MUTATIONS OF THE HFE GENE IN PATIENTS WITH OSTEOPOROSIS AND ARTHRITIS

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DECLARATION

This dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. No part of it has been presented for any degree or examination at any other University.

L McNAMARA

ABSTRACT

Iron is essential to virtually all forms of living organisms. In man, iron is implicated in a number of disease states and, in particular, iron overload is now recognised as a common disorder of iron metabolism and an important cause of morbidity. Hereditary haemochromatosis is an autosomal recessive disorder of iron metabolism characterised by excessive iron absorption from the upper gastrointestinal tract resulting in the accumulation of iron in various organs of the body. While hereditary haemochromatosis is associated with the development of diverse conditions including cardiomyopathy, diabetes, liver fibrosis and cirrhosis, penetrance is very variable. It represents one of the most common genetic disorders found in people of northern European descent. A number of genes have been shown to be implicated in the development of hereditary haemochromatosis, one of which, the HFE gene, is mutated in up to 80% of patients with the disease.

Osteoporosis is a disease characterised by low bone mass and structural deterioration of bone tissue leading to bone fragility and an increased susceptibility to fracture. It has been observed in most conditions associated with iron overload and the association between osteopoenia and hereditary haemochromatosis has been described, but little data exists concerning the pathogenesis of the disease and its relationship to the degree of iron overload.

The purpose of this study was to investigate the prevalence of the known HFE gene mutations in osteoporotic patients and, if found, to look for a correlation between these mutations and the severity of osteoporosis. Patients of northern or western European

ancestry attending an osteoporosis clinic were approached to take part in the study. An iron profile was performed on each patient and blood was drawn for DNA analysis. All patients were tested for the presence of the three common mutations of the gene, C282Y, H63D and S65C and for the presence of the intron 2 polymorphism (IVS2+4T→C). The whole of exon 2 of the HFE gene was sequenced in patients who were found to have a high transferrin saturation. Exon 2 was chosen for further investigation as that is the area of the gene which binds to the transferrin receptor.

The C282Y mutation was found to occur more frequently than in the general population. The prevalence of the H63D mutation was slightly higher, but not significantly so, and the prevalence of S65C was in line with previous reports. There was no correlation between the presence of these 3 mutations and the severity of osteoporosis in these patients. The presence of the intron 2 polymorphism was significantly higher than previously reported and correlated positively with less severe osteoporosis.

Hereditary haemochromatosis, if left untreated, is a potentially fatal disease. Treatment is simple and involves removal of the excess iron by regular phlebotomy. If the condition is diagnosed early and treatment instigated immediately, many of the diseases associated with it may never occur. The increased prevalence of mutations of the HFE gene found in this patient sample indicates that physicians should be aware of hereditary haemochromatosis as a causative factor in patients presenting with osteoporosis. The association of the intron 2

polymorphism with less severe osteoporosis needs to be confirmed by further investigation in a larger patient sample.

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CHAPTER 1

1. IRON

1.1 Introduction

No eukaryocytes are capable of survival without iron. Together with nickel, iron makes up the core of the earth and is the fourth most abundant element in its crust. Iron owes its biological importance to its unusual reactivity. There is no other element that exhibits the adaptability of iron in the electron transport reactions essential to life. The most important reaction is the reversible one electron oxidation and reduction reaction that allows iron to move between its two common oxidative states, the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. This reaction is exploited by most iron dependent enzyme systems involved in transport and storage of oxygen, electron transport and trans-membrane iron transport. It is responsible for the threat to human life posed by the excess amounts of iron found in acute and chronic iron overload. Iron is an important catalyst of oxyradical-mediated cellular and tissue injury, both of which are being increasingly recognised as pathological processes (Halliwell and Gutteridge, 1990). Despite convincing clinical evidence for the toxic effects of excess iron on the liver (Tavill and Bacon, 1986), the precise mechanisms involved are not well understood. *In vitro* work has been done showing that ionic iron stimulates lipid peroxidation and several theories of cellular and tissue injury due to iron-induced peroxidative damage to membrane phospholipids have been put forward (Ungemach, 1985; Rush *et al*, 1985). Lipid peroxidation is associated with mitochondrial and microsomal dysfunction in the liver in cases of experimental iron overload (Bacon and Britton, 1989), and may also be responsible for the increased

fragility of lysosomes that has been detected in liver samples from both iron-loaded humans and experimental animals. The vulnerability of the liver to the toxic effects of iron is due to its capacity to take up both specific and non-specific bound iron and also because absorbed iron is delivered directly to the liver via the portal vein. The toxic effect of excess iron has important health consequences due to the number of people affected with iron overload worldwide (See Section 1.4).

1.2 Iron Metabolism in Man

Iron metabolism can best be summarised as being the maintenance of iron-related physiological functions by balancing intake, transport, storage and loss of iron. Iron requirements and intake are governed by the need to maintain iron balance in the body relative to loss and requirements for growth. In addition to intake and loss, body iron stores play a major role in maintaining iron supplies for metabolic needs. When average iron status is assumed, the adult human has between 0.7 and 0.9mmoles of iron per kilogram of body weight (Bothwell *et al*, 1979). Red cell haemoglobin contains over 60% of this iron and some 25% is found in the form of storage iron, mainly in the liver. Other essential body iron consists of myoglobin in muscle and cell enzyme iron and a small amount is present in the circulation bound to the transport protein, transferrin. In normal individuals, up to 0.3mmoles/kg body weight of surplus iron is stored as ferritin and haemosiderin, mainly in the reticuloendothelial cells and hepatocytes (Heubers and Finch, 1987).

1.2.1 Internal Iron Exchange

Food iron is absorbed by the mucosal cells of the upper small intestine. When iron is released from these cells, it enters the portal circulation where it is bound to the transport protein, transferrin. In mammals, internal iron exchange is mediated by this protein and is largely concerned with three tissues: the erythron, which is involved in the synthesis and maintenance of haemoglobin, the reticuloendothelial system, which breaks down red cells and stores iron, and the parenchymal cells of the liver which act as an additional storage area for iron (Bothwell *et al* 1979) (Figure 1). Most of the iron absorbed by the gut is transported directly to the bone marrow where it is incorporated into haemoglobin.

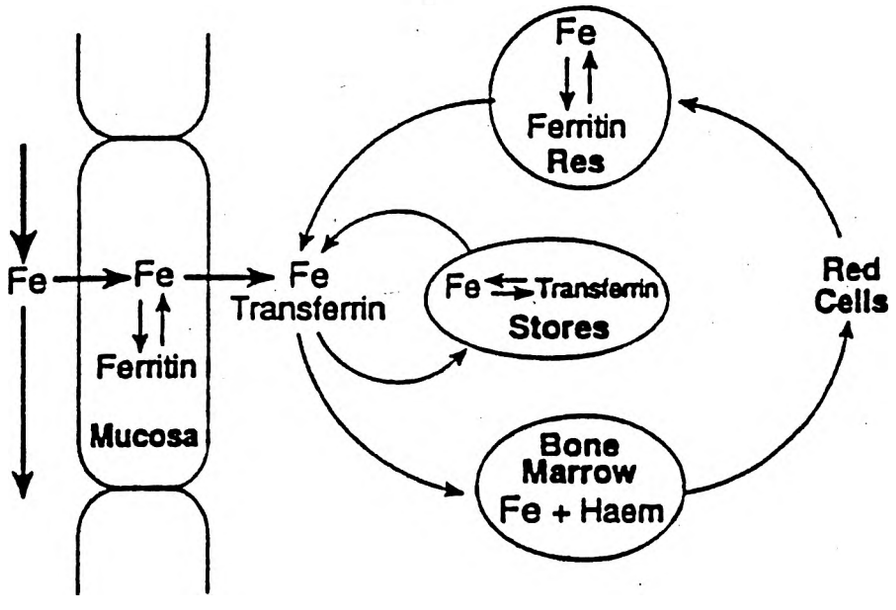


Figure 1: A model of internal iron exchange (MacPhail, 1998).

At the end of its life, the red cell is engulfed by the cells of the reticuloendothelial system, the iron removed and either stored or released back to the circulation to be picked up by transferrin once more. It is important to note that man has no effective excretory mechanism for iron and that this cycle is never reversed. Some loss of iron does take place, however, in the form of intracellular iron compounds lost during the normal turnover of epithelial cells in the skin, gastro-intestinal and genito-urinary tracts. These losses have been defined best in the adult male and amount to between 0.21 and 0.24μmoles/kg/day in normal individuals (Green *et al*, 1968). Under normal circumstances, these losses are relatively easily balanced by iron absorption. In the female, however, additional losses are incurred through menstruation, lactation and pregnancy, making the balance more difficult to maintain by absorption alone (Cole *et al*, 1971). Iron is distributed within the body via the plasma and the normal plasma concentration in adult males is around 2.1mmoles/l and 1.9mmoles/l in females, but exhibits marked diurnal and postprandial variation (Bothwell *et al*, 1979).

1.3 Iron Transport

The behaviour of iron in aqueous media, such as is present in living organisms, is largely determined by the oxidation-reduction reaction between Fe^{2+} and Fe^{3+} and the formation of hydroxides which are often insoluble and impermeable to cell membranes. In the aerobic extracellular environment, soluble Fe^{2+} is readily oxidised to the more insoluble Fe^{3+}, rendering it unavailable for cellular requirements. This problem has been overcome during evolution by the development of elaborate biological mechanisms that can bind Fe^{3+} and maintain it in a soluble form that can be utilised by cells. For this purpose, vertebrate species have consistently used transferrin, a specialised transport protein with two high affinity binding sites for iron. Under some circumstances, however, other proteins such as ferritin, lactoferrin and albumin may also play a role and, in states of iron overload when transferrin is saturated with iron, the plasma may contain iron that is either free or non-specifically bound to plasma proteins. Extracellular iron, may therefore, be divided into three categories, namely, transferrin-bound iron, specific non-transferrin-bound iron (eg ferritin) and non-specific non-transferrin-bound iron. Under normal circumstances, the first form, transferrin-bound iron, dominates iron transport.

1.3.1 Transferrin-Bound Iron

Transferrin, first isolated and characterised in 1947 by Laurell and Ingelman, is a glycoprotein with a molecular mass of approximately 77Kda. It is synthesised mainly in the liver and, although lymph nodes and peripheral blood lymphocytes may also be involved, synthesis by these cells is probably quantitatively unimportant (Morgan, 1981). Transferrin is distributed throughout most of the extracellular fluid of the body. It is continuously circulated from plasma to interstitial fluid and back to the blood via the

lymph vessels thereby fulfilling its major function of iron transport. Transferrin also provides for iron exchange between cells and extracellular fluid, is an essential protein for growth and differentiation in a number of cells and plays a significant role in inhibiting the growth and multiplication of bacteria. Each molecule of transferrin has the capacity to bind two atoms of Fe^{3+} plus one bicarbonate ion per metal ion. Binding of metal ions appears to stabilise the structure of the protein, making it more resistant to thermal and proteolytic attack than when it is in the iron-free form (Palmour and Sutton, 1971). The cell takes up mono- or diferric transferrin via specific receptor sites on the cell membrane. The transferrin-iron-receptor complex is endocytosed, the iron released and the apotransferrin-receptor complex is returned to the cell surface where the apotransferrin is released back into the circulation (Morgan, 1983) (Figure 2). The process by which the transferrin-iron-receptor complex is endocytosed into the cell has been well described (Morgan 1981). However, the process by which the released iron moves from the endocytosed vesicle into the cytoplasm is still not fully understood although recent work by Fleming and co-workers (1998) has shown that a recently discovered protein, DMT-1 (*Nramp2*), may be responsible for mobilising the iron from the acidified endosome. Transferrin is not catabolised during the processes involved with iron exchange, thus acting as a true carrier – taking iron up at one site, depositing it at another and becoming available to accept iron once more. Under normal circumstances, transferrin is approximately one third saturated with iron, leaving an abundance of iron free transferrin available to bind iron absorbed from the intestines or released from cells elsewhere in the body (Bothwell *et al*, 1979).

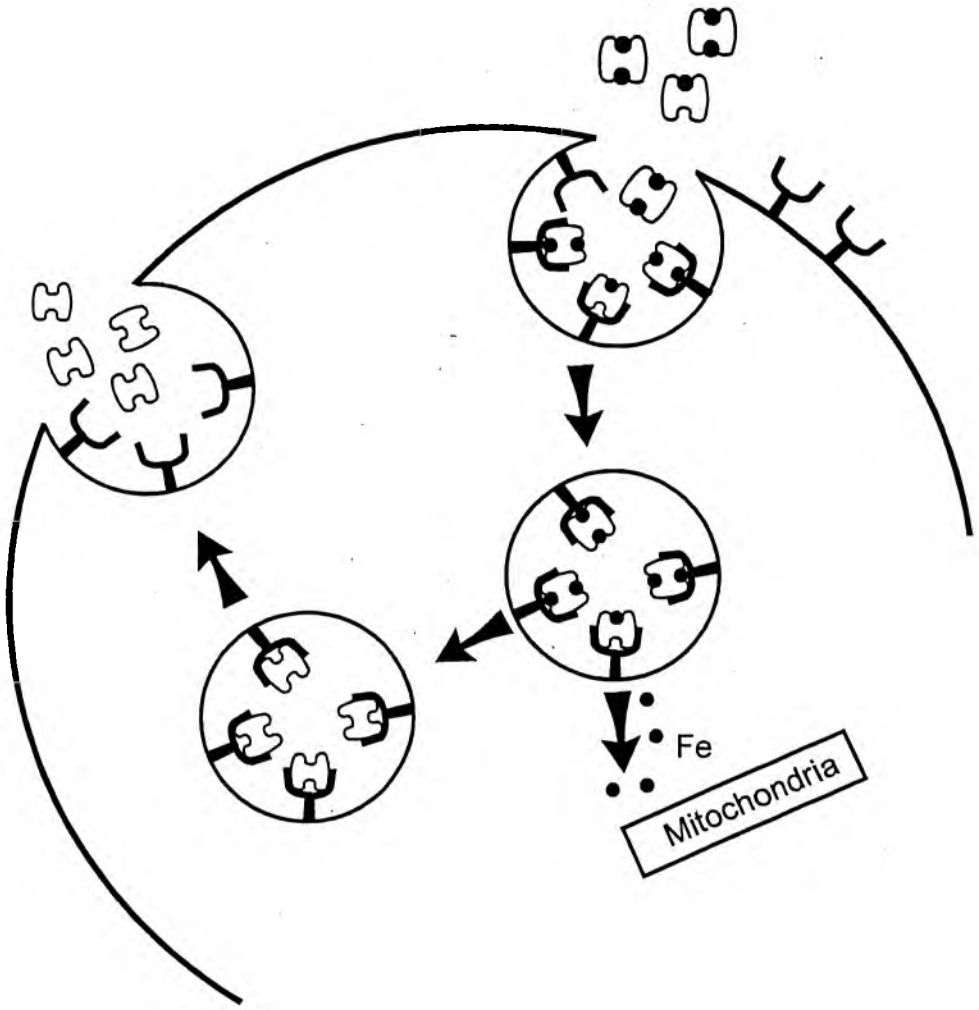


Figure 2: Cellular uptake of transferrin

Transferrin-iron complex

Apotransferrin

Receptors

1.3.2 Specific Non-Transferrin-Bound Iron

Several proteins that bind iron or iron-containing compounds are present in the plasma and may play a role in the extracellular transport of iron, especially in abnormal conditions such as iron overload and infection. These proteins are ferritin, lactoferrin, haemopexin, haptoglobin and albumin. The most abundant of these is ferritin and, although the quantity of iron carried by this protein is uncertain, it is probably quite low relative to that carried by transferrin (Garby and Noyes, 1959). Ferritin is the major iron storage protein, maintaining ferric iron in an available form. It is found in all tissues, but is most plentiful in liver, spleen and bone marrow.

Ferritin is found in the plasma of normal subjects in concentrations that relate to the amount of storage iron in the body. This relationship is the basis for the widespread use of the serum ferritin assay in the laboratory diagnosis of iron deficiency and overload. The amount of iron normally circulating in plasma ferritin is very low. With concentrations of between 15 and 300µg/l in adults (Walters *et al*, 1973), there is not enough iron to affect plasma iron concentration and turnover significantly. However, in iron overload and other diseases associated with liver cell damage, plasma ferritin may contain more iron. Ferritin levels of up to 68 000µg/l have been recorded in liver damage (AP MacPhail, personal communication), and, in this setting, may make an important contribution to the total plasma iron and iron uptake by the liver (Pootrakul *et al*, 1988).

