 0.2-2kb.

2.11.5 Optimisation of PCR in terms of Template DNA Concentration

Optimisation of Template DNA Concentration

1. Eight tubes (labeled 1-8) each containing the following PCR components were prepared:

Forward Primer [5 picomoles/µl]	2.5 µl
Reverse Primer [5 picomoles/µl]	2.5 µl
PCR Master Mix (Promega)	25 µl

2. Varying volumes of DNA template (i.e. product from Wizard Extraction procedure) was added to each of the nine tubes as follows (see Table 2.2):

Table 2.2: Preparation of tubes for Optimisation of PCR in terms of Template DNA Concentration

Tube Number	1	2	3	4	5	6	7	8
Volume of Wizard product added (µl)	10	12	15	16	17	18	19	20
Volume molecular biology grade water added (µl)	10	8	5	4	3	2	1	0
Final PCR volume (µl)	50	50	50	50	50	50	50	50

3. A negative control for PCR was also included. It contained every component above *excluding* the DNA (i.e. it contained forward and reverse primer, PCR master mix and 20µl of molecular biology grade water).

4. The contents of each of the tubes above were over layered with PCR mineral oil prior to PCR to prevent sample evaporation.

5. All tubes underwent PCR at the following conditions: 95°C for 5 minutes → Denaturation at 94°C for 45 seconds → Annealing at 55°C for 45 seconds → Extension at 72°C for 45 seconds (30 cycles).

6. Following PCR 10 µl of each PCR product was removed to a set of new tubes and 2 µl of 6X gel loading dye was added to each. The solution was then mixed by gentle pipetting.

7. 5 µl of each of the resultant mixtures was then loaded into wells of a 2% agarose gel (see section 2.7).

8. Molecular weight markers were prepared and used as detailed in section 2.8.

9. The gel then underwent electrophoresis at a rate of 5V/cm for 3 hours, after which time it was visualized over UV light on a transilluminator. All results were photographed and archived using the Syngene DigiGenius Documentation System

Procedure for Optimised Polymerase Chain Reaction (PCR):

1. For each DNA sample, a sterile tube with the following contents was prepared:

DNA Template (Product from Wizard Extraction procedure)	20 µl
Forward Primer [5 picomoles/µl]	2.5 µl
Reverse Primer [5 picomoles/µl]	2.5 µl
PCR Master Mix (Promega)	25 µl
FINAL VOLUME	50 µl

2. A separate set of tubes for each primer was prepared. i.e. the first set is set up for all samples using C282Y forward and reverse primers and the second set using H63D forward and reverse primers (see section 2.9 for primer solution preparation).

3. A negative control for PCR was also included. It contained every component above *excluding* the DNA.

4. The contents of each of the tubes above were over layered with PCR mineral oil prior to PCR to prevent sample evaporation.

5. All tubes underwent PCR at the following conditions: 95°C for 5 minutes → Denaturation at 94°C for 45 seconds → Annealing at 55°C for 45 seconds → Extension at 72°C for 45 seconds (30 cycles).

6. Following PCR, samples were stored frozen at -22°C for future analysis. Samples were completely thawed, immediately prior to use in restriction endonuclease digestions.

On random occasions one tube (for each set of primers) was prepared as above, but Promega Human Genomic DNA was utilized instead of DNA obtained via the Wizard® extraction procedure. This acted as a positive PCR/DNA control, in that it ensured that DNA obtained via the Wizard® kit method was of a comparable quantity and quality to that of Promega's human genomic DNA and therefore that which is required for subsequent analysis.

PCR was performed in a Hybaid Omn-E thermal cycler.

Following PCR, DNA or 'product' is available for subsequent genetic manipulation. In this study, product obtained from PCR was subjected to restriction enzyme digestion analysis in order to determine the presence/absence of the three main mutations responsible for hereditary haemochromatosis; C282Y, H63D and S65C.

2.12 Restriction Endonuclease Digestion

Restriction enzymes (also called restriction endonucleases), are enzymes that recognize short, specific DNA sequences. They cleave double-stranded DNA at specific sites that are within or adjacent to their recognition sequences. Isoschizomers are enzymes isolated from different sources that cleave within the same target sequences.

Each restriction enzyme has specific requirements to achieve optimal activity. Ideal storage and assay conditions favor the most activity and highest fidelity in a particular enzyme's function. Conditions such as temperature, pH, enzyme cofactor(s), salt composition and ionic strength affect enzyme activity and stability.

Restriction enzyme digestions should be carried out as per manufacturers instructions, as each manufacturer has optimized reaction conditions for their own enzymes.

In this study, the restriction enzymes *Rsa* I, *Nde* II and *Hinf* I were used to identify the three major haemochromatosis mutations C282Y, H63D and S65C respectively, following amplification by PCR. *Nde* II, is an isoschizomer of *Mbo* I, both of which have the following recognition sequence; ↓GATC. The recognition sequence of *Rsa* I is GT↓AC and that of *Hinf I* is G↓ANT C. Both *Nde II* and *Rsa I* restriction enzymes are examples of Type II enzymes, which means that they are binary in nature, consisting of a restriction endonuclease that cleaves a specific sequence of nucleotides and a separate methylase that modifies the same recognition sequence.

Following amplification, PCR products of 393bp were obtained using the C282Y primers mentioned earlier and were digested:

(1) with *Rsa* I, to give fragments at:

- 247bp and 146bp.....for normal non-mutated DNA (wild-type)
- 247p, 146bp, 117bp and 29bp.....for a C282Y carrier / heterozygote and
- 247bp, 117bp and 29bp.....for a mutant C282Y homozygote (Feder et al., 1996 & Jeffrey et al., 1999)

PCR products of 294bp are obtained using H63D primers and are digested:

(2) with *Nde* II, to give fragments at:

- 138bp, 99bp and 57bp.....for normal non-mutated DNA (wildtype)
- 237bp, 138bp, 99bp and 57bp.....for a H63D carrier / heterozygote and
- 237 bp and 57bp.....for a mutant H63D homozygote (Merryweather-Clarke et al., 1997a) and

(3) with *Hinf* I, to give fragments at:

- 147 bp and 80 bp.....for normal non-mutated DNA (wildtype)
- 227 bp, 147 bp and 80 bp.....for a S65C carrier / heterozygote and
- 227bp and 80 bp for a mutant S65C homozygote (correspondence with Kathryn Robson, Oxford, 2006).

All restriction endonucleases employed were used in accordance with the information supplied by the manufacturer (Promega), which recommends that;

Analytical scale restriction enzyme digests are performed in a volume of 20 µl on 0.2-1.5 µg of substrate DNA, using a 2- to 10-fold excess of enzyme over DNA. If an unusually large volume of DNA or enzyme is used, aberrant results may occur and may or may not be readily recognized.

The following is an example of a typical restriction enzyme digest. In a sterile tube, assemble in order:

Sterile, deionized water	16.3 µl
Restriction Enzyme 10X Buffer	2.0 µl
Acetylated BSA, 10 µg/µl	0.2 µl
DNA, 1 µg/µl	1.0 µl
Mix by pipetting, and then add:	
Restriction Enzyme, 10 u/µl	0.5 µl
Final Volume	20 µl

Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the optimum temperature for 1-4 hours.

Add 4 µl of 6X loading buffer and proceed to gel analysis. Note that overnight digests are usually unnecessary and may result in degradation of the DNA.

Experimental controls are important and the following controls were employed to help identify, understand and explain problems or inconsistencies in results:

(i) uncut experimental DNA
(ii) digest of commercially supplied control DNA
(iii) no-enzyme "mock" digest
(iv) 1 or 2 different size markers in more than 1 lane per gel.

Procedure for Restriction Enzyme Digestion for Mutational Analysis:

Following PCR, products were digested with *Rsa I* for C282Y mutational analysis, *Nde II* for H63D mutational analysis and *Hinf I* for S65C mutational analysis, in accordance with the following procedure.

1. For each DNA sample to be analysed a separate tube was prepared by assembling (in order) the following reagents:

Sterile, deionized water	12.3 µl
Restriction Enzyme 10X Buffer	2.0 µl
Acetylated BSA [10µg/ml]	0.2 µl
DNA Template *	5.0 µl
Mix by pipetting, and then add:	
Restriction Enzyme (*Rsa* I/*Nde* II/*Hinf* I)	0.5 µl
FINAL VOLUME	20 ul

* DNA Template refers to the products obtained for each sample following PCR with the relevant primers.

2. A 'control' which contained all components except the restriction enzyme was also prepared.

3. The tubes were then closed and centrifuged in a microcentrifuge for a few seconds. All tubes were incubated at 37°C for 4 hours.

4. Following incubation, 4 µl of 6X gel loading dye was added to each tube and the tube contents mixed by gentle pipetting.

5. 20 µl from each tube was loaded into different wells of a 2% agarose gel (see section 2.7).

6. Molecular weight markers were prepared and used as detailed in section 2.8.

7. The gel then underwent electrophoresis for 1 hour and 50 minutes at 155 volts (130 milliamps), after which time it was visualized under UV light on a transilluminator. All results were photographed and archived using the Syngene DigiGenius Documentation System

The same procedure was conducted for H63D and S65C mutation analysis. In the case of H63D and S65C analyses, the restriction enzymes used were *Nde* II and *Hinf* I, respectively.

2.13 Agarose Gel Electrophoresis

Agarose gels were prepared as detailed in section 2.7 and were used in both quantitation of DNA yield and in Restriction Endonuclease Digestion analysis for the haemochromatosis mutations C282Y, H63D and S65C.

Before usage, the previously prepared gels (in their units), were overlaid with the same TBE buffer used in gel preparation. Samples and molecular weight markers were then loaded into the wells and the gel underwent electrophoresis at either:

- 155 volts (130 milliamps) for 1 hour and 50 minutes (Restriction Enzyme Digestion Analysis).

Or

- 5V/cm until the bromophenol blue tracking dye has migrated at least 2 cm from the wells (Quantitation of DNA Yield).

2.14 Gel Analysis & Documentation

Ethidium bromide stained gels were visualized under UV light (365nm) on a Syngene DigiGenius documentation system (see Figure 2.0). The location of restriction fragments was compared visually to the 50bp-800bp fragments/increments of the 50bp DNA stepladder in order to determine the mutational status of each sample. A Lambda Hind III digest was used as a second molecular weight marker.

Figure 2.0: Syngene DigiGenius Documentation system. (http://www.syngene.com). Website accessed on 25th April 2005.

A number of samples (N=15) were also sent to external facilities for verification of results. The two external facilities that conducted genotyping were NUI Galway and the Centre for Liver Disease in Dublin. Both facilities use highly automated technologies such as real time PCR (RT-

PCR) for analysis of genetic mutations, which allows high sample throughput. Some results of external analysis conducted in Dublin are presented in Figures 3.13 to Figure 3.17.

2.15 Biochemistry Analysis

The following biochemical parameters; serum iron, UIBC, ferritin, HbA_{1c}, glucose, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed at Sligo General Hospital. Only samples that had documented were assayed. Details of the methods employed for routine testing of the aforementioned biochemistry parameters are outlined below.

Serum Iron:

Serum iron is greatly increased in haemochromatosis. Numerous photometric methods have been described for the determination of iron. All have the following common features:

- liberation of Fe^{3+} ions from the transferrin complex using acids or detergents
- reduction of Fe^{3+} ions to Fe^{2+} ions
- reaction of the Fe^{2+} ions to give a coloured complex.

Analysis of iron in this study was based on the BM-Iron Ferrozine Method for the BM/Hitachi "Modular" clinical chemistry analyzer. This is a colorimetric assay which does not involve deproteinization. Each blood sample is first added to a buffer/detergent, and then to an ascorbate/FerroZine solution which initiates the reaction. Under acidic conditions, iron is liberated from transferrin. Lipemic samples are then clarified by the detergent. Ascorbate causes the reduction of released Fe^{3+} ions to Fe^{2+} ions, which then react with FerroZine to form a coloured complex (see Figure 2.1). The colour intensity is directly proportional to the iron concentration and can be measured photometrically (Siedel et al., 1984).

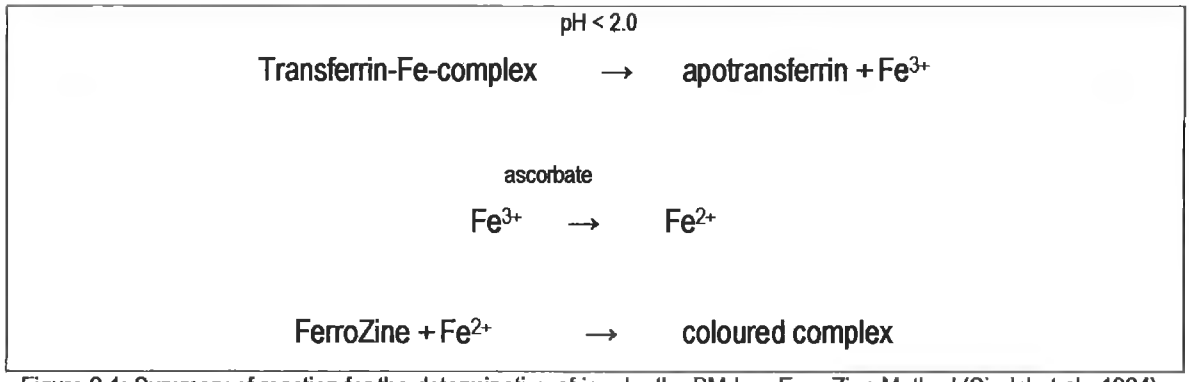


Figure 2.1: Summary of reaction for the determination of iron by the BM-Iron FerroZine Method (Siedel et al., 1984).

UIBC:

Transferrin is generally only 25 to 30 percent saturated with iron. The additional amount of iron that can be bound is the unsaturated iron binding capacity (UIBC). The sum of the serum iron and UIBC represents the total iron binding capacity (TIBC). UIBC is determined directly by saturating the transferrin at an alkaline pH with a known, but excess amount of iron. The excess iron is quantitated in the same manner as serum iron but at a pH above 7.5. The UIBC is determined by subtracting the amount of excess iron from that of the iron added. This procedure is a modification (by Roche) of the method of Goodwin (1966).

Serum is first added to an alkaline buffer/reactant solution containing a known concentration of iron to saturate the available binding sites on the transferrin. The FerroZine chromogen reacts only with Fe^{2+}; therefore, an iron reductant is added to ensure that all iron is present in the ferrous state. The excess unbound divalent iron reacts with FerroZine chromogen to form a magenta complex which is then measured spectrophotometrically (see Figure 2.2). The UIBC is equal to the difference measured in the concentrations of the added iron solution and the excess unbound iron. The TIBC is equal to the sum of the serum iron and the UIBC (Goodwin et al., 1966).

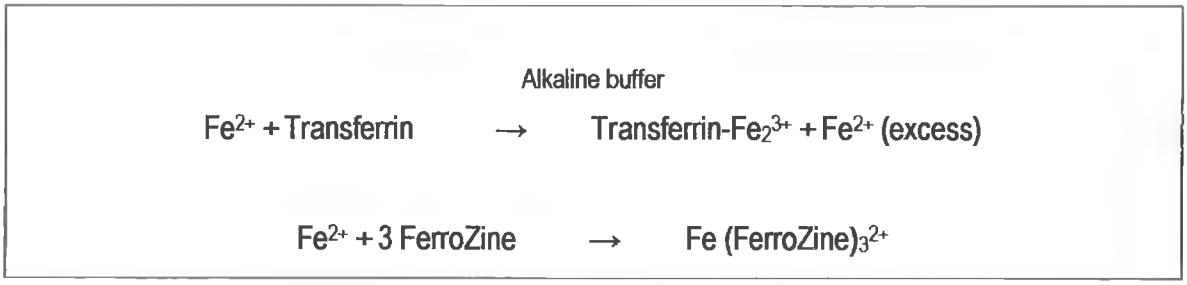


Figure 2.2: Summary of reaction for determination of UIBC (Goodwin et al., 1966).

Ferritin:

Serum ferritin concentrations reflect the level of storage iron in the body. The Abbott ARCHITECT™ Ferritin assay is a Chemiluminescent Microparticle Immunoassay (CMIA) for the quantitative determination of ferritin in human serum and plasma. In the first step the sample is combined with anti-ferritin coated paramagnetic microparticles. Any ferritin present in the sample binds to the anti-ferritin coated microparticles. In the second step, anti-ferritin acridinium labeled conjugate is added (after washing). Pre-Trigger and Trigger solutions are then added to the reaction mixture and the resulting chemiluminescence is measured as relative light units (RLUs). A direct relationship exists between the amount of ferritin in the sample and the RLUs detected by the ARCHITECT *i* optical system (Abbott Laboratories, List No. 6C11, 66-9281/R1).

HbA_{1c} / Glycated haemoglobin:

HbA_{1c} levels were determined in accordance with standard operating methods for the Menarini 'Hi-Auto A1c' Analyzer System at Sligo General Hospital. The principle of the test is that each sample of whole blood is first diluted by the autosampler, where it is separated by high-pressure liquid chromatography into HbA1ab, HbF, HbA1c, HbAO, HbS and HbC fractions. The eluted fractions are examined with a chromatic colorimeter, and the results analyzed by a microprocessor. The identification and the amount of the fractions (as peak ratios) are presented in the form of a print out (see Figure 2.3).

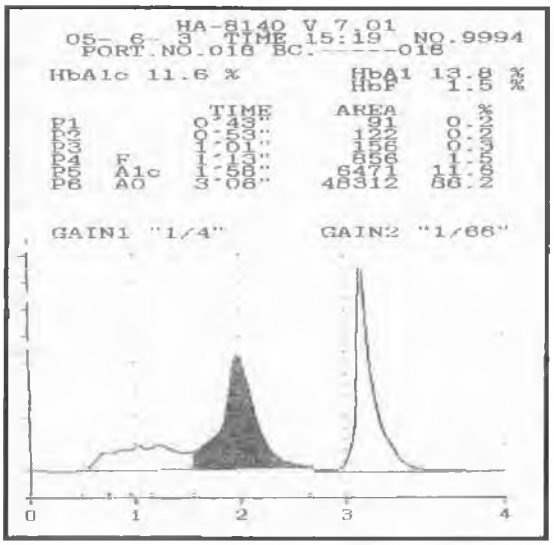


Figure 2.3: Typical Format of Result Printout from the Menarini Hi-Auto A1c analyser (HbA1c).

Aspartate aminotransferase (AST) & Alanine aminotransferase (ALT):

In this study, the following tests (which can be indicative of liver function) were conducted in accordance with standard methods for the Roche Modular Analyser and as per manufacturers instructions: Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST).
Analysis of AST is based on the following test principle (Bergmeyer *et al.*, 1986). When the sample is added to reagent 1 (buffer/coenzyme/enzyme) and α-ketoglutarate, the following reaction occurs

$$\alpha\text{-ketoglutarate} + \text{L-aspartate} \xleftrightarrow{\text{AST}} \text{L-glutamate} + \text{oxaloacetate}$$

The enzyme AST catalyses this equilibrium reaction. The increase in oxaloacetate is determined in an indicator reaction catalysed by malate dehyrogenase

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xleftrightarrow{\text{MDH}} \text{L-malate} + NAD^+$$

NADH is oxidized to NAD^+. The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of oxaloacetate and thus the AST activity.

Analysis of ALT is based on the following test principle (Bergmeyer et al., 1986). When the sample is added to reagent 1 (buffer/coenzyme/enzyme) and α-ketoglutarate, the following reaction occurs

$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xleftrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

The enzyme ALT catalyses this equilibrium reaction. The increase in pyruvate is determined in an indicator reaction catalysed by lactate dehyrogenase

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xleftrightarrow{\text{LDH}} \text{L-malate} + NAD^+$$

NADH is oxidized to NAD^+. The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of pyruvate and thus the ALT activity.

2.16 Risk Assessment

Gel and running buffer preparation involved use of ethidium bromide at a concentration of 0.5 µg/ml. Extreme caution was taken when working with this substance as it is a mutagen. Gloves were worn when handling gels or solutions containing this dye. Safety glasses were also used during gel and buffer preparation.

Gels were visualised on an ultraviolet transilluminator. When working with the aforementioned equipment, a full face UV protection shield was always worn. Excessive exposure to UV light can be carcinogenic.

Gloves were worn when handling blood / serum samples and waste materials contaminated with blood products were treated and disposed of as biohazardous waste.

2.17 Statistics / Data Analysis

Frequencies for genotypes and alleles (see Table 3.4) were calculated manually from the results presented in this thesis (Tables 3.0, 3.1 and 3.2) and in accordance with standard statistical formulae. Before comparing the allele frequencies of the two populations (diabetic and 'control'), calculations were performed to determine whether both populations were in Hardy-Weinberg equilibrium. A goodness-of-fit (Chi square) test was also used (a) to allow comparison between the observed and expected frequencies for both populations and (b) to allow comparison of genotype frequencies between the two populations. Appendix E contains all calculations done as part of the statistical analysis for this study. The data analysis conducted in this thesis matches statistical analysis done in recently published literature on haemochromatosis mutations (Malecki et al., 2003; Halsall et al., 2003).

2.18 Flow Diagram of Analysis for Present Study

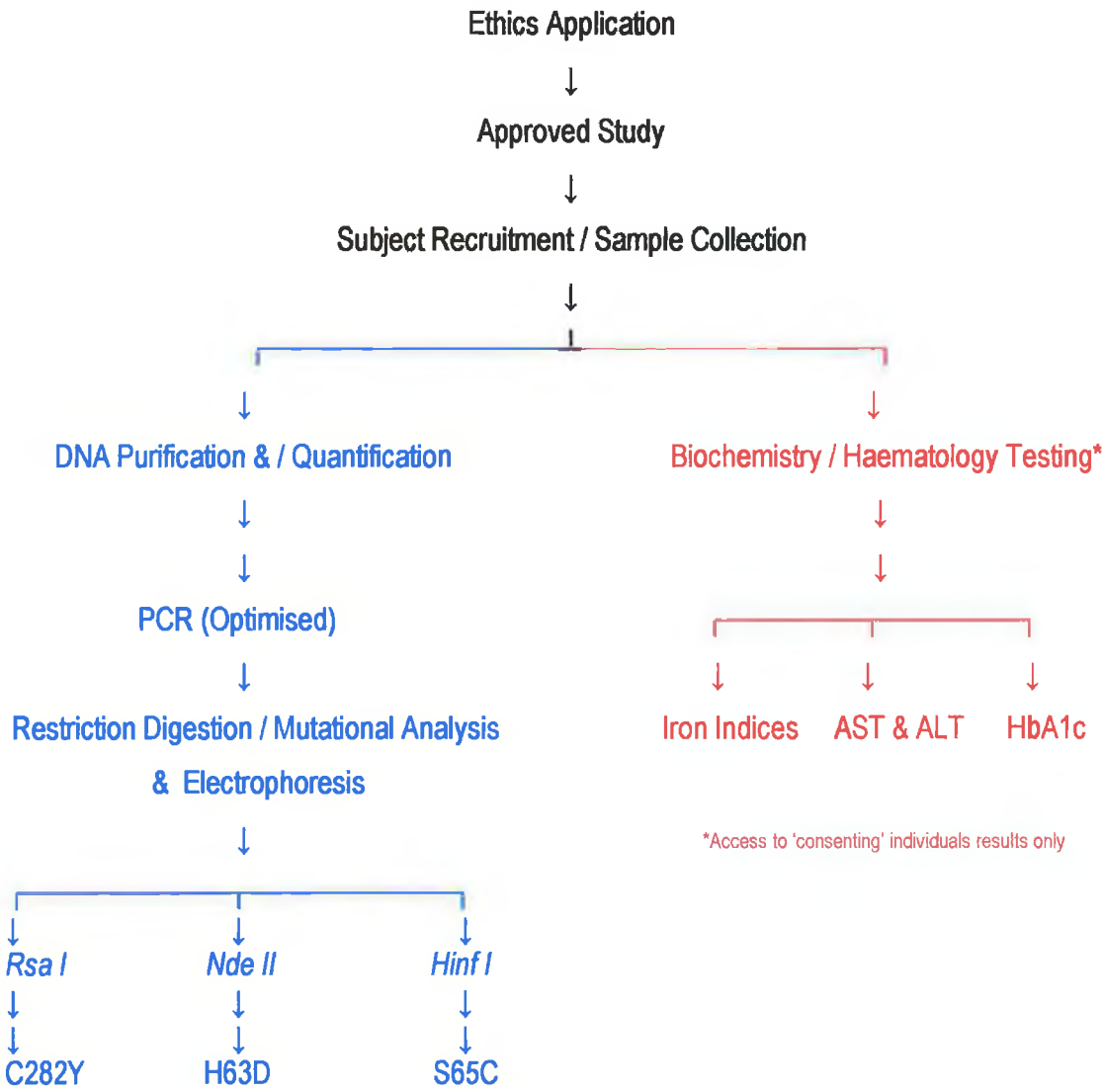


Figure 2.4: The diagram above shows the pathways of analysis for both genotype analysis (in blue) and biochemistry / haematology analysis (in red) of samples. Genotypic analysis was conducted in Research laboratories in the Institute of Technology, Sligo and Biochemistry / Haematology analysis was conducted in laboratories in Sligo General Hospital.

3.0 RESULTS

3.1 DNA Purification & Quantitation from Whole Blood

In order to conduct genetic analysis for the haemochromatosis mutations C282Y, H63D and S65C, it was first necessary to purify DNA from the subject's samples. DNA was extracted from 500 whole blood samples using a Wizard™ Genomic DNA Purification Kit from Promega (Cat # A1120). Purified DNA could then be quantified against DNA standards, to help ensure sufficient template was available for PCR.

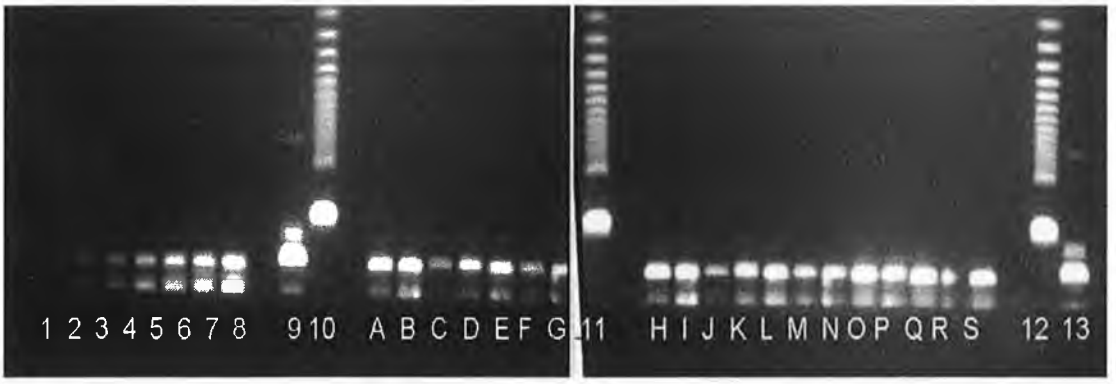


Figure 3.0: Quantitation of DNA Yield from whole blood post purification with Wizard™ Genomic DNA Purification kit.

Lanes 1 – 8 contain a range of DNA standards made from commercially available Human genomic DNA (Promega). The concentration range is as follows; 1-, 2-, 5-, 10-, 20-, 25-, 50- and 75 ng/µl (from left to right). Lanes 9 & 13 contain Lambda Hind III digest, which functions as a molecular weight marker. Lanes 10, 11 and 12 each contain 50 bp DNA Step Ladder for use as a molecular weight marker also. Lanes A to S represent 19 different whole blood samples that underwent the purification process. Comparison of the band intensities of samples, against those of the DNA standards, allows quantification of DNA yield post purification with Wizard™ Genomic DNA Purification kit.

3.2 *Genetic Analysis*

3.2.1 Optimisation of PCR

DNA obtained from the purification procedure serves as a template / source for PCR. A PCR reaction should be optimised in terms of (a) component concentrations (i.e. Taq polymerase, dNTP, $MgCl_2$, primer and template concentrations), (b) buffer levels and (c) run conditions (e.g. annealing and extension temperatures and times, cycle numbers etc.). Results for some of the components and run conditions that were optimised, are shown below.

3.2.2 Optimisation of PCR in terms of Magnesium Chloride ($MgCl_2$) concentration

In initial stages of PCR analysis, a Readymix™ Taq preparation (Sigma-Aldrich), was employed. As this product contains all the necessary components for a standard PCR reaction (with the exception of magnesium chloride / $MgCl_2$ and DNA template), it was unnecessary to optimise in terms of enzyme and dNTP concentrations or buffer capacity. It was however, essential to optimise in terms of $MgCl_2$ concentration. Results for the optimisation of magnesium chloride concentration are shown below (Figure 3.1). PCR was not successful at magnesium chloride concentrations below 1.5mM. It was successful at $MgCl_2$ concentrations between 2 mM and 4mM, however, it was decided to utilise a concentration of 1.5mM per PCR reaction to help minimise problems (like PCR inhibition) that can occur as a result of employing too high a concentration of magnesium chloride.

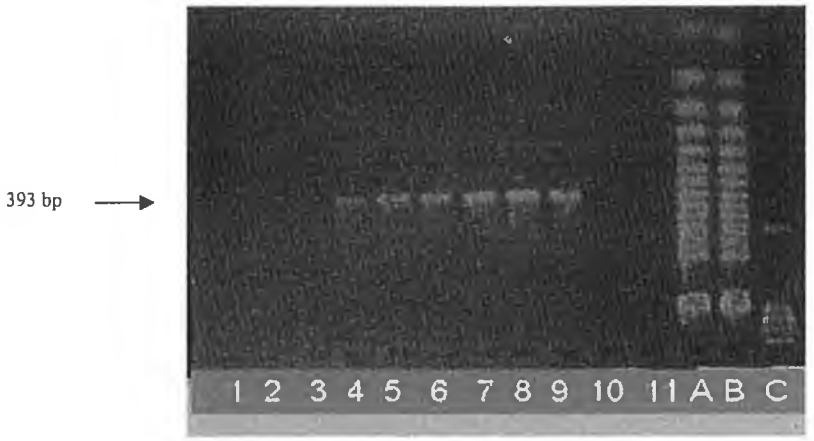


Figure 3.1: Optimisation of PCR in terms of Magnesium Chloride ($MgCl_2$) concentration.

Lanes 1 – 9 contain PCR reactions prepared with a range of magnesium chloride concentrations. The concentration range is as follows; 0-, 0.5-, 1-, 1.5-, 2-, 2.5-, 3-, 3.5- and 4 mM $MgCl_2$ per reaction (from left to right). Lanes A & B contain 50bp DNA Step Ladder to function as a molecular weight marker. Lane C contains Lambda Hind III digest to function as a second molecular weight marker. Lanes 10 and 11 are empty. Results presented above represent PCR carried out using the C282Y primer pair. (Note: Resolution of markers was not optimised at the time of image capture).

3.2.3 Optimisation of PCR in terms of Primer Annealing temperature

Another PCR parameter that was optimised was primer annealing temperature. Optimisation of annealing temperature ensures that specific primers bind optimally to template DNA. Results of experiments on optimisation of annealing temperature are shown in Figure 3.2 below. The optimum annealing temperature for the C282Y primer pair shown below, was 55°C (Lane 3). Identical optimisation work carried out on the H63D primer pair (not presented) reported an identical optimum annealing temperature for the aforementioned primer pair.

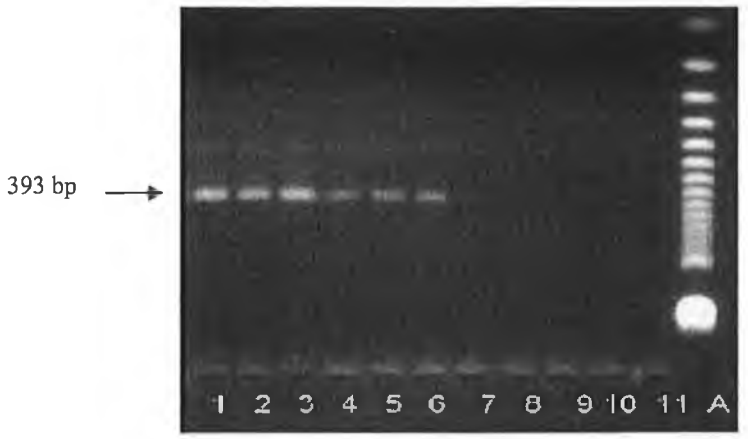


Figure 3.2: Optimisation of PCR in terms of Primer Annealing Temperature.

Lanes 1 to 11 contain PCR reactions performed (with Human genomic DNA), over a range of different primer annealing temperatures. The primer annealing temperature range was as follows; 50-, 54-, 55-, 56-, 57-, 59-, 61-, 63-, 65-, 68- and 70°C (from left to right). The data presented, pertains to optimisation of annealing temperature for the C282Y primer pair. Lane A contains 50bp DNA Step Ladder to function as a molecular weight marker. The size of the C282Y PCR product is 393 bp.

3.2.4 Optimisation of Primer Concentration & Template DNA Concentration

Similar experiments were run to determine optimum DNA concentrations and primer concentrations for PCR. Results from optimisation of primer concentration are not shown due to loss of photographic data from the documentation system. However, results of this optimisation were recorded in the lab notebook at the time of experimentation and optimum primer concentration for PCR was found to be 5 picomoles. The results below show that a volume of 20µl of product (obtained from extraction of DNA from whole blood, using Wizard™ Genomic DNA Purification kit), provided sufficient template DNA for successful PCR.

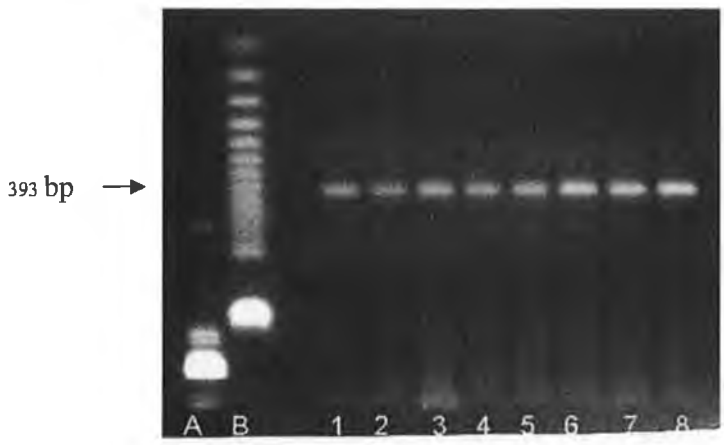


Figure 3.3: Optimisation of PCR in terms of Template DNA Concentration.

Lane A contains 50bp DNA Step Ladder to function as a marker of size. Lane B contains Lambda Hind III digest to function as a second marker of size. Lanes 1 to 8 represents PCR reactions that were conducted using varying volumes of 'extracted' template / DNA. The varying volumes of template used in PCR were; 10-, 12-, 15-, 16-, 17-, 18-, 19- and 20 µl. (Results presented above are representative of PCR carried out using C282Y primer pair only).

3.2.5 PCR Master Mix versus ReadyMix™ kit

During the course of optimisation procedures, it was decided to change from the Readymix™ PCR mix without $MgCl_2$ (Sigma-Aldrich), to a new product by Promega. The new product called PCR Master Mix is optimised in terms of all reaction components (except the template), and its use would therefore minimise the potential for error in PCR formulation that may arise with use of the previous product. From Figure 3.4 below, it is evident that both products produce comparable results.

Figure 3.4: Comparison of PCR carried out with two different component preparations; PCR Master Mix (Promega) & Readymix™ (Sigma-Aldrich).

Lanes A and D contain Lambda Hind III digest which functions as a molecular weight marker. Lanes B and C contain 50 bp DNA Step ladder which functions as a molecular weight marker also. Lanes 5, 6, 7 and 8 contain PCR reactions that were conducted using Readymix preparation (Sigma-Aldrich). Lanes 1, 2, 3 and 4 contain PCR reactions that were conducted using PCR Master Mix (Promega). DNA from four different individuals (from the diabetic population), was also used for PCR. DNA used in PCR reactions represented in lanes 1 & 5, came from sample number 4, those in lanes 2 & 6 came from sample number 5, those in lanes 3 & 7 came from sample number 8, and those in lanes 4 & 8 came from sample number 10.

3.2.6 Restriction Endonuclease Digestion / Mutational Analysis

Following PCR, the product was subjected to restriction endonuclease digestion with *Rsa I, Nde II* and *Hinf I*, which detected the presence / absence of the haemochromatosis mutations C282Y, H63D and S65C, respectively. All 250 samples from both the diabetic and 'control' populations were genotyped for the C282Y and H63D mutations. A selected group of 125 samples from each of the diabetic and 'control' populations underwent analysis for the S65C mutation.

(a) Restriction Digestion for the C282Y mutation using *Rsa I*

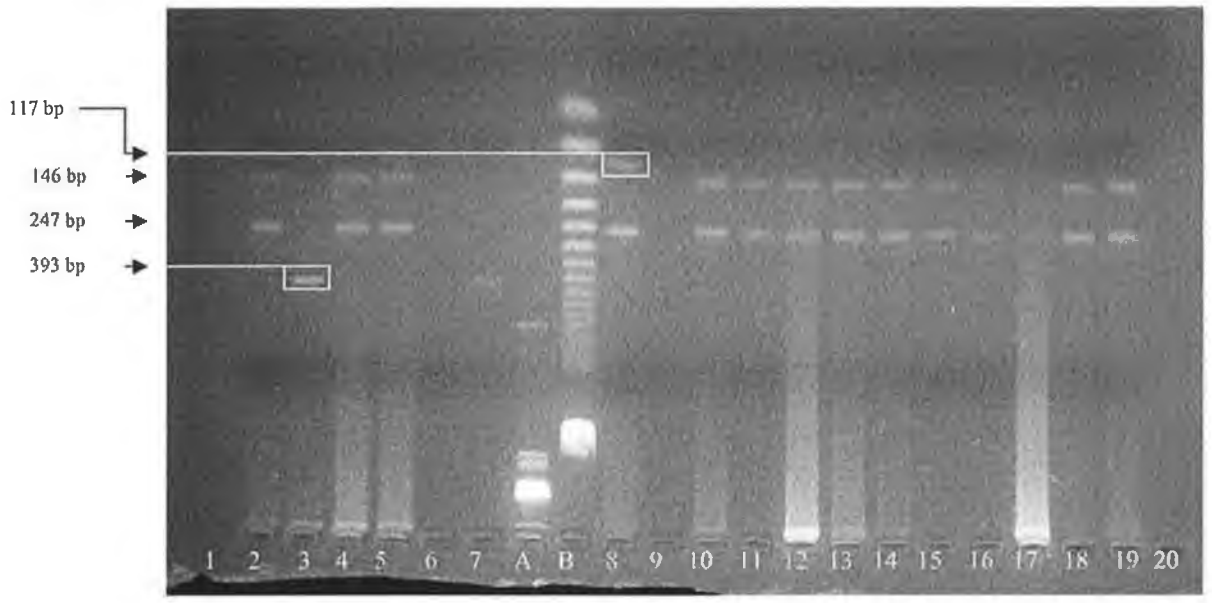


Figure 3.5: Typical Restriction Fragment Pattern indicating absence of the C282Y mutation

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which also functions as a molecular weight marker. Lane 8 contains a C282Y homozygote positive control demonstrating restriction fragments at 247 bp and 117 bp (the 29 bp fragment is not visible). ***Lanes 2, 4, 5, 10 – 16, 18 & 19 contain samples that lack the C282Y mutation*** (Restriction fragments at 247 bp and 146 bp). Lane 9 contains a negative control for PCR. Lanes 3 and 7 contain non-enzyme digested controls (undigested product fragment size of 393 bp). Results in Lanes 6 and 17 are unclear due to under-efficient purification (and as such were re-extracted and retested at a later date). Lanes 1 and 20 are empty.

Figure 3.6: Typical Restriction Fragment Pattern indicating a C282Y heterozygote

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which also functions as a molecular weight marker. Lane 7 contains a C282Y homozygote positive control demonstrating restriction fragments at 247 bp and 117 bp (the 29 bp fragment is not visible). ***Lane 8 contains a C282Y positive heterozygote control*** demonstrating restriction fragments at 247 bp, 146 bp and 117 bp (the 29 bp fragment is not visible). ***Lanes 10, 11, 13, 14 and 19 contain samples that are C282Y heterozygotes***. Lanes 2, 3, 4, 5, 6, 15 and 17 contain samples that lack the C282Y mutation (Restriction fragments at 247 bp and 146 bp). Lane 9 contains a negative control for PCR. Lanes 12, 16 and 18 contain non-enzyme digested controls (undigested product fragment size of 393 bp). Lanes 1 and 20 are empty.

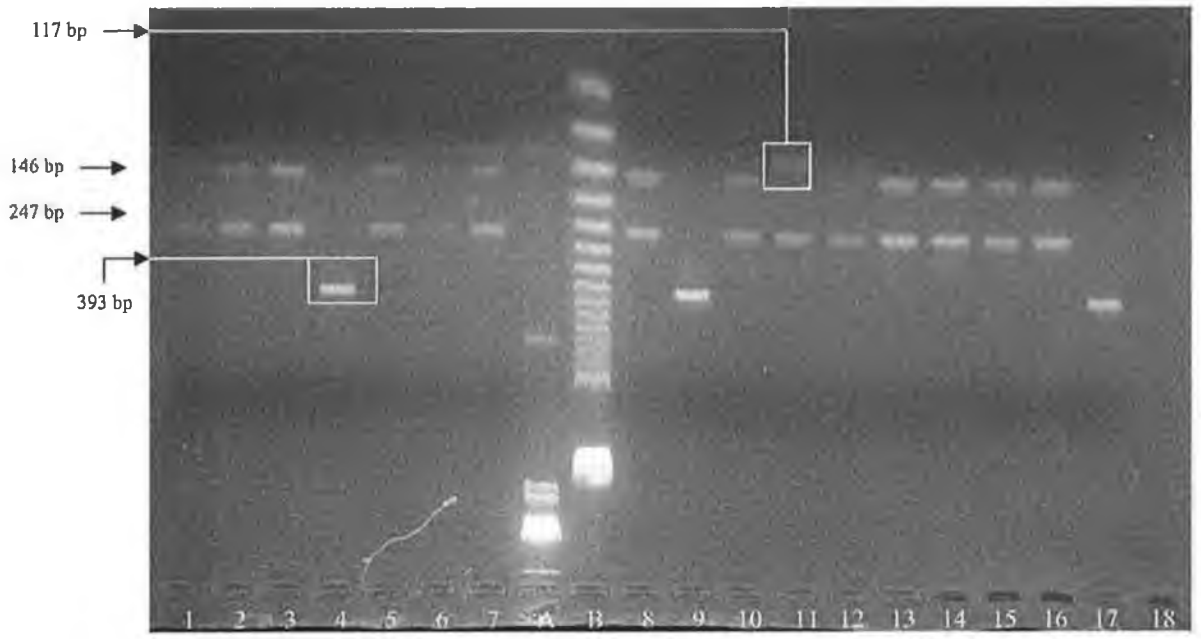


Figure 3.7: Typical Restriction Fragment Pattern indicating a C282Y homozygote

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which also functions as a molecular weight marker. Lane 7 contains a C282Y heterozygote positive control demonstrating restriction fragments at 247 bp, 146 bp and 117 bp (the 29 bp fragment is not visible). Lanes 2 and 12 contains C282Y heterozygotes. ***Lane 11 contains a C282Y homozygote*** (restriction fragments at 247 bp and 117 bp (the 29 bp fragment is not visible). Lanes 1, 3, 5, 6, 8, 10, 13, 14, 15 and 16 contain samples that lack the C282Y mutation (Restriction fragments at 247 bp and 146 bp). Lane 18 contains a negative control for PCR. Lanes 4, 9 and 17 contain non-enzyme digested controls (undigested product fragment size of 393 bp).

(b) Restriction Digestion for the H63D mutation using *Nde II*

Figure 3.8: Typical Restriction Fragment Pattern indicating absence of the H63D mutation

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which also functions as a molecular weight marker. Lane 10 contains a H63D heterozygote positive control demonstrating restriction fragments at 237 bp, 138 bp, 99 bp and 57 bp. Lanes 2 and 17 contain H63D heterozygotes. ***Lanes 4, 5, 6, 7, 13, 15, 16 and 19 contain samples that lack the H63D mutation*** as they demonstrate restriction fragments at 138 bp, 99 bp and 57 bp. Lane 11 contains a negative control for PCR. Lanes 3, 14 and 18 contain non-enzyme digested controls (undigested product fragment size of 294 bp). Results in Lanes 8 and 9 are unclear due to under-efficient purification (and as such were re-extracted and retested at a later date). Lanes 1 and 12 are empty.

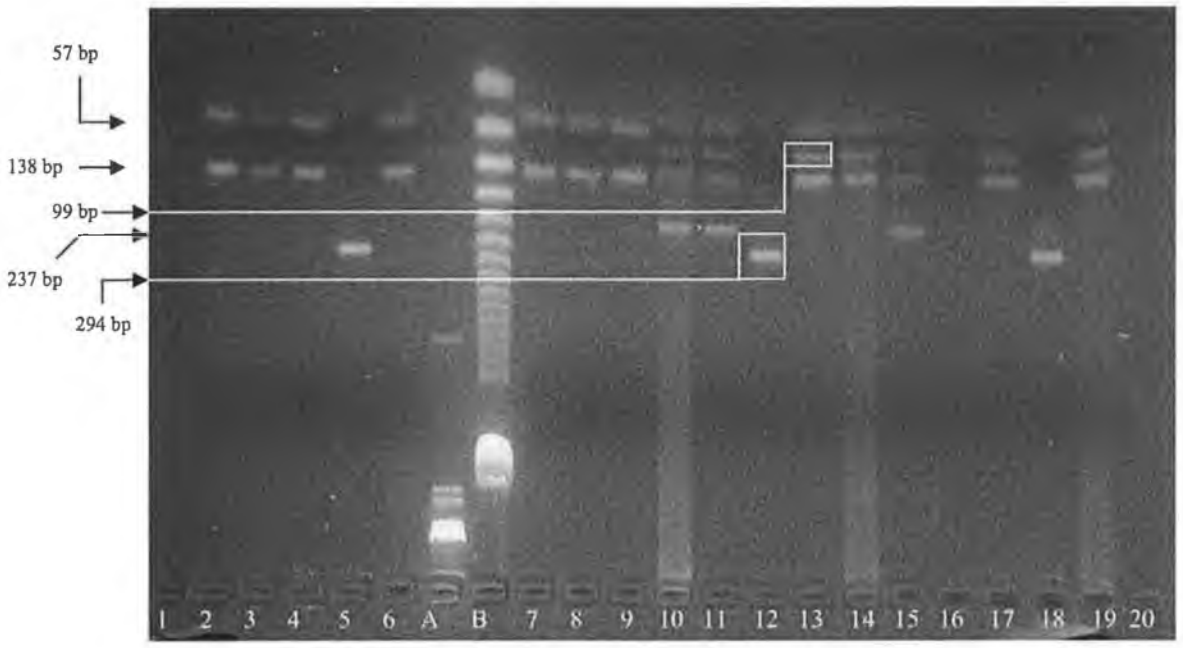


Figure 3.9: Typical Restriction Fragment Pattern indicating a H63D heterozygote

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which also functions as a molecular weight marker. Lane 10 contains a H63D heterozygote positive control demonstrating restriction fragments at 237 bp, 138 bp, 99 bp and 57 bp. ***Lanes 11 and 15 contain H63D heterozygotes.*** Lanes 13, 14, 17 and 19 contain samples that lack the H63D mutation as they demonstrate restriction fragments at 138 bp, 99 bp and 57 bp. Lane 16 contains a negative control for PCR. Lanes 12 and 18 contain non-enzyme digested controls (undigested product fragment size of 294 bp). Lanes 1 and 20 are empty. Note: Results in Lanes 2, 3, 4, 5, 6, 7, 8 and 9 pertain to S65C analysis that was run on the same gel.

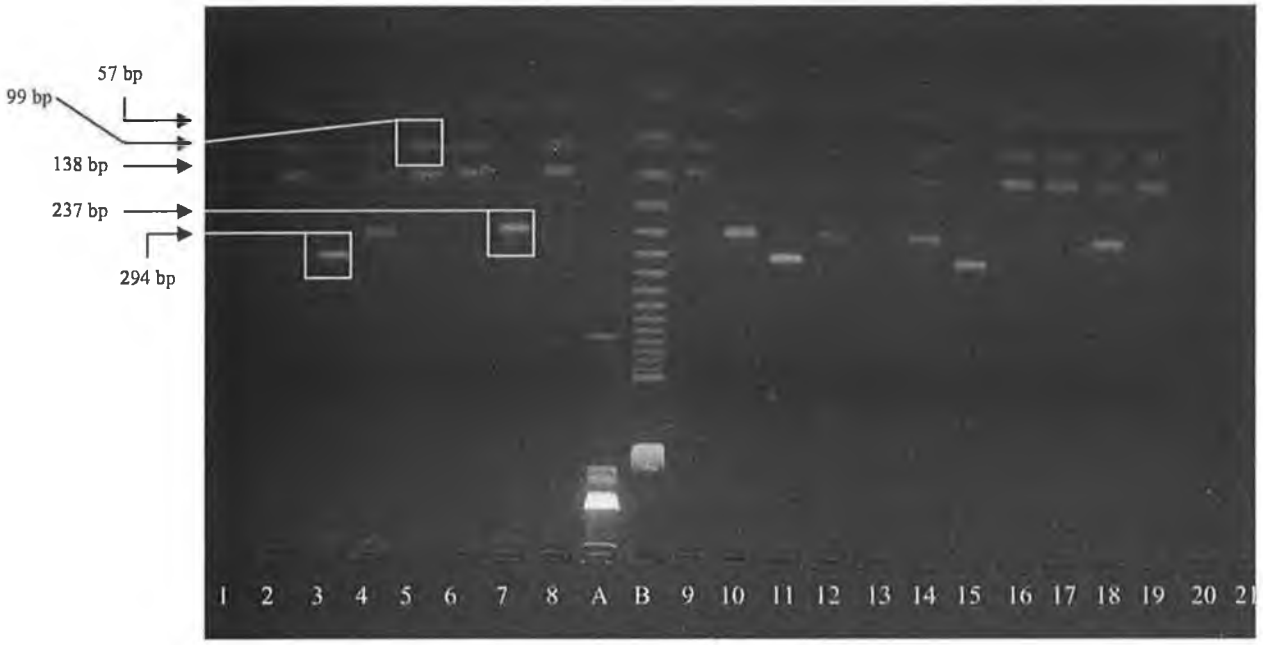


Figure 3.10: Typical Restriction Fragment Pattern indicating a H63D homozygote

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which also functions as a molecular weight marker. Lane 10 contains a H63D homozygote positive control demonstrating restriction fragments at 237 bp and 57 bp. ***Lane 7 contains a H63D homozygote***. Lanes 4, 12, 14 and 18 contain H63D heterozygotes. Lanes 2, 5, 6, 8, 9, 16, 17 and 19 contain samples that lack the H63D mutation as they demonstrate restriction fragments at 138 bp, 99 bp and 57 bp. Lane 13 contains a negative control for PCR. Lanes 3, 11 and 15 contain non-enzyme digested controls (undigested product fragment size of 294 bp). Lanes 1, 20 and 21 are empty.

(c) Restriction Digestion for the S65C mutation using *Hinf I*

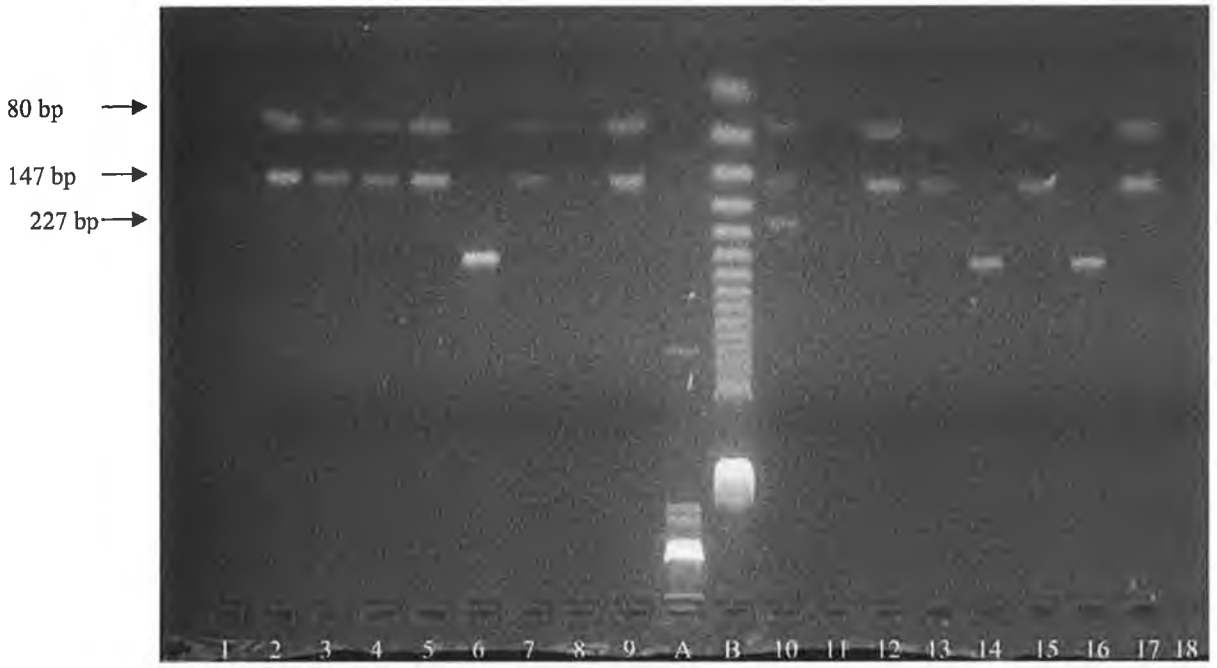


Figure 3.11: Typical Restriction Fragment Pattern indicating absence of the S65C mutation

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which functions as a molecular weight marker also. Lane 10 contains a S65C heterozygote positive control demonstrating restriction fragments at 80 bp, 147 bp and 227 bp. Lane 11 contains a negative control for PCR. ***Lanes 2, 3, 4, 5, 7, 8, 9, 12, 13, 15 and 17 contain samples, which lack the S65C mutation (restriction fragments at 80 bp and 147 bp).*** Lanes 6, 14 and 16 contain non-enzyme digested controls. Lanes 1 and 18 are empty.

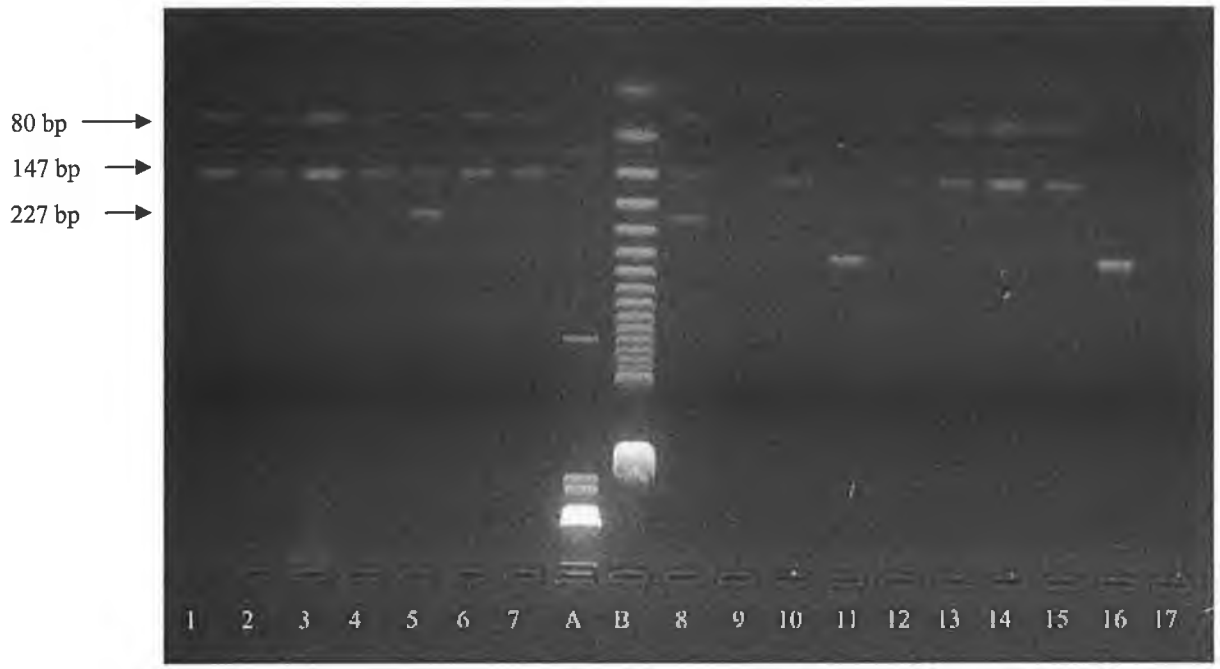


Figure 3.12: Typical Restriction Fragment Pattern indicating a S65C heterozygote

Lane A contains Lambda Hind III digest which functions as a molecular weight marker. Lane B contains a 50 bp DNA Step Ladder which functions as a molecular weight marker also. Lane 8 contains a S65C heterozygote positive control demonstrating restriction fragments at 80 bp, 147 bp and 227 bp. Lane 9 contains a negative control for PCR. ***Lane 5 contains a S65C heterozygote*** sample. Lanes 1, 2, 3, 4, 6, 7, 10, 12, 13, 14 and 15 contain samples, which lack the S65C mutation (restriction fragments at 80 bp and 147 bp). Lanes 11 and 16 contain non-enzyme digested controls. Lane 17 is empty.