1.3.3 Non-Specific Non-Transferrin-Bound Iron

Plasma transferrin has considerable reserves for coping with increasing amounts of incoming iron but these may be exceeded in pathological conditions such as acute iron poisoning, hereditary haemochromatosis, African iron overload and certain iron-loading anaemias. Brock and co-workers (1984), showed that the reticuloendothelial system continues to release iron in the presence of complete transferrin saturation, resulting in the appearance of iron in the plasma in a non-specific non-transferrin-bound form known as non-transferrin-bound iron (NTBI). NTBI has been directly implicated in the toxic effects of iron overload in patients with hereditary haemochromatosis (Gutteridge *et al*, 1985) and has been shown to be part of a pathway leading to hepatic injury in cases of African iron overload (McNamara *et al*, 1999). In addition, some tumour cells possess mechanisms that allow them to acquire and utilise NTBI without the mediation of transferrin. Although NTBI normally accounts for less than 1% of the total serum iron, it may account for up to 35% of serum iron in iron overloaded patients (Batey *et al*, 1978). Non-transferrin-bound iron is cleared much more rapidly and efficiently from the plasma by the liver than is transferrin-bound iron suggesting that this plasma pool may be a significant source of hepatic iron (Wright *et al*, 1986).

1.4 Iron Overload

Iron overload, for many years considered a rarity, is now recognised as a common disorder of iron metabolism. Iron overload has been implicated as an etiological factor in, amongst other diseases, cirrhosis, congestive heart failure and oesophageal cancer. It is now also widely recognised that individuals with severe iron overload show increased evidence of hepatocellular carcinoma and that it is a significant cause of death (Mandishona *et al*, 1998). Few notable symptoms precede advanced injury to the internal organs and early diagnosis and treatment is important. Iron overload may occur as a genetic disease, as in hereditary haemochromatosis and possibly African iron overload, or as a secondary condition as in the iron-loading anaemias.

1.4.1 Hereditary Haemochromatosis

Haemochromatosis, from the Greek words *haima* for blood and *chromatos* for colour, was a generic term coined in 1889 by von Recklinghausen to denote iron storage disease associated with widespread tissue injury. It represents one of the most common genetic disorders affecting people of northern and western European descent and studies carried out in Australia and the United States have estimated population frequencies of 1:300 and 1:200 respectively (Leggett *et al*, 1990; Edwards *et al*, 1982). In 1996, Feder and co-workers discovered a candidate gene in which two missense mutations were closely correlated with the development of the disease. Following on this, studies by Merryweather-Clarke and co-workers (1997), in which they screened 5956 European chromosomes for the mutations described by Feder, established that they were most prevalent in northern European populations with the highest allele frequency being in the United Kingdom and Denmark. It has recently been estimated that the mutations first

appeared between 60 and 70 generations ago, assuming a mean generation time of 20 years, this time equates to 600 – 700 AD (Thomas *et al*, 1998).

Hereditary haemochromatosis is an autosomal recessive disorder of iron metabolism characterised by abnormally increased absorption of iron from a normal diet. This excess iron slowly accumulates and is deposited in various organs of the body over many years. As the major site of iron storage, the liver is a conspicuous victim of excess iron deposition and mild to moderate hepatomegaly develops fairly early, followed by fibrosis and cirrhosis. In the heart, congestive cardiomyopathy is the most common defect that occurs with iron overload but other problems have been described, including pericarditis and angina (Liu and Olivieri, 1994). Dysfunction of the endocrine system is common in patients with iron overload and many patients develop *diabetes mellitus* requiring insulin therapy. Pituitary dysfunction with reduced gonadotropin levels is common and parathyroid activity is frequently compromised. Arthropathy and osteoporosis are frequently seen and are often the presenting features. Cutaneous iron deposition can damage the skin and enhance melanin production resulting in hyperpigmentation in some patients. In symptomatic patients, treatment involves the removal of excess iron as quickly as possible. Iron is best removed from the body by weekly or twice-weekly phlebotomy of 500ml. Since 500ml of blood contains approximately 250mg of iron and 20g or more of storage iron may be present, 2 – 3 years of weekly phlebotomy are usually required for its removal. Once the serum ferritin and transferrin saturation have returned to normal, one phlebotomy every 3 months is usually enough as maintenance therapy. In a retrospective evaluation of phlebotomy therapy in patients with hereditary haemochromatosis, Bomford and Williams (1976) compared 85 patients receiving

phlebotomy with 26 untreated patients. The 5-year survival rate with therapy was increased from 33% to 89%. With removal of iron, patients lose their lethargy, the liver and spleen decrease in size, biochemical liver tests return to normal, skin pigmentation decreases and cardiac failure may be reversed. Carbohydrate tolerance improves in 30 – 40% of patients, resulting in a reduction in insulin requirements or drug therapy. However, removal of excess iron has little or no effect on hypogonadism, arthropathy, osteoporosis or portal hypertension. Hepatic fibrosis may decrease but cirrhosis is irreversible and hepatocellular carcinoma occurs in about 25 - 30% of patients (Powell *et al*, 1994). If the disease is diagnosed early enough and treatment instigated timeously however, disorders associated with the condition may never develop and life expectancy is normal. In light of its frequency and the potential for safe and effective treatment, haemochromatosis has been dubbed "the genetic disorder of the twenty-first century" (Barton and Bertoli, 1996).

1.4.1.1 The HFE gene

Even after more than two decades of intensive research, the genetic complexity of hereditary haemochromatosis is still unfolding. More than 20 years ago, it was described as an autosomal recessive disorder associated with the human leukocyte antigen (HLA)-A3 complex (Simon *et al,* 1976) and, subsequently in 1978, Cartwright and co-workers linked it to HLA-A on the short arm of chromosome 6. In 1996, Feder and co-workers identified a 250-kilobase region located more than 3 megabases telometric from the major histocompatibility complex on chromosome 6 that was identical by descent in 85% of hereditary haemochromatosis patients. In this region, they identified a gene related to the major histocompatibility complex class I family that they called HLA-H but which

was subsequently re-named HFE (Bodmer *et al*, 1997). HFE is a 12146bp protein composed of 7 exons encoding a 343 amino acid protein. It has three extra-cellular domains analogous to the α1, α2 and α3 domains of other MHC Class I proteins. In contrast to other members of this family however, the α1 and α2 domains are non-polymorphic. β_2 microglobulin is a separate protein and interacts with the HFE gene product in a non-covalent manner in the α3 homologous region for co-expression at the cell surface. In addition, HFE contains a membrane spanning region and a short cytoplasmic tail (Figure 3). The main mutation reported by Feder and co-workers (1996) is a cysteine to tyrosine substitution at amino acid 282 in exon 4 of the gene (C282Y) and the second is a histidine to aspartate change at amino acid 63 in exon 2 (H63D). Douabin and co-workers subsequently reported a third mutation in 1997, a serine to cysteine substitution at amino acid 65 also in exon 2 (S65C). A number of other uncommon mutations and polymorphisms have been identified in the HFE gene. Only some of these are associated with the development of hereditary haemochromatosis, and they are largely confined to individual families or a handful of individuals (Pointon *et al*, 2000). A number of diallelic intronic polymorphisms have also been described in introns 1, 2, 4 and 5. In connection with the intron 2 polymorphism (IVS2+4T→C), it has been noted that chromosomes that carry the C282Y mutation have thymine (wild type) exclusively at this locus and in chromosomes that carry the H63D mutation, thymine is found in 88 – 100% of cases (Pointon *et al*, 2000). These polymorphisms are not thought to be in any way implicated with the disease, although some of them have been used to establish haplotypes within the HFE gene (Beutler and West, 1997).

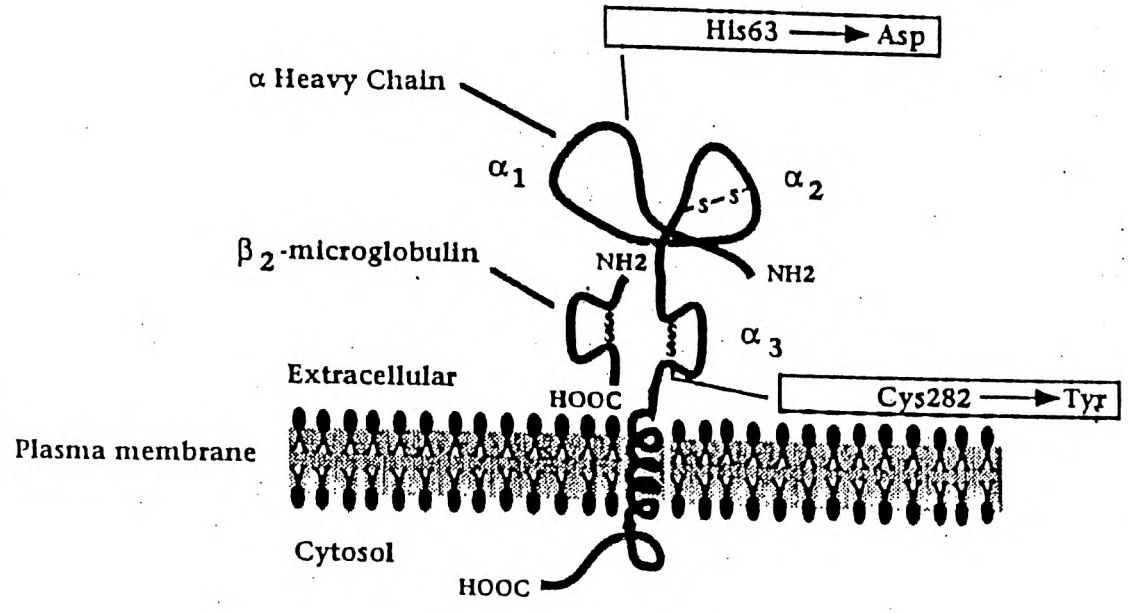


Figure 3: A model of the HFE protein. The approximate positions of the C282Y and H63D mutations are marked. (Feder *et al*, 1996).

The C282Y mutation has been well characterised as being the main mutation responsible for haemochromatosis in all the population studies performed throughout the world. It accounts for 80-90% of hereditary haemochromatosis chromosomes with between 81% and 90% of patients being homozygous (Feder *et al*, 1996). The functional relevance of the C282Y mutation has been clarified by cell transfection assays (Feder *et al*, 1997). These studies showed that cysteine 282 is one of four cysteine residues that are invariant in MHC class I molecules. It forms a critical disulphide bridge in the α_3 domain and the presence of the mutation prevents binding to β_2-microglobulin, disrupting intracellular protein trafficking and resulting in intracellular sequestration of the HFE protein. It has further been demonstrated that the wild-type (normal) HFE protein forms a stable complex with the transferrin receptor and decreases the affinity of transferrin binding. The C282Y mutation nearly completely eliminates this interaction (Feder *et al*, 1998).

The role of the H63D mutation in hereditary haemochromatosis is not clear. It has been reported that, among non-C282Y chromosomes, 21% – 43% and possibly up to 73% bear the H63D substitution indicating that this mutation is increased among hereditary haemochromatosis probands (Borot *et al*, 1997; Beutler, 1997). Merryweather-Clarke and co-workers (1997) found this mutation to be present in almost all populations and reported a worldwide allele frequency of 8.1% with the highest occurrence being amongst Basques (30.4%). No chromosome that bears the C282Y mutation has ever been found to contain the H63D mutation. Compound heterozygotes (C282Y/H63D) account for approximately 7% of haemochromatosis sufferers and the lower than expected frequency of H63D homozygotes found amongst probands compared with the H63D frequency in the population is evidence of the incomplete penetrance of this mutation.

This mutation may therefore be considered as a genetic variant that increases the risk of developing a milder form of haemochromatosis (Beutler, 1997). The presence of the H63D mutation has no effect on the association of HFE with β_2-microglobulin and the protein is expressed normally on the cell surface where it forms a stable complex with the transferrin receptor. However, studies in tissue culture have shown that wild-type HFE decreases the affinity of the transferrin receptor for transferrin and that when the H63D mutation is present, it does not have this effect (Feder *et al*, 1998). The loss of HFE-repressor function for transferrin uptake could result in increased cellular uptake of iron.

Population studies have shown that the occurrence of the S65C mutation is relatively low with a gene frequency of 0.016 (Beutler *et al*, 2000). It has been shown that the S65C substitution is significantly higher among hereditary haemochromatosis chromosomes that are not carriers of the C282Y or H63D substitutions (Mura *et al*, 1999). The S65C mutation has never been found in individuals homozygous for the C282Y or H63D mutations nor in compound heterozygotes for these two mutations, indicating that they may be mutually exclusive. The functional significance of the S65C mutation is not known, although one can hypothesise that the S65C substitution, located so close to the H63D, may not be able to reduce the affinity of transferrin receptor for transferrin as efficiently as the wild-type, as demonstrated for the H63D mutant protein.

In a considerable number of patients with haemochromatosis, including some with a clear-cut genetic pattern, complete sequencing of the coding region of the HFE gene and the intron/exon boundaries failed to reveal any abnormality (Pietrangelo *et al*, 1999). Two other proteins have recently been put forward as a site of defect in patients with

haemochromatosis. A recently reported missense mutation in the gene encoding ferroportin, a transmembrane transport protein, has been shown to be associated with the development of autosomal-dominant haemochromatosis, not linked to HFE, in a large Italian family (Montosi *et al*, 2001). A homozygous nonsense mutation in the gene encoding transferrin receptor-2 (TFR2) has also been identified in a cohort of patients with non-HFE haemochromatosis (Camaschella *et al*, 2000).

A number of other hereditary conditions associated with iron overload have been described, these include:

i) African iron overload in sub-Saharan Africa, which is thought to result from the interaction between an HLA-independent genetic component and environmental factors (Gordeuk *et al*, 1992).

ii) Juvenile haemochromatosis, an autosomal recessive disorder leading to severe iron loading in the second and third decade of life. The gene for this disease has been mapped to chromosome 1q (Roetto *et al*, 1999).

It would, therefore, not be surprising if further genes were found to be involved. Iron overload has not been thoroughly investigated in Caucasian populations who are not of northern European descent and the study of such populations may well reveal further HFE mutations or other genes associated with iron metabolism. Major advances in the understanding of hereditary haemochromatosis and iron metabolism have been made since the identification of the HFE gene, but there is still much to be uncovered.

1.5 Osteoporosis

Osteoporosis, or porous bone, is a disease characterised by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures. The term osteoporosis refers to more severe bone loss, with or without fragility fractures, than the term osteopenia, which means too little bone in the absence of fragility fractures. Osteomalacia, meaning soft bone, contains too little mineral and this condition may co-exist with either of the previous two (Schnitzler, 1995).

Osteoporosis is the most common metabolic bone disease and, although it can affect both sexes, the vast majority of sufferers are elderly white women. Ageing is associated with an annual bone loss of about 1% in both women and men (Genant *et al*, 1988). Over and above this age-related bone loss, women suffer additional peri- and post-menopausal bone loss between the ages of 45 and 65 years. Individuals with low peak bone mass at skeletal maturity (age 25 - 30 years) are particularly at risk of developing osteoporosis later. Men are rarely affected by hypogonadal osteoporosis because the andropause generally sets in some 15 years later than the menopause. Asian populations are also affected but blacks only rarely (Figure 4). The disease is very widespread and one third of females aged 65 years have had at least one vertebral fracture (Riggs and Melton, 1986).

Bone may be lost slowly or rapidly and the loss may or may not be spontaneously reversible. The bone turnover, or re-modelling process, goes on throughout life. Osteoclasts remove minute amounts of bone, which leaves small erosion cavities on the

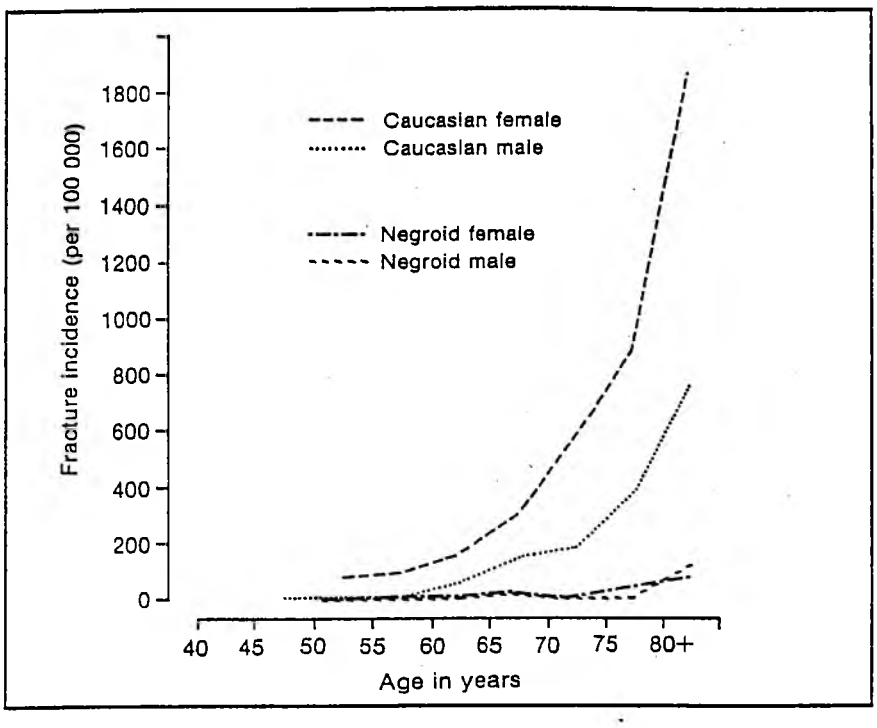


Figure 4: Annual incidence of femoral neck fractures in the Johannesburg population aged 45 years and over (Solomon, 1979).

internal surface of the bone. This raw surface attracts osteoblasts that refill the cavities with new bone. In healthy young adults, bone formation promptly follows erosion and the amount of bone formed equals that removed so no bone is lost during the re-modelling cycle. With ageing, however, less bone is formed than is lost so a little is lost with each cycle. This bone loss is slow and not spontaneously reversible. On the other hand, rapid bone loss may occur through a sudden increase in the creation of new remodelling cycles, i.e. erosion sites. This happens at menopause and under certain other circumstances (Table 1). This rapid loss recovers partly when resorption switches back to formation on removal of the cause. Bone mass is under strong genetic control, but the specific genes and allelic variants contributing to bone mass and osteoporotic risk are not well defined (Zmuda *et al*, 1999). Trabecular bone (vertebral bodies) is lost more rapidly than cortical bone (shafts of limb bones) because of the greater surface area in trabecular bone. For this reason, spinal fractures are the first osteoporotic fractures to manifest.

DEXA, which stands for dual x-ray absorptiometry, has become the gold standard for measurement of bone mineral density or bone mass. The technique analyses bone mineral content by measuring the attenuation of an X-ray beam passed through the patient's body. The more bone a patient has, the more radiation energy is absorbed and the weaker is the emerging beam which is measured in the detector. The X-ray source emits two energy peaks, one measures soft tissue density, the other measures both soft tissue and bone density. The soft tissue value is then subtracted from that of soft tissue plus bone and the resultant bone density value printed on a reference curve (Figure 5). Low bone mineral density (BMD) is the most important risk factor for future fragility fractures (Johnston and Melton, 1995). At any age, every decrease of BMD by one standard

Age-related factors	Genetic factors	Medical factors	Lifestyle factors
Oestrogen deprivation	Low bone mass in either or both parents	Oophorectomy	Alcohol abuse
Prolonged inactivity	White or Asian extraction	Hysterectomy	Inactivity
Calcium and vitamin D deficiency		Corticosteroid therapy	Heavy smoking
		Hyperparathyroidism	High protein diet
		Thyrotoxicosis	Low body weight or anorexia nervosa
		Anticonvulsant therapy	Excessive exercise
		Gastrectomy	
		Small bowel resection or exclusion	
		Myeloma or metastases	
		Vitamin C deficiency	
		Hereditary haemochromatosis	

Table 1: Factors that aggravate bone loss

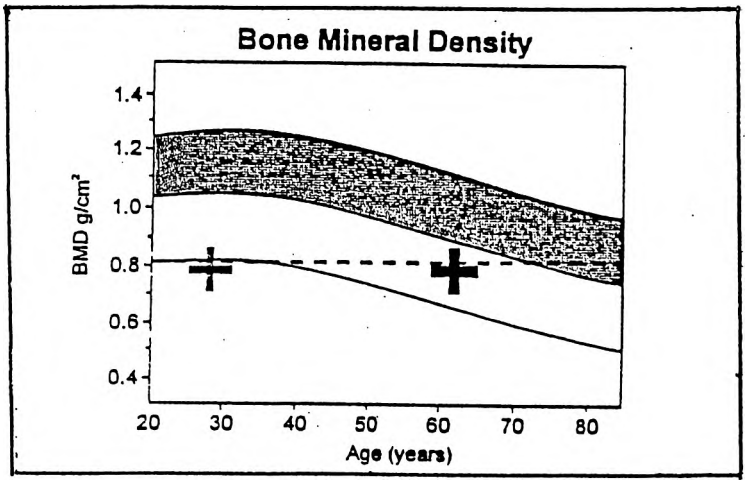


Figure 5: The computer-generated printout of a DEXA scan gives the normal reference curve and the patient's result (cross at age 62). The middle curve represents the mean of a normal population. The area between the upper and lower curves represents the normal range.

deviation, i.e. 1 T score, below the young normal mean, doubles the risk of fracture. In the interpretation of a DEXA value, it matters less whether this falls within the age-related normal range, than that it be above the fracture threshold, i.e. the lowest young normal limit (the stippled line in Figure 5).

The World Health Organisation (WHO) has defined the following categories of bone loss:

0 – 1.2 SD below the young normal mean	-	normal
1.2 – 2.5 SD below the young normal mean	-	osteopenia
> 2.5 SD below the young normal mean	-	osteoporosis
>2.5 below the young normal mean plus low impact fractures	-	severe osteoporosis

(WHO Study Group, 1994).

Prevention of the development of osteoporosis is of the utmost importance because complete cure of the established condition is not possible. Prevention is divided into primary prevention from birth to attainment of peak bone mass at age 30 years, and secondary prevention from the peri-menopause onwards. By definition primary prevention is aimed at the entire population and secondary prevention at targeted groups identified by risk factors such as:

- female sex	- alcohol abuse	-low spinal bone mineral density
- slim build	- heavy smoking	- calcium deficiency
- family history	- inactivity	- vitamin D deficiency

The overall concept is:

i) to achieve as high a peak bone mass as possible, and

ii) to maintain the high level of peak bone mass for as long as possible.

Studies have shown that high calcium intake during growth is associated with greater peak bone mass than is low calcium intake and that exercise also increases peak bone mass (Slemenda *et al*, 1990). Secondary prevention at the menopause is particularly important in women with risk factors for osteoporotic fractures.

1.6 Osteoporosis and Iron Overload

The skeletal system may be profoundly affected by chronic liver disease resulting in a variety of conditions that may include osteomalacia, osteoporosis and osteonecrosis (Pais MJ, 1980). The association between osteoporosis and haemochromatosis was first noted by Delbarre in 1960. Subsequent studies have reported that the frequency of osteoporosis in patients suffering from hereditary haemochromatosis varies between 28% and 45% (Sinigaglia *et al*, 1997; Diamond *et al*, 1989). Osteoporosis is usually described in patients with primary haemochromatosis although it has been observed in secondary haemochromatosis (de Vernejoul *et al*, 1982). Haemochromatotic patients with osteoporosis are usually asymptomatic, although spinal abnormalities may lead to fracture and vertebral collapse, resulting in back pain and deformity. The exact mechanism leading to the development of osteoporosis in this group is not known although both inadequate bone formation and excessive bone resorption are thought to operate. It has been suggested that excess iron may impair osteoblastic activity with a diminution in the synthesis of osteoid matrix (Diamond *et al*, 1989). Iron-associated osteopathy has been reported to be associated with hypogonadism, vitamin C deficiency, hyperparathyroidism and iron excess (Wapnick *et al*, 1971; de Vernejoul *et al*, 1984; Diamond *et al*, 1989; Pawlotsky *et al*, 1999).

Osteoporosis has also been observed in other conditions associated with iron overload such as African iron overload in sub-Saharan Africa. This disease, associated with hepatocellular carcinoma (Mandishona *et al*, 1998), has also been shown to be associated with osteoporosis (Grusin and Samuel, 1957; Seftel *et al*, 1966). Several of the various recognised causes of osteoporosis could conceivably play a part in the pathogenesis of

the disease seen in these subjects. However, studies done by Lynch and co-workers (1967) suggest that the osteoporosis seen in these patients is due to ascorbic acid deficiency resulting from severe iron overload. Although there appears to be a genetic component to the development of African iron overload (Gordeuk *et al*, 1992), it is worth noting that mutations of the HFE gene are not found in patients with this disease (McNamara *et al*, 1998).

1.7 The Aim of this Dissertation

Hereditary haemochromatosis is an enigmatic genetic disease. A number of studies have shown that the penetrance of the common C282Y mutation of the HFE gene is extremely variable, to the extent that some individuals may be homozygous for the mutation and yet never develop iron overload (Crawford *et al*, 1998; Olynyk *et al*, 1999; Adams and Chakrabarti, 1998). While some of the many clinical manifestations of hereditary haemochromatosis are obviously due to iron overload, others are not. For example, severe iron loading of the pancreas is a common manifestation of the disease and fibrosis, caused by the excess iron, is the usual explanation for the diabetes that develops as a result. However, despite the term "bronze diabetes" used to describe hereditary haemochromatosis in the early days, the occurrence of diabetes in symptomatic patients varies between 30 and 60% (Beutler *et al*, 2001) and many patients with severe iron overload are not diabetic. Since hereditary haemochromatosis presents in so many differing ways and with commonplace conditions such as diabetes and arthritis, the diagnosis is frequently missed (Espinosa-Morales and Escalante, 1998; Eyres *et al*, 1992; Goldschmidt *et al*, 1987). It is, therefore, not surprising that screening Caucasian patients attending, for example, a diabetic clinic will reveal a number with previously undiagnosed haemochromatosis (Conte *et al*, 1998).

The Metabolic Bone Diseases Clinic at the Johannesburg Hospital deals with patients presenting with a primary diagnosis of osteoporosis, the majority of whom also have osteoarthritis. Both these diseases can be manifestations of hereditary haemochromatosis and a study was therefore undertaken at this clinic to test the following two hypotheses:

i) Measuring iron status and screening for the mutations of the HFE gene in osteoporotic patients of western or northern European origin attending the clinic would reveal a number of previously undiagnosed haemochromatotics.

ii) Since the relationship between iron overload in haemochromatosis and osteoporosis and arthritis is not clear, some patients might have the haemochromatosis genotype without being iron overloaded.

Two additional investigations were included:

i) The association between an intron 2 polymorphism in the HFE gene (IVS2+4T→C), the H63D mutation and osteoporosis, and

ii) DNA analysis of exon 2 of the HFE gene in 3 additional patients who had phenotypic features of hereditary haemochromatosis, including arthritis, but who lacked the classical genotypes associated with the disease.

The methods used to investigate these hypotheses included:

i) Measurements of iron status. Transferrin saturation greater than 50% was taken as a marker of iron overload as this is the most consistent feature of hereditary haemochromatosis and is central to the pathogenesis of iron overload (Sections 1.4.1, 2.1 and 2.2). The measurement of non-transferrin-bound iron was used to confirm the presence of unbound or "free" iron in these patients (Sections 1.3.3 and 2.3).

ii) Clinical information derived from patient records, including DEXA scans (Section 1.5), clinical and demographic data.

iii) DNA analysis. The previously described mutations of the HFE gene associated with hereditary haemochromatosis (Section 1.4.1.1) were identified using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) (Sections 2.4.3 and 2.4.4). Exon 2 of the HFE gene, the region coding for the α_1 domain of the protein (Section 1.4.1.1), was sequenced to look for additional mutations or polymorphisms in selected patients with high transferrin saturation or other features suggestive of hereditary haemochromatosis (Sections 2.4.5 and 2.4.6). In addition, DNA analysis was used to investigate a polymorphism (IVS2+4T→C) in intron 2 of the HFE gene which is associated with the H63D mutation (Section 1.4.1.1). Statistical analysis was used to investigate the relationship between this polymorphism and osteoporosis.

CHAPTER 2

2. METHODS

2.1 The Measurement of Iron Status

The iron status of individuals has traditionally been assessed by a combination of measurements that reflect the amount of iron in different body compartments (Bothwell *et al*, 1979). The haemoglobin concentration represents the iron in the red cell compartment, the serum iron and transferrin saturation measure the iron in circulation and the serum ferritin concentration is directly related to the amount of iron in the storage compartment. Other measurements include the free erythrocyte protoporphyrin, which is an indirect measurement of the availability of iron to the bone marrow, the serum transferrin receptor, which is inversely related to the concentration of intracellular iron and serum transferrin, which is inversely related to iron stores. In iron overload, there is abundant iron for haemoglobin synthesis and the concentration of haemoglobin and free erythrocyte protoporphyrin is normal. However, the excess iron present in both storage sites and the circulation is reflected by high concentrations of serum ferritin and serum iron, resulting in saturation of transferrin. Once transferrin becomes approximately two thirds saturated with iron, non-transferrin bound iron starts to appear in the serum.

2.2 Serum Ferritin, Serum Iron, Iron Binding Capacity and Transferrin Saturation

An enzyme-linked immunosorbent assay (ELISA) was used for measuring serum ferritin (Conradie and Mbhele, 1980). Rabbit anti-ferritin IgG was adsorbed onto polyvinyl chloride (PVC) microtitre plates. Ferritin from standards, controls or samples binds to the IgG and is then detected by a conjugate consisting of rabbit anti-ferritin IgG and horseradish peroxidase, which, in turn, reacts with a chromagen to form a coloured complex. Standards in the range 0 - 64μg/l were run in triplicate with every assay, which also included low, medium and high controls of known value. Serum samples were routinely diluted 1:10 in buffer containing 2% neutral (non-immunised) rabbit serum and run in triplicate. The rabbit serum is necessary in the diluent because of the occurrence of heterophile anti-bovine IgG antibodies in most human sera. Samples with results that fell outside the range of the assay were further diluted 1:20 and 1:100 and repeated.

Serum iron and iron binding capacity measurements were performed according to standard methods (International Committee for Standardisation in Haematology, 1978a and b).

The transferrin saturation was calculated by dividing the serum iron by the total iron binding capacity and multiplying by 100.

2.3 The Measurement of Non-Transferrin-Bound Iron

A high-pressure liquid chromatography (HPLC) based method for the measurement of NTBI was used in this study because of its extreme sensitivity and selectivity. The method is suitable for small quantities of serum and, in addition, the use of a flow cell allows measurements of only the desired substance without interference from any other substances that may be present. This technique has been well validated in our laboratory (McNamara *et al*, 1999).

2.3.1 The Principles of High Pressure Liquid Chromatography

Chromatography basically involves separations due to differences in the equilibrium distribution of sample components between two different phases. One of these phases is a moving or mobile phase (a flowing stream of solvent), and the other is a stationary phase (the particles inside a separation column). The sample components migrate through the chromatographic system only when they are in the mobile phase. The velocity of migration of a component is a function of equilibrium distribution. The components having distributions favouring the stationary phase migrate more slowly than those having distributions favouring the mobile phase do. Separation then results from different velocities of migration as a consequence of differences in equilibrium distributions.

2.3.2 Method for the Measurement of Non-Transferrin-Bound Iron

2.3.2.1 Iron contamination

As mentioned before, iron is the fourth most plentiful element in the earth's crust. Due to the ubiquitous nature of the metal, contamination of the assay with iron from outside sources can be a problem. The following steps were taken in an attempt to overcome this.

1. Sample collection: Blood was collected directly into an iron-free tube, the serum separated into plastic cryo-tubes and stored at –20°C until required.
2. Glassware: Plastic-ware was used whenever possible. When the use of glass was unavoidable, it was made iron-free by soaking in a solution of 10% potassium dichromate and 25% sulphuric acid for 48 hours, then rinsing thoroughly in de-ionised water and drying in a hot air oven.
3. Water: Even though de-ionised water was used throughout the assay, it was nonetheless treated with Chelex-100 ion exchange resin (Biorad, SA Scientific, Johannesburg, SA) prior to use. This resin contains paired iminodiacetate ions coupled to a styrene divinylbenzene support. It has high selectivity and is capable of removing all metal ions from liquids. Approximately 50gm of Chelex was used per litre of water. The water-resin mixture was stirred on a magnetic stirrer for 2 hours, the resin removed by filtration and the water stored at room temperature until required.
4. Commercial reagents used were all either Analytical or HPLC grade.
5. HPLC system: To have a truly iron-free HPLC system, all parts made of stainless steel such as the pumps, injection system, tubing and columns should be replaced with inert materials such as glass or Teflon. This is, however, an expensive and specialised procedure. In our assay we overcame this problem in three ways.

Firstly, a polyethylene column was used rather than steel. Secondly, a high affinity chelator in the mobile phase served to remove any residual iron contamination in the buffer and column and, thirdly, the entire system was passivated with nitric acid prior to use. To do this, the column and detector were disconnected and the tubing connected with a union. A minimum of 200mls iron-free water was flushed through the system, followed by 100mls 6N nitric acid and a further 200mls of water. The injector was switched several times during the procedure.