The following Tables (Tables 3.0 – 3.4), record the incidence of the three haemochromatosis mutations in both the diabetic and 'control' populations.

Table 3.0: ***Incidence of C282Y mutations in the Diabetic and Control Populations.***

Mutational Status	Sample Identification Numbers at each genotype	
	Diabetic Population	***Control Population***
C282Y homozygous	164. **(N = 1)**	C48, C92, C184. **(N = 3)**
C282Y heterozygous	3, 4, 32, 34, 35, 57, 58, 63, 67, 71, 72, 81, 86, 88, 90, 103, 104, 113, 117, 122, 139, 152, 156, 161, 167, 171, 182, 191, 196, 205, 206, 212, 215, 219, 225, 238, 250. **(N = 37)**	C8, C10, C11, C25, C28, C30, C33, C46, C47, C58, C66, C73, C79, C80, C100, C103, C104, C107, C108, C128, C130, C134, C156, C157, C158, C159, C162, C177, C181, C185, C192, C198, C211, C214, C219, C222, C229, C236, C237, C238, C239, C242, C244, C246, C249. **(N = 45)**
No C282Y mutation	1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 36, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 59, 60, 61, 62, 64, 65, 66, 68, 69, 70, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 85, 87, 89, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 105, 106, 107, 108, 109, 110, 111, 112, 114, 115, 116, 118, 119, 120, 121, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 153, 154, 155, 157, 158, 159, 160, 162, 163, 165, 166, 168, 169, 170, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 183, 184, 185, 186, 187, 188, 189, 190, 192, 193, 194, 195, 197, 198, 199, 200, 201, 202, 203, 204, 207, 208, 209, 210, 211, 213, 214, 216, 217, 218, 220, 221, 222, 223, 224, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249. **(N = 211)**	C1, C4, C5, C6, C7, C9, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C26, C27, C29, C31, C32, C34, C35, C36, C37, C38, C39, C40, C41, C42, C43, C44, C45, C49, C50, C51, C52, C53, C54, C55, C56, C57, C59, C60, C61, C62, C63, C64, C65, C67, C68, C69, C70, C71, C72, C74, C75, C76, C77, C78, C81, C82, C83, C84, C85, C86, C87, C88, C89, C90, C91, C93, C94, C95, C96, C97, C98, C99, C101, C102, C105, C106, C109, C110, C111, C112, C113, C114, C115, C116, C117, C118, C119, C120, C121, C122, C123, C124, C125, C126, C127, C129, C131, C132, C133, C135, C136, C137, C138, C139, C140, C141, C142, C143, C144, C145, C146, C147, C148, C149, C150, C151, C152, C153, C154, C155, C160, C161, C163, C164, C165, C166, C167, C168, C169, C170, C171, C172, C173, C174, C175, C176, C178, C179, C180, C182, C183, C186, C187, C188, C189, C190, C191, C193, C194, C195, C196, C197, C199, C200, C201, C202, C203, C204, C205, C206, C207, C208, C209, C210, C212, C213, C215, C216, C217, C218, C220, C221, C223, C224, C225, C226, C227, C228, C230, C231, C232, C233, C234, C235, C240, C241, C245, C247, C248, C250. **(N = 199)**

Table 3.0 shows the incidence of C282Y mutations in the Diabetic and Control Populations. The individual genotypes exhibited by all samples that were tested, is also detailed in the Table above. Results were unavailable for Sample Number(s): 37 (Diabetic Population); C2, C3 and C243 (Control Population). Therefore only 249 diabetic samples and 247 control samples were analysed for C282Y instead of 250 samples for each group (see Discussion).

Table 3.1: ***Incidence of H63D mutations in the Diabetic and Control Populations.***

Mutational Status	Sample Identification Numbers at each genotype	
	Diabetic Population	***Control Population***
H63D homozygous	14, 44, 50, 55, 60, 97, 109, 114, 173, 183, 189, 202, 228. **(N = 13)**	C4, C5, C17, C26, C121, C148, C213, C234, C240. **(N = 9)**
H63D heterozygous	7, 8, 11, 12, 18, 20, 21, 23, 24, 26, 29, 30, 36, 40, 45, 46, 48, 51, 54, 57, 59, 62, 64, 69, 74, 76, 80, 83, 106, 107, 110, 113, 120, 122, 124, 127, 131, 142, 145, 146, 149, 163, 165, 166, 174, 176, 179, 181, 186, 193, 196, 198, 209, 218, 222, 227, 230, 236, 237, 240, 241, 242, 248. **(N = 63)**	C1, C7, C12, C23, C34, C38, C42, C44, C45, C53, C58, C63, C67, C69, C71, C73, C75, C85, C86, C87, C91, C94, C99, C110, C112, C116, C117, C123, C125, C126, C127, C132, C135, C137, C138, C141, C142, C145, C151, C156, C164, C165, C171, C173, C179, C180, C190, C191, C194, C197, C201, C215, C223, C229, C236, C238, C242. **(N = 57)**
No H63D mutation	1, 2, 3, 4, 5, 6, 9, 10, 13, 15, 16, 17, 19, 25, 27, 28, 31, 32, 33, 34, 35, 37, 38, 39, 41, 42, 43, 47, 49, 52, 53, 56, 58, 61, 63, 65, 66, 67, 68, 70, 71, 72, 73, 75, 77, 78, 79, 81, 82, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 105, 108, 111, 112, 115, 116, 117, 118, 119, 121, 123, 125, 126, 128, 129, 130, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 143, 144, 147, 148, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 164, 167, 168, 169, 170, 171, 172, 175, 177, 178, 180, 182, 184, 185, 187, 188, 190, 191, 192, 194, 195, 197, 199, 200, 201, 203, 204, 205, 206, 207, 208, 210, 211, 212, 213, 214, 215, 216, 217, 219, 220, 221, 223, 224, 225, 226, 229, 231, 232, 233, 234, 235, 238, 239, 243, 244, 245, 246, 247, 249, 250. **(N = 173)**	C2, C3, C6, C8, C9, C10, C11, C13, C14, C15, C16, C18, C19, C20, C21, C22, C24, C25, C27, C28, C29, C30, C31, C32, C33, C35, C36, C37, C39, C40, C41, C43, C46, C47, C48, C49, C50, C51, C52, C54, C55, C56, C57, C59, C60, C61, C62, C64, C65, C66, C68, C70, C72, C74, C76, C77, C78, C79, C80, C81 C82, C83, C84, C88, C89, C90, C92, C93, C95, C96, C97, C98, C100, C101, C102, C103, C104, C105, C106, C107, C108, C109, C111, C113, C114, C115, C118, C119, C120, C122, C124, C128, C129, C130, C131, C133, C134, C136, C139, C140, C143, C144, C146, C147, C149, C150, C152, C153, C154, C155, C157, C158, C159, C160, C161, C162, C163, C166, C167, C168, C169, C170, C172, C174, C175, C176, C177, C178, C181, C182, C183, C184, C185, C186, C187, C188, C189, C192, C193, C195, C196, C198, C199, C200, C202, C203, C204, C205, C206, C207, C208, C209, C210, C211, C212, C214, C216, C217, C218, C219, C220, C221, C222, C224, C225, C226, C227, C228, C230, C231, C232, C233, C235, C237, C239, C241, C243, C244, C245, C246, C247, C248, C249, C250. **(N = 184)**

Table 3.1 shows the incidence of the H63D mutations in the Diabetic and Control Populations. The individual genotypes exhibited by all samples that were tested, is also detailed in the Table above. Results were unavailable for Sample Number: 22 (Diabetic Population). Therefore only 249 diabetic samples analysed for H63D instead of 250 (see Discussion)

Table 3.2: ***Incidence of S65C mutations in the Diabetic and Control Populations.***

Mutational Status	Sample Identification Numbers at each genotype	
	Diabetic Population	*Control Population*
S65C homozygous	- **(N = 0)**	- **(N = 0)**
S65C heterozygous	54 **(N = 1)**	C8, C78, C124, C145. **(N = 4)**
No S65C mutation	1, 2, 3, 6, 7, 10, 11, 13, 15, 16, 18, 21, 22, 26, 27, 28, 31, 32, 35, 36, 38, 39, 41, 42, 43, 44, 45, 50, 51, 52, 56, 59, 63, 64, 65, 66, 69, 70, 71, 72, 74, 77, 80, 81, 85, 88, 89, 90, 92, 94, 97, 99, 100, 102, 103, 106, 107, 110, 113, 114, 117, 118, 120, 122, 123, 127, 128, 129, 131, 132, 133, 134, 137, 138, 140, 143, 144, 146, 147, 150, 151, 152, 156, 157, 159, 161, 163, 166, 167, 168, 172, 173, 176, 177, 179, 183, 184, 186, 190, 194, 195, 198, 199, 200, 203, 204, 209, 210, 211, 218, 219, 223, 228, 230, 233, 235, 236, 241, 243, 246, 249, 250. **(N = 123)**	C1, C4, C5, C9, C12, C14, C17, C20, C24, C25, C29, C30, C33, C34, C37, C40, C46, C47, C49, C53, C55, C57, C58, C60, C61, C62, C67, C68, C73, C75, C76, C79, C82, C83, C84, C86, C87, C91, C93, C95, C96, C98, C101, C104, C105 C108, C109, C111, C112, C115, C116, C119, C121, C125, C126, C130, C135, C136, C139, C141, C142, C148, C150, C153, C154, C155, C158, C160, C162, C164, C165, C169, C170, C171, C174, C175, C178, C180, C181, C182, C183, C187, C188, C189, C191, C192, C193, C196, C197, C201, C202, C205, C206, C207, C208, C212, C213, C214, C215, C216, C217, C220, C221, C222, C224, C225, C226, C227, C229, C231, C232, C234, C237, C238, C239, C240, C242, C244, C245, C247, C248. **(N = 121)**

Table 3.2 shows the incidence of the S65C mutations in the Diabetic and Control Populations. The individual genotypes exhibited by all samples that were tested, is also detailed in the Table above. One sample was inadvertently analysed in duplicate (18), this meant that only 124 diabetic samples were analysed for S65C instead of 125 (see Discussion).

Table 3.3: ***Incidence of compound heterozygosity for the haemochromatosis mutations C282Y, H63D and S65C in the Diabetic & 'Control' Populations***

Genotype	Sample Identification Number, Number & Frequency at each Genotype	
	Diabetic Population	**Control Population**
C282Y/H63D compound heterozygotes	57, 113, 122, 196 (N=4) Frequency = 0.8%	C58, C73, C156, C229, C236, C238, C242 (N=7) Frequency = 1.41%
C282Y/S65C compound heterozygotes	-	C8 (N=1) Frequency = 0.27%
H63D/S65C compound heterozygotes	54 (N=1) Frequency = 0.27%	C145 (N=1) Frequency = 0.27%

Table 3.3 (above) shows the incidence of C282Y/H63D-, C282Y/S65C- and H63D/S65C- compound heterozygosity in both the Diabetic and Control Populations. The various samples at each genotype are identifiable by their sample identification numbers (sample numbers are also included and are in parentheses). The frequency of each genotype in the two populations is also reported.

Table 3.4: Allele & Genotype Distribution of the HFE gene in the Diabetic & 'Control' populations

	Allele Frequency				Genotype Frequency
Codon 282	*wt*	*C282Y*	x^2 = 0.58* & 0.0643♠	*wt/wt*	*wt/C282Y & C282Y/C282Y*
			1df p = <0.05*♠		
*Diabetic Population**	459 (92.2%)	39 (7.8%)		211 (84.74%)	38 (15.26%)
'Control' Population♠	443 (89.7%)	51 (10.3%)		199 (80.57%)	48 (19.43%)
Codon 63	*wt*	*H63D*	x^2 = 4.74* & 2.805♠	*wt/wt*	*wt/H63D & H63D/H63D*
			1df p = >0.05* & p = <0.05♠		
*Diabetic Population**	409 (82.1%)	89 (17.9%)		173 (69.48%)	76 (30.52%)
'Control' Population♠	425 (85%)	75 (15%)		184 (73.6%)	66 (26.4%)
Codon 65	*wt*	*S65C*	x^2 = 0.5091* & 0.033♠	*wt/wt*	*wt/S65C & S65C/S65C*
			1df p = <0.05*♠		
*Diabetic Population**	246 (99.19%)	2 (0.81%)		123 (99.19%)	1 (0.81%)
'Control' Population♠	246 (98.4%)	4 (1.6%)		121 (96.8%)	4 (3.2%)

Table 3.4 shows the allele frequencies of the three haemochromatosis mutations in both the Diabetic and Control Populations (indicated by the codon where the mutation occurs; i.e. the C282Y mutation occurs in codon 282, the H63D mutation occurs in codon 63 and the S65C mutation occurs in codon 65). The Table also details the genotype frequencies for each of the three mutations in the two populations. Appendix E contains all statistical calculations. No statistically significant differences in the HFE genotype frequencies were found between the two populations.

Key:
wt = wildtype
x^2 = chi- squared
df = degrees freedom

Table 3.5 Results of External Genetic Testing

Sample Number	Genotype
34	C282Y/Normal*
37	Normal/Normal*
38	Normal/Normal*
41	Normal/Normal*
49	Normal/Normal*
50	H63D/H63D*
53	Normal/Normal*
56	Normal/Normal*
64	H63D/Normal*
108	Normal/Normal*
C8	S65C/C282Y compound heterozygote♣
C54	S65C heterozygote♣
C78	S65C heterozygote♣
C124	S65C heterozygote♣
C145	S65C/H63D compound heterozygote♣

Results were validated by re-testing of 15 samples at two external genetic facilities. Both facilities are accredited for testing and use highly automated technologies such as lightcyclers for analysis of genetic mutations. Some results of external analysis conducted by Dublin are presented in Figures 3.13 to 3.17.

Key: * = Genotyping was carried out by the Cytogenetics Unit in the National University of Ireland (NUI), Galway.
♣ = Genotyping was carried out by the Centre for Liver Disease, Eccles Street, Dublin (c/o Dr. Sharon Barrett).

3.3 *Biochemistry Analysis*

Table 3.6 which follows, details the results of the various biochemistry parameters that were analysed in individuals who gave written consent for study participation (N=26 individuals from the Diabetic Population).

Table 3.6: ***Results of Biochemistry analysis for individuals who consented to study participation (N = 26).***

Sample Number	Gender	Age	Iron (Fe)	TIBC	Transferrin Saturation	Ferritin	AST	ALT	HbA1c	Genotype
		years	*µmole/L*	*µmole/L*	*%*	*µg/L*	*IU/L*	*IU/L*	*%*	
67	F	68	18	51.7	35	21.4	38	31	8.6	C282Y heterozygote■
77	M	58	14	50.4	28	171.9	21	39	8.1	Wildtype●
60	M	59	15	54.9	27	128.9	26	37	7.1	H63D homozygote■
37	M	67	12	46.3	26	145.8	20	17	9.7	Wildtype▲
39	F	76	13	62.3	21	72.5	39	37	7	Wildtype●
54	M	77	17	43.5	39	97.7	27	21	9.1	H63D/S65C compound heterozygote●
52	M	51	27	57.4	47	161.4	21	27	7	Wildtype●
47	M	82	15	62.8	24	52	26	19	6.9	Wildtype■
34	M	50	37	54.6	68	196.6	18	23	9.2	C282Y heterozygote■
38	F	51	12	55.5	22	22.4	24	18	7	Wildtype●
72	F	46	28	44.9	62	43.2	16	24	9.5	C282Y heterozygote●
175	M	40	15	68.4	22	119.6	-	-	11.6	Wildtype■
74	M	65	14	40.2	35	73.9	21	30	7.8	H63D heterozygote●

Table 3.6 continued

68	M	71	21	48.5	43	260.8	23	28	7.9	Wildtype■
76	M	72	11	71.2	15	111.5	31	38	8.3	H63D heterozygote■
55	M	76	12	42.2	28	115.1	20	18	7.5	H63D homozygote■
66	M	61	14	58.1	24	13.4	26	33	8.9	Wildtype●
56	F	55	19	54.6	35	95.4	36	37	6.4	Wildtype●
42	F	53	14	55.4	25	32.3	26	30	8.5	Wildtype●
51	F	74	21	50.1	42	88.1	34	38	7.7	H63D heterozygote●
78	M	76	22	52.5	42	436.4	24	21	6.1	Wildtype■
222	M	73	-	-	-	-	15	16	7	H63D heterozygote■
49	M	68	20	57.9	35	59.2	21	18	8.2	Wildtype■
41	F	52	16	68.7	23	46	21	24	8.2	Wildtype●
50	M	20	17	49.5	34	41.5	23	20	9.2	H63D homozygote●
46	M	50	16	61.5	26	141.2	27	42	9.1	H63D heterozygote■
Results Range			11 - 37	40.2 – 68.7	15 - 68	13.4 – 436.4	15 - 39	16 - 39	6.4 – 11.6	
Mean of All Results			16.92	52.43	31.85	105.7	24	26.39	8.14	
Mean of Male Results			16.61	51.11	31.28	112.56	21.67	24.83	8.26	
Mean of Female Results			17.63	55.4	33.13	52.66	29.25	29.88	7.86	
Reference Range (Sligo General Hospital)			106 – 283	40 – 75	< 30	21.61 – 274.66	5 – 37	5 – 40	3.8 – 5.0 *	

Key: * DCCT aligned

■ Genotyped for the C282Y & H63D mutations only ▲ Genotyped for the H63D mutation only.

● Genotyped for the C282Y, H63D & S65C mutations Male Reference Range = Female Reference Range =

Table 3.7: ***Mean Values for iron indices for each haemochromatosis genotype exhibited in the 26 consenting diabetic individuals that underwent biochemistry analysis.***

	Iron (µmole/L)	TIBC (µmole/L)	Transferrin Saturation (%)	Ferritin (µg/L)	AST (IU/L)	ALT (IU/L)	HbA1c (%)
C282Y heterozygote (N=3) [Sample numbers: 67, 34, 72]	27.67	50.4	55	87.07	24	26	9.1
H63D homozygote (N=3) [Sample numbers: 60, 55, 50]	14.67	48.87	29.67	95.17	23	25	7.93
H63D heterozygote (N=6) [Sample numbers 54, 74, 76, 51, 222, 46]	15.8*	53.3*	31.4*	102.48*	25.83	30.83	8.17
Wildtype (N=14) [Sample numbers 77, 37, 39, 52, 47, 38, 175, 68, 66, 56, 42, 78, 49, 41]	16.71	55.63	29.79	120.65	25.23♠	26.77♠	7.96

Table 3.7 shows the mean values for iron indices exhibited by each haemochromatosis genotype.

* = only 5 out of 6 individuals had test results available, therefore results presented are a mean of 5 values.

♠ = only 13 out of 14 individuals had test results available, therefore results presented are a mean of 13 values.

Note: One S65C/H63D compound heterozygote was identified within this group. This individuals iron indices values were included when calculating the mean values for the H63D heterozygote genotype (row three above). No mean results for the S65C heterozygote genotype are presented, as there was only one S65C heterozygote identified.

Figure 3.13: Lightcycler / RT-PCR graph for C282Y amplification (Examples of External Results / Analysis conducted by Centre for Liver Disease, Dublin).

The figure below shows results of 5 samples that were amplified for C282Y mutation analysis in duplicate. The sample numbers corresponding to each line are detailed on the left-hand side of the printout (inside the blackened box). Results of approximately 45 cycles of amplification are shown.

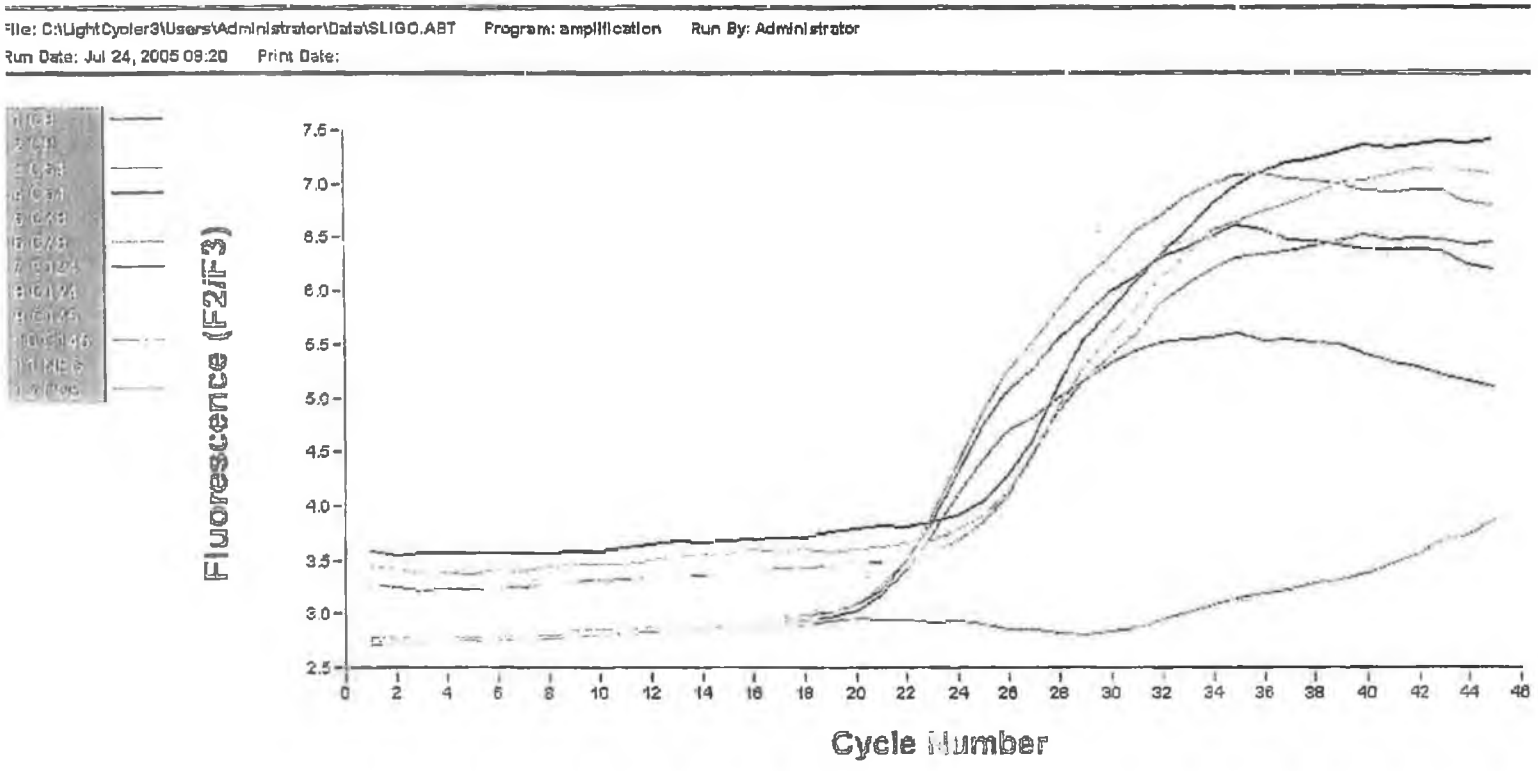


Figure 3.14: Lightcycler / RT-PCR graph for H63D amplification (Examples of External Results / Analysis conducted by Centre for Liver Disease, Dublin).

The figure below shows results of 5 samples that were amplified for H63D mutation analysis in duplicate. The sample numbers corresponding to each line are detailed on the left-hand side of the printout (inside the blackened box). Results of approximately 45 cycles of amplification are shown.

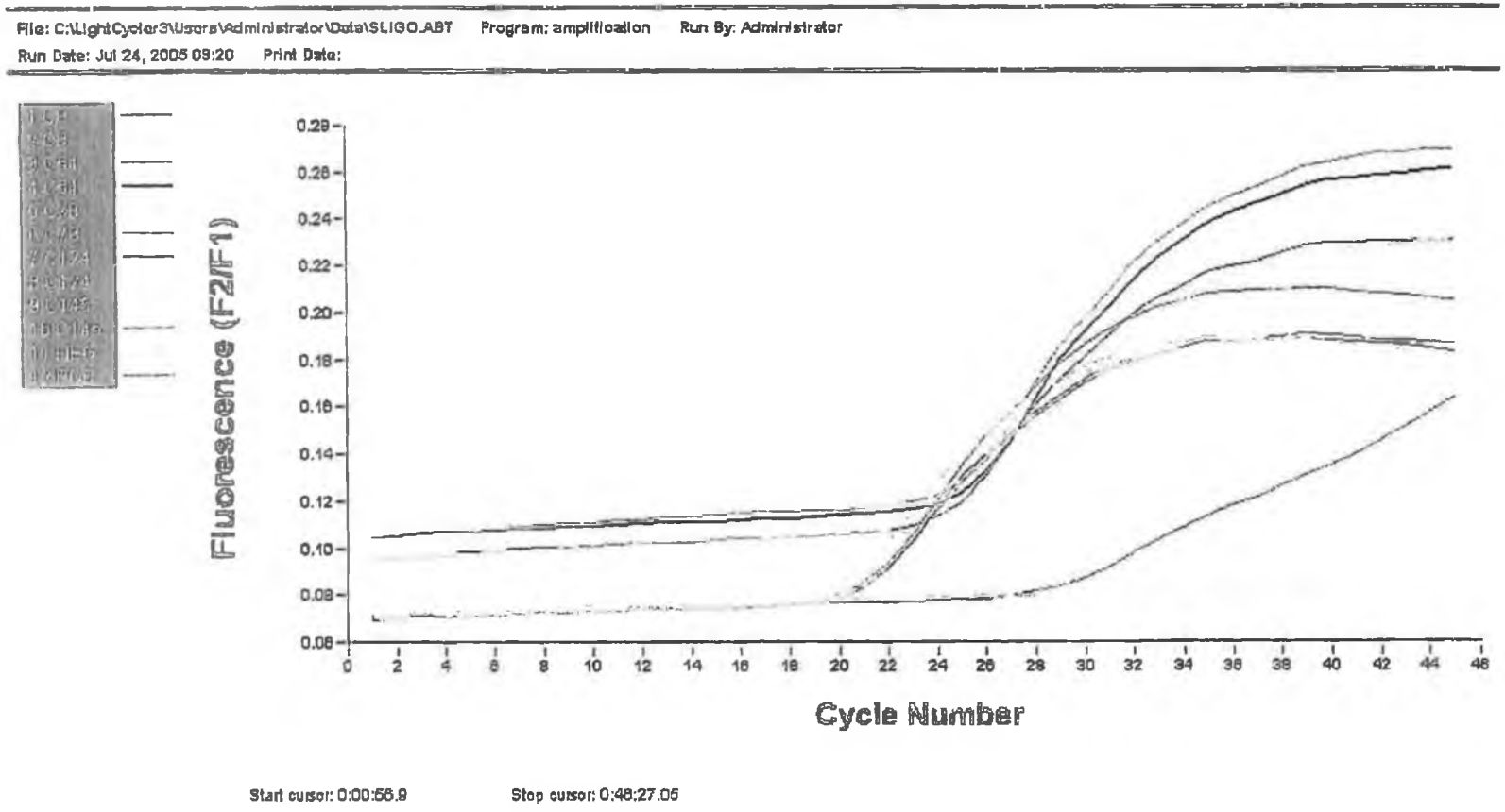


Figure 3.15: Lightcycler / RT-PCR graph for H63D negative sample (Examples of External Results / Analysis conducted by Centre for Liver Disease, Dublin).