2.3.2.2 Principle of the assay:

The method of analysis used in this study was adapted from that of Al-Refaie and colleagues (1992). In the first step of the assay, an iron chelator with sufficient binding affinity to collect the low molecular weight iron and iron non-specifically bound to proteins in serum without competing for iron bound to transferrin and ferritin, is required. Nitrilo-triacetic acid (NTA) is a ligand that fulfils these requirements. At concentrations of up to 100mM, NTA has been shown not to compete for transferrin-bound iron, while at a concentration of 80mM it is capable of removing 98% of iron non-specifically bound to albumin (Singh *et al*, 1990). Although NTA is capable of removing iron from ferritin, the process is extremely slow and requires many hundreds of hours and high concentrations (>1M) of the ligand (Pape *et al*, 1968). Excess NTA is therefore added to the serum samples where it binds all low molecular weight and non-specifically bound iron, thus converting all the iron of interest to an iron-NTA complex. This mixture is then centrifuged through a low molecular weight cut off filter to remove everything but the NTA-iron complex. The filtrate is then injected directly into the HPLC. The mobile

phase of the HPLC contains a high affinity iron chelator, L_1, which removes and converts the NTA-bound iron to a coloured complex with a peak absorbance at 460nm. This is termed "on-column derivitisation" and complete complexation takes place immediately if the chelator is present at, or above, 2mM in the mobile phase (Singh *et al*, 1990).

2.3.2.3 Assay procedure:

Standards:

A 1mg/ml stock iron standard was prepared by adding 1gm polished electrolytic iron wire to 20ml 7M re-distilled hydrochloric acid and placing in a boiling bath. Once the wire had dissolved, the solution was made up to 1 litre with de-ionised iron-free water. A working standard of 100mM iron for this assay was made freshly for each assay by taking 55.8µl of the stock standard and making it up to 10ml with 80mM NTA. A standard curve was generated by diluting the working standard to a range of 1µM - 10µM in 80mM NTA and injecting 20µl of each onto the column. Each measurement was done in duplicate and both points plotted on the graph (Figure 6).

Samples:

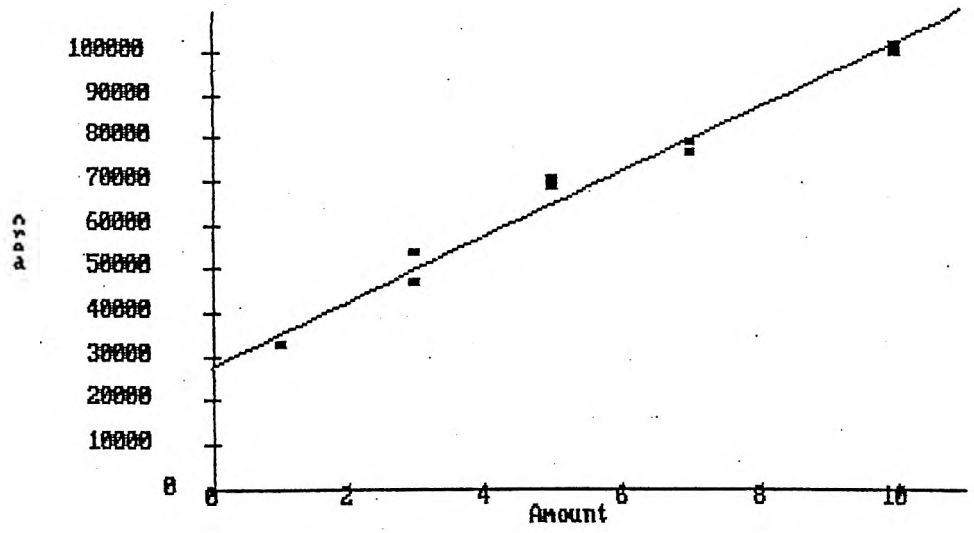
25µl 800mM NTA (Sigma Chemical Corporation, St Louis, USA) was added to 225µl serum, mixed thoroughly and allowed to stand for 20 minutes. 150µl of this mixture was then placed in a 10 000 molecular weight cut off filter with a 0.2cm^2 membrane area (Millipore Corporation, Bedford, USA) and centrifuged at 14 000rpm for 15 minutes. 20µl of the filtrate was then injected directly onto the HPLC. To eliminate periodic contamination from the filters, they were pre-washed with 100µl 10mM NTA followed by 100µl iron-free water and then air dried before use.

---------- fe-1 ----------

Expected retention time (min): 2.315

Absolute search window (min): 0.000
Relative search window (%) : 10.00

Level	Amount	Area	Area/Amount	Used?
1	1.000000	32070.705078	32070.705078	Yes
2	1.000000	32018.197266	32018.197266	Yes
3	3.000000	52937.500000	17645.833984	Yes
4	3.000000	46705.296875	15568.432617	Yes
5	5.000000	67833.992188	13566.798828	Yes
6	5.000000	69722.140625	13944.427734	Yes
7	7.000000	78102.796875	11157.541992	Yes
8	7.000000	75769.242188	10824.177734	Yes
9	10.000000	99028.007813	9902.800781	Yes
10	10.000000	100927.00781	10092.701172	Yes



Calibration equation: 7385.809082x + 27105.281250
Curve type: Linear
Weighting: Equal
Correlation coefficient: 0.958137

Figure 6: An example of a non-transferrin-bound iron standard curve.

Mobile Phase:

5mM 4-Morpholinpropansulfonic acid (MOPS) (Roche Diagnostics SA (Pty) Ltd, Johannesburg, SA) was mixed with 5mM 1,2 dimethyl-3-hydroxy-pyrid-4-one (L_1) and 5% acetonitrile. The pH was adjusted to 7.0 with concentrated NaOH and the solution vacuum filtered through a 22μM filter and de-gassed for 10-20 minutes. The L_1 was purchased from Professor RC Hider, Kings College, London.

Column:

The column was a 8mm x 10cm Novapak C-18 silica column (Microsep (Pty) Ltd, Johannesburg, SA), with a volume of 5ml and a particle size of 4μm.

The flow rate was 1ml/minute.

The L_1-iron peak eluted from the column at between 2.4 and 2.6 minutes as shown in Figures 7a and 7b. Figure 7c shows the sample peak (A) overlaid on a 5μM iron standard peak (B), demonstrating that the elution times in both cases were the same.

The HPLC system used in our laboratory is a Waters Quartenary system with a variable wavelength detector and chromatography data processor utilising APEX software (Microsep (Pty) Ltd, Johannesburg, SA).

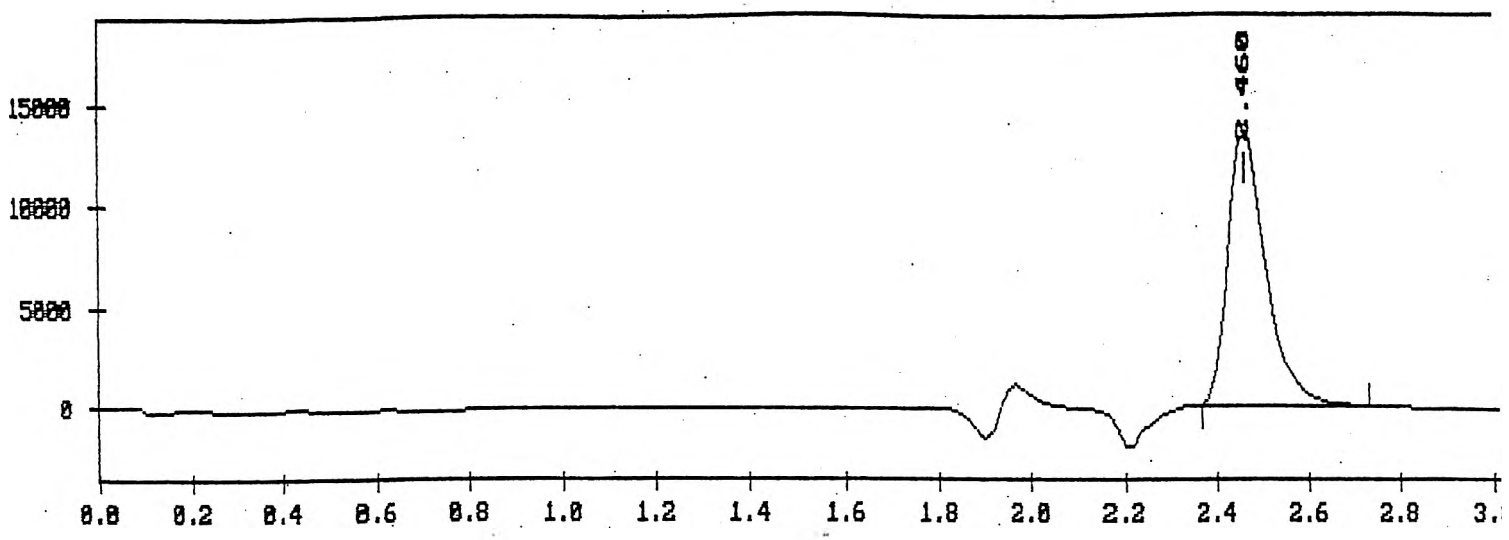


Figure 7a: Chromatographic data of a 7μM iron standard

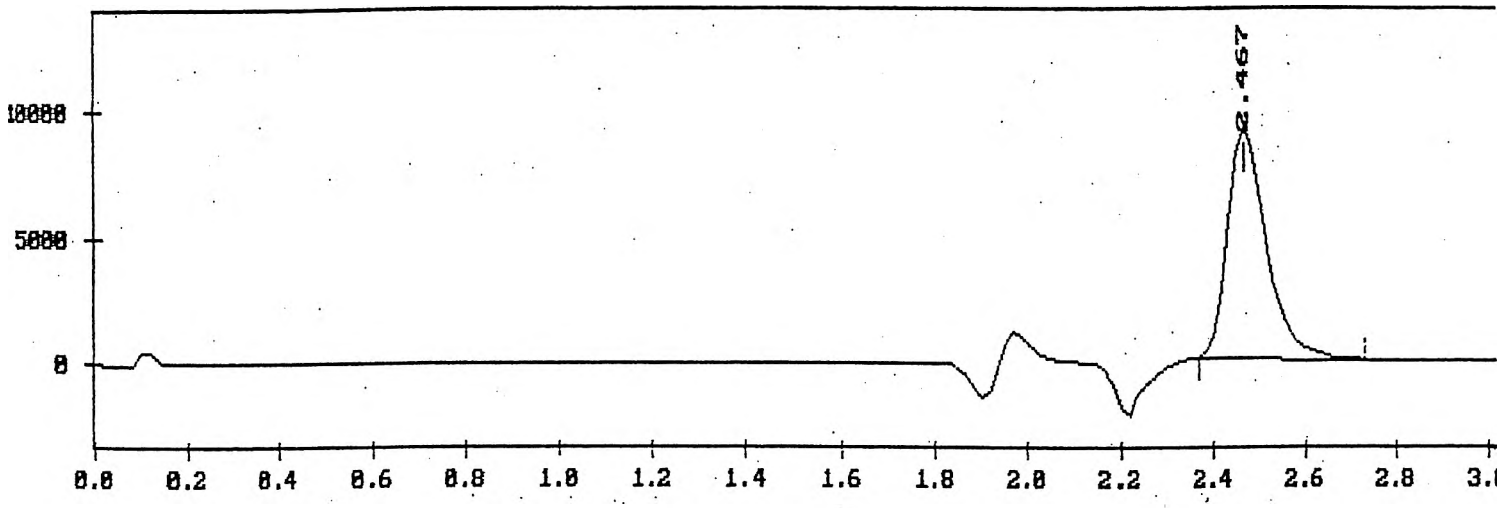


Figure 7b: Chromatographic data of a sample containing non-transferrin-bound iron

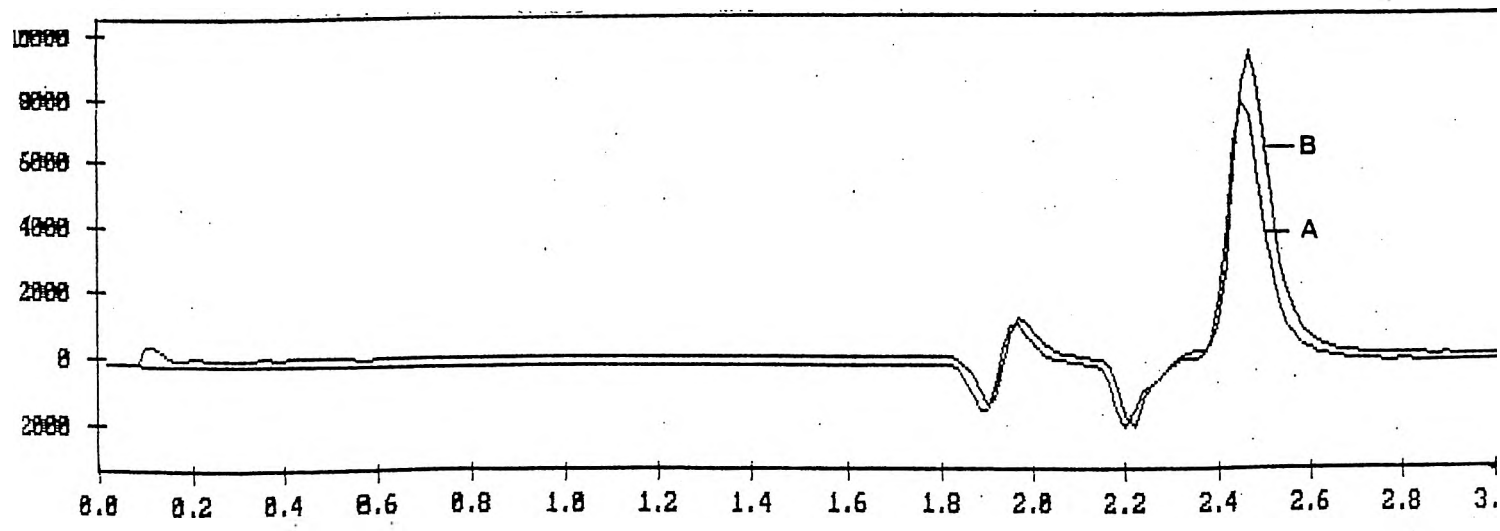


Figure 7c: Sample peak (A) overlaid on a 5μM iron standard peak (B)

2.4 DNA Analysis

2.4.1 DNA Extraction

DNA was extracted from whole blood using an adaptation of the method described by Miller and co-workers (1988).

2.4.1.1 Reagents

Unless otherwise stated, all chemicals were obtained from Merck Chemicals (Pty) Ltd, Johannesburg, South Africa.

- Lysing Buffer: 10mls 1M Tris (hydroxymethylaminomethane) pH 8.0, 5mls 1M magnesium chloride ($MgCl_2$) and 10mls Triton X-100 were made up to 1 litre with distilled water, autoclaved and stored at 4°C until required. Immediately prior to use, 109.5gm sucrose was added and dissolved by stirring.
- T20E5: A solution of 20mM Tris and 5mM ethylenediamenetetraacetic acid was made up in distilled water, the pH adjusted to 8.0 and stored at room temperature.
- Saturated sodium chloride (NaCl): 40gm NaCl was added slowly to 100mls distilled water and stirred. The solution was stored at room temperature.
- Proteinase K mixture: The proteinase K was obtained from Roche Diagnostics South Africa (Pty) Ltd, Randburg, South Africa. The mixture was made freshly for every assay according to the following table:

Final Concentration	Stock Solution	Volume Used
1% sodium dodecyl sulphate	10%	800µl
2mM EDTA	0.5M	32µl
2mg/ml Proteinase K	10mg/ml	1600µl
Distilled water		5600µl

2.4.1.2 Method:

- Blood samples were collected in EDTA tubes.
- 10mls of whole blood was thoroughly mixed with approximately 45mls ice-cold lysing solution and centrifuged at 4°C for 10 minutes at ±2300rpm.
- The supernatant was decanted and discarded. The pellet was re-suspended in ±20mls lysing solution and the tube placed on ice for 5 minutes before being centrifuged for a further 10 minutes.
- The supernatant was decanted and discarded and the pellet re-suspended in 3mls T20E5. 200µl 10% sodium dodecyl sulphate (SDS) was then added followed by 500µl of proteinase K mix. The solution was thoroughly mixed and the tube incubated at 42° – 50°C overnight.
- The following day, 1ml of saturated NaCl was added and the tube placed on ice for 5 minutes before being centrifuged at 4°C for 30 minutes at ±2300rpm. The resulting pellet consists of proteins precipitated by the salt and the DNA should be present in the supernatant.
- The supernatant was transferred to a clean tube and 20mls room temperature ethanol added. At this stage the DNA was usually visible as a white aggregate. If none could be seen, the tube was placed at –70°C for 30 minutes. The DNA was then spooled out, placed in a microfuge tube and washed gently in ice cold 70% ethanol.

- The tube was centrifuged briefly at 2000rpm to pellet the DNA. As much ethanol as possible was removed and the open tube left at room temperature until the last visible traces of ethanol had evaporated. It is very important not to allow the pellet of DNA to dry completely; otherwise it becomes very difficult to dissolve. 500 - 1000µl sterile water was then added and the tube placed on a rocking platform until the DNA had completely dissolved. The solution was then stored at -20°C until required.

2.4.2 Quantification of DNA

2.4.2.1 Spectrophotometry:

The absorbance of the DNA solution was measured at 260nm and 280nm. The ratio of A_{260} to A_{280} should be greater than 1.75. A lower ratio is an indication that significant amounts of protein remain in the preparation. DNA can be quantified at 260nm because a solution with an optical density of 1.0 (using a 10mm path length cell) contains approximately 50µg/l of DNA.

2.4.2.2 Agarose gel electrophoresis:

Agarose gels are cast by melting the agarose in a buffer until a clear solution is achieved. The melted solution is then poured into a mould and allowed to harden. When hardened, the agarose forms a matrix, the density of which is 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 towards the anode. The DNA is then visualised by staining the gel with the fluorescent dye, ethidium bromide (Sharp *et al*, 1973) and

viewing under ultra-violet light. A permanent record can be made by photographing the gel using a Polaroid instant camera.

2.4.2.2.1 Method:

Unless otherwise stated, all chemicals were obtained from Merck Chemicals (Pty) Ltd, Johannesburg, South Africa.

A stock solution of Tris-Borate (TBE) buffer was prepared by dissolving 108gm Tris, 55gm Boric acid and 40ml 0.5M EDTA (pH8.0) in 1 litre of water. This buffer was diluted 1:10 for use and a 0.8% gel prepared using molecular grade agarose (Whitehead Scientific, Cape Town, South Africa). A 10mg/ml ethidium bromide (Sigma-Aldrich, Atlasville, South Africa) solution was made in water and added to the gel mixture in the proportion 3µl/100ml and thoroughly mixed just prior to pouring. A 10mg/ml solution of Herring Sperm DNA (Roche Diagnostics South Africa (Pty) Ltd, Johannesburg, South Africa) was prepared in water. The DNA was sheared by sonication at maximum amplitude for 9 cycles of 2 minutes with 30 second intervals, the sample was placed in a boiling bath for 10 minutes and then on ice for 5 minutes. A standard gel was prepared by mixing 1 – 10ug DNA with loading buffer (5gm sucrose, 50mM EDTA 0.01gm bromophenol blue (ICN Biomedicals Inc, Ohio, USA) and 1gm Ficoll Type 400 (Sigma-Aldrich, Atlasville, South Africa)) and running at 90V for 30 minutes. 5µl of each sample was mixed with 1 drop of loading buffer and run the same way. The gels were then viewed on an ultra violet transilluminator (Spectroline, New York, USA) and photographed using a Polaroid CU5 Land Camera with a 5-inch lens. The samples were

then compared with the standard gel and an approximate estimation of the concentration of DNA in the samples made.

2.4.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalysed by a DNA polymerase. These oligonucleotides typically have different sequences and are complementary to sequences that lie on opposite strands of the template DNA and also flank the segment of DNA that is to be amplified. The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is then repeated many times. The products of one round of amplification serve as templates for the next so each successive cycle essentially doubles the amount of desired DNA product (Figure 8).

2.4.3.1 Reagents:

- Primers: The primers used for PCR of the C282Y and H63D mutations were those originally published by Feder and co-workers (1996). They were ordered from MWG-Biotech, Munich, Germany. The primers for PCR of the S65C mutation were the same as those for the H63D mutation. The primer sequences were as follows:

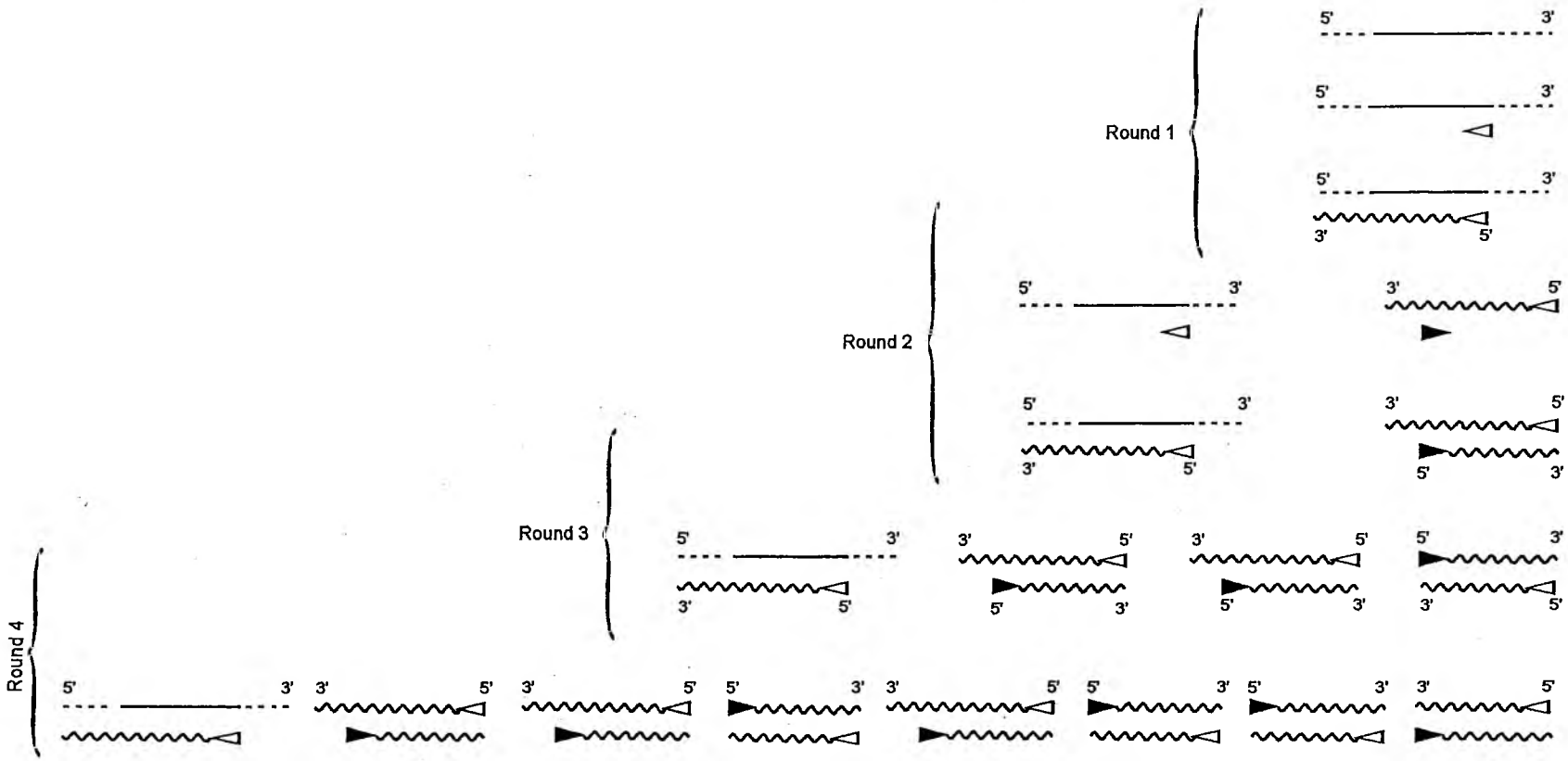


Figure 8: The steps involved in the first few rounds of a PCR reaction. The original template (top line) is single-stranded DNA and the forward and reverse primers are shown as ► and ◄ respectively. (Sambrook *et al*, 1989).

C282YF - 5'-TGG CAA GGG TAA ACA GAT CC-3'

C282YR - 5'-CAC AGG CAC TCC TCT CAA CC-3'

H63DF - 5'-ACA TGG TTA AGG CCT GTT GC-3'

H63DR - 5'-GCC ACA TCT GGC TTG AAA TT-3'

Previously published primers for PCR of the whole of exon 2 of the HFE gene (Barton *et al*, 1999) did not work in our hands. A new reverse primer was therefore designed to include a section of intron 2 and used along with the existing H63D forward primer, which encompasses a section of intron 1. The sequence of the newly designed reverse primer was as follows:

EXON2R - 5'-GAT GAA AAG CTC TGA CAA CC-3'

This primer was purchased from TIB MolBiol, Berlin, Germany.

PCR of the region spanning the C282Y mutation produced a 390 base pair (bp) product, that of the H63D and S65C produced a 208bp product and that of the whole of exon 2 produced a 345bp fragment. The primers were all diluted to a concentration of 25pmol/µl.

- Taq Polymerase: BioTaq (Whitehead Scientific, Cape Town, South Africa) is a thermostable DNA polymerase purified from *Thermus aquaticus* (Chien *et al*, 1976). It was used at a concentration of 1 unit per reaction.
- Magnesium Chloride ($MgCl_2$): This was supplied with the BioTaq as a 50mM stock solution. The concentration of $MgCl_2$ required for the assay was optimised at 1.5mM by performing a titration using concentrations from 0.5mM to 3.5mM.

- dNTPs: A premixed solution of the sodium salts of dATP, dCTP, dGTP and dTTP each at a concentration of 10mM in water was purchased from Roche Diagnostics South Africa (Pty) Ltd.
- Reaction buffer: A 10X stock solution of 670mM Tris, 160mM ammonium sulphate ($(NH_4)_2SO_4$) and 0.1% Tween-20 was supplied with the BioTaq.

2.4.3.2 Assay procedure:

The polymerase chain reaction is capable of amplifying as little as a single molecule of DNA. Precautions therefore have to be taken to guard against accidental contamination of the reaction mixture. The reactions were carried out in a reserved area that was cleaned regularly with 0.1M hydrochloric acid (HCl). All pipette tips and tubes were autoclaved before use. Automatic pipettes were wiped with 0.1M HCl and disposable gloves were worn. All reagents were prepared and stored in small aliquots at –20°C until required. Microfuge tubes containing reagents to be used were centrifuged briefly before being opened. Controls using DNA from patients known to be homozygous for the C282Y mutation and heterozygous for the H63D mutation were included in every run. A negative control containing all the components of the reaction except template DNA was also included.

- For a total reaction volume of 25µl a mixture was made up as follows:

10X buffer	-	2.5µl
50mM $MgCl_2$	-	1.5µl
Forward primer	-	1µl
Reverse primer	-	1µl
dNTP mix	-	0.5µl

Taq polymerase - 0.2µl

Sterile water - 17.3µl

These volumes were multiplied by the number of samples and then 24µl added to each sample tube.

- 1µl of target DNA was then added to each tube and the mixture overlaid with 1 drop of light mineral oil (Sigma-Aldrich, Atlasville, South Africa).
- The PCR conditions were the same for the C282Y, H63D and S65C mutations and were as follows:

  35 cycles of 1 minute @ 94°C, 1 minute @ 59°C and 1 minute @ 72°C followed by 1 cycle of 10 minutes @ 72°C.
- PCR of exon 2 was performed using Promega PCR Mastermix (Whitehead Scientific, Cape Town, SA). This consisted of 50 units/ml of Taq DNA Polymerase supplied in a proprietary reaction buffer (pH8.5), 400µM each of dGTP, dATP, dTTP and dCTP plus 3mM $MgCl_2$. 12.5µl of the Mastermix was added to 1µl template DNA plus 1µl each of forward and reverse primer and nuclease free water was added to a total volume of 25µl. The conditions for PCR of exon 2 were as follows: 1 cycle of 95°C for 2 minutes, 60°C for 1 minute and 72°C for 1 minute, followed by 30 cycles of 1 minute each at 94°C, 60°C and 72°C and 1 cycle of 5 minutes at 72°C.
- 5µl of PCR product was analysed on a 3% agarose gel (Figures 9a, 10a and 11a).

PCR of some of the samples produced very small amounts of product. In these cases the PCR reaction was repeated exactly as before, depending on the mutation being looked for, using 1µl of the product in place of template DNA.

2.4.4 Restriction Enzyme Digestion:

Restriction enzymes are a class of enzymes called restriction endonucleases. These enzymes, isolated from bacteria, received their name because they restrict viral infection by degrading the invading nucleic acid. Three classes of restriction endonucleases have been discovered, Type II being the ones most widely used. Type II enzymes recognise a specific sequence and produce a double stranded break precisely within the sequence by binding specifically to, and cleaving, double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition sequence.

The C282Y mutation in the HFE gene creates a GTAC sequence that is recognised by the restriction enzyme RsaI (Feder *et al*, 1996). In homozygotes for this mutation, restriction enzyme digestion produces two fragments of 279 and 111 base pairs respectively. A heterozygote produces three fragments of 390, 279 and 111 base pairs respectively. The H63D mutation causes a GATC site to be changed to GATG. The GATC site would normally be recognised by the restriction enzyme NdeII (MboI) (Feder *et al*, 1996), a homozygote therefore produces only one fragment of 208 base pairs and a heterozygote three fragments of 208, 141 and 67 base pairs respectively. The S65C mutation changes the sequence GAGTC to GTGTC removing a HinfI recognition site (Douabin *et al*, 1999). Homozygotes are, therefore, the same as for the H63D mutation and a heterozygote produces three fragments of 208, 146 and 62 base pairs respectively. The polymorphism in intron 2 creates an MaeII site, A↓CGT (Douabin *et al*, 1999) and also an RsaI site, GT↓AC (Beutler and West, 1997). Using our primers, which encompassed an existing MaeII site, digestion of the unmutated gene produced two fragments of 235

and 110bp. A homozygote produced 3 fragments of 188, 110 and 47bp respectively and a heterozygote the same but including a 235bp fragment.

2.4.4.1 Method:

- All restriction endonucleases were purchased from Roche Diagnostics South Africa (Pty) Ltd.
- Digestions for the C282Y, H63D and S65C mutations were performed according to the manufacturer's instructions using 20µl of PCR product. Reactions were incubated at 37°C for 90 minutes. 1 drop of ficoll dye was added to each tube after digestion and the contents loaded on to a 3% agarose gel containing ethidium bromide. The gel was run in TBE buffer at 90 – 100V for ±90 minutes and then visualised as described previously (Section 2.4.2.2.1) (Figures 9b, 10b and 11b).
- Digestion with MaeII was carried out for 1 hour at 50°C. In an attempt to visualise the 47bp fragment, 5µl of the digested product was run on a 5% acrylamide gel at ±40V for 90 – 120 minutes. The gel was stained by gentle agitation in 200ml water plus 20µl ethidium bromide for 60 – 90 minutes and photographed as described previously. However, the results were ambiguous and difficult to interpret (Figure 12). Digestions were then repeated using RsaI and the digested product run on a 2% agarose gel containing ethidium bromide. Although the 47bp product was very faint on this gel, results were interpreted using known homozygote and heterozygote controls that had been identified by sequencing (Appendix 1) (Figure 13).