The figure below shows results of an amplification carried out on a sample that was negative for the H63D mutation. The sample number and name corresponding to the H63D negative sample is 11 NEG (inside the blackened box on the left-hand side). The temperature range over which the test was run is shown on the X axis.

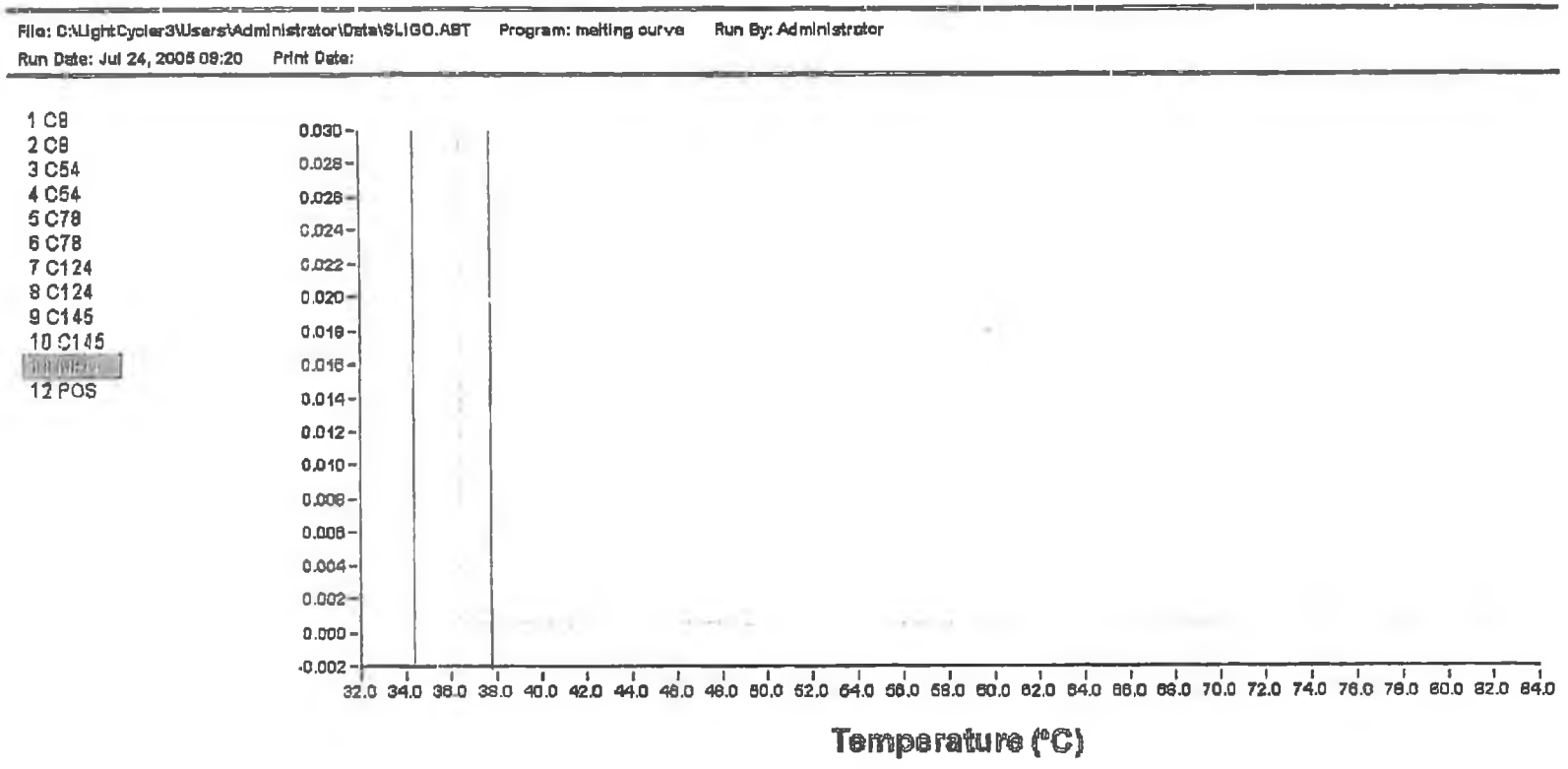


Figure 3.16: Lightcycler / RT-PCR graph for sample C54 [C282Y amplification] (Examples of External Results / Analysis conducted by Centre for Liver Disease, Dublin).

The Figure below shows results of an amplification for C282Y, of an individual sample (sample number C54). The sample in question was found to lack the C282Y mutation.

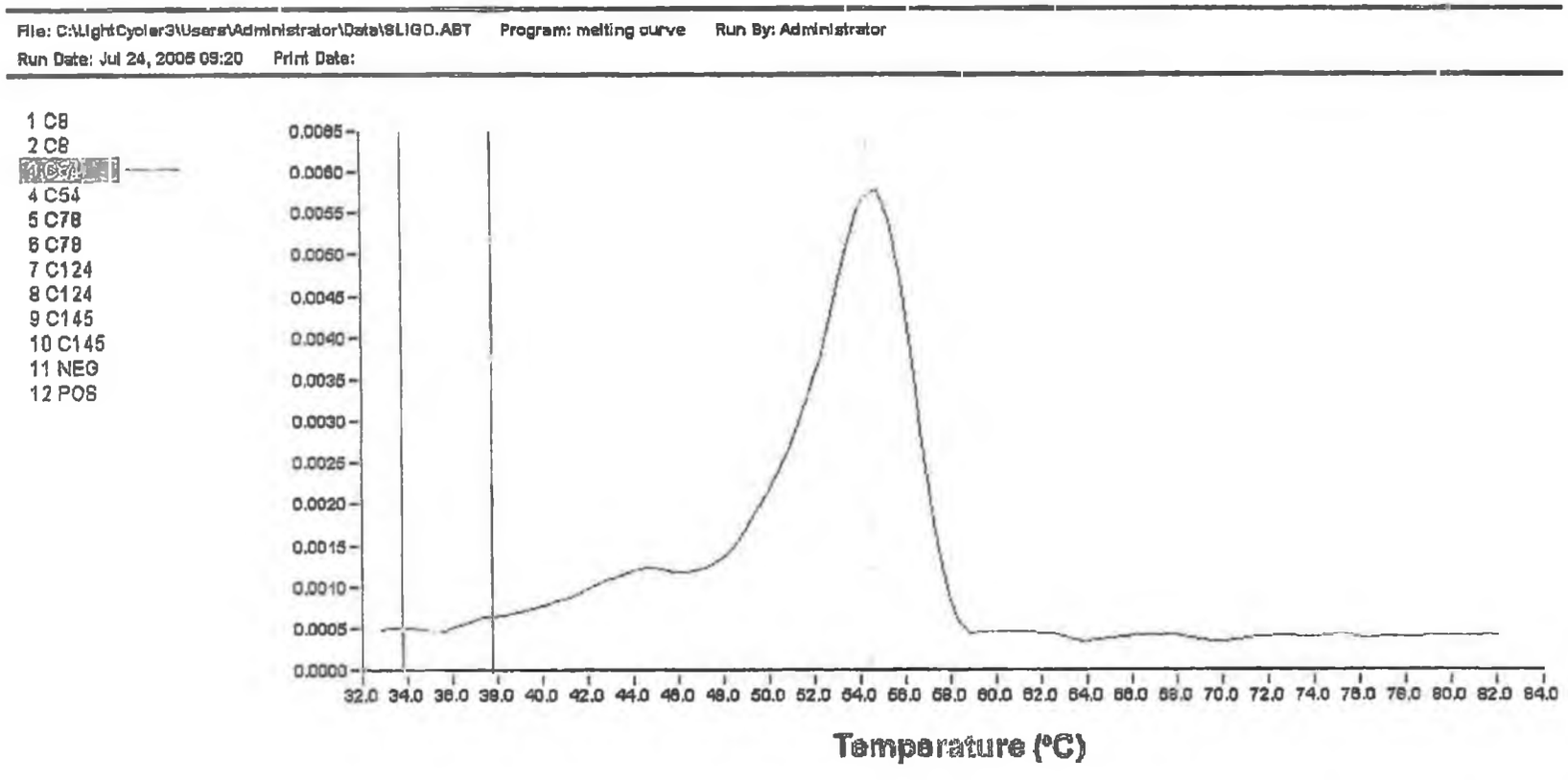
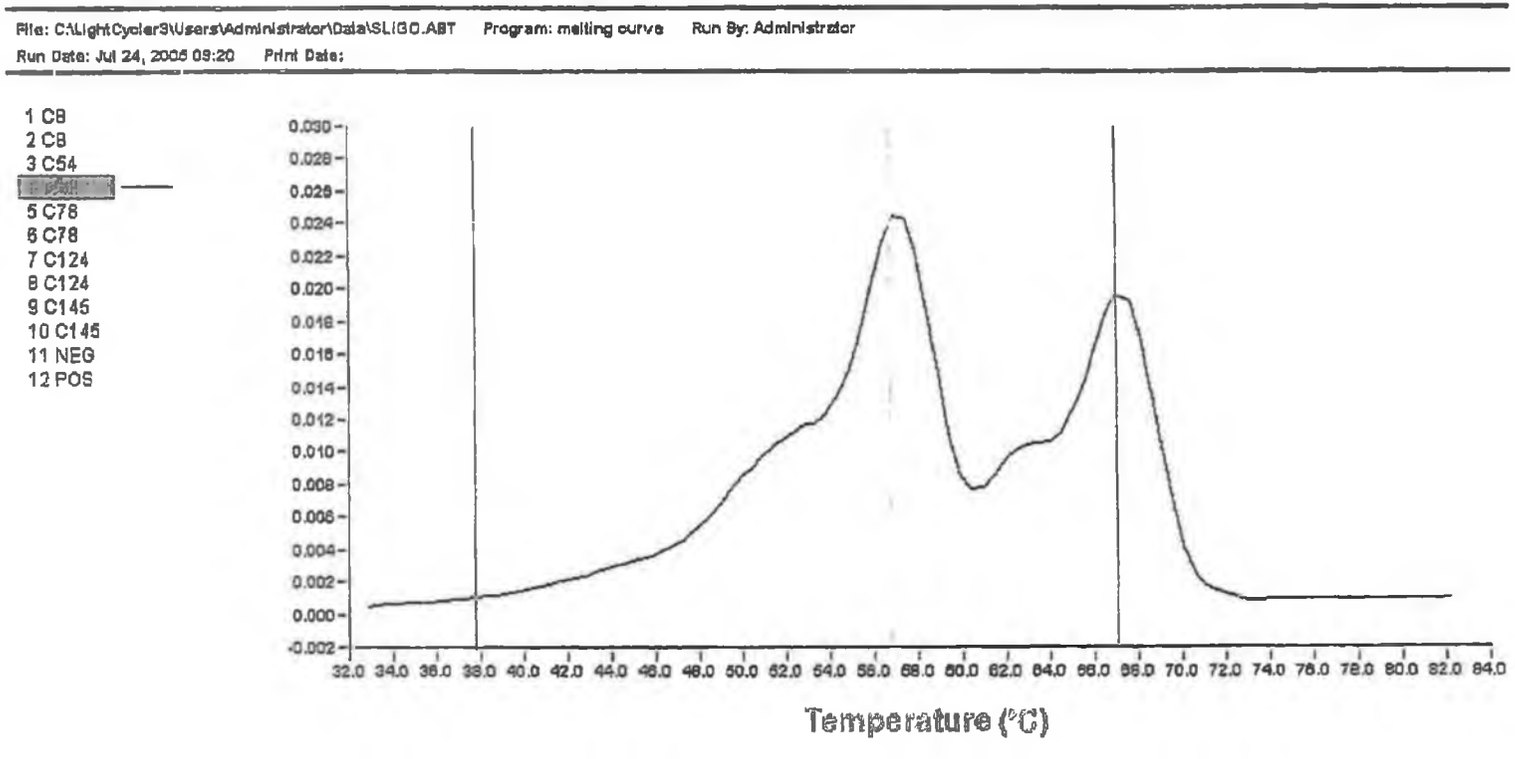


Figure 3.17: Lightcycler / RT-PCR graph for sample C54 [H63D amplification] (Examples of External Results / Analysis conducted by the Centre for Liver Disease, Dublin).

The Figure below shows results of an amplification for H63D, of an individual sample (sample number C54). The sample in question was found to lack the H63D mutation.



4.0 DISCUSSION

Discussion

The objectives of this study were to establish PCR assays for C282Y, H63D and S65C mutations in the HFE (hereditary haemochromatosis) gene, to determine the incidence of the three main genetic mutations for hereditary haemochromatosis in the North West population of ireland and to establish whether there is an increased frequency of these mutations in diabetic individuals from the same region.

The importance of this study lies in the fact that it will provide baseline data, which can be used to assess whether there is a need to implement a regional screening program for haemochromatosis, a condition that once diagnosed, is fully treatable and manageable. Early diagnosis (even before phenotypic expression) can help avoid development of complications such as diabetes and arthritis and decrease morbidity. Taking into account the co-existence of diabetes in haemochromatosis and vice versa (diabetes occurs at a relatively high rate in individuals with haemochromatosis) and the fact that some of the highest levels of iron in drinking water are found in the North West region (NWHB Reports, 2001), it is reasonable to propose an investigational study into the haemochromatosis mutation levels in the region.

The main finding of the study was that there was no significant difference in the incidence of HFE genotypes between the Diabetic and Control populations. C282Y and H63D allele frequencies in the control population were favourably comparable to other studies on similar populations. A higher incidence of H63D heterozygosity was found in diabetic individuals than in controls. Unexpectedly, the incidence of the S65C mutation (in control individuals) reported in the present study is higher than that reported in the general Irish population.

4.1 Sample Collection

The first stage in the study was recruitment of research subjects, which took place in the Out Patient department of Sligo General Hospital. Individuals attending the Diabetic Clinic were approached, given an information sheet about the study and asked if they were interested in participating. Those people that consented to participation signed consent forms recording their decision to do so and also gave blood samples, which were taken by phlebotomist, registrar or Medical consultant. Due to

time constraints, a limited number of willing participants for this phase of the study and the limited number of people (with concurrent heavy work loads) available for subject recruitment, only 26 diabetic samples were obtained in this manner. The majority of these were enlisted by the project Researcher. It was therefore decided to decrease total sample size from N = 1530 (original ethics application, see Appendix A) to N = 500 (N = 250 in each of the diabetic and 'control' populations). In order to reach test population sizes, supplemental samples had to be obtained from the laboratories of Sligo General Hospital. For the Diabetic Population: The remaining two hundred and twenty four samples were obtained by selecting them randomly from samples, which entered the lab for routine HbA1c testing. All samples (that were obtained through random selection) for genetic analysis were blinded to the researcher. 'Control' samples (N = 250) were obtained similarly, with the exception that they were samples that entered the lab for routine haematology testing.

To summarize, the first problem encountered within this study was at the stage of research subject recruitment. Only a very limited number of samples were obtained by the recruitment strategy initially decided upon during study design. The original sample number envisaged for the study (N=1530 individuals; 765 per diabetic and 'control' group, which was statistically calculated) had to be revised down and samples obtained in the manner detailed above. For future reference, in order to meet the quota of samples set down during study design, it is essential to have a sufficient number of qualified staff that are committed and resourced to collecting the correct sample size. Following sample receipt, the first requisite was to purify DNA from all 500 whole blood samples.

4.2 DNA Purification from whole blood

Prior to genetic analysis, DNA was extracted from all study samples by a Wizard™ kit procedure. Results demonstrated that if the purification procedure was successful, sufficient template DNA would be produced for use in PCR (see section 3.1). The Wizard™ kit instructions state that the DNA pellet obtained from the procedure should be rehydrated in a 100 μl volume of DNA Rehydration solution, however it was decided to decrease this volume to 50 μl in order to concentrate the available DNA. The rationale behind this decision was that the samples were stored frozen, for a period of up to a four months before being extracted, a factor that can cause a decrease in DNA

concentration (Wizard™ kit instructions). This was an important finding as there is little evidence of this requirement in published literature.

DNA obtained through the Wizard kit extraction procedure can be quantified (see Figure 3.0) but was not routinely done in this study due to mainly budgetary and time constraints. This meant that some samples (approximately 10%) had to be re-extracted at a later date due to insufficient template for original PCR analysis. DNA purification from fresh whole blood samples would have produced more favourable results in terms of lowering the percentage of samples that required re-extraction. Repeat testing due to poor DNA purification may cause unacceptable delays in a routine diagnostic service and therefore use of fresh specimens is advised.

A second type of DNA purification was also tried as part of this research, but was not chosen as the final method of extraction for reproducibility problems. The aforementioned method involved extraction of DNA from whole blood using a resin called Chelex® 100 (Biorad) and a method used by Walsh et al., (1991). Both purification methods are more favorable than traditional procedures, which can involve phenol-chloroform extraction and/or ethanol precipitation (Budowle & Baechtel, 1990; Sambrook et al., 1989) as well as inorganic procedures that employ high salt concentration (Dykes, 1988), excess proteinase K digestion (Grimberg et al., 1989) and the use of glass wool (Vogelstein & Gillespie, 1979). The Chelex extraction procedure is a favourable method of purification as it is simple, rapid, involves no organic solvents and does not require multiple tube transfers for most types of samples (Walsh et al., 1991). The Wizard™ kit was the chosen method for DNA extraction in this research because of its pre-prepared, high quality reagents, ease of use, high reproducibility of results and because only basic technologies were needed.

The DNA purification methods attempted and utilized in this study compare favourably with methods used in published research on haemochromatosis mutation analysis. Other commercially available DNA purification kits like QIAamp® Blood Mini kit (Qiagen GmbH, Germany) and Isolate 1 DNA extraction kit (Cruachem Ltd.), have been used in HFE genotyping studies (Gomez-Llorente et al., 2004; Datz et al., 1998), as well as variations on the Chelex extraction procedure (Byrnes et al., 2001; Ryan et al., 1998; Stott et al., 1999). Other DNA purification methods such as methanol

extraction (Barry et al., 2005) and standard alkaline lysis (Elsea & Leykam, 2000) have also been utilised in studies on haemochromatosis.

The use of automated technology or genetic analysers for rapid DNA quantification, had it been available, would ensure that unnecessary re-extractions or more lengthy quantification procedures could be avoided. The results for 15 samples were verified through re-testing by two external facilities. This formed part of the validation process for the 'in-house' assay. .Both external test facilities (Galway & Dublin) use automated systems for genotyping and are accredited for genetic testing.

4.3 PCR Optimisation

Once sufficient DNA template was available for PCR (following extraction), other factors, which can affect the success of PCR, were investigated. As initial attempts at PCR were conducted using a ReadyMix™ kit without magnesium chloride / $MgCl_2$ (Sigma-Aldrich), it was necessary to optimise in terms of $MgCl_2$ concentration. From Figure 3.1 it is evident that PCR was unsuccessful at $MgCl_2$ concentrations below 1.5 mM. Although PCR was successful at $MgCl_2$ concentrations between 2 mM and 4mM, it was decided to utilise a concentration of 1.5mM per PCR reaction to help minimise problems (like PCR inhibition) that can occur as a result of employing too high a concentration of magnesium chloride. This is in agreement with current practices / literature (Barry et al., 2005; Gomez-Llorente et al., 2004; Elsea & Leykam, 2000; Datz et al., 1998; Sambrook et al., 1989).

To further refine assay conditions, primer annealing temperature was evaluated and optimised. PCR reactions were set up and run at a series of different annealing temperatures between 50°C and 70°C. The range of temperatures used for annealing optimisation work was decided on, following manual calculation of the melting temperatures (T_m) of all four primers. The optimum annealing temperature for a primer is thought to be approximately 5°C below its melting temperature. Calculation of T_m for each primer pair helped decide the temperature range used in optimisation of annealing temperature (see methods section 2.11.3). Figure 3.2 shows that the optimum annealing temperature for the C282Y primer pair was 55°C. Similar work was carried out on the H63D primer pair and it was found that it had an identical optimum annealing temperature to that of the C282Y

primer pair (results are not shown). The annealing temperature used in this study was within the temperature range used in other studies on HFE mutation analysis, albeit with slightly different primers (Gomez-Llorente et al., 2004; Barry et al., 2005; Stott et al., 1999; Datz et al., 1998). Primer concentration was also investigated and the optimum concentration for PCR was found to be 5 picomoles of each primer pair. Again, this concentration was within the range used in other studies on haemochromatosis (Barry et al., 2005; Stott et al., 1999)

Template DNA concentration was also evaluated to ensure optimal PCR conditions. Results indicate that a 20 µl volume of product from the DNA purification procedure produces sufficient DNA to ensure further analysis of PCR product is successful (see Figure 3.3). Other HFE studies used similar volumes of DNA from their respective extraction procedures (Gomez-Llorente et al., 2004). To optimise in terms of any other PCR reaction components would have been superfluous, as they came pre-optimised in the ReadyMix™ kit.

During the analytical period a new PCR Master mix (Promega) came onto the market and was optimised in terms of all PCR reaction components save template concentration. Figure 3.4 illustrates that both products produced comparable results. It was decided that PCR Master mix (Promega) should be employed instead of the ReadyMix™ kit, to help ensure minimal interference in PCR efficiency which may occur as a result of small variations in the concentration of magnesium chloride.

All of the findings of these optimisation trials yielded results that fall within the accepted limits as set out in the literature. However, it should be noted that every laboratory should take literature values as guidelines and optimise their own systems.

4.4 Restriction Endonuclease Digestion

Once the PCR conditions had been optimised in terms of reaction component(s) and run conditions, it was possible to use PCR product for further analysis. In this study, PCR product was subjected to endonuclease digestion with various restriction enzymes in order to determine the presence /

absence of C282Y, H63D and S65C, the three main mutations responsible for hereditary haemochromatosis. All digestions were carried out in accordance with manufacturers instructions.

To detect the C282Y mutation, PCR product (393 bp) was digested with the enzyme *Rsa I*, which produces a characteristic pattern of restriction fragments, all of which have definite sizes. Bands were visually evaluated, with those produced in samples being sized by comparison of their position to those of molecular weight markers. Two different molecular weight markers were employed in this research; they were a 50 bp DNA Step Ladder (Promega) and a Lambda Hind III marker (Sigma-Aldrich). The 50bp DNA Step Ladder has 16 DNA fragments ranging from 50 base pairs (bp) to 800 bp, in exactly 50 bp increments. The Lambda Hind III digest has characteristic bands of size 125 bp, 564 bp, 2027 bp, 2322 bp, 4361 bp, 6559 bp, 9416 bp and 23130 bp (only the smallest bands are evident on gel results presented). C282Y homozygotes produce bands of the following sizes; 29 bp, 117 bp and 247 bp. Carriers of the C282Y mutation produce bands of 29 bp, 117 bp, 146 bp and 247 bp. Samples that lack the C282Y mutation will produce restriction fragments of 146 bp and 247 bp (Feder et al., 1996; Jeffrey et al., 1999). See Figures 3.5 to 3.7 for typical restriction fragment patterns associated with the C282Y mutation.

The H63D mutation was detected by subjecting PCR product (294 bp) to enzyme digestion with *Nde II*. Digestion produced bands of characteristic size. H63D homozygotes had restriction fragments of 57 bp and 237 bp and carriers of the mutation had fragments of 57 bp, 99 bp, 138 bp and 237bp. Samples that lacked H63D had restriction fragment sizes of 57 bp, 99 bp and 138 bp (Merryweather-Clarke et al., 1997a). Figures 3.8 to 3.10 show typical restriction patterns associated with the H63D mutation.

The final mutation assesed was the S65C mutation. Digests were performed with the restriction enzyme *Hinf I* to establish the presence / absence of the mutation. Samples that lacked the S65C mutation produced two restriction fragments (at 80 bp and 147 bp), whereas carriers of the mutation produced an extra restriction fragment (at 80 bp, 147 bp and 227 bp) (see Figures 3.11 and 3.12).

The rationale behind the usage of the four primers employed in the present study is detailed in section 1.7 and Figure 1.5 (Literature Review).

All enzymes and primers employed in this research have been utilised in published literature on the mutational analysis of C282Y, H63D and S65C.

4.5 Quality Control (pre- and post- PCR)

A rigorous QC/QA program is essential for any laboratory procedure, but especially so in genetic analysis. Duplicate samples give insight to the reproducibility or precision of the analysis, 'blanks' or negative controls test each step of the analysis and help prevent false positive results and positive controls monitor the efficiency of the reaction to prevent false negative results.

Cross contamination should be minimised by strict separation of steps in separate rooms, minimal air flow between rooms and no movement of equipment and supplies between restricted areas. Working areas should be clean with UV lights and disposable gloves being employed and separate lab coats worn in each defined area.

A typical recommendation for allocation of lab space for PCR states that at least two separate areas be defined for pre- and post- PCR manipulations. The first defined area should be used for pre-PCR work such as weighing of chemicals, preparation of buffers and stocks for PCR, DNA extraction and preparation of PCR. The second defined area should be used for post-PCR work like electrophoresis, processing, sequencing and restriction analysis of PCR products and for loading of amplified products for second stage amplification. Segregation of lab space and equipment for each phase of work is helpful in preventing undesirable contamination of stocks and samples by PCR products and vice versa (Innis et al., 1990). Because of limited space, adherence to these guidelines was difficult. However, every attempt possible, was made to ensure the guidelines were observed at all times.

In this research, two defined areas were utilised to minimise cross-contamination during analysis and a different set of micropipettes and a different lab coat were used in each of the two areas. Quality in the extraction procedure and in PCR was assured by analysing random samples in duplicate. Negative controls for both procedures were also employed. The extraction procedure was conducted using 300 µl of molecular biology grade water instead of 300 µl of whole blood and therefore

functioned as a 'blank' or negative control. A negative control for PCR was included in each run performed. This control contained all the components of a PCR reaction with the exception of template DNA (whose volume in the reaction was replaced with molecular biology grade water). On random occasions a tube was also prepared for PCR using commercially available human genomic DNA as a source of template DNA. The PCR product of this tube could then be visually compared (following electrophoresis), with products obtained from samples that were extracted or purified as part of this research by Wizard™ kit purification. Positive control samples for C282Y homozygosity, C282Y/H63D compound heterozygosity, S65C heterozygosity and S65C/H63D compound heterozygosity were sourced and run alongside population samples, in both PCR and restriction endonuclease digestion procedures. Samples that were positive for C282Y homozygosity and C282Y/H63D compound heterozygosity were obtained through the laboratories of Sligo General Hospital following external genotyping by the Cytogenetics unit of NUI Galway. Dr. Sharon Barrett of the Centre for Liver Disease in Dublin generously supplied S65C heterozygote and S65C/H63D compound heterozygous positive controls.

Another quality control procedure that was used in this research was validation of results through external testing of samples by two accredited genotyping institutions. Ten samples from the Diabetic population were sent to the Cytogenetics unit of NUI Galway for genotyping in terms of the C282Y and H63D haemochromatosis mutations. Five S65C carriers that were identified in this study were also sent to the Centre for Liver Disease in Dublin for confirmatory testing (four samples were from the 'control' population and 1 sample was from the diabetic population). These five samples were genotyped on a lightcycler for the three main haemochromatosis mutations C282Y, H63D & S65C. Results of all external analysis from both Galway and Dublin, were identical to those obtained in this study and therefore validate the methodologies employed in this study (Table 3.5). Figures 3.13 to 3.17 inclusive, are examples of results graphs obtained for HFE mutation analysis by the Centre for Liver Disease in Dublin. Figure 3.13 shows results of a C282Y amplification run, Figure 3.14 shows results of a H63D amplification run and Figure 3.15 shows results for a H63D negative sample. Figures 3.16 and 3.17 show results (C282Y and H63D) for one of our samples analysed (sample number C54). The fifteen samples that were sent to external institutions were all genotyped by highly automated technology such as lightcyclers in a real time PCR (RT-PCR) set-up. External validation of the method is very costly at approximately 120 euro per test, but it highlighted that the fundamental methodologies employed in this research are comparable in terms of reliability to the more automated

technologies in use today. Sincere thanks is extended to Dr. Sharon Barrett (Dublin), who analysed samples free of charge.