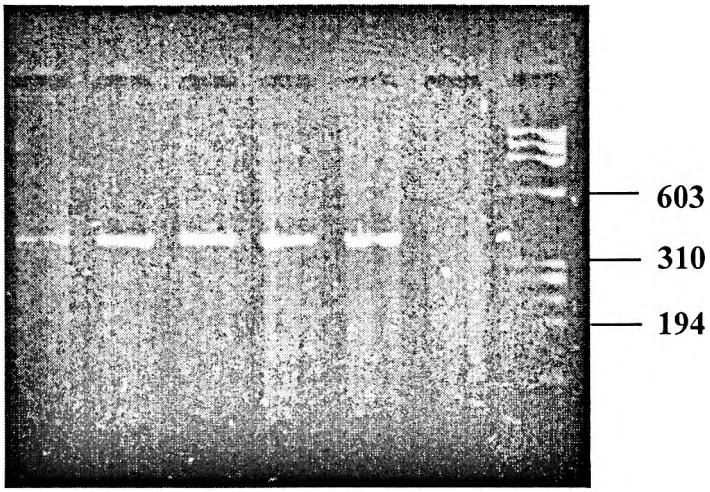


Figure 9a: PCR product of a 390bp fragment containing C282Y mutation
Lane 7 contains a molecular weight marker

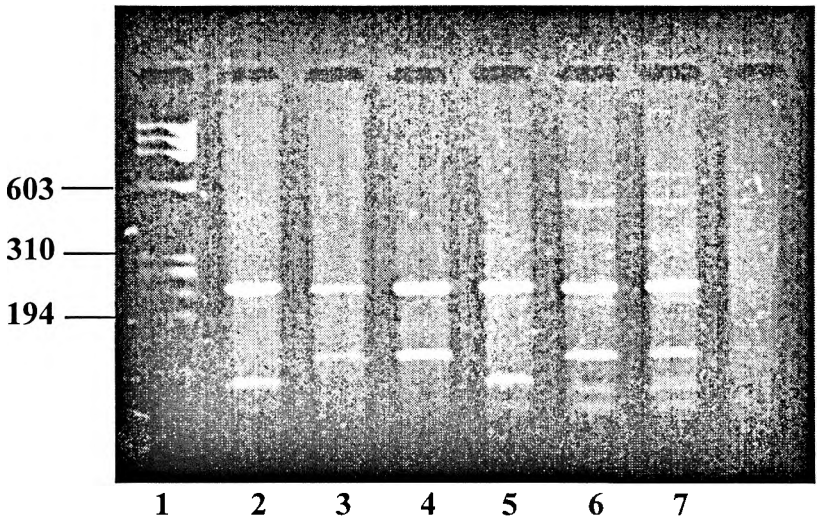


Figure 9b: Restriction digest of fragment containing C282Y mutation
Lane 1 contains a molecular weight marker

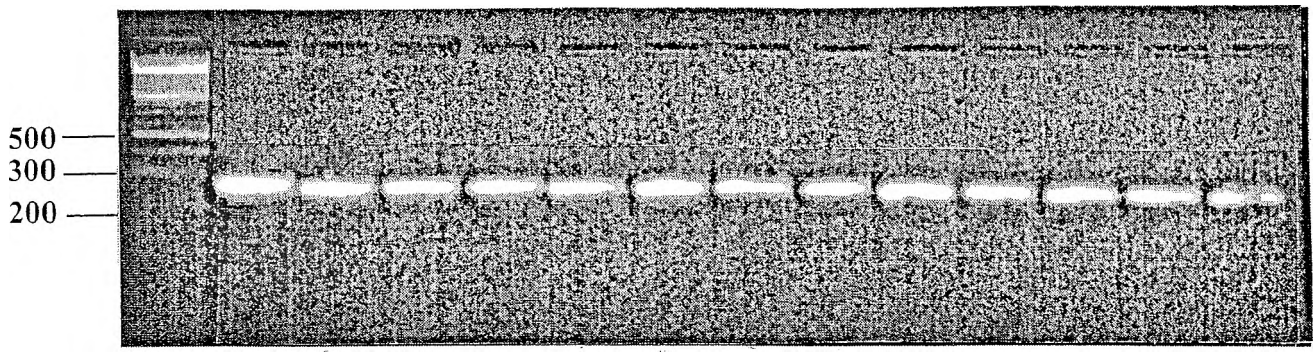


Figure 10a: PCR product of a 208bp fragment containing H63D mutation

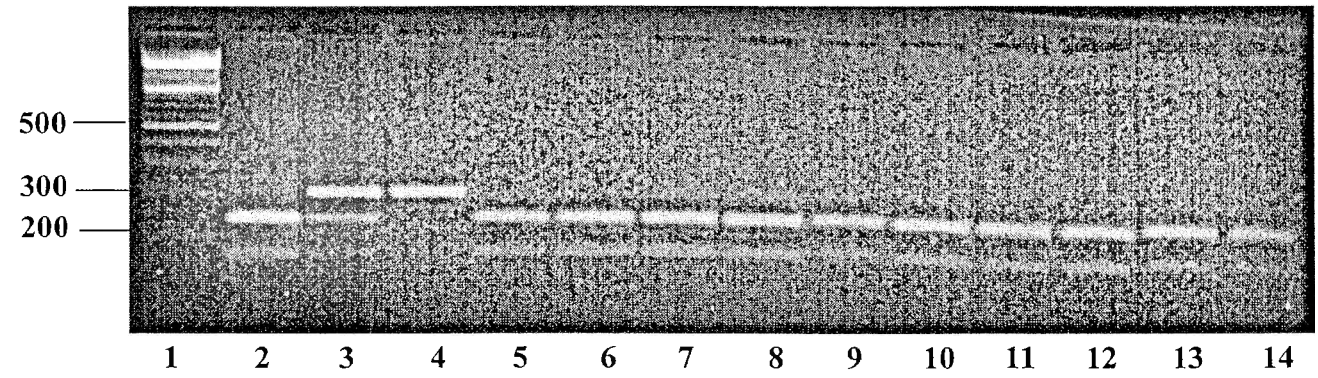


Figure 10b: Restriction digest of fragment containing H63D mutation
Lane 1 contains a molecular weight marker
Lane 3 is a heterozygote and Lane 4 is a homozygote.
Lanes 2 and 5-14 contain the wild type (normal)

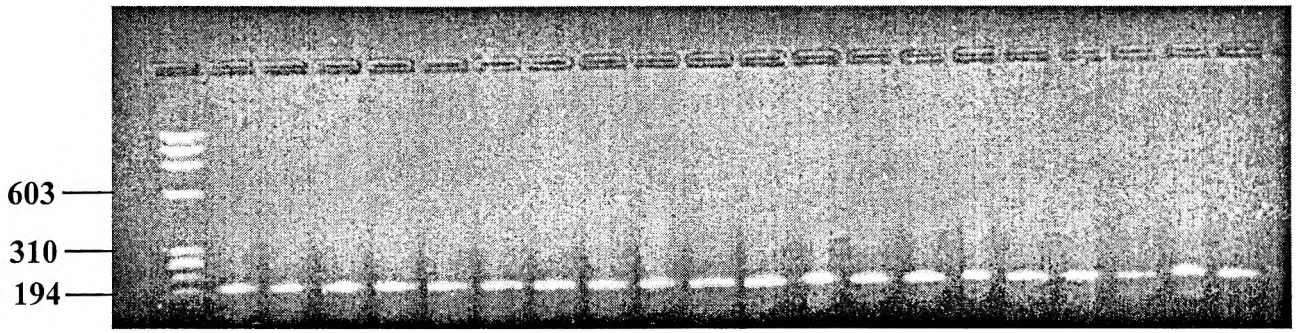


Figure 11a: PCR product of a 208bp fragment containing S65C mutation
Lane 1 contains a molecular weight marker

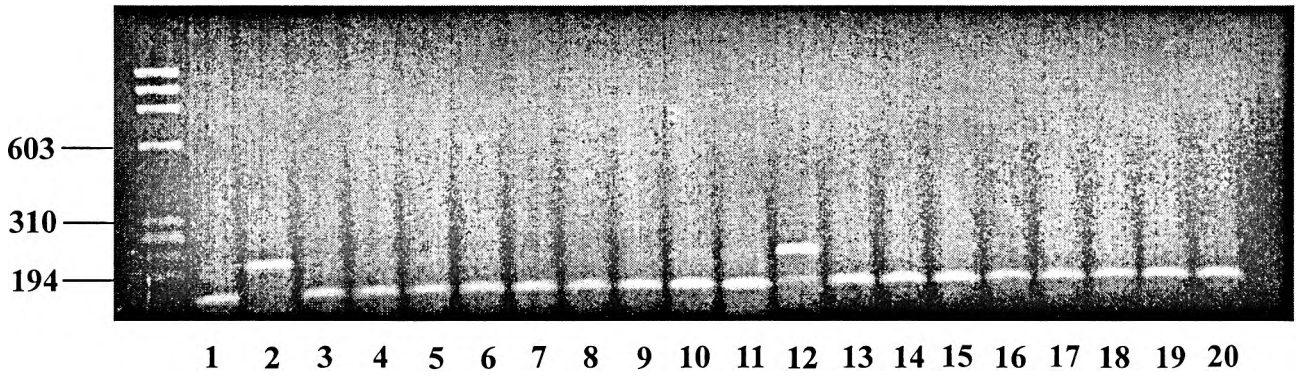


Figure 11b: Restriction digest of fragment containing S65C mutation
Lane 1 contains a molecular weight marker
Lane 2 shows a homozygote and Lane 12 contains a heterozygote.

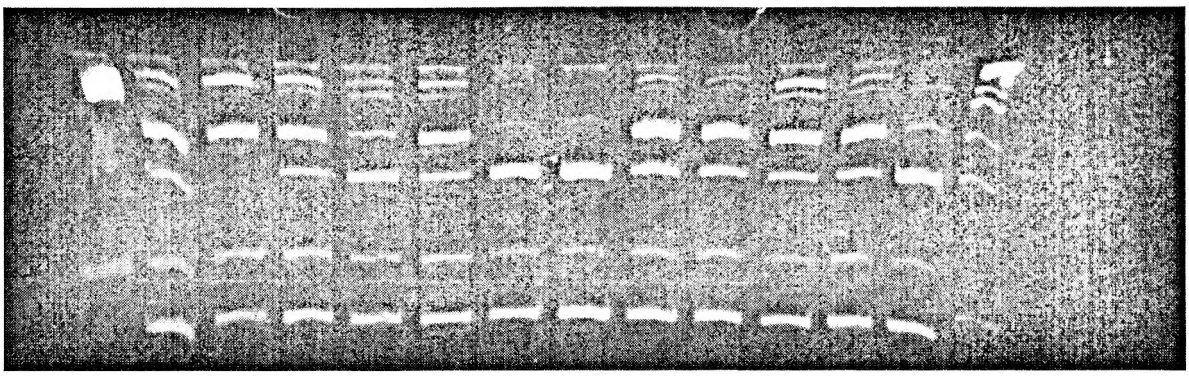


Figure 12: 5% acrylamide gel of MaeII digestion of exon 2

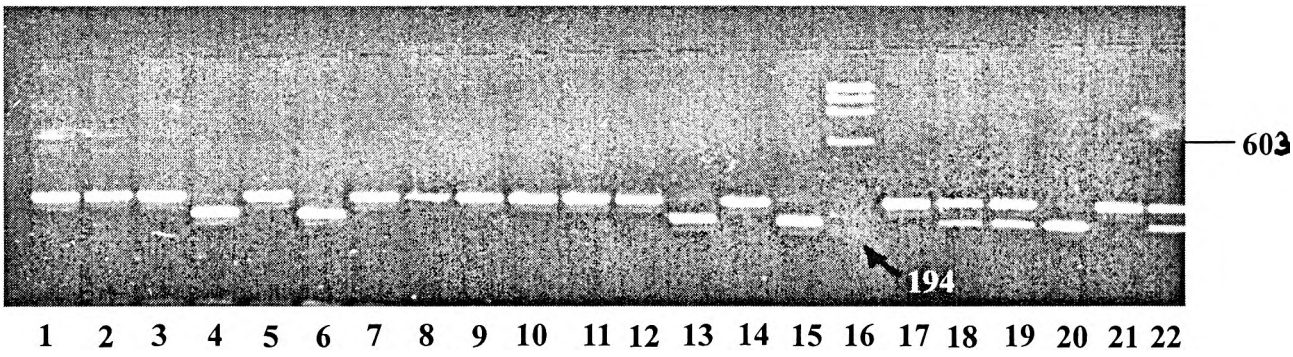


Figure 13: 2% agarose gel of Rsal digestion of exon 2
Lanes 4, 6, 13, 15 and 20 show homozygotes
Lanes 18, 19 and 22 show heterozygotes
The remaining lanes show the wildtype
Lane 16 contains a molecular weight marker

2.4.5 Manual DNA Sequencing

Sequencing was performed using a Sequenase Version 2.0 DNA Sequencing Kit obtained from AEC-Amersham (Pty) Ltd, Sandton, South Africa.

2.4.5.1 Principle of the method:

The sequencing of DNA has undergone rapid improvement since the introduction of chain-termination DNA sequencing (Sanger *et al*, 1977). The chain-termination method involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at only one site where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analogue that will not support continued DNA elongation. The chain-terminating nucleotide analogues are the 2', 3'-dideoxynucleoside 5'-triphosphates (ddNTPs). These lack the 3'-OH group necessary for DNA chain elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalysed polymerisation will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP give complete sequence information. A radioactively labelled nucleotide is also included in the synthesis, so the labelled chains of various lengths can be visualised by autoradiography after separation by high-resolution electrophoresis.

PCR makes use of two primers, deoxynucleoside triphosphates (dNTPs) and DNA polymerase to produce multiple copies of a specific DNA sequence. When complete, most of the dNTPs and primers remain intact and these interfere with normal sequencing methods, which also make use of primers and nucleotides. The PCR product is therefore

treated with two enzymes to remove these unwanted materials prior to being used for sequencing. One removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The other removes the remaining dNTPs from the PCR mixture that would interfere with the labelling step of the sequencing process.

2.4.5.2 Assay procedure

- 2.5µl PCR product was added to 1 unit of Exonuclease I and 2 units of Shrimp Alkaline Phosphatase (SAP) in a microfuge tube. The solution was mixed and incubated at 37°C for 15 minutes. The Exonuclease and SAP were then inactivated by heating at 80°C for a further 15 minutes.
- 1µl of either forward or reverse primer (10pm/ml) was added to the 4.5µl treated product plus 4.5µl water to bring the total volume to 10µl.
- The solution was denatured at 100°C for 2 – 3 minutes and then plunged immediately into ice for 5 minutes.
- While cooling, 2.5µl each of ddG, ddA, ddT and ddC termination mix was placed into separate tubes, which were capped and placed at 37°C. 7-deaza-dGTP, a nucleotide analogue for dGTP, was used in this reaction to eliminate compressed regions in the sequence.
- The following was then added to each tube of treated PCR product:

  2µl 5X Sequenase reaction buffer (200mM Tris HCl, pH 7.5; 100mM $MgCl_2$: 250mM NaCl)

  1µl 0.1M dithiothreitol (DTT)

  2µl labelling mix (7.5µM each of 7-deaza-dGTP, dCTP and dTTP) diluted 1:5

  0.5µl (5µCi) ^{35}S (AEC-Amersham (Pty) Ltd)

2μl Sequenase DNA polymerase

- The tubes were then thoroughly mixed and placed on ice for 30 minutes.
- 1μl of Mn buffer (0.15M sodium iso-citrate, 0.1M manganese chloride ($MnCl_2$)) was then added to each tube and thoroughly mixed.
- 3.5μl of this mixture was then placed into each of the tubes containing the termination mixture and the tubes placed at 37°C for a further 30 minutes.
- The reaction was stopped by adding 4μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) and the samples stored on ice until required.
- All samples were sequenced using both the forward and reverse primers.

2.4.5.3 Gel electrophoresis:

The quality of the gel electrophoresis is often the factor that limits the extent of sequence information that can be determined in a single sequencing experiment. The length of time the gel is run determines the region of sequence that is readable. Under optimal conditions, 200 or more bases can be read starting at the bottom of the gel. The following precautions were therefore observed when preparing sequencing gels.

- All reagents were of electrophoresis grade.
- Solutions of monomers were made freshly for every gel.
- Gels were prepared the day before required so that complete polymerisation could be ensured.
- Before initial use, the glass electrophoresis plates were soaked in 2M sodium hydroxide (NaOH) for 1 hour. Before the gels were poured the plates were cleaned by being soaked overnight in a solution of 1% 7X-PF detergent (ICN

Biomedicals Inc, USA). They were then scrubbed with a soft brush and thoroughly rinsed before being allowed to air dry. Plates were then rubbed thoroughly three times with 100% ethanol and finally with ice-cold iso-propanol. Approximately 2ml of Gel Slick glass plate coating (AT Biochem, USA) was applied in a thin layer on to one of the plates and allowed to dry. The plates were assembled with 0.4mm spacers, clamped together and laid flat for pouring.

2.4.5.3.1 Reagents and method:

- 20X glycerol tolerant buffer (GTB): 216gm Tris, 72gm Taurine (United States Biochemicals, Ohio, USA) and 4gm EDTA were dissolved in 1 litre of water and autoclaved.
- Acrylamide and bis-acrylamide were purchased from BioRad Laboratories, California, USA and the urea from Merck Chemicals (Pty) Ltd, SA. 6% gels were mixed as follows:

Gel Conc (%)	Acrylamide/ Bis-acrylamide	7M Urea	20X GTB	H_2O
6%	5.7gm/0.3gm	42.0gm	5ml	±50ml

- The chemicals were dissolved with gentle heat and the volume adjusted to 100ml. The solution was filtered and de-gassed. Just before pouring, 1ml 10% ammonium persulphate and 25µl N,N,N',N'-tetramethylethylenediamine (TEMED) (BioRad Laboratories, California, USA) were added to the solution and thoroughly mixed.

- The gel solution was allowed to flow between the plates by gravity. When full, sharks tooth combs were inserted, flat side down, and the gel allowed to polymerise overnight.
- The following day, the gel was pre-run in 1X GTB buffer at 55W for ±30 minutes.
- The combs were then removed and re-inserted teeth side down to form the wells for the samples.
- The top of the gel was washed with 1X GTB buffer using a syringe.
- The samples were denatured at 75°C for 5 minutes and then 2.5µl of each was immediately loaded onto the gel.
- The gel was run at 55W for 2 – 3 hours, until the loading dye had run off the bottom.
- The top plate was then removed and the gel soaked in a mixture of 15% methanol and 7.5% acetic acid for 5 minutes to remove the urea.
- The gel was then transferred onto filter paper, covered with plastic film and dried on a vacuum dryer for 1 hour 45 minutes at 80°C.
- The dried gel was autoradiographed at room temperature for 48 – 64 hours using Hyperfilm (AEC-Amersham (Pty) Ltd, SA) (Figures 14, 15, 16 and 17).

2.4.6 Automated DNA Sequencing

Automated sequencing was performed to confirm the presence of the intron 2 polymorphism. Both the sense and anti-sense strands of the 345bp product (exon 2) were sequenced on an Applied Biosystems 377 instrument. The primers were the same as those used for amplification. Results are shown in Appendix 1.