Two problems were encountered during the genetic analysis phase of the study. After 3 months of optimisation work, analysis was unavoidably delayed due to a breakdown of the Hybaid Omne E thermal cycler. The cycler was immediately returned to the suppliers for repair of the central heating element of the block, which took approximately two months. In the interim, a replacement cycler of a different type was eventually appropriated and work was resumed. However, following complete repair and return of the Hybaid thermal cycler, it was then necessary to repeat all work done on the replacement model to avoid any inter model variations. Prior to initiation of testing, (and at regular intervals thereafter), equipment calibration / maintenance should be and was conducted, to ensure both reproducibility in testing and quality of any results so generated.

The second problem encountered was one regarding Tris-borate EDTA (TBE) buffers (Sigma-Aldrich) which were used in the initial months of analysis for both gel preparation and as a running buffer for electrophoresis. It was found that the borate in the aforementioned buffers often precipitated out of solution irreversibly, even when prepared and stored in accordance with the manufacturers recommendations and this adverse event affected sample resolution. This finding was later confirmed by reports received by Sigma-Aldrich Technical services department. It was therefore decided by the author to discontinue the use of these products in analysis and to replace them with a TBE 10X concentrate buffer by Promega. In terms of reagents, it is advisable to try products from multiple manufacturers before making a final selection. This action aids the discovery of potential problems that may affect assay quality that could have been avoided.

4.6 Genetic Analysis

The results of this study report an incidence rate for C282Y homozygosity of 1 in 249 (0.4%) and 1 in 83 (1.22%) for the tested diabetic and 'control' populations, respectively. C282Y heterozygosity occurred at a rate of 1 in 7 (14.86%) in diabetic individuals and 1 in 6 (18.22%), in individuals from the 'control' population. Homozygosity for the H63D mutation occurred in individuals from the diabetic and 'control' populations at rates of 1 in 20 (5.22%) and 1 in 28 (3.6%), respectively. H63D heterozygosity was reported at a rate of 1 in 4 (25.30%) in diabetic individuals and 1 in 5 (22.8%) in

'control' individuals. Subsections from both the diabetic and 'control' populations were also genotyped for the S65C mutation, where it was found to occur with an incidence of 1 in 124 (0.81%) in diabetics and 1 in 32 (3.2%) in 'control' individuals (see Table 4.2). Allele and genotype frequencies for both populations are shown in Table 3.4.

4.6.1 C282Y mutations

Table 3.0 shows the incidence of the C282Y mutation in the two populations tested. Genotypic results were available for 249 out of 250 people in the diabetic population and 247 out of 250 people in the 'control' population. Insufficient DNA concentrations in the remaining four samples meant that inconclusive genetic results were obtained. In a routine hospital setting a further sample would be obtained from the individual, however, as samples were anonymous in nature, it was impossible to acquire new blood samples from these four individuals. Time constraints in the analytical phase meant that the only other alternative – i.e. acquisition and testing of four new samples, could not be pursued. Of the 249 diabetic individuals screened for C282Y, one was C282Y homozygous (0.4%), 37 were heterozygotes (14.86%) and 211 had no C282Y mutation (84.74%). Screening of the 'control' population showed that 3 individuals were C282Y homozygotes (1.22%), 45 were heterozygotes (18.22%) and 199 lacked the C282Y mutation (80.56%).

Results from external genetic testing by Centre for Liver Disease, Dublin were available for sample numbers C8, C54, C78, C124 and C145. All five samples were found to lack the C282Y mutation (see Table 3.5). These external results were in complete agreement with results obtained in this study.

Results from this study report higher C282Y homozygote frequencies (in a control population) than those reported in the following studies. Beutler et al., (2002a) reported a 1/375 (0.27%) C282Y homozygote frequency in a population from California, while Olynyk et al., (1999) found a 1/200 (0.5%) frequency in a population from Western Australia and Jackson et al., (2001) found a 1/147 (0.68%) frequency in blood donors from South Wales, for the same genotype. A study in Canada (Arya et al., 1999) reported a C282Y homozygote frequency of 1/327 (0.31%). Jackson et al., (2001) found a C282Y homozygote incidence of 1/147 (0.68%). Studies in the U.S., Austria and France

reported C282Y homozygote frequencies of 0.45%, 0.4% and 0.57% respectively (Barry et al., 2005; Datz et al., 1998; Deugnier et al., 2002). One reason to explain the high levels of this genotype in Ireland, may be the postulated Celtic / North West European origin of the mutation.

Data from studies in Ireland reports that up to 93% of hereditary haemochromatosis patients are C282Y homozygotes. Byrnes et al., (1999) conducted a retrospective analysis of Irish neonates and identified a C282Y allele frequency of 11%. These results correspond to a C282Y homozygote genotype frequency of 1/83 (1.2%), which is the highest reported C282Y allele frequency worldwide. A different study in Ireland reported a C282Y homozygote frequency of 1.24% (Murphy et al., 1998), which is almost identical to the same genotype frequency found in this research.

An article by Kelleher & Crowe (2001) reported an incidence of 1/5 (20%) for C282Y heterozygosity in the general Irish population.

The results of this study show agreement with this data by reporting C282Y homozygosity and C282Y heterozygosity incidences of 1.22% and 18.22% respectively, in the 'control' population (see Table 4.2). Other studies have reported lower frequencies (12.66%, 8.3%, 8.6%) for the C282Y heterozygous genotype in control populations (Jackson et al., 2001; Barry et al., 2005; ARUP Laboratories, 2002).

Results presented in this thesis, report a genotype frequency for C282Y homozygosity in diabetics of 0.4%. This frequency is similar to that reported for a diabetic population in the U.K., where C282Y homozygosity was found to occur at a frequency of 0.49% (Singh et al., 1992). Other studies have reported higher frequencies for the same genotype in diabetic populations but have larger sample sizes than those of this study). In Italian and Danish studies frequencies of 1.34% and 1.26%, respectively, were reported (Conte et al., 1998; Ellervik et al., 2001). A study by Ellervik et al., (2001) reported a C282Y heterozygote frequency of 9.4% in diabetic individuals. Data from the present study found a C282Y heterozgote frequency of 14.86% in the diabetic population. Florkowski et al., (1999) reported higher C282Y homozgote and lower C282Y heterozygote frequencies (of 0.8% and 7.4%, respectively), in a study they conducted with diabetic individuals, when compared to data from

the present study. One reason suggested to account for the variations in C282Y homozygosity levels reported in diabetic individuals, is the different sample sizes used in each study.

The following allele frequencies for C282Y in control populations have been reported: 3.2% (Malecki et al., 2005), 8.23% (Jackson et al., 2001) and 5.69% (Barry et al., 2005). The C282Y allele frequency for the control population found in this study was 10.3% (see Table 3.4). The C282Y allele frequency for the diabetic population in this study was reported as 7.8%. This allelic frequency is higher than that reported in diabetic population from Poland (Malecki et al., 2005). However, it should be noted that the study by Malecki et al., (2005) looked at type II diabetics specifically whereas no such distinction was made in this study (i.e. the diabetic population may have been made up of both type I and type II diabetics).

4.6.2 H63D mutations

Table 3.1 shows the incidence of the H63D mutation in both the diabetic and 'control' populations. Genotypic results were available for 249 out of 250 diabetic individuals and all 250 'control' individuals. Again, the reason for the incompleteness of testing in the diabetic group, being inconclusive genetic results (due to insufficient DNA concentration in the sample), and time constraints in the analytical phase. Of the 249 diabetics screened for H63D, 13 were H63D homozygotes (5.22%), 63 were heterozygotes (25.30%) and 173 lacked the H63D mutation (69.48%). In the 'control' population, 9 homozygotes (3.6%) and 57 heterozygotes (22.8%) were identified. The remaining 184 'control' individuals did not have the H63D mutation (73.6%). Table 4.2 details the frequencies for all genotypes.

Results from external genetic testing by Centre for Liver Disease, Dublin were available for sample numbers C8, C54, C78, C124 and C145. Only one sample (number C145) was found to carry the H63D mutation, but in compound heterozygosity with the S65C mutation (see Table 3.5). The clinical significance of this genotype is still uncertain.

The frequency of H63D homozygosity (in a control population) reported in this research (3.6%) was similar to that of other studies. Studies in the U.K, Ireland and the U.S. found H63D homozygote

frequencies of 2.38%, 3.67% and 3.09%, respectively (Jackson et al., 2001; Ryan et al., 1998; Barry et al., 2005). Frequencies for H63D heterozygosity in our 'control' population (22.8%) were comparable to levels reported in three of the aforementioned studies. That is, 23.8% (Jackson et al., 2001), 24.77% (Ryan et al., 1998) and 19.34% (Barry et al., 2005). The H63D allele frequency in controls found in this research i.e. 15% (see Table 3.4) is comparable to that found in both a British population (15.3%) and a U.S. population (14.07%) (Jackson et al., 2001; Barry et al., 2005) and is higher than that (11.2%) found in a Polish population (Malecki et al., 2005).

Ellervik et al., (2001) reported H63D homozygote and heterozygote frequencies of 2.1% and 20%, respectively in a diabetic population. A different study by Florkowski et al., (1999) found a H63D homozygote frequency of 2.3% and a H63D heterozygote frequency of 26.7% in diabetics. Results from the present study report slightly higher frequencies of 5.22% and 25.30%, respectively for the same genotypes, in the diabetic population. H63D allele frequencies in our Diabetic population (17.9%) were slightly higher than those reported in a type II diabetic population by Malecki et al., (14.4%) in 2003.

Results from this study show that there was a higher frequency of H63D heterozygosity in the diabetic population than in the control population (Table 3.1). This difference was not statistically significant (see Appendix E).

4.6.3 S65C mutations

Table 3.2 shows the incidence of the S65C mutation in a subsection of both the diabetic and 'control' populations. The aim was to establish the S65C mutation incidence rate in 125 individuals from each of the two aforementioned test populations. As Table 3.2 indicates, only 124 results (out of 125), were obtained for the diabetic subsection tested. The reason being, that one sample was inadvertently analysed in duplicate (sample number 18), and a replacement sample could not be genotyped before the allowed analytical period elapsed. The incidence rate of S65C heterozygosity or S65C 'carrier' rate was found to be 0.81% and 3.2% for the diabetic and 'control' populations, respectively (see Table 4.2).

The five samples found to carry the S65C mutation were sent to the Centre for Liver Disease, Dublin for genotyping and therefore external validation. All five samples were confirmed to be S65C heterozygotes or carriers of the mutation (see Table 3.5). Analysis for the S65C mutation was not a service on offer / carried out by the genetic facility in Galway. Therefore the ten samples that were analysed in the Galway facility were only genotyped for C282Y and H63D.

Data from an unpublished research Masters study, reports a S65C carrier incidence of approximately 1/56 (3 S65C carriers identified out of a population of 168) in an Irish population. However, the study population was biased in that it consisted of samples chosen from a population previously referred for haemochromatosis mutation testing (personal correspondence with Dr. S. Barrett, Centre for Liver Disease, Dublin).

Interestingly, results of this study have identified a higher carrier incidence of the S65C mutation than that reported in the aforementioned study. A S65C carrier incidence of approximately 1/32 (3.2%) was found in the 'control' population. However, only one individual in the diabetic population carried the same mutation (0.81%). These results suggest that the S65C mutation may be unrelated to diabetes mellitus.

The S65C variant has been reported to interfere with H63D analysis for some methods (lightcycler & Stott duplex method), such that H63D/S65C compound heterozygotes appear as H63D homozygotes. Therefore H63D homozygotes detected by the aforementioned methods must be further analysed to out rule interference by S65C, which can contribute to erroneous results (King & Barton, 2004). A study by Douabin et al., (1998) found the S65C mutation in 5 out of 160 control chromosomes, which represents an allele frequency of 3.2% for this mutation in the general population. The allelic frequency for S65C reported in the control population in this study was 1.6% (see Table 3.4). This frequency compares favourably to that reported by Barry et al., in 2005 (1.8%). Different studies reported that the S65C mutation was present in up to 1.5% of the European population (Beutler et al., 2000; Jazwinska et al., 1996). S65C allele frequencies between 1.7 and 2.2% (in control populations) have also been reported, but there has been no association of the S65C allele with a phenotype of high transferrin saturation (Arya et al., 1999).

4.6.4 Compound Heterozygotes

It is apparent from analysis of results in Tables 3.0 to 3.2, that there were three C282Y/H63D compound heterozygotes (0.8%) in the diabetic population (sample numbers 113, 122 and 196) whereas there were seven (1.41%) in the 'control' population (sample numbers C58, C73, C156, C229, C236, C238 and C242). Only one C282Y/S65C compound heterozygote (0.27%) was identified ('control population' sample number C8). Two H63D/S65C compound heterozygotes (0.27%) were found, one in each of the diabetic and 'control' populations, sample numbers 54 & C145 (see Tables 3.3 and 4.2).

Kelleher & Crowe (2001) reported an incidence of 1/25 for C282Y/H63D compound heterozygosity in the general Irish population. The genotype frequency of C282Y/H63D compound heterozygosity in a control population has been reported at 2.38% (Jackson et al., 2001) and 1.98% (Barry et al., 2005), which is higher than the frequency reported for the same genotype in this study. Barry et al., also reported H63D/S65C compound heterozygote frequency of 0.65% in a control population. Although these frequencies for compound heterozygosity are higher than those reported in this study, it is important to note that this may have been due to larger sample sizes. Both Ellervik et al., (2001) and Florkowski et al., (1999) reported higher C282Y/H63D compound heterozygote frequencies of 1.1% and 2.1%, respectively, than results obtained in the present study in a similar population.

The incidence of C282Y/H63D compound heterozygotes in this study was lower that that reported by Kelleher & Crowe (2001), occurring at approximately 1/36 in the 'control' population and 1/84 in the diabetic population. A reason suggested to account for this difference is the variance in sample size between the two studies. The present study has a relatively small sample size in comparison to some other 'population' studies on haemochromatosis.

Only four studies have looked at the three hereditary haemochromatosis mutations in the general population (Mura et al., 1999; Beutler et al., 2000, Barry et al., 2005 & Čimburová et al., 2005). Another two studies evaluated all three mutations but were biased in their exclusion of certain genotypes (Remacha et al., 2000 & Holmstrom et al., 2002). None have been conducted on the Irish population to date.

4.6.5 Haemochromatosis Allele Frequencies

Tables 4.0 and 4.1 below, summarise the allele frequencies in diabetic and control populations, and indicate how data from the present study compares to published literature.

Table 4.0: HFE allele frequencies in Diabetic Populations

Population	Reference	Allele Frequencies (%)		
		C282Y	*H63D*	S65C
Ireland (North West)	***Present Study***	***7.8***	***17.9***	***0.81***
Poland	Malecki et al., 2003	1.8	3.2	-
Canada	Arya et al., 1999		24.8 & 14.7% (high & low transferrin saturation groups)	1.7 & 2.2% (high & low transferrin saturation groups)
UK	Jackson et al., 2001	8.23	15.3	-

It is evident from an extensive review of literature that there are a very limited number of studies which investigate the allele frequencies of the haemochromatosis mutations in diabetic populations. From Table 4.0 it can be seen that the C282Y allele frequency found in the present study is comparable to that found in the U.K (Jackson et al., 2001), but much higher than that found in Canada (Arya et al., 1999). Similarly, the allele frequency found in the present study for H63D is comparable to the frequency reported by the same study in the U.K (Jackson et al., 2001) and higher than frequencies reported in both Poland and Canada, respectively (Malecki et al., 2003; Arya et al., 1999). Allele frequencies for S65C were found to be lower than those reported in another study on a diabetic population (Arya et al., 1999).

Table 4.1: HFE allele frequencies in Control Populations

Population	Reference	Allele Frequencies (%)		
		C282Y	H63D	S65C
Ireland (North West)	***Present Study***	***10.3***	***15***	***1.6***
USA (Caucasian)	Barry et al., 2005	5.69	14.07	1.8
Russia	Potekhina et al., 2005	3.5	15.2	0
Norway	Milman et al., 2005	6.6	11.2	1.5
Denmark	Milman et al., 2005	5.7	12.8	1.5
Finland	Milman et al., 2005	3.6	9.8	1.6
Faroe Islands	Milman et al., 2005	8.0	17.5	1.0
Czech Republic	Čimburová et al., 2005	3.4	14.9	1.25
Northern Spain	Altes et al., 2004	3.0	20.0	1.0
Tunisia	Sassi et al., 2004	0.09	15.17	-
Croatia	Ristic et al., 2003	3.3	14.5	1.8
Slovenia	Ristic et al., 2003	4.0	14.5	0.5
Russia	Mikhailova et al., 2003	3.7	13.3	1.7
Northern Italy	Mariani et al., 2003	3.2	13.4	1.3
Italy	Pietrapertosa et al., 2003	1.5	14.0	0.5
Poland	Malecki et al., 2003	3.2	11.2	-
Sweden	Holmstrom et al., 2002	6.2	11.4	1.6
Sweden	Beckman et al., 2001	2.0	7.9	3.0
Mediterranean Basin	Campo et al., 2001	0.15	18.6	0.15
France	Aguilar-Martinez et al., 2001	3.0	16.9	-
UK	Merryweather-Clarke et al., 2000	8.1	15.2	-
Germany	Merryweather-Clarke et al., 2000	3.8	13.2	-
Austria	Merryweather-Clarke et al., 2000	3.7	12.9	-
Bulgaria	Merryweather-Clarke et al., 2000	0.0	23.0	-
Greece	Merryweather-Clarke et al., 2000	1.3	13.5	-
Spain	Merryweather-Clarke et al., 2000	3.6	30.4	-
USA (Caucasian)	Beutler et al., 2000	6.3	15.2	1.6
USA (Hispanic)	Beutler et al., 2000	2.7	12.4	0.6
USA (Asian)	Beutler et al., 2000	0.2	3.3	0
USA (Black)	Beutler et al., 2000	1.1	5.1	0.7
France	Mura et al., 1999	7.7	14.0	1.95
Scotland	Miedzybrodzka et al., 1999	8	15.7	-
Ireland	Ryan et al., 1998	14	17.9	-
Portugal	Porto et al., 1998	2.8	23.0	-

Results from studies on haemochromatosis allele frequencies in control populations are shown in Table 4.1. The C282Y allele frequency reported by the present study is comparable to other studies on general populations in Ireland and the U.K (Ryan et al., 1998; Merryweather-Clarke et al., 2000). It is important to note that the general Irish population (and that of the North West region also), remain the highest-ranking populations in terms of worldwide C282Y allele frequency. The allele frequency for H63D reported by the present study is comparable to published frequencies for the same mutation in all 34 countries detailed in Table 4.1. Allele frequencies for S65C in studies in the US (Barry et al., 2005; Beutler et al., 2000), Finland (Milman et al., 2005), Sweden (Holmstrom et al., 2002; Beckman et al., 2001), France (Mura et al., 1999), Croatia (Ristic et al., 2003) and Russia (Mikhailova et al., 2003) are comparable to the frequency reported in the present study.

4.6.6 Recommendations of Practice Guidelines for the Diagnosis of Haemochromatosis by Genetic Testing: Clinical Implications

Draft Best Practice Guidelines (King & Barton, 2004) for the molecular genetic diagnosis of hereditary haemochromatosis state the following:

In the case of diagnostic referral (i.e patient affected), if genetic analysis yields a:

(a) C282Y homozygote, then a diagnosis of hereditary haemochromatosis is made.
(b) C282Y/H63D compound heterozygote, then some individuals with this genotype will have iron overload but to a lesser extent than C282Y homozygotes (Lyon & Frank, 2001). Five per cent of patients with hereditary haemochromatosis have this genotype and so does 2% of the general population in the UK.
(c) C282Y heterozygote, then the individual is a carrier of the C282Y mutation and a diagnosis of hereditary haemochromatosis is unlikely.
(d) H63D homozygote, the individual has a slight risk of developing iron overload (Mura et al., 2000; Burke et al., 2000; Beutler et al., 2000). This genotype is present in approximately 2% of the population but its significance remains unclear.
(e) H63D heterozygote, the individual is a carrier of the H63D mutation and a diagnosis of hereditary haemochromatosis is unlikely.

In the case of predictive referral (i.e. patient currently unaffected), if genetic analysis yields a:

(a) C282Y homozygote, then the person is at risk of developing haemochromatosis and indices of iron overload like ferritin and transferrin saturation should be monitored on a yearly basis
(b) C282Y/H63D compound heterozygote & C282Y heterozygote, individuals are at risk of developing haemochromatosis and indices of iron overload should be monitored every three (compound heterozygotes) and five years (C282Y heterozygotes).
(c) H63D homozygote, the individual has a slight risk of hereditary haemochromatosis
(d) H63D heterozygote, there is no increased risk of developing iron overload.

To summarise, it is recognised that the methods employed in this work are slower and more time-consuming than currently available automated methods (e.g. multiplex multicolour analysis for simultaneous detection of the three main haemochromatosis mutations, Gómez-Llorente et al., 2004), but they may be more cost-effective in situations where technical and scientific time can be factored as cost neutral. Highly specialised and expensive equipment such as light cyclers were unavailable at the testing institutes (I.T. Sligo & Sligo General Hospital) and although sample throughput was low, the basic principles of genotyping were well learned and successfully applied. These basic principles may now be applied to testing for other genetic mutations.

The main advantage of genotypic testing is that is provides a result, which remains the same, irrespective of the stage of iron accumulation, and is not influenced by dietary intake or tissue damage. It must be noted, however, that it is still uncertain whether the majority of people homozygous for C282Y will eventually develop clinical symptoms of the disorder (Dooley & Worwood, 2000). Genetic testing alone is only part of the picture and genetic counseling must go hand in glove with the analytical process.

4.7 Biochemistry Analysis

Biochemical measures of iron status are used to screen for haemochromatosis, with tests for transferrin saturation (serum iron concentration divided by total Iron-binding capacity, multiplied by 100) and serum ferritin being recommended (Powell et al., 1998).

In this study, various indices of iron overload such as serum iron, ferritin, Unbound Iron Binding Capacity (UIBC) and calculated TIBC (Total Iron Binding Capacity) were assessed. Parameters associated with diabetics such as glycated haemoglobin (HbA1c) were also analysed and AST and ALT levels were investigated on the basis that they can be reflective of liver function (caused by excessive iron deposition in the case of haemochromatosis). Many studies have been conducted which correlate biochemistry testing (for indices of body iron stores), with data from genetic analysis (Gleeson et al., 2004; O'Brien et al., 1990; Ellervik et al., 2001; Kankova et al., 2002; Barton et al., 2005).

Data could only be presented for individuals who consented to study participation. Figure 3.6 contains biochemistry results for twenty-six research subjects (18 male & 6 female), ranging in age from 20 to 82 years. At the time of testing, none of the subjects had serum iron levels or TIBC (total iron binding capacity) levels outside the reference ranges of Sligo General Hospital. Four females (sample numbers 67, 72, 56 and 51) and eight males (sample numbers 54, 52, 34, 74, 68, 78, 49 and 50), had transferrin saturation levels outside the hospital reference range for their gender. Only one male (sample number 78) had ferritin levels elevated above the normal reference range. When aspartate aminotransferase (AST) and alanine aminotransferase levels were assessed, four females (sample numbers 67, 39, 56 and 51) were out with the normal hospital reference levels for AST. None of the males had AST values outside the reference ranges. One male (sample number 46) and three females (sample numbers 39, 56 and 51) had elevated ALT levels. All twenty-six individuals had glycated haemoglobin (HbA1c) levels outside hospital non-diabetic reference ranges that are aligned with Diabetes Control and Complications Trial (DCCT). This simply meant that all 26 individuals had not managed their diabetes effectively in the three months preceeding the test. There is no specific value of HbA1c below which the risk of diabetic complications is elimated completely.

Serum iron is greatly increased in haemochromatosis. None of the population tested in this study had serum iron levels outside the normal hospital ranges.

Elevated fasting serum transferrin saturation and persistently raised serum ferritin concentration can be suggestive of haemochromatosis. Numerically, local biochemical testing methods will dictate what constitutes elevated transferrin saturation. A result of 45% or greater is a sentinel of

haemochromatosis and is the value considered generally acceptable for genetic testing, especially to facilitate early detection of haemochromatosis (King & Barton, 2004). TIBC measures the total capacity of an individual's blood to transport iron and correlates with the amount of the protein transferrin in the blood. It quantifies the amount of iron blood would carry, if the transferrin were fully saturated. Transferrin saturation is the most specific and sensitive test for iron accumulation. It is calculated from the serum iron concentration and the TIBC. The percentage saturation is calculated using the following formula: (100 x serum iron / TIBC). Values greater than 50% in women and 55% in men, is indicative of iron loading due to haemochromatosis (Dooley & Worwood, 2000). Two individuals tested in this study (one male and one female, sample numbers 34 and 72), had transferrin saturation values above this range and both were identified as C282Y heterozygotes. Ten other individuals had transferrin saturation levels higher than the reference range for Sligo General Hospital (sample numbers 67, 54, 52, 74, 68, 56, 51, 78, 49 and 50). Half of these ten samples were carriers of HFE mutations. Raised transferrin saturation levels act as an early indicator of iron accumulation (Edwards & Kushner, 1993), but is not necessarily raised in young C282Y homozygotes.

While TIBC and transferrin essentially measure the same thing, they are typically used in conjunction with serum iron, to evaluate the iron status of individuals. In iron overload / haemochromatosis serum iron will be high and TIBC will be low or normal. The gold standard for the measurement of transferrin-iron saturation requires colorimetric measurement of iron and immunological measurement of transferrin. Transferrin-iron saturation may then be calculated using the molecular weight of transferrin (Crawford & Hickman, 2000; Gambino et al., 1997). Direct measurement of transferrin is less likely to suffer from interferences.

Genetic haemochromatosis characterized by transferrin saturation >45% is linked to C282Y homozygosity. Low ferritin is an excellent indicator of iron deficiency because ferritin is an acute phase protein, however, high ferritin levels are not necessarily synonymous with iron overload (Vantyghem et al., 2005).

Serum ferritin levels do not surpass the upper limit of normality until hepatic iron concentrations are elevated, after this they increase disproportionately with the degree of liver damage (Dooley &

Worwood, 2000). A recent study states that 10% of type II diabetes mellitus patients with high ferritin levels had transferrin saturation levels greater than those considered normal i.e. 40%. Serum ferritin should be cautiously evaluated in individuals with type II diabetes as it may falsely indicate 'normal iron stores' (Fernandez-Real et al., 2002).

Because serum ferritin is an acute-phase reactant, elevated levels may be a result of chronic disease (hepatitis C), inflammation, alcohol abuse, malignancies (tumours) or iron overload. It is therefore necessary to interpret high serum ferritin carefully in the context of the presence or absence of the aforementioned conditions. Dietary iron supplements are known to increase ferritin levels and some diseases (like infections, late-stage cancers, lymphomas, and severe inflammation), whilst not directly affecting the body's iron storage may cause artificially high ferritin levels. Such information highlights the limitations of serum ferritin as an indicator test for haemochromatosis. Serum ferritin levels above 300 µg/L in men and post-menopausal women and ≥ 200 µg/L in pre-menopausal women, is indicative of primary iron overload (Imperatore et al., 2004). The introduction of the serum ferritin assay by Addison et al., in 1972 provided the drive awaited for, to allow for the study of haemochromatosis. The test has not proved as valuable as originally hoped in the detection of iron accumulation in the early stages (Dooley & Worwood, 2000).

Available evidence from screening studies strongly suggest that approximately 75% of C282Y homozygotes have biochemical expression of iron overload (O'Neil & Powell, 2005). In C282Y homozygote populations, 50% of women and 20% of men did not have increased ferritin levels. Based on multivariate analysis, highly significant associations with increased ferritin levels in C282Y homozygotes, were male sex, increasing age and the absence of a non-expressor in the family (Lazarescu et al., 2005).

Liver function tests measure liver injury rather than liver function. Therefore, if a substance or molecule causes liver injury it will be reflected by changes in the normal levels of liver enzymes. Inflammation of hepatic cells results in elevated levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST). ALT is an enzyme produced in hepatic cells and is the most sensitive marker for liver cell damage. If hepatic cells are damaged, ALT leaks out of the cells and into the bloodstream thus causing an elevation in ALT levels. AST, a ubiquitous enzyme, also

reflects hepatic cell damage and ratios between AST and ALT are useful to physicians in assessing the etiology of liver enzyme abnormalities. (http://www.gastromd.com/lft.html).

In Ireland (for a diagnosis of haemochromatosis), transferrin saturation is considered elevated when $\geq$ 52%, serum ferritin when it is $\geq$ 300 μg/L and ALT and AST when they are >40 IU/L (Ryan et al., 2002).

The results of this study showed that one male had serum ferritin levels elevated above the aforementioned range (sample number 74), but was identified as not having any of the two haemochromatosis mutations of C282Y or H63D. Another male had relatively high serum ferritin levels of 260.8 μg/L, but was also identified as lacking the C282Y and H63D mutations. This finding is not unusual as ferritin is an acute phase reactant and is raised in such conditions as moderate alcohol consumption.

Out of the twenty-six individuals that underwent biochemistry testing for this study, three were found to be C282Y heterozygotes (samples 67, 34 and 72), six were H63D heterozygotes (samples 54, 74, 76, 51, 222 and 46), and two were H63D homozygotes (samples 55 and 50).

The biochemical effect of the H63D mutation is unknown, as it does not affect assembly or expression of HFE gene (Lebron et al., 1998 and 1999). Biochemistry results from this study, although very limited, support this statement. From Table 3.7 it can be seen that the majority of mean values for H63D homozygotes and heterozygotes (for the biochemistry parameters tested), were within the standard reference ranges for 'normal' individuals. The mean transferrin saturation levels were higher in C282Y heterozygotes and H63D heterozygotes than in wildtype individuals that underwent biochemistry testing. Mean AST and ALT levels were higher in H63D heterozygotes than in wildtype individuals. Mean serum iron levels were higher in C282Y heterozygotes than in individuals that had the wildtype genotype. It is important to note that the biochemistry results presented are based on a single blood sampling with no knowledge if it was taken in the fasting state. Repeat testing of a second 'fasting' sample for all 26 individuals is required for confirmation of the results obtained.

The diabetic parameter of glycated haemoglobin was also assessed in this study. Glycation is the post-translational, non-enzymic covalent chemical linkage of glucose onto proteins through amino groups. It occurs in tissues that are exposed to glucose and the extent of protein glycation is dependent on the level of exposure to glucose. At the time of measurement the percentage glycation of haemoglobin depends on the average age of the erythrocytes in the specimen, with the percentage of HbA_{1c} being higher in older cells. The mean life of erythrocytes is 117 days in non-diabetics, with a gender difference of about 11 days less in females. Glycated haemoglobin level is an index of mean blood glucose over the preceeding 120 days and therefore functions as an indicator of how well diabetes has been controlled over this time period (Jeffcoate, 2003). Results for all twenty-six individuals in the present study were out with the normal reference range utilized in the hospital which has been aligned with those set down in the Diabetes Control and Complications Trial (DCCT).

Studies have shown that differences in the ferritin levels related to particular genotypes of C282Y and H63D in diabetic individuals were not detected (Kankova et al., 2002). A recent review by Halsall et al., (2003) suggests that there is little evidence for a strong association between serum ferritin and the development of diabetes independent of HFE status (Eshed et al., 2001). This would explain the lack of success for screening for haemochromatosis in type II diabetes mellitus cohorts using biochemical markers as disease indicators (George et al., 1995; Turnbull et al., 1997). A study in Ireland stated that when screening diabetics for haemochromatosis, it is important to remember that persistently high ferritin has a relatively low positive predictive value (16.6%) and that normal transferrin saturation levels do not exclude a diagnosis (O'Brien et al., 1990).

Biochemical results from this small study would show agreement with the statement, that biochemical markers are not as reliable / successful as indicators of haemochromatosis as genotyping, for detecting haemochromatosis, especially in diabetic individuals.

4.8 Haemochromatosis & Diabetes

Four lines of scientific evidence favour the hypothesis that iron plays a role in diabetes (type II).

(1) The first piece of evidence is that an increased prevalence of haemochromatosis was found among unselected patients with type II diabetes mellitus. Phelps et al.,(1989) and Conte et al., (1998) reported that diabetes confers an increased risk for haemochromatosis, which was 2.4% and 1.34% higher in Australian and Italian populations respectively. Different studies have described an increased frequency of the C282Y mutation in type II diabetics (Kwan et al., 1998; Moczulski et al., 2001). However, the evidence for this is not always consistent, with some studies reporting no significant difference in the prevalence of the C282Y mutation between individuals with type II diabetes and controls Frayling et al., 1998; Braun et al., 1998; Dubois-Laforgue et al., 1998; Fernandez-Real et al., 1999). In a Spanish population the frequency of H63D was significantly higher in individuals with type II diabetes (Fernangez-Real et al., 1999). This diversity of opinion may just reflect the diversity of populations tested. Results from the present study do not show an increased prevalence of haemochromatosis in diabetic individuals compared with control individuals.

(2) Frequent blood donations leading to decreased iron stores has been demonstrated to constitute a protective factor in the development of diabetes (Ascherio et al., 2001). This finding is important given the high prevalence of increased iron stores in countries of the Western world and the observation that increased iron stores appear to predict an increased incidence of type II diabetes Salonen et al., 1999).

(3) The next piece of evidence is that iron stores may influence insulin action in type II diabetes, in that it significantly increases insulin sensitivity and significantly decreases HbA1c blood levels in diabetics who undergo phlebotomy (Fernandez-Real et al., 1999). Higher iron stores are associated with an increased risk of type II diabetes in women (Jiang et al., 2004).

(4) The final pieces of evidence are that patients with insulin-resistant hepatic iron overload (IR-HIO) who undergo phlebotomy can prevent significant tissue damage (Fernandez-Real et al., 2002) and that insulin resistant features are often seen in patients with chronic hepatitis C virus. In these

individuals, iron stores and family history of diabetes (amongst other things), were found to predict the development of diabetes (Petit et al., 2001).

Some studies have reported that C282Y and H63D allele frequencies do not differ significantly between control and diabetic populations (Singh et al., 1992; Kankova et al., 2002). Other studies have found more patients with diabetes were C282Y homozygotes than controls (Ellervik et al., 2001), whilst others still have identified a higher prevalence of H63D homozygosity and heterozygosity in individuals with type II diabetes mellitus (Malecki et al., 2003). No difference in the frequency of C282Y/H63D compound heterozygosity between diabetics and controls was found by Ellervik et al., 2001. Halsall et al., (2003) suggests that C282Y gene frequency, number of C282Y homozygotes and C282Y/H63D compound heterozygotes are unlikely to be different in people with typical type II diabetes mellitus, than in age- and gender- matched controls. Table 4.2 below, compares the incidence of haemochromatosis genotypes in the Diabetic and 'Control' Populations tested in this study.

Table 4.2: Incidences of haemochromatosis genotypes in the tested Diabetic & 'Control' Populations

	Diabetic Population	*Control Population*
C282Y homozygotes	0.4%	1.22%
C282Y heterozygotes	14.86%	18.22%
No C282Y mutation	84.74%	80.56%
H63D homozygotes	5.22%	3.6%
H63D heterozygotes	25.30%	22.8%
No H63D mutation	69.48%	73.6%
S65C homozygotes	0%	0%
S65C heterozygotes	0.81%	3.2%
No S65C mutation	99.19%	96.8%
C282Y/H63D compound heterozygotes	0.8%	1.41%
C282Y/S65C compound heterozygotes	0%	0.27%
H63D/S65C compound heterozygotes	0.27%	0.27%

Allele and genotype distributions for both populations are shown in Table 3.4. Before comparisons between allele frequencies (for all 3 haemochromatosis mutations) of the two populations were

made, it was determined whether both populations were in Hardy-Weinberg equilibrium /HWE. The 'Control' population was in HWE for all three loci (C282Y, H63D and S65C). The Diabetic population was in HWE for the C282Y and S65C loci only. Calculations for allele and genotype frequencies, as well as those for chi square testing can be found in Appendix E. From statistical calculations it is evident that there was a significant difference between the observed and the expected H63D allele frequencies in the Diabetic population (the calculated chi square value was greater than the chi square value in statistical tables at a probability or p value of 0.05 with 1 degree freedom). This means that there is only a 5% probability that the calculated chi square value occurred by chance. A chi square test was also carried out to determine if there was a statistically significant difference between the five genotype frequencies, (C282Y homozygote, C282Y heterozygote, H63D homozygote, H63D heterozygote and S65C heterozygote) of the diabetic and control populations. No such difference was found.

There is still no conclusive evidence for a genetic association between the two haemochromatosis and diabetes, and as such screening for haemochromatosis in diabetic individuals remains controversial. In the U.S. there are an estimated 16 million individuals with diabetes. Iron overload may cause a significant amount of this diabetes. If this problem is based on iron, it can be treated, the diabetes arrested and in some cases reversed, then this population should be but is not being tested for iron overload (Iron Overload Diseases Association, 2006). A study by Conte et al., (1998) postulated that such a screening program would be beneficial to individuals with type II diabetes mellitus. Malecki et al., (2003) also stated that heterozygote carriers of HFE mutations are at a higher risk of developing type II diabetes.

There is still much debate as to whether screening strategies should be based on genotyping or assessment of biochemical parameters indicative of iron overload, or a combination of both (Clark, 2002).

Genotyping identifies individuals who are at risk of developing haemochromatosis and distinguishes between C282Y homozygotes, heterozygotes and C282Y/H63D compound heterozygotes who each have varying risks of developing the disorder. Genotyping may also prevent the need for invasive procedures like liver biopsies in many asymptomatic individuals. One study states that genotype analysis was shown to be more cost-effective compared with phenotype analysis by serum iron

studies in siblings and children of affected patients (El-Serag et al., 2000). Because the prevalence of haemochromatosis defined by biochemical studies and liver biopsy (among diabetics) has been reported to be as low as 0.1% and as high as 9.5% (George et al., 1995; Phelps et al., 1989; Czink et al., 1991; Turnbull et al., 1997; O'Brien et al., 1990), genotyping should be identified and used as a more accurate method of determining disease prevalence.

5.0 CONCLUSIONS

5.1 Conclusion

Three assays were developed for the HFE gene mutations C282Y, H63D and S65C, based on PCR, agarose gel electrophoresis and restriction enzyme digestion of amplicons. PCR assays were validated internally, by the use of multiple controls and externally, by confirmatory genotyping by independent genetic facilities.

The present study determined the incidence of the three haemochromatosis mutations C282Y, H63D and S65C in a population from the North West of Ireland. It is one of the first studies to investigate (a) the incidence of all three HFE mutations in a population from the North West region of Ireland and (b) the incidence of the same three mutations in a Diabetic population from the same region.

Incidences for C282Y homozygosity and C282Y heterozygosity in the control population were similar to those in the general Irish population as reported by Kelleher & Crowe, (2001). The incidence for C282Y/H63D compound heterozygosity was lower than those reported by the aforementioned authors. The H63D allele frequency in the diabetic population was found to be slightly higher than that reported by Malecki et al., (2005). Unexpectedly, the results of this study also determined that there was an S65C carrier frequency of one in thirty-two for the control population, which is thought to be the highest incidence discovered to date, in the general Irish population.

The present study investigated whether there was an increased incidence of the three haemochromatosis mutations in a diabetic population from the same region of Ireland. Results showed that there was a slight increase in the incidence of the H63D heterozygous genotype in diabetics when compared to controls, but this was not statistically significant. Incidences for C282Y heterozygosity were comparable in both groups (Figure 4.2). However, statistical analysis of data from the present study showed that there was no evidence for significant differences between the frequencies of all five HFE genotypes in the 'control' and diabetic populations. Further research into the HFE mutational incidence in the North West population of Ireland is needed, to help inform policy on screening / diagnostic strategies and such studies should employ much larger sample sizes. In the majority of genetic testing facilities, highly automated analysers facilitate rapid mutational analysis of large numbers of samples. However, it should be

noted that automated systems are not always ideal, with some methods incorrectly diagnosing individuals with the wrong genotype (H63D genotype versus S65C genotype).

Biochemical indices of iron overload were also examined in a subsection of twenty-six Diabetic individuals. Results showed that biochemical markers are not as reliable as indicators of haemochromatosis, as genotyping, for detecting haemochromatosis especially in diabetic individuals.

Emerging scientific evidence has shown a bi-directional relationship between iron metabolism and diabetes (type II), - iron affects glucose metabolism, and glucose metabolism impinges on several iron metabolic pathways (Fernandez-Real et al., 2002). Future studies into HFE mutations (existing and yet to be discovered), may contribute to the search for a link between the two conditions.

More research is needed to ascertain why such phenotypic variability exists amongst individuals with haemochromatosis, and as to why biochemical indicators of iron overload and genotype data are not always matched in their diagnosis of the disorder.

Genetic testing allows the early identification of individuals (whether diabetic or non-diabetic), who are at risk of developing clinical disease before pathologic iron storage occurs. This fact, in conjunction with the availability of a facile treatment for haemochromatosis, makes screening for the condition recommendable, especially in the Irish population, where the incidence of haemochromatosis alleles is at its highest, worldwide. Diagnosing a genetic disorder not only allows for disease-specific management options, but also has implications for the affected individual's entire family. Genotyping ensures 'overdiagnosis' rather than 'underdiagnosis', which in itself is less acceptable in terms of increasing the risk of mortality/morbidity and the development of complicating conditions.

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APPENDIX A

RESEARCH ETHICS COMMITTEE (REC)
SLIGO GENERAL HOSPITAL

APPLICATION FORM

INSTRUCTIONS: Please complete in type. Please place a circle around Yes/No options as appropriate. A version of this form is available on disc from the administrator of the REC and on www.ref-sligo.ie.

It is essential that this form is completed fully and sent with relevant enclosures. You should not simply refer to the protocol but complete the form with the information requested. Please complete the checklist before sending. Where a question is not applicable it is important to make this clear and not to leave it blank. It is important that the language used in this application is clear and understandable to lay members. All abbreviations should be explained.

When a Pharmaceutical Industry sponsored study has been approved a fee of **€650** will be levied to cover administration expenses.

TITLE OF PROJECT:
Analysis of Haemochromatosis Mutations in the North West Population

Applicant's Checklist

Please indicate if the following have been enclosed by underlining or placing a circle round Yes/No/Not applicable options.

Application Form (1 signed original, 14 copies) *	Yes	
Full Protocol (15 copies)*	Yes	
Principal Investigator c.v. (1 copy)*	Yes	
Application Fee of €650		Not applicable
Research subject consent form incl. version no. and date (use REC form www.ref-sligo.ie/ethics.htm) (15 copies)	Yes	
Research subject info. sheet incl. version no. and date (15 copies)	Yes	
Advertisement for research subjects (15 copies)		Not applicable
GP/consultant information sheet or letter (15 copies)	Yes	
Interview schedules for research subjects (15 copies)		Not applicable
Letters of invitation to research subjects (15 copies)		Not applicable
Questionnaire Finalised/Not yet finalised (15 copies)	Yes	
Investigative brochure or data sheet for IND (1 copy)		Not applicable
Letter of Medical Indemnity (1 copy)		Not applicable
Letter of Confirmation of Insurance (1 copy)		Not applicable
Irish Medical Board Approval for IND (1 copy)		Not applicable

* Must be submitted

SECTION 1	**Details of applicant(s)**

1. Short title of project *(including any version dates):*

Analysis of Haemochromatosis Mutations in the North West Population

Full title:

Analysis of Haemochromatosis Mutations in the North West Population

2. Principal Investigator *(who will be responsible for dealing with the REC)*

[2 Co-Principal Investigators. Co-Principal Investigator (1), will deal with the REC]

Surname: (1) Williams (2) Bird

Forename: (1) John (2) Jeremy

Title: (1) Dr. (2) Dr.

Present appointment of applicant: (1) Head of Biochemistry, Sligo General Hospital.
(2) Lecturer / Science Academic Staff, Institute of Technology, Sligo.

Qualifications: (1) FAMLS, FIMLS, PhD, MBA.
(2) C.Biol, B.Sc., MSc., M.I.Biol, PhD (Genetics)

Office Address: (1) Biochemistry Department, Level 4, Sligo General Hospital, The Mall, Sligo.
(2) School of Science, Office Number B2025, I.T. Sligo, Ballinode, Sligo.

Tel: (1) 071-74561 (Office) ; 087-2331826 (Mobile)
(2) 071-55436 (Office) ; 071-9167787 (Home)

Fax: (1) 071-74658
(2) 071-46802 (Science Fax.)

E-Mail: (1) john.williams@nwhb.ie
(2) bird.jerry@itsligo.ie

3. **Co-Investigators** *(only the listed co-investigator(s) may perform the procedures indicated in this protocol. They may NOT amend the protocol).*

Co-Investigators are called Collaborators for the purpose of this study

Please state name(s), title, present appointment, qualifications:

- Dr. Donal Murray, Consultant Physician, SGH.
- Dr. Shukri Ramadan, Medical Registrar, SGH.
- Ms. Lydia Kirk, Current MSc Researcher, I.T. Sligo. (BSc. (Hons) Toxicology, 1.1)

---

4. **Who is sponsoring the study?**

Contact name: Dr. Richard Thorn. (Director of Institute of Technology, Sligo)

Organisation: Institute of Technology, Sligo (I.T. Sligo)

Address: Ballinode, Sligo.

Tel: 071-9155200

Fax: 071-9144096 (College Fax.)

E-Mail: thorn.richard@itsligo.ie

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5. Will researchers be paid for taking part in the study? *Yes*

If yes, what is the payment to the researchers per research subject?
1 MSc Research Student Is Involved who will be paid € 9523
per year for 2 years

---

6. Has any funding bee obtained or sought by the investigator in respect of this study e.g. research grants? *Yes*

If Yes, **a. Funding applied for** *Yes*

b. Funding secured *Yes*

c. Where will the research funds be lodged?
I. T. Sligo

d. Does the Investigator have any direct personal involvement with the sponsoring organisation? (e.g. financial, shareholding etc.)

No

---

7. Proposed start date and duration of the study

1st October 2003 (24 Months Duration)

SECTION 2 **Details of project**

*This section must be completed fully. A copy of the protocol should be enclosed with the application form, but it is **not** sufficient to complete questions by referring to the protocol.*

8. Is the study part of a Multi-Centre project? No

If yes:

a. **Which centres are involved in the study:** N/A

b. **Which Research Ethics Committees have been approached and what is the outcome to date?** N/A

c. **Who will have the overall responsibility for the study?** N/A

d. **Who has control of the data generated?** N/A

9. **Location of the research project:** Sligo General Hospital & I.T. Sligo

10. **Aims and objectives of project**

- To analyse the incidence of the two major hereditary haemochromatosis mutations, C282Y and H63D (of the HFE gene), in a population from the North West region.
- To compare the levels of the two aforementioned mutations in the 'normal' (non-diabetic) population to those in a diabetic population, from the same region.
- To compare biochemical iron indices of individuals carrying the C282Y mutation from various water supply catchments within the North West region.

11. **Scientific background of study**

Hereditary Haemochromatosis (HH) is a common genetic disorder which causes abnormalities in iron metabolism within the body. The disorder is characterized by inappropriate absorption of iron and its subsequent deposition in the

parenchymal cells of major organs.[1] The pathology of haemochromatosis is entirely a function of iron overload, i.e. when the body's normal capacity for binding and storing iron is exceeded; the element exists in an unbound state, which makes it extremely reactive and therefore toxic.[2] Excessive absorption and storage of dietary iron primarily affects the following organs; liver, heart, pancreas, endocrine organs, skin and joints,[3] where it is deposited and accumulates, resulting in impairment of organ structure and function, that can lead to cirrhosis, diabetes mellitus, cardiac dysfunction, arthritis, hypogonadism and premature death (Edwards *et al.*, 1980).

It is only in recent decades that the basic pathophysiology of haemochromatosis was discovered to lie in a defective gene, which controls the intestines mucosal barrier to iron absorption. In 1996, investigators at Mercator Genetics Corporation (Menlo Park, California), identified the gene responsible for hereditary haemochromatosis.[4] The gene was described as the HLA-H gene, but has since been renamed by the Nomenclature Committee of the Genome Database, as the HFE gene. It can be found on the short arm of chromosome 6 of the human karyotype. HH is considered an autosomal recessive disorder because both copies of the HFE gene, residing on the two homologs of the autosomal chromosome 6, need to have a particular mutation in order for the disorder to present itself. Two specific mutations of the HFE gene, when present in the appropriate arrangement can lead to the development of HH.[5] Clinical genetic analysis has identified both of these mutations; one occurs at the 845 nucleotide locus causing substitution of tyrosine for cysteine in codon 282; and one occurs at the 187 nucleotide locus, causing substitution of aspartic acid for histidine in codon 63. These mutations are designated C282Y and H63D, respectively (Feder *et al.*, 1996). Homozygosity for the C282Y mutation is closely linked to the disorder of HH, as it accounts for 93% of HH chromosomes in Ireland, 90% in the U.K and 100% in Australia.[6,7] Homozygous mutation at the 845 locus has high penetrance (phenotypic expression of a gene) when untreated, due to the sufficient time iron has to be absorbed. Iron overload is unlikely in the 845 heterozygous state-but is possible under certain circumstances like high intake of ascorbic acid or alcohol, or concurrent thalassemia minor (Felitti, 1999). Homozygosity at the 187 locus is also unlikely to cause iron overload, but is possible. The H63D mutation may contribute to hepatic iron overload, but rarely in the absence of the C282Y mutation (Bacon *et al.*, 1999[8]; Moirand *et al.*, 1999[9]). This mutational status is described as compound heterozygosity for the C282Y/H63D mutations. Byrnes *et al.*, (2001), investigated the prevalence of C282Y and H63D mutations within the Irish population, and an allele frequency of 11% for C282Y and 15% for H63D was found to exist.[10] The highest frequency of C282Y alleles worldwide has been reported in the Irish population, with 1 in 20 people having the genetic susceptibility to develop significant iron overload.[11]

Studies by Adams *et al.*, (1995)[12,13], have concluded that early diagnosis of HH through genetic analysis of the aforementioned gene mutations, and implementation of plans that monitor and treat hereditary haemochromatosis, i.e. measurement of biochemical indices of iron overload and phlebotomy therapy, are cost-effective and support widespread population screening for the disorder. Early detection of HH in its presymptomatic phase (ideally in children with a familial history of haemochromatosis), may mean that the disorder never

develops to the stage of phenotypic expression, by which time major therapeutic advantage has already been lost. HH can be fatal if left undetected and without treatment (Bothwell et al., 1995), but if therapy is initiated before tissue injury occurs, patients have a normal life expectancy.[14] This proves that early diagnosis of individuals is therefore crucial, and represents a major opportunity for chronic disease prevention.

Diabetes is a condition classically associated with hereditary haemochromatosis. In addition to the effect of iron on the pancreatic beta cells, there may also be a genetic link between the two conditions, which would explain the frequent occurrence of diabetes in siblings of patients with haemochromatosis and diabetes. Nelson et al., (1979), reported that the diabetes experienced in patients with haemochromatosis, was similar to that observed in patients with type II diabetes, with normal/exaggerated glucagons response but impaired response to glucose. Studies by O'Brien et al., (1990), and Adams et al., (1991), showed that between 20 % and 80 % of cases with hereditary haemochromatosis also have diabetes mellitus, depending on the population studied. Results from studies in Denmark (Ellervik et al., 2001), lend support to the theory that hereditary haemochromatosis is a disease often overlooked in patients with late-onset type I diabetes mellitus, a late manifestation of iron overload. These results also suggest that there is an association between C282Y homozygosity and type I diabetes. To date there has been no comprehensive information on the prevalence of the two mutations in the BMW region and its association with Diabetes Mellitus type 1 and type 2. There is a necessity to understand the allele frequency in the regional population, and the association between penetrance rates of the disease and homozygosity. There may be specific regional risk factors involved within the North West catchment including increased iron loading due to water quality characteristics, which may play a role in the prevalence of hereditary haemochromatosis. Recently an association has been demonstrated between heterozygosity for the C282Y mutation and cardiovascular death in women (Roest et al, 1999).

Many techniques in molecular biology have been employed in the detection of C282Y and H63D mutations of the HFE gene. Merryweather-Clarke *et al.*, (1997) used restriction fragment length polymorphism (RFLP) analysis in conjunction with PCR, to determine the prevalence of haemochromatosis mutations in European chromosomes.[15] Liang *et al.*, (2000) detected the C282Y and H63D mutations through the analysis of single-nucleotide extension (SNE) products by capillary electrophoresis.[16] Beutler *et al.*, (2000) performed allele-specific oligonucleotide hybridization using peripheral-blood DNA amplified by PCR, to analyse samples for C282Y and H63D mutations.[17] In this study, PCR methodology and other accepted techniques in molecular biology, will be used to detect the aforementioned gene mutations associated with HH, in both 'normal' and diabetic populations of the North West, through the genotyping of DNA obtained from buccal smears and blood samples.

12. Brief outline of project:

The objectives of the study are to determine and compare the incidence of the two major mutations responsible for hereditary haemochromatosis, (i.e. C282Y and H63D of the HFE gene), in a diabetic population and a non-diabetic or 'normal' population, from the North West region.
Recruitment of the study population (both diabetics and non-diabetics) will be on a voluntary basis, and will be conducted by study investigators, in the Outpatient Department and the Diabetic Clinic of Sligo General Hospital. As part of the recruitment process, individuals will be given Information Sheets to help them decide on whether they would like to be involved in the study or not, and what will be required of them, should they decide to participate. Study participation will be confirmed by way of documented consent, following which, all study participants will be required to give both a blood and a buccal cell sample. Completion of a simple questionnaire will also be necessary.

The sample size for the study is 1530, comprised of n = 765, for each of the diabetic and non-diabetic / 'normal' populations. The sample size for each of the aforementioned populations was calculated using the sample size formula for a single proportion or percentage, which allows for 80% power and a 5% two-sided significance level, and an increase/decrease of 100% from the prevalence documented for the general Irish population (Kelleher and Crowe, Irish Medical Times, 2001).

Genetic analysis in the form of PCR (Polymerase Chain Reaction), will be carried out on DNA extracted from blood and or buccal cell samples, in order to determine the incidence of the C282Y and H63D mutations, responsible for hereditary haemochromatosis

Biochemical analyses will also be carried out on collected blood samples as part of the study, and the following tests will be conducted:

Serum Iron, Serum Ferritin, Transferrin Saturation, Total Iron Binding Capacity (TIBC), Full Blood Count, Liver Function Tests (LFT's), fasting venous blood sugar level and glycosylated haemoglobin. In addition to these tests, blood samples from female participants will undergo pregnancy testing, in order to ensure the relevant individuals are excluded from the study, therefore avoiding any unnecessary risks.

Statistical analyses will be carried out on the data obtained from genetic and biochemical testing, and will be used to compare the incidence of hereditary haemochromatosis in a diabetic population to that of a non-diabetic or 'normal' population from the same region (i.e. the North West). Biochemical iron indices of individuals carrying the C282Y mutation will also be analysed in order to determine whether a link exists between C282Y homozygosity and iron levels in water, from varying water supply catchments within the region.

13. Study design (e.g. RCT, cohort, case control, epidemiological analysis)

The population sample size for the study, was calculated using the sample size formula for a single proportion or percentage, which allows for 80% power and a 5% two-sided significance level, and an increase/decrease of 100% from the prevalence documented for the general Irish population (Kelleher and Crowe, 2001).

Randomisation of study subject's for both populations (diabetic and non-diabetic), was achieved as a result of the volunteer nature for study participation.

Study participants from the diabetic population will be case-matched by age (ten year age classes), and gender, to study participants in the non-diabetic or control population.

14. Size of the study (including controls) : 1530 individuals/subjects

Will the study involve:

(a) Human Subjects *Yes*

i) How many patients will be recruited? – 765 individuals/subjects

ii) How many controls will be recruited? - 765 individuals/subjects

iii) What is the primary end point?

To determine and compare the incidence of the major mutations for hereditary haemochromatosis, C282Y and H63D, in a diabetic population and a non-diabetic population from the North West region.

iv) How was the size of the study determined?

The population sample size for the study, was calculated using the sample size formula for a single proportion or percentage, which allows for 80% power and a 5% two-sided significance level, and an increase/decrease of 100% from the prevalence documented for the general Irish population (Kelleher and Crowe, 2001). Randomisation of study subject's for both populations (diabetic and non-diabetic), was achieved as a result of the volunteer nature for study participation. Study participants from the diabetic population will be case-matched by age (ten year age classes), and gender, to study participants in the non-diabetic or control population.

v) Was there formal statistical input into the overall design of the study?