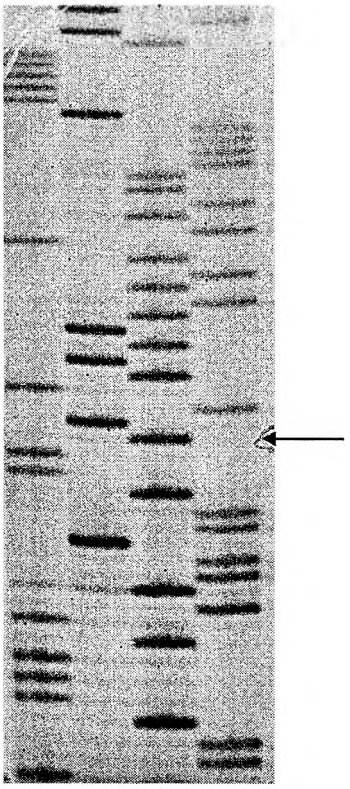


Figure 14a: Sequencing gel showing a homozygous C282Y mutation (C→T) (arrow)

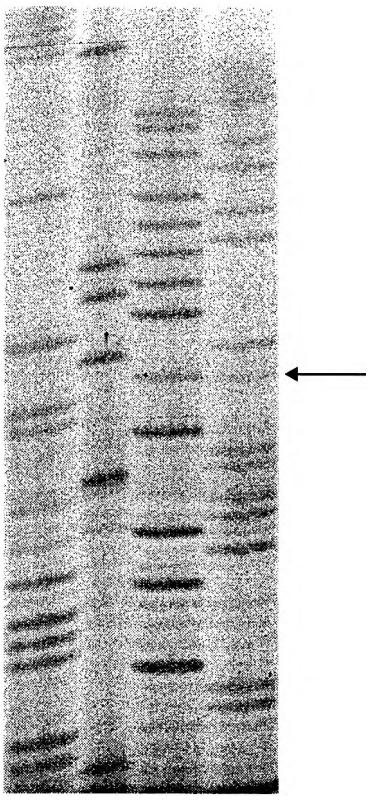


Figure 14b: Sequencing gel showing a heterozygous C282Y mutation (C and T) (arrow)

The reverse primer was used to generate both these sequences.

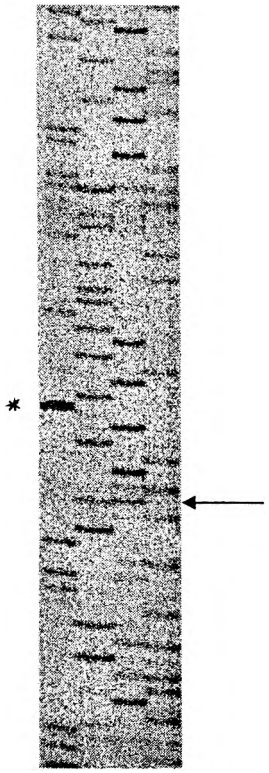


Figure 15: Sequencing gel showing a heterozygous S65C mutation (A and T) (arrow)
This sequence shows a normal H63D site (*)

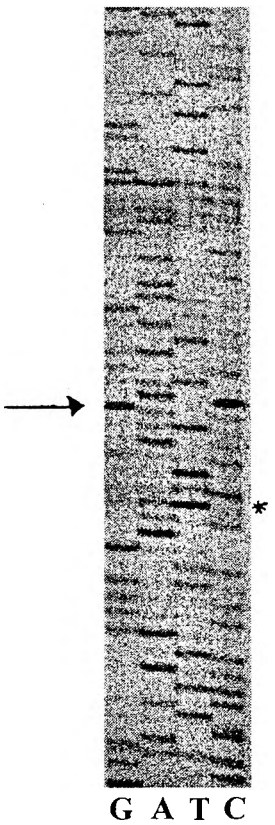


Figure 16: Sequencing gel showing a heterozygous H63D mutation (G and C) (arrow)
This sequence shows a normal S65C site (*)

The reverse primer was used to generate these sequences.

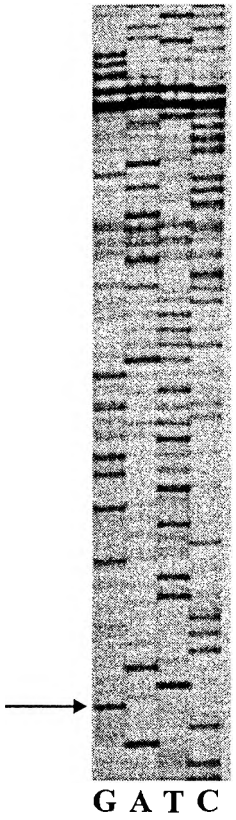


Figure 17a: Sequencing gel showing a homozygous intron 2 polymorphism (a → g) (arrow)

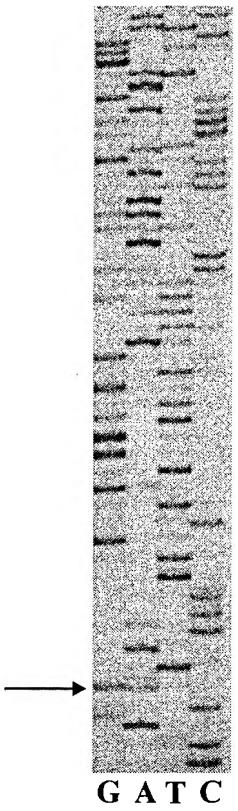


Figure 17b: Sequencing gel showing a heterozygous intron 2 polymorphism (a and g) (arrow)

CHAPTER 3

3. RESULTS

The work described in this dissertation was conducted in the Department of Medicine, University of the Witwatersrand, South Africa. Approval for the studies was obtained from the Committee for Research on Human Subjects (Medical), University of the Witwatersrand (Protocol No: M990715). Informed consent was obtained from all the subjects according to the guidelines laid down by the above committee.

3.1 Study Subjects

Two groups of patients were studied. The first group consisted of 50 unrelated Caucasians attending the Metabolic Bone Diseases Clinic at the Johannesburg Hospital, South Africa. Forty women with ages ranging from 24 to 80 years (mean 63.3 ± 12.5) and 10 men with ages ranging from 25 to 76 years (mean 55.7 ± 15.4) took part in the study. Subjects who were willing to participate in the study were selected on the basis of their western or northern European ancestry. The majority were of Celtic origin (Table 2). DEXA scan ratings at first attendance, if available, for both the hip and lumbar spine were noted (Table 3), an iron profile was performed on each person and a detailed family history taken where possible.

The second study group consisted of 3 unrelated male Caucasians. These three patients had been referred for further evaluation due to abnormal iron studies in the absence of the classical hereditary haemochromatosis genotype.

Number	Patient Identification	Ancestry
1	HHAO 02	Irish
2	HHAO 04	Portuguese/Dutch/German
3	HHAO 05	Irish
4	HHAO 06	Scottish/English/Swedish
5	HHAO 07	English/Italian/German
6	HHAO 08	English/Welsh
7	HHAO 09	English
8	HHAO 10	Scottish/Welsh
9	HHAO 11	Welsh/Irish/Scottish/French
10	HHAO 12	German/Irish
11	HHAO 13	English
12	HHAO 14	Irish
13	HHAO 15	Scottish/English/German
14	HHAO 16	French/Dutch
15	HHAO 17	Russian/Polish/English
16	HHAO 18	English
17	HHAO 19	Irish/French
18	HHAO 20	German
19	HHAO 21	Irish
20	HHAO 23	Irish/Scottish
21	HHAO 24	Dutch/Irish
22	HHAO 25	Dutch
23	HHAO 26	Welsh
24	HHAO 27	English
25	HHAO 28	Irish/English/French
26	HHAO 29	English/Welsh/Scottish
27	HHAO 30	Irish/English
28	HHAO 31	Irish/Lithuanian
29	HHAO 32	English/Irish/German
30	HHAO 33	English/Irish
31	HHAO 34	German/English/French
32	HHAO 35	Scottish
33	HHAO 36	Swedish/Dutch
34	HHAO 37	Swedish
35	HHAO 38	French/English
36	HHAO 39	Scottish/Irish/English
37	HHAO 40	English
38	HHAO 41	German/Scottish
39	HHAO 42	Scottish
40	HHAO 43	Scottish
41	HHAO 44	English/German
42	HHAO 45	Welsh/German
43	HHAO 46	Irish/English
44	HHAO 48	English
45	HHAO 49	English
46	HHAO 50	English
47	HHAO 52	Norwegian/German/English
48	HHAO 57	German/Dutch/French/Irish
49	HHAO 58	English
50	HHAO 59	English/French/Dutch

Table 2: Ancestral details of Study Group 1

			DEXA T Scores		
			Lumbar Spine	Femur	
Number	Patient ID	Fractures	L1 – L4	Neck	Total
1	HHAO 02	No	-1.00	-2.28	-1.82
2	HHAO 04	No	-3.39	-2.82	-3.26
3	HHAO 05	Yes	+0.39	-2.23	-2.17
4	HHAO 06	No	-1.74		
5	HHAO 07	Yes	-0.42	-1.93	-2.40
6	HHAO 08	No	-2.32	-3.52	-2.87
7	HHAO 09	No	-3.63	-3.98	-3.63
8	HHAO 10	Yes	-4.65	-3.73	-3.79
9	HHAO 11	No	-2.69	-3.19	-2.77
10	HHAO 12	No	-2.08	-1.83	-1.55
11	HHAO 13	Yes	-4.58	-2.99	-2.56
12	HHAO 14	Yes	-3.23	-3.74	-2.74
13	HHAO 15	No	-1.23	-3.04	-2.18
14	HHAO 16	Yes	-3.69	-3.42	-3.79
15	HHAO 17	Yes	-2.32	-2.28	-0.50
16	HHAO 18	No	-2.38	-1.95	-1.10
17	HHAO 19	No	-5.53	-4.17	-3.80
18	HHAO 20	No	-3.76	-2.79	-2.20
19	HHAO 21	Yes	-3.20	-3.76	-3.28
20	HHAO 23	No	-2.08	-2.88	-2.65
21	HHAO 24	Yes	-3.42	-3.04	-2.27
22	HHAO 25				
23	HHAO 26	Yes	-0.40	-3.15	-1.23
24	HHAO 27	No	+0.78	-1.02	-0.26
25	HHAO 28	No	-2.35	-1.06	-0.87
26	HHAO 29	No	-4.00	-1.91	-0.30
27	HHAO 30	No	-1.76	-2.96	-2.68
28	HHAO 31	No	-2.50	-2.68	-2.23
29	HHAO 32	No	-1.84	-2.47	-1.51
30	HHAO 33	Yes	-3.64	-3.30	-2.92
31	HHAO 34				
32	HHAO 35	No	-2.80	-1.48	-1.07
33	HHAO 36	No	-2.84	-2.09	-2.24
34	HHAO 37	Yes	-2.29	-3.38	-2.91
35	HHAO 38	No	-2.00	-1.44	-1.43
36	HHAO 39	No	-2.24	-2.78	-1.89
37	HHAO 40	No	-2.25	-1.87	-1.52
38	HHAO 41	No	-3.97	-1.52	-0.84
39	HHAO 42	No	-1.32	-0.59	-0.80
40	HHAO 43	No	-2.34	-2.66	-1.87
41	HHAO 44	No	-2.35	-1.74	-0.61
42	HHAO 45	No	-0.71	-2.14	-2.18
43	HHAO 46	Yes	-2.93	-3.39	
44	HHAO 48	No	-2.49	-1.83	-1.78
45	HHAO 49	No	-3.50	-3.41	-3.29
46	HHAO 50	No	-5.78	-3.38	-3.28
47	HHAO 52				
48	HHAO 57	No	-2.21	-1.83	-0.80
49	HHAO 58				
50	HHAO 59				

Table 3: Study Group 1 DEXA T scores. Where scores are absent, the DEXA was either not done or scores were skewed due to calcification or other causes.

Patient 1 (age 31) complained of severe lethargy but had no other clinical features of hereditary haemochromatosis apart from a repeatedly raised fasting transferrin saturation of between 48% and 68%. His serum ferritin was normal. Patient 2 (age 65) was diagnosed with hereditary haemochromatosis in 1980 on liver biopsy and high transferrin saturation (74%). He underwent repeated weekly venesection at that time and was on a regular 3 monthly phlebotomy programme at the time of the study. He has severe arthritis of the hands, typical of hereditary haemochromatosis, as well as arthritis of the joints of the feet and ankles. Patient 3 (age 52) was diagnosed with hereditary haemochromatosis 10 years ago when he presented with non-insulin-dependent diabetes. The diagnosis of haemochromatosis was confirmed on liver biopsy. He underwent a vigorous phlebotomy programme and was on maintenance phlebotomy at the time of the study. He has severe intermittent reactive arthritis affecting his metacarpophalangeal joints, knees and ankles. There were no clinical features of rheumatoid arthritis and the rheumatoid factor was negative. All three patients were Caucasian and of north western European origin. DEXA scans were not available for this group.

3.2 Osteoporosis and Arthritis

Thirty patients out of the fifty included in the study had arthritis, 29 had osteoarthritis predominantly affecting the spine but also the hands and knees. One patient (HHAO 09) had rheumatoid arthritis affecting all joints except the temperomandibular. DEXA scans were available for 45 out of the 50 patients. Based on the WHO criteria (Section 1.5), 23 patients were osteoporotic and 12 were osteopenic. The presence of low impact fractures combined with DEXA scores >-2.50 in the remaining 10 showed them to be severely osteoporotic.

3.3 Study Group 1

3.3.1 Iron Profile

The results of the iron profile in this group are summarised in Table 4. None of the subjects had iron deficiency anaemia, however 11 (22%) had a serum ferritin indicative of depleted iron stores (<12μg/l). One patient had evidence of iron deficient erythropoeisis (HHAO 30). Only one subject had iron studies typical of hereditary haemochromatosis (HHAO 02), however, 8 additional patients had a transferrin saturation greater than 50%. Non-transferrin bound iron was present in all 9 and there was a positive correlation with transferrin saturation (rho=0.698, $p < 0.02$). Ferritin values were normal in all subjects except for HHAO 02 who was subsequently identified as a C282Y homozygote.

3.3.2 HFE Gene Analysis

All 50 subjects were screened for the most common mutations of the HFE gene (C282Y, H63D) as well as the S65C mutation using PCR and RFLPs. These results are shown in Table 5. Altogether 19 patients (38%) were found to carry one or other of these 3 mutations. Three patients were homozygous (6%) and 1 patient (2%) was heterozygous for the C282Y mutation. Thirteen patients were heterozygous for the H63D mutation (26%), 1 patient was heterozygous for the S65C mutation (2%) and there was 1 C282Y/H63D compound heterozygote (2%). Only one of the homozygous patients (HHAO 02) had an iron profile typical of hereditary haemochromatosis. One of the other homozygotes (HHAO 10) was found to have been previously diagnosed as having hereditary haemochromatosis and was on a regular phlebotomy programme.

Number	Patient ID	Hb (g/100ml)	Iron (µg/100ml)	Tf Saturation (%)	Ferritin (µg/l)
1	HHAO 02	13.8	217	90	>1000
2	HHAO 04	12.5	102	26	46
3	HHAO 05	16.8	109	35	17
4	HHAO 06	13.8	71	23	6
5	HHAO 07	15.4	138	48	4
6	HHAO 08	13.4	100	39	47
7	HHAO 09	13.9	38	23	239
8	HHAO 10	17.0	53	62	11
9	HHAO 11	13.2	54	21	2
10	HHAO 12	13.4	74	34	3
11	HHAO 13	15.7	76	31	32
12	HHAO 14	14.8	74	26	210
13	HHAO 15	12.4	122	47	28
14	HHAO 16	16.1	100	35	43
15	HHAO 17	14.0	70	24	7
16	HHAO 18	15.7	113	42	33
17	HHAO 19	15.9	135	52	133
18	HHAO 20	14.0	122	52	10
19	HHAO 21	15.2	120	16	76
20	HHAO 23	12.5	87	22	21
21	HHAO 24	13.4	74	25	26
22	HHAO 25	12.5	114	37	31
23	HHAO 26	15.0	85	29	75
24	HHAO 27	14.8	99	28	83
25	HHAO 28	14.9	111	39	20
26	HHAO 29	12.7	91	27	28
27	HHAO 30	12.5	45	12	2
28	HHAO 31	11.5	163	58	22
29	HHAO 32	12.9	80	19	2
30	HHAO 33	13.2	78	25	4
31	HHAO 34	12.7	169	52	20
32	HHAO 35	15.0	95	36	91
33	HHAO 36	11.5	87	23	27
34	HHAO 37	15.2	72	17	37
35	HHAO 38	17.7	59	16	46
36	HHAO 39	14.9	178	57	33
37	HHAO 40	13.9	119	37	21
38	HHAO 41	14.8	81	28	14
39	HHAO 42	15.2	102	29	26
40	HHAO 43	16.2	130	45	32
41	HHAO 44	16.2	104	30	2
42	HHAO 45	15.6	106	23	49
43	HHAO 46	16.4	124	36	12
44	HHAO 48	15.5	114	38	20
45	HHAO 49	16.3	132	37	33
46	HHAO 50	15.7	112	35	62
47	HHAO 52	11.5	131	54	12
48	HHAO 57	17.1	144	43	145
49	HHAO 58	13.7	114	38	45
50	HHAO 59	13.0	216	75	51

Table 4: Iron status of Study Group 1.