Yes

vi) What method of analysis will be used?

Relevant Statistical analysis allowing comparison of populations.

(b) Patient Records *Yes*

i) How many records will be examined?

765

ii) How many control records will be examined?

765

iii) What is the primary end point?

To determine and compare the incidence of the major mutations for hereditary haemochromatosis, C282Y and H63D, in a diabetic population and a non-diabetic population from the North West region.

iv) How was the size of the study determined?

The population sample size for the study, was calculated using the sample size formula for a single proportion or percentage, which allows for 80% power and a 5% two-sided significance level, and an increase/decrease of 100% from the prevalence documented for the general Irish population (Kelleher and Crowe, 2001). Randomisation of study subject's for both populations (diabetic and non-diabetic), was achieved as a result of the volunteer nature for study participation.Study participants from the diabetic population will be case-matched by age (ten year age classes), and gender, to study participants in the non-diabetic or control population.

v) Will information relevant to this study ONLY be extracted?

Yes

SECTION 3	**Recruitment of subjects**

15. How will the subjects in the study be:

i) selected?

Positive for Diabetes Mellitus

ii) recruited?

Voluntary Recruitment at Dr. Donal Murrays Diabetic Clinic in SGH

iii) what inclusion criteria will be used?

Positive for diabetes mellitus
Aged greater than 20 years
Reside in the North West Region
Of a Celtic or North European Origin

iv) what exclusion criteria will be used?

Recent Excessive Blood Loss
Recent Blood Transfusion
Non- Celtic Or non North European Origin
Pregnant
Under the age of 20 years

16. How will the control subjects group (if used) be: *(Type N/A if no controls)*

i) selected?

Non-diabetic or "Normal" population (age & gender matched to the diabetic population).

ii) recruited?

Voluntary Recruitment in Outpatient Department in Sligo General Department

iii) what inclusion criteria will be used?

Aged greater than 20 years
Reside in the North West Region
Of a Celtic or North European Origin

iv) what exclusion criteria will be used?

Recent Excessive Blood Loss
Recent Blood Transfusion
Non- Celtic Or non North European Origin
Pregnant
Under the age of 20 years

---

17. Will there be payment to research subjects of any sort? *Yes*

If yes, how much per subject and for what? *N/A*

SECTION 4	Consent

18. Is *written* consent to be obtained? *Yes*

If yes, please attach a copy of the consent form to be used.

If no written consent is to be obtained, please justify.

See Consent Form Attached

19. Please attach a copy of the written information sheet or letter to be given to the subject.

If no Information Sheet is to be given, please justify.

See research subject information sheet

20. Will any of the subjects or controls be from one of the following special populations?

Infants (<1 year):no

Children (1-18 years): no

Elderly (>59 years): yes

Pregnant women: no

Unconscious or severely ill: no

Mentally disabled: no

Mentally retarded: no

Prisoners: no

Students: yes

If yes, please specify and justify:

Study Participants must be 20 years of age or older. This would include elderly people & students who make up a large proportion of the Local Population

21. What special arrangements have been made to deal with the issues of consent for the subjects of special populations? Normal follow up procedures

SECTION 5	Details of interventions

22. Will the study involve the use of Investigational New Drugs (IND) or new device? No

No

If yes, complete the following:

i. **Drug name:** N/A

ii. **IND#:** N/A

iii. **Company:** N/A

iv. **Phase:** N/A

I II III IV

23. Will the study involve the use of an existing product outside the terms of its product licence?

No

If yes, specify (e.g. different populations, change of labelling): N/A

and also complete the following:

i. **Drug name: N/A**

ii. **IND#: N/A**

iii. **Company: N/A**

iv. **Phase: N/A** I II III IV

24. Will the study involve the use of a drug inside its product licence?

No

If yes, complete the following:

v. **Drug name: N/A**

vi. **IND#: N/A**

vii. **Company: N/A**

25. Will the study involve laboratory/clinical procedures NOT part of ordinary management? *Yes*

If yes, please explain: PCR assay for mutations C282Y and H63D, routine serological testing

---

26. Will the study involve the withholding of treatment as a result of taking part in the research study? *No*

If yes, please explain: N/A

---

27. Will the study involve the clinical experimental use of radiation or radioisotopes?

No

---

28. Will the study involve the use of biohazardous or infectious agents?

No

If yes, please explain:

N/A

---

29. How will the health of the participants be monitored during and after the study?

Normal Clinical Procedures

---

30. What medical examinations will persons selected for inclusion in the study undergo before participating in the study?

Diabetes Testing & diabetes testing

SECTION 6	Risks and ethical problems

31. Are there any potential hazards? *Yes*

If yes, please give details, and give the likelihood and details of precautions taken to meet them, and arrangements to deal with adverse events.

Vaccination of workers of Hepatitis A and B

---

32. Is this study likely to cause any discomfort or distress? *Yes*

If yes, please give details and justify.

Diagnosis of C282Y homozygosity (emotional distress)

---

33. Are there any particular ethical problems or considerations that you consider to be important or difficult with the proposed study? yes

Please give details

Genetic councelling of C282Y homozygotes

---

34. Will treatments provided during the study be available if needed after termination of the study?

N/A

---

35. Will information be given to the patient's General Practitioner? *Yes*

Please note: permission should always be sought from research subjects before doing this.

If yes, please enclose an information sheet/letter for the GP.

If no, please justify:

See G.P. Letter attached

---

36. If the study is on hospital patients, will consent of all consultants whose patients are involved in this research be sought? *Yes*

If no, please justify:

N/A

SECTION 7	**Compensation and confidentiality**

Product liability and consumer protection legislation make the supplier and producer (manufacturer) or any person changing the nature of a substance, e.g. by dilution, strictly liable for any harm resulting from a consumer's (subject or patient) use of a licensed product.

---

37. Have arrangements been made to provide indemnity and/or compensation in the event of a claim by, or on behalf of, a subject for non negligent harm?

(Please indicate N/A if not applicable)

No

If yes, please give details of compensation arrangements with this application.

N/A

---

38. In cases of equipment or medical devices, have appropriate arrangements been made with the manufacturer to provide indemnity?

(Please indicate N/A if not applicable)

N/A

If yes, please give details and enclose a copy of the relevant correspondence with this application.

N/A

---

39. Will the study include the use of any of the following?

Audio/video recording	No
Observation of patients	No

If yes to either:

i) **How are confidentiality and anonymity to be ensured?N/A**

ii) **What arrangements have been made to obtain consent for these procedures?**

N/A

---

40. What steps will be taken to safeguard confidentiality of personal records?

Personal records held in a secure environment in SGH. Access limited to predefined personnel involved in study / research

---

41. Will medical records be examined by research worker(s) not employed by the NWHB?

No

---

42. Will the study data be held on a computer?

Yes

If yes, will the data be held so that participants cannot be identified from computer files (i.e. no name, address, PCN or other potential identifier)?

Yes

Will records linking study participant ID numbers with identifying features be stored confidentially?

Yes

---

43. What steps will be taken to safeguard the information relating to specimens and the specimens themselves?

Normal steps for safe guarding

PLEASE ENSURE THAT YOU COMPLETE THE CHECKLIST ON THE FRONT COVER OF THE APPLICATION FORM AND ENCLOSE ALL RELEVANT ADDITIONAL DOCUMENTS.

SECTION 8	**Declaration**

DECLARATION

The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

I agree to supply annual review and final reports on the pro forma provided, and to advise my sponsor, the REC and any local researchers taking part in the project of any adverse or unexpected events that may occur during this project.

Signature of Principal Researcher: .....

Date: 17/11/22

CLINICAL TRIAL PROTOCOL

TITLE:

Analysis of Haemochromatosis Mutations in the North West Population

INVESTIGATORS:-

Dr. John Williams, (Head of Biochemistry, Sligo General Hospital)

Dr. Jeremy Bird, (Lecturer, Institute of Technology, Sligo)

Dr. Donal Murray, MD, MRCP, MRCGP, (Consultant Physician, Sligo General Hospital)

Dr. Fsukri Ramadan (Medical Registrar, Sligo General Hospital)

Lydia Kirk, BSc. (Current MSc. Researcher, Institute of Technology, Sligo)

1.0 PRECIS.

The objectives of the study are to determine and compare the incidence of the two major mutations responsible for hereditary haemochromatosis, (i.e. C282Y and H63D of the HFE gene), in a diabetic population and a non-diabetic or 'normal' population, from the North West region.

Recruitment of the study population (both diabetics and non-diabetics) will be on a voluntary basis, and will be conducted by study investigators, in the Outpatient Department and the Diabetic Clinic of Sligo General Hospital. As part of the recruitment process, individuals will be given Information Sheets to help them decide on whether they would like to be involved in the study or not, and what will be required of them, should they decide to participate. Study participation will be confirmed by way of documented consent, following which, all study participants will be required to give both a blood and a buccal cell sample. Completion of a simple questionnaire will also be necessary.

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Genetic analysis in the form of PCR (Polymerase Chain Reaction), will be carried out on DNA extracted from blood and or buccal cell samples, in order to determine the incidence of the C282Y and H63D mutations, responsible for hereditary haemochromatosis

Biochemical analyses will also be carried out on collected blood samples as part of the study, and the following tests will be conducted:

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Statistical analyses will be carried out on the data obtained from genetic and biochemical testing, and will be used to compare the incidence of hereditary haemochromatosis in a diabetic population to that of a non-diabetic or 'normal' population from the same region (i.e. the North West). Biochemical iron indices of individuals carrying the C282Y mutation will also be analysed in order to determine whether a link exists between C282Y homozygosity and iron levels in water, from varying water supply catchments within the region.

2.0 INTRODUCTION.

Hereditary Haemochromatosis (HH) is a common genetic disorder which causes abnormalities in iron metabolism within the body. The disorder is characterized by inappropriate absorption of iron and its subsequent deposition in the parenchymal cells of major organs.[1] The pathology of haemochromatosis is entirely a function of iron overload, i.e. when the body's normal capacity for binding and storing iron is exceeded; the element exists in an unbound state, which makes it extremely reactive and therefore toxic.[2] Excessive absorption and storage of dietary iron primarily affects the following organs; liver, heart, pancreas, endocrine organs, skin and joints,[3] where it is deposited and accumulates, resulting in impairment of organ structure and function, that can lead to cirrhosis, diabetes mellitus, cardiac dysfunction, arthritis, hypogonadism and premature death (Edwards *et al.*, 1980).

It is only in recent decades that the basic pathophysiology of haemochromatosis was discovered to lie in a defective gene, which controls the intestines mucosal barrier to iron absorption. In 1996, investigators at Mercator Genetics Corporation (Menlo Park, California), identified the gene responsible for hereditary haemochromatosis.[4] The gene was described as the HLA-H gene, but has since been renamed by the Nomenclature Committee of the Genome Database, as the HFE gene. It can be found on the short arm of chromosome 6 of the human karyotype. HH is considered an autosomal recessive disorder because both copies of the HFE gene, residing on the two homologs of the autosomal chromosome 6, need to have a particular mutation in order for the disorder to present itself. Two specific mutations of the HFE gene, when present in the appropriate arrangement can lead to the development of HH.[5] Clinical genetic analysis has identified both of these mutations; one occurs at the 845 nucleotide locus causing substitution of tyrosine for cysteine in codon 282; and one occurs at the 187 nucleotide locus, causing substitution of aspartic acid for histidine in codon 63. These mutations are designated C282Y and H63D, respectively (Feder *et al.*, 1996). Homozygosity for the C282Y mutation is closely linked to the disorder of HH, as it accounts for 93% of HH chromosomes in Ireland, 90% in the U.K and 100% in Australia.[6,7] Homozygous mutation at the 845 locus has high penetrance (phenotypic expression of a gene) when untreated, due to the sufficient time iron has to be absorbed. Iron overload is unlikely in the 845 heterozygous state-but is possible under certain circumstances like high intake of ascorbic acid or alcohol, or concurrent thalassemia minor (Felitti, 1999). Homozygosity at the 187 locus is also unlikely to cause iron overload, but is possible. The H63D mutation may contribute to hepatic iron overload, but rarely in the absence of the C282Y mutation (Bacon *et al.*, 1999[8]; Moirand *et al.*, 1999[9]). This mutational status is described as compound heterozygosity for the C282Y/H63D mutations. Byrnes *et al.*, (2001), investigated the prevalence of C282Y and H63D mutations within the Irish population, and an allele frequency of 11% for C282Y and 15% for H63D was found to exist.[10] The highest frequency of C282Y alleles worldwide has been reported in the Irish population, with 1 in 20 people having the genetic susceptibility to develop significant iron overload.[11]

Studies by Adams *et al.*, (1995)[12,13], have concluded that early diagnosis of HH through genetic analysis of the aforementioned gene mutations, and implementation of plans that monitor and treat hereditary haemochromatosis, i.e. measurement of biochemical indices of iron overload and phlebotomy therapy, are cost-effective and support widespread population screening for the disorder. Early detection of HH in its presymptomatic phase (ideally in children with a familial history of haemochromatosis), may mean that the disorder never develops to the stage of phenotypic expression, by which time major therapeutic advantage has already been lost. HH can be fatal if left undetected and without treatment (Bothwell et al., 1995), but if therapy is initiated before tissue injury occurs, patients have a normal life expectancy.[14] This proves that early diagnosis of individuals is therefore crucial, and represents a major opportunity for chronic disease prevention.

Diabetes is a condition classically associated with hereditary haemochromatosis. In addition to the effect of iron on the pancreatic beta cells, there may also be a genetic link between the two conditions, which would explain the frequent occurrence of diabetes in siblings of patients with haemochromatosis and diabetes. Nelson et al., (1979), reported that the diabetes experienced in patients with haemochromatosis, was similar to that observed in patients with type II diabetes, with normal/exaggerated glucagons response but impaired response to glucose. Studies by O'Brien et al., (1990), and Adams et al., (1991), showed that between 20 % and 80 % of cases with hereditary haemochromatosis also have diabetes mellitus, depending on the population studied. Results from studies in Denmark (Ellervik et al., 2001), lend support to the theory that hereditary haemochromatosis is a disease often overlooked in patients with late-onset type I diabetes mellitus, a late manifestation of iron overload. These results also suggest that there is an association between C282Y homozygosity and type I diabetes. To date there has been no comprehensive information on the prevalence of the two mutations in the BMW region and its association with Diabetes Mellitus type 1 and type 2. There is a necessity to understand the allele frequency in the regional population, and the association between penetrance rates of the disease and homozygosity. There may be specific regional risk factors involved within the North West catchment including increased iron loading due to water quality characteristics, which may play a role in the prevalence of hereditary haemochromatosis. Recently an association has been demonstrated between heterozygosity for the C282Y mutation and cardiovascular death in women (Roest et al, 1999).

Many techniques in molecular biology have been employed in the detection of C282Y and H63D mutations of the HFE gene. Merryweather-Clarke *et al.*, (1997) used restriction fragment length polymorphism (RFLP) analysis in conjunction with PCR, to determine the prevalence of haemochromatosis mutations in European chromosomes.[15] Liang *et al.*, (2000) detected the C282Y and H63D mutations through the analysis of single-nucleotide extension (SNE) products by capillary electrophoresis.[16] Beutler *et al.*, (2000) performed allele-specific oligonucleotide hybridization using peripheral-blood DNA amplified by PCR, to analyse samples for C282Y and H63D mutations.[17] In this study, PCR methodology and other accepted techniques in molecular biology, will be

used to detect the aforementioned gene mutations associated with HH, in both 'normal' and diabetic populations of the North West, through the genotyping of DNA obtained from buccal smears and blood samples.

3.0 OBJECTIVE (S) OF STUDY

3.1 To analyse the incidence of the two major hereditary haemochromatosis mutations, C282Y and H63D (of the HFE gene), in a population from the North West region.

3.2 To compare the levels of the two aforementioned mutations in the 'normal' (non-diabetic) population to those in a diabetic population, from the same region.

3.3 To compare biochemical iron indices of individuals carrying the C282Y mutation from various water supply catchments within the North West region.

4.0 STUDY DESIGN & METHODOLOGY

4.1 LOCATION OF STUDY

Recruitment of individuals for participation in the study, biochemical analysis of biological samples (i.e. blood and buccal smear samples), and analysis of the mutational status of biological samples by PCR will be conducted at the following facility:

Sligo General Hospital,
The Mall,
Sligo,
Co. Sligo,
Ireland.

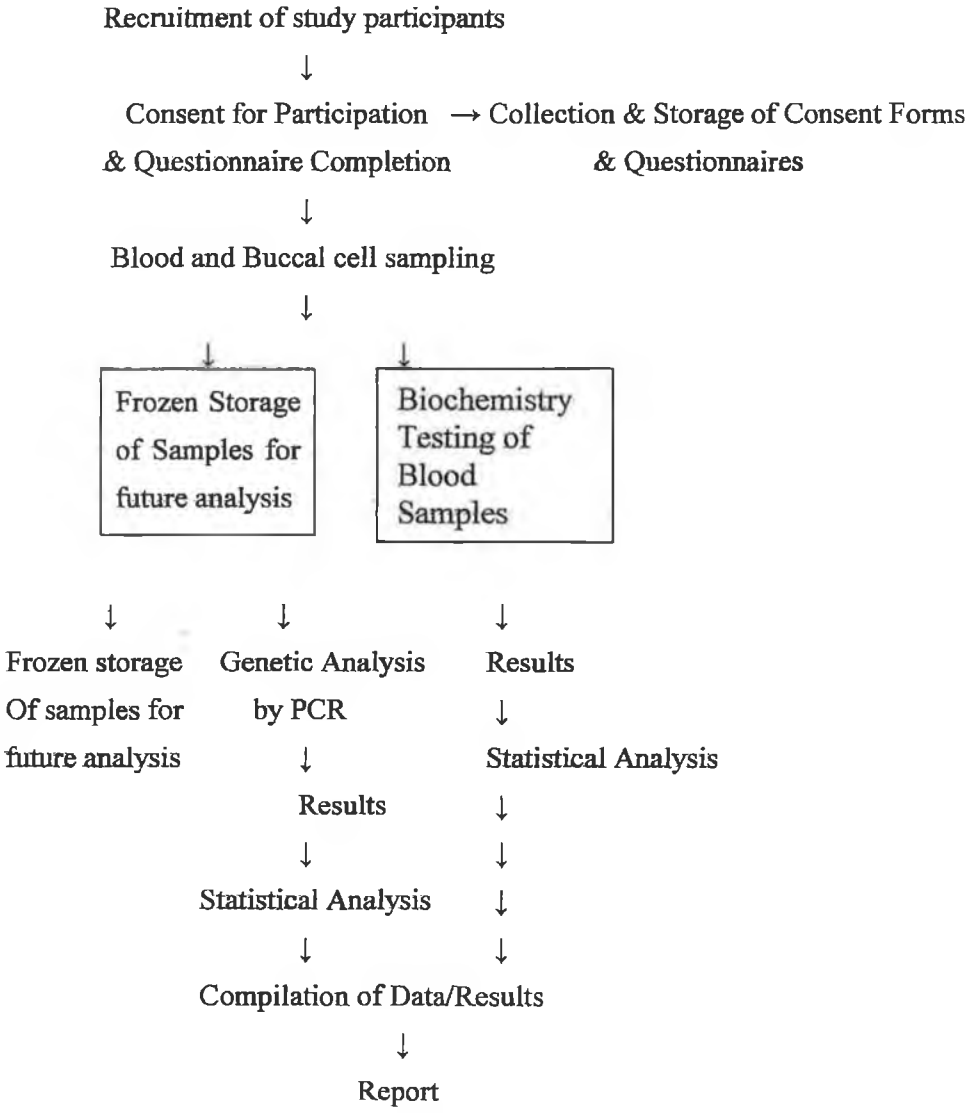
4.2 PERSONNEL RESPONSIBLE FOR THE STUDY.

Co-Principal Investigator (1): Dr. John Williams (External MSc. Research Supervisor)
Co-Principal Investigator (2): Dr. Jeremy Bird (Internal MSc. Research Supervisor)

Collaborators:

Consultant Physician: Dr. Donal Murray, MD, MRCP, MRCGP.
Medical Registrar: Dr. Shukri Ramadan.
MSc. Research Student: Ms. Lydia Kirk, BSc.

4.3 STUDY SEQUENCE.



4.4 PROCEDURES

4.4.1 SAMPLE COLLECTION PROCEDURES

Blood samples will be collected from study participants, by suitably qualified personnel utilising normal medical procedures already in place at SGH.

Buccal cell samples will be obtained using a mouth wash collection procedure.

4.4.2 DNA EXTRACTION PROCEDURE

DNA will be extracted from blood and buccal cell samples using methods that incorporate Chelex 100 resin.

4.4.3 PROCEDURE FOR GENETIC ANALYSIS

DNA extracted from blood/buccal cell samples will be amplified by PCR. Subsequent restriction digestion and gel electrophoresis of DNA fragments will help identify the presence of C282Y and H63D mutations. Southern blotting techniques will confirm the presence of the individual mutations.

4.4.4 PROCEDURE FOR BIOCHEMISTRY ANALYSIS

The biochemical tests listed in section 1.0 of this protocol will be conducted in compliance with Standard Operating Methods (S.O.M.'s), currently in place in the laboratories of SGH.

4.4.5 STATISTICAL ANALYSIS

Suitable statistical analysis will be carried out on results obtained from genetic and biochemical analyses, to allow comparisons between the diabetic and the non-diabetic or 'normal' population.

4.4.6 STUDY DESIGN

The population sample size for the study, was calculated using the sample size formula for a single proportion or percentage, which allows for 80% power and a 5% two-sided significance level, and an increase/decrease of 100% from the prevalence documented for the general Irish population (Kelleher and Crowe, 2001).

Randomisation of study subject's for both populations (diabetic and non-diabetic), was achieved as a result of the volunteer nature for study participation.

Study participants from the diabetic population will be case-matched by age (ten year age classes), and gender, to study participants in the non-diabetic or control population.

5.0 INCLUSION & EXCLUSION CRITERIA

Inclusion criteria for study participants in the:

Diabetic Population	***Non-Diabetic Population***
Positive for Diabetes Mellitus	Aged greater than 20
Aged greater than 20	Reside in the North West region
Reside in the North West region	Celtic / Northern European Origin
Celtic / Northern European Origin	-

Exclusion criteria for study participants in the:

Diabetic Population	***Non-Diabetic Population***
Recent Excessive Blood Loss	Recent Excessive Blood Loss
Recent Blood Transfusion	Recent Blood Transfusion
Pregnant	Pregnant
Non-Celtic / Non-North European Origin	Non-Celtic / Non-North European origin
Under the age of 20	Under the age of 20

6.0 SUBJECT MONITORING & CRITERIA FOR THEIR WITHDRAWAL

The general health of study participants will be monitored in accordance with normal procedures in place in SGH, at times when blood and buccal cell sampling occur.

Participation is on a voluntary basis. Study Participants are free to withdraw from the study at any time. Withdrawal will not affect the standard of care received. Individuals who choose to withdraw from the study are asked to notify any of the Study Investigator's, as to their decision.

7.0 HUMAN SUBJECT PROTECTIONS

7.1 RATIONALE FOR SUBJECT SELECTION

Inclusion and Exclusion criteria (see Section 5.0) have been defined on the basis of published data. See Section 10.0 for References.

Individuals of non-Celtic origin are excluded from the study due to the fact that studies have shown that the incidence of hereditary haemochromatosis is at its highest in people of a Celtic origin.[18,19,20,21.]

8.0 ADVERSE EVENT REPORTING AND DATA MONITORING

Should adverse events occur during the course of the study period, they must be recorded in writing and brought to the attention of the Co-Principal Investigator (1), immediately.

9.0 CONSENT PROCESS & DOCUMENTS

On recruitment of study subjects, every individual will receive a Research Subject Information Sheet, which explains what the study is about and what will be expected of people who decide to participate. Patient Information sheets will be approved by SGH ethics committee prior to their distribution, and a copy of the approved document will be maintained in a clearly identifiable Study Folder for the entire duration of the study period.

Individuals who chose to participate in the study will be required to sign a consent form indicating their decision. Consent forms will be collected immediately prior to blood and buccal cell sampling. All completed consent forms will be maintained in a clearly identifiable Study Folder.

Participants will also be asked to complete a simple questionnaire of personal details relevant to the study. All completed questionnaires should be collected along with completed consent forms

prior to sampling of blood and buccal cells, and will be maintained in a clearly identifiable Study Folder.

Because the study is not being conducted by any study participant's own G.P, a letter will be sent to them informing them of their patient's consent to participate in the study. A Research Subject Information Sheet will also be sent.

10.0 REFERENCES

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11. Kelleher & Crowe, Article in the Irish Medical Times, Nov. 23rd, 2001, Vol.45, No.47, pp 28-29.

12. Adams, P.C., Kertesz, A.E, Valberg, L.S., (1995). *Screening for Haemochromatosis in children of homozygotes : prevalence and cost-effectiveness.* Hepatology, **22**, 1720-1727.

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11.0 DOCUMENTATION

11.1 All raw data recorded on raw data forms will be submitted to the Co-Principal (1) Study Investigator, who will maintain them for the duration of the study in a clearly identifiable Study Folder, and be responsible for their correct archival after completion of the study.

12.0 CONFIDENTIALITY

The highest standards of confidentiality will be applied to any personal information of study subject's collected. Any information about study participants which leaves the hospital will have the names of subject's removed, so that they are not recognisable.

13.0 FATE OF STUDY RESULTS/DATA

The results obtained from this study will form part of a publicly available MSc Research project submitted by Ms. Lydia Kirk, B.Sc. to the awarding body of HETAC. They may also be published in peer-reviewed journals.

14.0 PROTOCOL AMENDMENTS

14.1 No changes, revisions or modifications must be made to this study protocol unless approved by the Co-Principal Study Investigator's: any such changes must be agreed by the Research Ethics Committee (SGH) and recorded in an addendum giving reasons for the alterations.

15.0 PROTOCOL DEVIATIONS

15.1 The details and procedures set out in the protocol must be followed at all times during the conduct of the study. Should any deviation from the authorised protocol occur it must be recorded and reported in writing to the Co-Principal (1) Study Investigator immediately.

16.0 PROTOCOL APPROVAL

16.1 Approved by: ..... Date: .....

CO-PRINCIPAL INVESTIGATOR (1)

..... Date: .....

CO-PRINCIPAL INVESTIGATOR (2)

..... Date: .....

Collaborating Consultant Physician

..... Date: .....

ON BEHALF OF Research Ethics Committee (SGH)

North Western Health Board
Sligo General Hospital
The Mall, Sligo
Telephone (071) 71111. Fax (071) 74645

CONSENT FORM

Consent by subject for participation in Research Protocol

ɔcol Number:
ınt Identification Number for this trial:
of Protocol: "*Analysis of Haemochromatosis Mutations in the North West Population*"

or(s) Directing Research:

1. I confirm that I have received a copy of the Research Subject Information Sheet for the above study. I have read it and I understand it. I have received an explanation of the nature, purpose, duration and foreseeable effects and risks of the study and what my involvement will be and the possible benefit of taking part.

2. I have had time to consider whether to take part in this study and I have had the opportunity to ask questions. I have also had time to consider what will be required of me, should I decide to participate in the study.

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. I understand that my taking part in this study will be kept confidential, and my name or personal details will not be disclosed outside the hospital and GP surgery.

4. I have, to the best of my knowledge, informed the investigator of my medical history, previous participation in clinical trials, medication and any consultations that I have had with a doctor over the past 4 months.

5. I understand that my GP, Dr....., will be informed by Dr..... .....that I am taking part in this study.

6. I understand that sections of any of my medical notes may be looked at by responsible individuals from Sligo General Hospital or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records for the purposes of this study.

7. I agree to take part in the above study.

ıe of patient	Date		Signature
ıe of person taking consent	Date		Signature
Principal Investigator (1)	Date		Signature

1 copy for patient, 1 copy for investigator, 1 copy for medical records

Research Subject Information Sheet

1. Study title

"Analysis of Haemochromatosis mutations in the North West Population"

2. Invitation paragraph

You are being invited to take part in the research study mentioned above. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information.

3. What is the purpose of the study?

Haemochromatosis is a condition characterised by the excessive absorption of dietary iron from the digestive tract. Over time, the excess iron accumulates in tissues throughout the body, leading to symptoms and signs of iron overload. These may include diabetes mellitus, liver disease (cirrhosis), heart failure, skin pigmentation, joint pains, and sexual dysfunction/ loss of libido.

Haemochromatosis is usually an inherited disorder and is most common in Caucasian people. Recent studies have revealed that Ireland has the highest incidence of Hereditary Haemochromatosis in the world, where it is believed that 1 in 20 people have the genetic susceptibility to develop significant iron overload. Two mistakes in the gene called HFE cause the condition of Hereditary Haemochromatosis.

The primary goal of screening is to detect the presence of the gene mistakes responsible for the Hereditary Haemochromatosis before it produces any symptoms or causes complications. The aim of this study is to determine the number of non-diabetic people in the North Western region who possess the gene mistakes responsible for the disorder, and also to determine the type of mistake in the gene. The same determination will be carried out on people with Diabetes mellitus in the same region.

Specific attention is being given to diabetics, as Diabetes mellitus is reported in 10-40% of patients with Haemochromatosis.

4. Why have I been chosen?

You have been chosen to take part in this study because you reside in the BMW region and are in good general health.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive.

6. What will happen to me if I take part?

Should you decide to participate in this research all that will be required of you is a quick visit to Sligo General Hospital for completion of a short questionnaire and to allow us to obtain blood sample and a cheek cell sample. The entire procedure will take no longer than 20 minutes to complete.

7. What are the alternatives for diagnosis or treatment?

Participation in this study is voluntary. In the event that it is discovered that you have Hereditary Haemochromatosis, the research doctor may suggest you speak to your GP about future phlebotomy therapy (regular donation of blood to help decrease body stores of iron). This should prevent the development of many complications of the disorder.

8. What are the possible benefits of taking part?

The main benefit of participating in this study is that you will find out whether or not you have the disorder. Haemochromatosis could be asymptomatic for many years and its complications could be preventable if the disease is diagnosed early.

9. What are the possible risks of taking part?

Because your participation requires a blood sample to be taken, potential exists for slight bruising at the site of the vein puncture.

It is possible that insurance companies might "Load" your premium if you apply for insurance and you are known to have haemochromotosis, however, people who are diagnosed and treated for haemochromotosis before complications would have a normal life expectancy.

10. What if something goes wrong?

In the event that unforeseen circumstances arise, your doctors are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

11. Will my taking part in this study be kept confidential?

Yes, all information which is collected during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital/surgery, will have your name and address removed so that you cannot be recognised from it.

12. What will happen to the results of the research study?

Results of the study will be collated within two years from study initiation and may be published after this time in a reputable medical forum.

13. Who is organising and funding the research?

A seed fund grant from the Institute of Technology, Sligo, will help fund this study.

14. Who has reviewed the study?

The Research Ethics Committee (REC) of Sligo General Hospital has reviewed the study prior to its commencement.

If you have any queries you can contact the REC at:

Research Ethics Committee
Sligo General Hospital (SGH)
The Mall
Sligo

15. Contact for Further Information

Dr. Donal Murray, Consultant Physician, SGH, Tel.:071-9171111, Extn. 4119

Dr. John Williams, Head of Biochemistry, SGH, Tel.:071-9171111, Extn.4561

Dr. Shukri Ramadan, Medical Registrar, SGH, Tel.: 071-9171111, Bleep 252

Dr. Jeremy Bird, Lecturer, I.T. Sligo, Tel: 071-9155436

Ms. Lydia Kirk, MSc. Researcher, I.T. Sligo, Tel: 071-9155414

Thank you for taking part in this study!

North Western Health Board
Sligo General Hospital
The Mall, Sligo
Telephone (071) 71111. Fax (071) 74645

Dr. John Williams (Head of Biochemistry Dept.,) &
Co-Principal Investigator (1),
Haemochromatosis Study,
Sligo General Hospital,
The Mall,
Sligo.

Name of GP,
Address of GP.

DATE:

RE: Participation of Patient in Clinical Study

Study Title:

'Analysis of Haemochromatosis mutations in the North West Population'.

Dear GP,

This letter is to inform you of your patient ..... consent to participate in the aforementioned study. Please find enclosed a Research Subject Information Sheet (for your information), as given to said person to help them decide whether to participate in the study or not. Please feel free to contact me if you require any further information about the study.

Regards,

.....
Dr. John Williams (SGH)

PATIENT QUESTIONNAIRE

Please complete all the following questions using block capital letters or by using a tick '√ ' to indicate the appropriate response(where applicable).

1. Surname: .....

2. Forename: .....

3. Address: .....

.....

.....

.....

4. Age: 20-30yrs ☐ 31-41yrs ☐ 42-52yrs ☐ 53-63yrs ☐

63+yrs ☐

5. Do you suffer from diabetes mellitus? Yes..... ☐
No..... ☐
Don't Know... ☐

6. Are you currently taking / or have in the past week taken?:

Vitamin C supplements: Yes ☐ No ☐
Iron supplements: Yes ☐ No ☐

7. Are you taking the above supplements:

Following your GP's recommendation..... ☐
Through personal choice..... ☐
Not Applicable..... ☐

8. Have you been diagnosed as having the condition of haemochromatosis?

Yes ☐ No ☐

9. To the best of your knowledge, has any member of your family been diagnosed as having haemochromatosis?

Yes ☐ No ☐ Don't know ☐

If you answered 'Yes' to the above, please indicate in the space provided below, the number and type of relatives that have been diagnosed (e.g. 1 Aunt, 2 Brothers etc). Please fill in 'N/A' in the space below if you answered either 'No' or 'Don't know'.

.....
.....
.....
.....

10. Have you ever donated blood? Yes☐ No☐
11. Are you currently taking any medication? Yes☐ No☐

If you answered 'Yes' to the above question, please list the medication you are taking in the space provided below.

.....
.....
.....
.....

12. Do you suffer from any of the following? (Tick 'Yes' or 'No' box as appropriate).

	Yes	No
Extreme Fatigue		
Joint Pain		
Arthritis		
Impotence(loss of libido)		
Skin Bronzing/Discoloration		
Heart Fluttering		
Depression		
Abdominal Pain		
Liver disease		
Gallbladder disease		
Loss of body hair (including baldnes)		
Weight loss		
Weight gain		
Elevated Cholesterol		
Slow maturation (delayed physical development)		

13. Name & Address of your G.P: .....
.....
.....
.....
.....
.....
.....

Note: Questionnaire's should be returned to your Research Doctor.

Thank you for taking the time to complete this questionnaire. Your assistance is greatly appreciated.

APPENDIX B

...Station Approach
...0ZF Gateshead

Certificate of Ana...

TAGN

Customer:

Dr Jeremy Bird, Institute of Technology

Customer Number:	19025
Order Number:	000037338
Order ID:	31121-005
Order Date:	21/11/2003
Comments:	

OligoName: C282YF — Oligo Number: 31121A1C06 1/4

-TGG CAA GGG TAA ACA GAT CC-3'

...elivered:	11.4	OD	No. Bases:			20	Synthesis Scale:		0.04 umol standard
	315.6	µg	GC %:			50.0	Purification:		RP-Column
	48.6	nmol					Delivery Form:		Lyophilised
...onc.:	-	µM	A	C	G	T	Quality Control:		DMT Monitoring
...W:	6499		7	4	6	3	Lyophilised		
...m:	57.3								

...NA

...OligoName: C282YR — Oligo Number: 31121A1D06 2/4

...AC CTC CTC AGG CAC TCC TC-3'

...elivered:	10.6	OD	No. Bases:			20	Synthesis Scale:		0.04 umol standard
	348.6	µg	GC %:			60.0	Purification:		RP-Column
	55.6	nmol					Delivery Form:		Lyophilised
...nc.:	-	µM	A	C	G	T	Quality Control:		DMT Monitoring
...W:	6273		3	10	2	5	Lyophilised		
...:	61.4								

...NA

...ligoName: H63DF — Oligo Number: 31121A1E06 3/4

...A TGG TTA AGG CCT GTT GC-3'

...ivered:	8.7	OD	No. Bases:			20	Synthesis Scale:		0.04 umol standard
	258.0	µg	GC %:			50.0	Purification:		RP-Column
	39.9	nmol					Delivery Form:		Lyophilised
...c.:	-	µM	A	C	G	T	Quality Control:		DMT Monitoring
...:	6472		4	4	6	6	Lyophilised		
	57.3								

...A

Eurofins MWG Operon
Unit F Gateshead

Certificate of Analysis

JobName: H63DR

Oligo Number: 21121A1H04 4/4

5' GCT GTC GTT GTG ATT TTC C-3'

Delivered:	9.5	OD	No. Bases:	22	Synthesis Scale:	1.04	µmol standard
	309.5	µg	GC %:	45.5	Purification:	RP-Column	
	43.7	nmol			Delivery Form:	Lyophilised	
	-	µM	A	C	G	T	Quality Control:	DMT monitoring
	7087		1	4	6	11	Lyophilised	
	58.4							

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May 9, 2005

Lydia Kirk
Centre for Sustainability
New Business Innovation Centre
Institute of Technology Sligo
Ballinode, Ireland

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APPENDIX D

Research & Education Foundation
Sligo General Hospital, Sligo, Ireland.
Tel: + 353 - 71 - **74678**. Fax: + 353 - 71 - **74679**.
www.**ref-sligo.ie**

Ms. Lydia Kirk
MSc Researcher
School of Science
IT Sligo
Sligo

December 9th 2004

Re. Application for Research & Education Foundation Research Seed Grant 2004

Dear Lydia,

It gives me great pleasure to announce that you have been successful in your application for a research grant of €5,000. An independent committee has reviewed your application "*Analysis of Haemochromatosis mutations in the North West population*" and found your project eligible for a grant.

The Foundation wishes you every success with your study and hopes that you will present a poster of your project for our Annual Multidisciplinary Research Conference.

A cheque with the above sum will be forwarded to you within the next few weeks.

Yours sincerely,

Mette Jensen Kavanagh
Development Officer

APPENDIX E

Statistics

Before comparing the allele frequencies of two populations, it is important to determine whether each population is in Hardy-Weinberg equilibrium.

The Hardy-Weinberg principle (HWP) (also Hardy-Weinberg equilibrium (HWE), or Hardy-Weinberg law) states that, under certain conditions, after one generation of random mating, the genotype frequencies at a single gene locus will become fixed at a particular equilibrium value. It also specifies that those equilibrium frequencies can be represented as a simple function of the allele frequencies at that locus.

In the simplest case of a single locus with two alleles **A** and **a** with allele frequencies of p and q respectively, the HWP predicts that the genotypic frequencies for the **AA** homozygote to be p^2, the **Aa** heterozygote to be $2pq$ and the other **aa** homozygote to be q^2. The Hardy-Weinberg principle is an expression of the notion of a population in "genetic equilibrium" and is a basic principle of population genetics.

When a population is in Hardy-Weinberg equilibrium for a given genetic locus, it means that there is random mating (with respect to that locus), no selection, no mutation, no gene flow and the population is large enough to avoid the random effects of genetic drift (http://en.wikipedia.org/wiki/Hardy-Weinberg_principle. Accessed on 7th March 2006).

If populations are not in Hardy-Weinberg equilibrium then it is more likely that differences in allele frequencies of the two populations is due to reproductive isolation.

For data analysis of the research presented in this thesis, the steps listed below were followed:

(1) Individual genotypes were scored
(2) Genotype frequencies were calculated
(3) Allele frequencies were calculated
(4) Using the observed allele frequencies, the expected genotype frequencies under Hardy-Weinberg equilibrium were calculated

(5) A goodness-of-fit test (Chi-square) was used to compare the expected and observed frequencies (http://grows.uns.edu/analysis/data_analysistext.htm. Accessed on 7th March 2006).

(6) The null hypothesis (H_o) is that the population is in Hardy-Weinberg equilibrium. The alternative hypothesis (H_a) is that the population is not in Hardy-Weinberg equilibrium.

Tables 3.0 to 3.2 & Table 4.0 were sources of data for statistical analysis (i.e. observed numbers).

Hardy-Weinberg equilibrium

1) For the C282Y locus

(a) in the Diabetic Population:

Genotype	C282Y homozygote **AA**	C282Y heterozygote **Aa**	No C282Y mutation **aa**	Total
Observed Number(s)	1	37	211	249

Allele frequency calculation:

$$p = \frac{2 \times \text{observed number (AA)} + \text{observed number (Aa)}}{2 \times (\text{observed number (AA)} + \text{observed number (Aa)} + \text{observed number (aa)})}$$

$$p = \frac{2 \times 1 + 37}{2 \times (1 + 37 + 211)}$$

$$p = \frac{39}{498}$$

$p = 0.078$ = C282Y allele frequency; as a % = 7.8%

and: $q = 1 - p$

$q = 1 - 0.078$

$q = 0.922$ = wildtype allele frequency; as a % = 92.2%

Aside:

Overall allele number is: 249 x 2 = 498

7.8% of 498 = 39 = the number (N) and

92.2% of 498 = 459 = the number (N)

Therefore the Hardy-Weinberg expectation is:

Expected number (AA) = $p^2n = (0.078)^2 \times 249 = 1.52$

Expected number (Aa) = $2pqn = 2 \times 0.078 \times 0.922 \times 249 = 35.81$

Expected number (aa) = $q^2n = (0.922)^2 \times 249 = 211.67$

Chi-square (x^2) test states:

$$x^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$x^2 = \frac{(1 - 1.52)^2}{1.52} + \frac{(37 - 35.81)^2}{35.81} + \frac{(211 - 211.67)^2}{211.67}$$

$x^2 = 0.178 + 0.040 + 0.002$

$x^2 = 0.58$

The 5% significance level for 1 degree of freedom (1df) = 3.84 (from statistics tables)

Since $x^2 = 0.58$ and 0.58 is < 3.84, the null hypothesis (i.e. the population is in Hardy-Weinberg equilibrium) is accepted.

→ *Therefore, the diabetic population is in HWE for the C282Y locus.*

(b) in the 'Control' Population:

Genotype	C282Y homozygote **AA**	C282Y heterozygote **Aa**	No C282Y mutation **aa**	Total
Observed Number(s)	3	45	199	247

Allele frequency calculation:

$$p = \frac{2 \times \text{observed number (AA)} + \text{observed number (Aa)}}{2 \times (\text{observed number (AA)} + \text{observed number (Aa)} + \text{observed number (aa)})}$$

$$p = \frac{2 \times 3 + 45}{2 \times (3 + 45 + 199)}$$

$$p = \frac{51}{494}$$

$p = 0.103$ = C282Y allele frequency; as a % = 10.3%

and: $q = 1 - p$

$q = 1 - 0.103$

$q = 0.897$ = wildtype allele frequency; as a % = 89.7%

Aside:

Overall allele number is: 247 x 2 = 494

10.3% of 494 = 51 = the number (N) and

89.7% of 494 = 443 = the number (N)

Therefore the Hardy-Weinberg expectation is:

Expected number (AA) = $p^2n = (0.103)^2 \times 247 = 2.62$

Expected number (Aa) = $2pqn = 2 \times 0.103 \times 0.897 \times 247 = 45.64$

Expected number (aa) = $q^2n = (0.897)^2 \times 247 = 198.74$

Chi-square (x^2) test states:

$$x^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$x^2 = \frac{(3 - 2.62)^2}{2.62} + \frac{(45 - 45.64)^2}{45.64} + \frac{(199 - 198.74)^2}{198.74}$$

$$x^2 = 0.055 + 0.009 + 0.0003$$

$$x^2 = 0.0643$$

The 5% significance level for 1 degree of freedom (1df) = 3.84 (from statistics tables)

Since $x^2 = 0.0643$ and 0.0643 is < 3.84, the null hypothesis (i.e. the population is in Hardy-Weinberg equilibrium) is accepted.

→ *Therefore, the control population is in HWE for the C282Y locus.*

2) For the H63D locus

(a) in the Diabetic Population:

Genotype	H63D homozygote **AA**	H63D heterozygote **Aa**	No H63D mutation **aa**	Total
Observed Number(s)	13	63	173	249

Allele frequency calculation:

$$p = \frac{2 \times \text{observed number (AA)} + \text{observed number (Aa)}}{2 \times (\text{observed number (AA)} + \text{observed number (Aa)} + \text{observed number (aa)})}$$

$$p = \frac{2 \times 13 + 63}{2 \times (13 + 63 + 173)}$$

$$p = \frac{89}{498}$$

$p = 0.179$ = C282Y allele frequency; as a % = 17.9%

and: $q = 1 - p$

$q = 1 - 0.179$

$q = 0.821$ = wildtype allele frequency; as a % = 82.1%

Aside:

Overall allele number is: 249 x 2 = 498

17.9% of 498 = 89 = the number (N) and

82.1% of 498 = 409 = the number (N)

Therefore the Hardy-Weinberg expectation is:

Expected number (AA) = $p^2n = (0.179)^2 \times 249 = 7.98$

Expected number (Aa) = $2pqn = 2 \times 0.179 \times 0.821 \times 249 = 73.19$

Expected number (aa) = $q^2n = (0.821)^2 \times 249 = 167.84$

Chi-square (x^2) test states:

$$x^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$x^2 = \frac{(13 - 7.98)^2}{7.98} + \frac{(63 - 73.19)^2}{73.19} + \frac{(173 - 167.84)^2}{167.84}$$

$$x^2 = 3.16 + 1.42 + 0.16$$

$$x^2 = 4.74$$

The 5% significance level for 1 degree of freedom (1df) = 3.84 (from statistics tables)

Since $x^2 = 4.74$ and 4.74 is > 3.84, the null hypothesis (i.e. the population is in Hardy-Weinberg equilibrium) is rejected.

→ *Therefore, the diabetic population is not in HWE for the H63D locus.*

(b) in the 'Control' Population:

Genotype	C282Y homozygote **AA**	C282Y heterozygote **Aa**	No C282Y mutation **aa**	Total
Observed Number(s)	9	57	184	250

Allele frequency calculation:

$$p = \frac{2 \times \text{observed number (AA)} + \text{observed number (Aa)}}{2 \times (\text{observed number (AA)} + \text{observed number (Aa)} + \text{observed number (aa)})}$$

$$p = \frac{2 \times 9 + 57}{2 \times (9 + 57 + 184)}$$

$$p = \frac{75}{500}$$

$p = 0.15$ = C282Y allele frequency; as a % = 15%

and: $q = 1 - p$

$q = 1 - 0.15$

$q = 0.85$ = wildtype allele frequency; as a % = 85%

Aside:

Overall allele number is: 250 x 2 = 500

15% of 500 = 75 = the number (N) and

85% of 500 = 425 = the number (N)

Therefore the Hardy-Weinberg expectation is:

Expected number (AA) = $p^2n = (0.15)^2 \times 250 = 5.625$

Expected number (Aa) = $2pqn = 2 \times 0.15 \times 0.85 \times 250 = 63.75$

Expected number (aa) = $q^2n = (0.85)^2 \times 250 = 180.625$

Chi-square (x^2) test states:

$$x^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$x^2 = \frac{(9 - 5.625)^2}{5.625} + \frac{(57 - 63.75)^2}{63.75} + \frac{(184 - 180.625)^2}{180.625}$$

$$x^2 = 2.025 + 0.72 + 0.06$$

$$x^2 = 2.805$$

The 5% significance level for 1 degree of freedom (1df) = 3.84 (from statistics tables)

Since $x^2 = 2.805$ and 2.805 is < 3.84, the null hypothesis (i.e. the population is in Hardy-Weinberg equilibrium) is accepted.

→ *Therefore, the control population is in HWE for the H63D locus.*

3) For the S65C locus

(a) in the Diabetic Population

Genotype	C282Y homozygote **AA**	C282Y heterozygote **Aa**	No C282Y mutation **aa**	Total
Observed Number(s)	0	1	123	124

Allele frequency calculation:

$$p = \frac{2 \times \text{observed number (AA)} + \text{observed number (Aa)}}{2 \times (\text{observed number (AA)} + \text{observed number (Aa)} + \text{observed number (aa)})}$$

$$p = \frac{2 \times 0 + 1}{2 \times (0 + 1 + 123)}$$

$$p = \frac{1}{124}$$

$p = 0.0081$ = C282Y allele frequency; as a % = 0.81%

and: $q = 1 - p$

$q = 1 - 0.0081$

$q = 0.9919$ = wildtype allele frequency; as a % = 99.19%

Aside:

Overall allele number is: 124 x 2 = 248

0.81% of 248 = 2 = the number (N) and

99.19% of 248 = 246 = the number (N)

Therefore the Hardy-Weinberg expectation is:

Expected number (AA) = $p^2n = (0.0081)^2 \times 124 = 0.0081$

Expected number (Aa) = $2pqn = 2 \times 0.0081 \times 0.9919 \times 124 = 1.99$

Expected number (aa) = $q^2n = (0.9919)^2 \times 124 = 122$

Chi-square (x^2) test states:

$$x^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$x^2 = \frac{(0 - 0.0081)^2}{0.0081} + \frac{(1 - 1.99)^2}{1.99} + \frac{(123 - 122)^2}{122}$$

$$x^2 = 0.0081 + 0.493 + 0.008$$

$$x^2 = 0.5091$$

The 5% significance level for 1 degree of freedom (1df) = 3.84 (from statistics tables)

Since $x^2 = 0.5091$ and 0.5091 is < 3.84, the null hypothesis (i.e. the population is in Hardy-Weinberg equilibrium) is accepted.

→ *Therefore, the diabetic population is in HWE for the S65C locus.*

(b) in the 'Control' Population:

Genotype	C282Y homozygote **AA**	C282Y heterozygote **Aa**	No C282Y mutation **aa**	Total
Observed Number(s)	0	4	121	125

Allele frequency calculation:

$$p = \frac{2 \times \text{observed number (AA)} + \text{observed number (Aa)}}{2 \times (\text{observed number (AA)} + \text{observed number (Aa)} + \text{observed number (aa)})}$$

$$p = \frac{2 \times 0 + 4}{2 \times (0 + 4 + 121)}$$

$$p = \frac{4}{250}$$

$p = 0.016$ = C282Y allele frequency; as a % = 1.6%

and: $q = 1 - p$

$q = 1 - 0.016$

$q = 0.984$ = wildtype allele frequency; as a % = 98.4%

Aside:

Overall allele number is: 125 x 2 = 250

1.6% of 250 = 4 = the number (N) and

98.4% of 250 = 246 = the number (N)

Therefore the Hardy-Weinberg expectation is:

Expected number (AA) = $p^2n = (0.016)^2 \times 125 = 0.032$

Expected number (Aa) = $2pqn = 2 \times 0.016 \times 0.984 \times 125 = 3.936$

Expected number (aa) = $q^2n = (0.984)^2 \times 125 = 121.032$

Chi-square (x^2) test states:

$$x^2 = \sum \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

$$x^2 = \frac{(0 - 0.032)^2}{0.032} + \frac{(4 - 3.936)^2}{3.936} + \frac{(121 - 121.032)^2}{121.032}$$

$$x^2 = 0.032 + 0.001 + 0.00000846$$

$$x^2 = 0.033$$

The 5% significance level for 1 degree of freedom (1df) = 3.84 (from statistics tables)

Since $x^2 = 0.033$ and 0.033 is < 3.84, the null hypothesis (i.e. the population is in Hardy-Weinberg equilibrium) is accepted.

→ *Therefore, the control population is in HWE for the S65C locus.*

The preceeding data is presented in Table form in the Results section (see Table 3.6).

Additional Statistics:

A Chi square test was also performed to determine whether there was a difference between the genotype frequencies of the Diabetic & Control populations. Where genotype frequencies occurred at a rate of 1 per population, they were excluded from the main chi square calculation (see Table below) as they are considered not significant and a Fisher's exact test must be performed (i.e. C282Y homozygous and S65C heterozygous genotypes). The calculation was

carried out on Minitab where raw data was used, different sample sizes were ignored and control results were converted to allow for minor differences in 'n'. The control group was taken as giving the expected outcome.

Table: Chi square test data transcribed from Minitab analysis

Genotype	**Observed**	**Expected**	**Chi square**	**Cumulative Probability**	**P Value**
C282Y heterozygote	37	45.00	3.83158	0.852774	0.147226
H63D homozygote	13	9.00			
H63D heterozygote	63	57.00			
Genotype		**Ex for Obs n**			
C282Y heterozygote	37	45.40	4.05255	0.868175	0.131825
H63D homozygote	13	8.96			
H63D heterozygote	63	56.80			

Results show that there is no difference between the genotype frequencies of Diabetics and Controls.

Each of the five genotypes was also taken separately and the two populations compared using two proportions. Confidence intervals (95%) were also calculated and transcribed from Minitab (see below).

Test and Confidence Interval for Two Proportions

(a) C282Y homozygotes:

Sample	X	N	Sample p
1	1	249	0.004016
2	3	247	0.012146

Difference = p (1) – p (2)
Estimate for difference: -0.00812968
95% CI for difference: (-0.0238876, 0.00762821)

Test for difference: = 0 (vs not = 0): z = -1.01 P value = 0.312

Note: The normal approximation may be inaccurate for small samples (i.e. when frequency is 1 per population, Minitab does Fisher's exact test).

Fisher's exact test: P value = 0.371.

(b) C282Y heterozygotes:

Sample	X	N	Sample p
1	37	249	0.148594
2	45	247	0.182186

Difference = p (1) – p (2)
Estimate for difference: -0.0335919
95% CI for difference: (-0.0989297, 0.0317460)
Test for difference: = 0 (vs not = 0): z = -1.01 P value = 0.314

(c) H63D homozygotes:

Sample	X	N	Sample p
1	13	249	0.052209
2	9	250	0.036000

Difference = p (1) – p (2)
Estimate for difference: 0.0162088
95% CI for difference: (-0.0198003, 0.0522180)
Test for difference: = 0 (vs not = 0): z = 0.88 P value = 0.378

(d) H63D heterozygotes:

Sample	X	N	Sample p
1	63	249	0.253012
2	57	250	0.228000

Difference = p (1) – p (2)

Estimate for difference: 0.0250120

95% CI for difference: (-0.0499572, 0.0999813)

Test for difference: = 0 (vs not = 0): z = 0.65 P value = 0.513

(e) S65C heterozygotes:

Sample	X	N	Sample p
1	1	124	0.008065
2	4	125	0.032000

Difference = p (1) – p (2)

Estimate for difference: -0.0239355

95% CI for difference: (-0.0585731, 0.0107021)

Test for difference: = 0 (vs not = 0): z = -1.35 P value = 0.178

Note: The normal approximation may be inaccurate for small samples (i.e. when frequency is 1 per population, Minitab does Fisher's exact test).

Fisher's exact test: P value = 0.370.

All the P values in (a) to (e) show non-significance at the 95% confidence level.

Additional Statistics:

Chi square test to determine whether there is a difference between the genotype frequencies of the Diabetic & Control populations.

Ho: there is a difference between the genotype frequencies of Diabetics and Controls
Ha: there is no difference between the genotype frequencies of Diabetics and Controls

The Control population data will serve as the expected values in the Chi square test

Data:

Genotype	Diabetic Population			Control Population		
	No. in population with genotype	Total Population Size (Tested Population)	% Population	No. in population with genotype	Total Population size (Tested Population)	% Population
C282Y Homozygote	1	249	0.4	3	247	0.0122
C282Y Heterozygote	37	249	14.86	45	247	0.1822
H63D Homozygote	13	249	5.22	9	250	0.036
H63D Heterozygote	63	249	25.3	57	250	0.228
S65C Heterozygote	1	124	0.81	4	125	0.032

Genotype	% Population (Diabetic)	Total Population Size (Control)	Expected Number (Control)	Observed Number (Control)	Difference (O - E)	$\frac{(O-E)^2}{E}$
C282Y Homozygote	0.4	247	0.988	3	2.012	4.1
C282Y Heterozygote	14.86	247	36.704	45	8.296	1.88
H63D Homozygote	5.22	250	13.05	9	- 4.05	1.26
H63D Heterozygote	25.3	250	63.25	57	- 6.25	0.62
S65C Heterozygote	0.81	125	1.0125	4	2.9875	8.82
					Chi square value / x^2 =	***16.68***

Degrees of freedom (df) = (Number of genotypes – 1) = 4

The critical value for x^2 distribution with 4 df and and α level of 0.05 is : 9.49 (from Chi square tables).

The observed statistic of 16.68 is greater than the critical value, even at $\alpha = 0.01$, therefore the null hypothesis (Ho) is rejected and the alternative hypothesis is accepted.

That is: There is no difference between the genotype frequencies of Diabetics and Controls.