Number	Patient Number	Genotype	Intron 2 Polymorphism
1	HHAO 02	C282Y/C282Y	normal
2	HHAO 04	normal	normal
3	HHAO 05	normal	normal
4	HHAO 06	H63D/-	heterozygous
5	HHAO 07	H63D/-	homozygous
6	HHAO 08	normal	normal
7	HHAO 09	normal	homozygous
8	HHAO 10	C282Y/C282Y	normal
9	HHAO11	normal	heterozygous
10	HHAO12	normal	normal
11	HHAO13	normal	normal
12	HHAO14	normal	homozygous
13	HHAO15	H63D/-	heterozygous
14	HHAO16	normal	normal
15	HHAO17	normal	heterozygous
16	HHAO18	normal	normal
17	HHAO19	normal	normal
18	HHAO20	normal	normal
19	HHAO21	C282Y/C282Y	normal
20	HHAO23	normal	heterozygous
21	HHAO24	normal	heterozygous
22	HHAO25	normal	normal
23	HHAO26	normal	heterozygous
24	HHAO27	H63D/-	homozygous
25	HHAO28	normal	heterozygous
26	HHAO29	normal	heterozygous
27	HHAO30	normal	homozygous
28	HHAO31	normal	normal
29	HHAO32	normal	normal
30	HHAO33	normal	heterozygous
31	HHAO34	H63D/-	homozygous
32	HHAO35	normal	normal
33	HHAO36	normal	normal
34	HHAO37	S65C/-	heterozygous
35	HHAO38	H63D/-	heterozygous
36	HHAO39	H63D/-	heterozygous
37	HHAO40	normal	heterozygous
38	HHAO41	H63D/-	heterozygous
39	HHAO42	normal	normal
40	HHAO43	normal	heterozygous
41	HHAO44	normal	heterozygous
42	HHAO45	H63D/-	heterozygous
43	HHAO46	normal	normal
44	HHAO48	H63D/-	heterozygous
45	HHAO49	H63D/-	homozygous
46	HHAO50	H63D/-	normal
47	HHAO52	H63D/-	heterozygous
48	HHAO57	C282Y/-	heterozygous
49	HHAO58	C282Y/H63D	heterozygous
50	HHAO59	normal	normal

Table 5: Genotype in Study Group 1.

The remaining homozygote and the compound heterozygote (HHAO 21 and HHAO 58) had normal iron status and no history of excessive blood loss. The only C282Y heterozygote had a slightly raised transferrin saturation (43%). Only three of the H63D heterozygotes had a transferrin saturation >50%.

There were 9 (18%) patients with a raised transferrin saturation (>50%). Two of these were C282Y homozygotes and were not investigated further. None of the raised transferrin saturations could be explained by mutations of the HFE gene. The whole of exon 2, including the intron/exon boundaries, was therefore sequenced to look for a possible explanation. Analysis of the sequences revealed 1 homozygote and 2 heterozygotes for an intron 2 polymorphism (IVS2+4T→C). The rest of the patient cohort was then screened for this polymorphism. Twenty-nine patients (58%) were shown to have it, 7 of whom were homozygous. Three of the homozygotes and 9 of the heterozygotes for the polymorphism carried none of the other mutations. Twelve of the thirteen (92%) H63D mutants carried the polymorphism, as did the C282Y/H63D heterozygote. The S65C heterozygote was also heterozygous for it. These results are also shown in Table 5. The association of the intron 2 polymorphism with the H63D mutation was statistically significant (chi square = 8.98; p = 0.01). No further abnormalities in exon 2 of the HFE gene were detected in this group.

3.4 Study Group 2

All subjects had raised serum iron levels (>150μg/100ml) and normal ferritin levels. Repeated transferrin saturation tests constantly revealed results greater than 55% (Table 6). Of these three patients, one was heterozygous for the H63D mutation, one was heterozygous for the C282Y mutation and one carried none of the three common mutations. The whole of exon 2 including the intron/exon boundaries was sequenced revealing no additional mutations. However, the H63D heterozygote was also shown to have the intron 2 polymorphism, IVS2+4T→C (Table 7).

Number	Patient Number	Hb (g/100ml)	Iron (μg/100ml)	Tf Sat (%)	Ferritin (μg/l)
1	HHAO 03	15.4	171	65	20
2	HHAO 47	16.4	186	58	8
3	HHAO 53	15.5	160	66	10

Table 6: Iron status of Study Group 2.

Number	Patient Number	Genotype	Intron 2 Polymorphism
1	HHAO 03	normal	normal
2	HHAO 47	H63D/-	heterozygous
3	HHAO 53	C282Y/-	normal

Table 7: Genotype in Study Group 2.

CHAPTER 4

4. DISCUSSION

4.1 Study Group 1

4.1.1 Iron Profile

In normal individuals, there is a reasonably close correlation between the serum ferritin concentration and the amount of body iron stores. Ferritin levels <12μg/l are indicative of decreased iron stores, which can result from a number of causes. These include increased blood loss, lack of available food iron or impairment in iron absorption. However, ferritin values may be highly unreliable in the presence of an inflammatory process. Eight out of the 11 subjects with a low serum ferritin in this study had arthritis with associated inflammation making interpretation of their serum ferritin levels difficult. It is also possible that there might be blood loss in these patients as a result of treatment with anti-inflammatory drugs. The patient with evidence of iron deficient erythropoeisis (HHAO 30) had a duodenal ulcer that could be the cause of increased blood loss.

The degree of saturation of serum transferrin is a function of both the serum iron and the transferrin concentration (Section 1.3.1). The iron-binding capacity of transferrin is normally only about one-third saturated but there is a considerable circadian variation with a morning peak and an evening trough. This is mainly due to the variation in the donation of iron to transferrin by the cells of the macrophage system (Heubers and Finch, 1987). Of the 8 subjects in this study with transferrin saturation >50%, only 2 could be explained by mutations of the HFE gene (HHAO 02 and HHAO 10). There were also

some untreated patients with mutations of the gene whose transferrin saturations were normal (HHAO 21, 47 and 58). This is in line with the incomplete penetrance of the gene (Section 1.4.1.1) and with observations made by Crawford and co-workers (1998), who concluded that 17.3% of homozygous individuals do not express a phenotypic profile of haemochromatosis and that this finding is particularly common in women.

4.1.1.1 Non-Transferrin-Bound Iron

In the present study, using the method described in Chapter 2, concentrations of NTBI up to 0.5μM were indistinguishable from zero, and a positive NTBI result was therefore taken to be one greater than 0.5μM. This has been reported previously (Porter *et al*, 1996; McNamara *et al*, 1999).

In a normal healthy individual, the plasma concentration of NTBI typically does not exceed 1μM/l and is often undetectable (Anderson, 1999). Levels of up to 9.0μM/l have been detected in patients with β-thalassaemia (Al-Refaie *et al*, 1992), and of up to 8.0μM/l in cases of African iron overload (McNamara *et al*, 1999). NTBI was originally reported only in patients with a transferrin saturation exceeding 100%, but as detection methods have improved over the years it has been detected in the serum of patients with raised transferrin saturation but with unsaturated transferrin still present. Free iron is able to catalyse reactions leading to the production of highly toxic oxygen radicals which cause damage to lipids, proteins and DNA within cells, leading to such events as DNA strand breakage and disruption of calcium ion metabolism (Halliwell and Cross, 1994). It has been suggested that excess iron may impair osteoblastic activity and previous studies have shown that patients with normal serum ferritin levels and mildly elevated transferrin

saturation indices showed some abnormalities in bone formation rates (Diamond *et al*, 1989).

4.1.2 HFE Gene Analysis

The prevalence of the C282Y and H63D mutations of the HFE gene has been studied in Caucasian patients attending clinics specialising in the management of the well-recognised features of hereditary haemochromatosis such as diabetes and cirrhosis (Conte *et al*, 1998; Bacon *et al*, 1999). This is the first report of an increased frequency of the C282Y mutation occurring in patients attending an osteoporosis clinic. The allele frequency of the C282Y mutation found in this study of 50 osteoporotic subjects was 8%. This is significantly greater (chi square = 4.528; p = 0.033), than that reported in an analysis of European chromosomes (Merryweather-Clarke *et al*, 1997) and in South Africans of European descent (de Villiers *et al*, 1999). The allele frequency of the H63D mutation was 15.2% in our study group, slightly higher than the 13.6% reported by Merryweather Clarke and co-workers (1997), but not statistically significant. The finding of a single S65C mutation is in line with that found in control subjects by other workers (Mura *et al*, 1999; Barton *et al*, 1999) and probably falls within the normal occurrence in the general population. Statistical analysis showed no correlation between severity of osteoporosis and the presence of any of the 3 common HFE gene mutations.

The allele frequency of the intron 2 polymorphism (IVS2+4) was 39%. This is significantly higher (chi square = 15.00; p = <0.001) than previously reported (Beutler *et al*, 1997; Höhler *et al*, 1999). Analysis of variance showed that the presence of this polymorphism in the heterozygous state was associated with lower DEXA T scores in the

femur ($p = 0.04$). However, this relationship was not seen in the lumbar spine ($p = 0.193$) nor was there any relationship between the severity of osteoporosis overall in the presence or absence of the polymorphism (chi square = 1.966; $p = 0.5$). This finding might warrant further investigation in a larger patient sample to determine whether there is a possibility of linkage disequilibrium with another disease-related mutation.

4.2 Study Group 2

Two of these patients (HHAO 47 and 53) had clinical features compatible with a diagnosis of hereditary haemochromatosis and were on a regular phlebotomy programme. The finding of a raised transferrin saturation and non-transferrin-bound iron in spite of a normal or low serum ferritin is characteristic of patients with haemochromatosis who are on a vigorous bleeding programme. The third patient (HHAO 03) had very few clinical features of haemochromatosis.

The phenotypic expression of hereditary haemochromatosis in these patients could not be explained by the known mutations of the HFE gene or by any other abnormalities in exon 2 of the gene. It is possible that the iron overload in these patients may be due to an abnormality elsewhere in the HFE gene or in other recently described genes such as ferroportin and transferrin receptor 2 (See Section 1.4.1.1) or by interaction with other, as yet unknown, disease related genes.

CHAPTER 5

5.1 CONCLUSION

In this group of patients attending an osteoporosis clinic, a higher than expected frequency of mutations of the HFE gene was found. Hereditary haemochromatosis is easily treated by phlebotomy to remove excess iron, thus preventing the possibly fatal consequences of cirrhosis, diabetes and cardiomyopathy, amongst others, which characterise the fully developed disease. This finding emphasises the need for physicians dealing with osteoporosis to include screening for hereditary haemochromatosis in their diagnostic work up. Although the prevalence of HFE gene mutations was higher in this group, there was no indication that any of the mutations studied affected the severity of osteoporosis.

Phenotypic expression of mutations of the HFE gene is extremely varied. This was demonstrated in this group of patients with osteoporoses some of whom carried the mutations but were not iron overloaded. Although continuous blood loss due to the high consumption of non-steroidal anti-inflammatory drugs may account for the absence of iron overload in these patients, poor penetrance of these mutations is well described.

CHAPTER 6

6. REFERENCES

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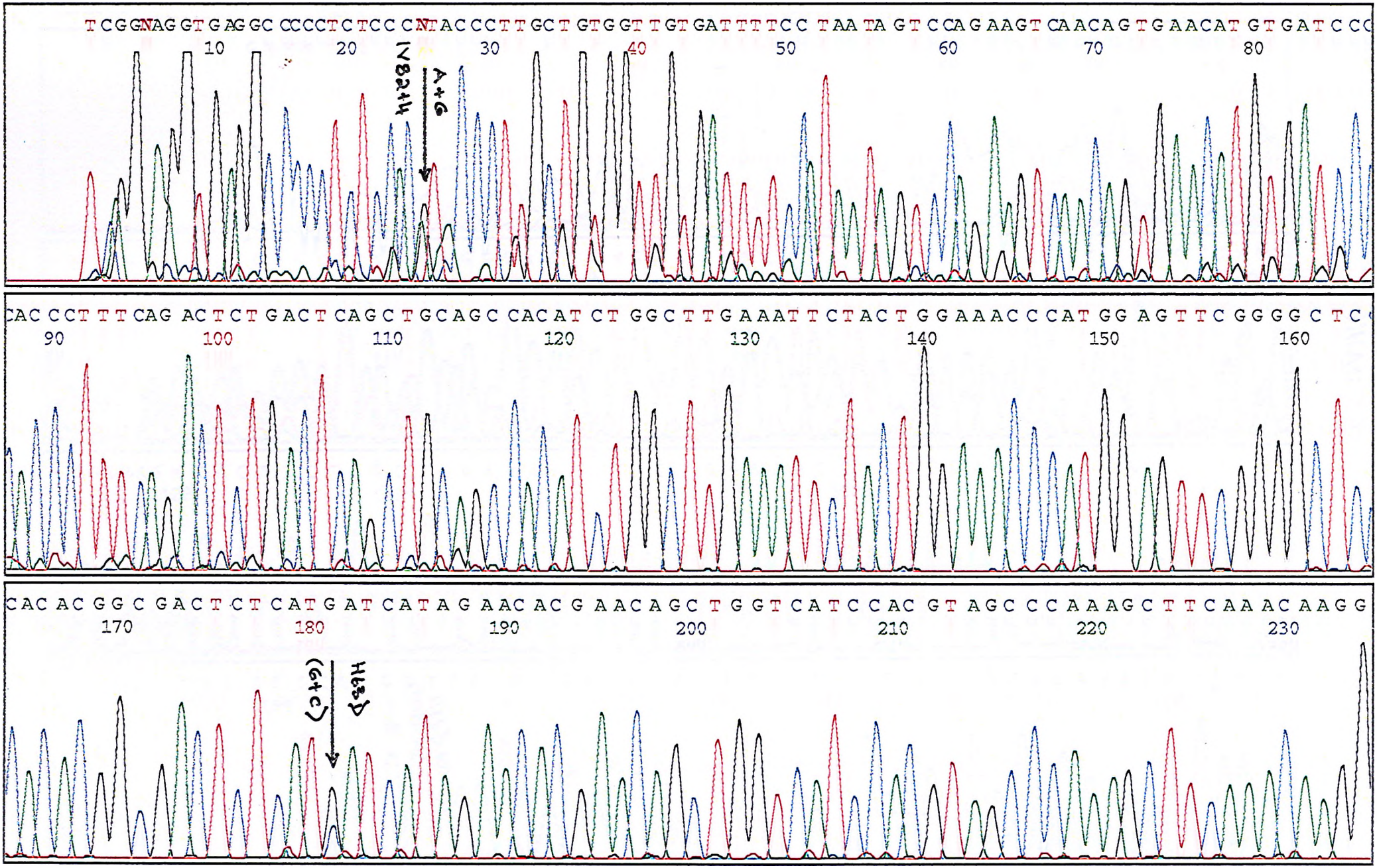
APPENDIX 1

Appendix 1 contains two automated sequence graphs and a section of the HFE gene (Genbank Accession Z92910).

The first sequence graph (HHA0 39) shows a heterozygous intron 2 polymorphism (IVS2+4T→C). The reverse primer was used to generate this sequence so the polymorphism is shown as an A→G change. This sequence also shows a heterozygous H63D site. Both sites are marked with vertical black arrows.

The second sequence (HHA0 30) shows a homozygous intron 2 polymorphism. The reverse primer was also used to generate this sequence. This sequence also shows a normal H63D site. Both sites are marked with vertical black arrows.

The portion of the HFE gene shows exon 2 and parts of intron 1 and 2. The primers used to generate the PCR product are marked and the position of the intron 2 polymorphism is underlined.


TCGGNAGGTGAGGCCCCTCTCCCNTACCCTTGCTGTGGTTGTGATTTCCTAATAGTCCAGAAGTCAACAGTGAACATGTGATCCC
10
20
30
40
50
60
70
80
V82M
A→G
CACCCTTTCAGACTCTGACTCAGCTGCAGCCACATCTGGCTTGAAATTCTACTGGAAACCCATGGAGTTCGGGGCTC
90
100
110
120
130
140
150
160
CACACGGCGACTCTCATGATCATAGAACACGAACAGCTGGTCATCCACGTAGCCCAAAGCTTCAAACAAGG
170
180
190
200
210
220
230
(G→C)
H63D

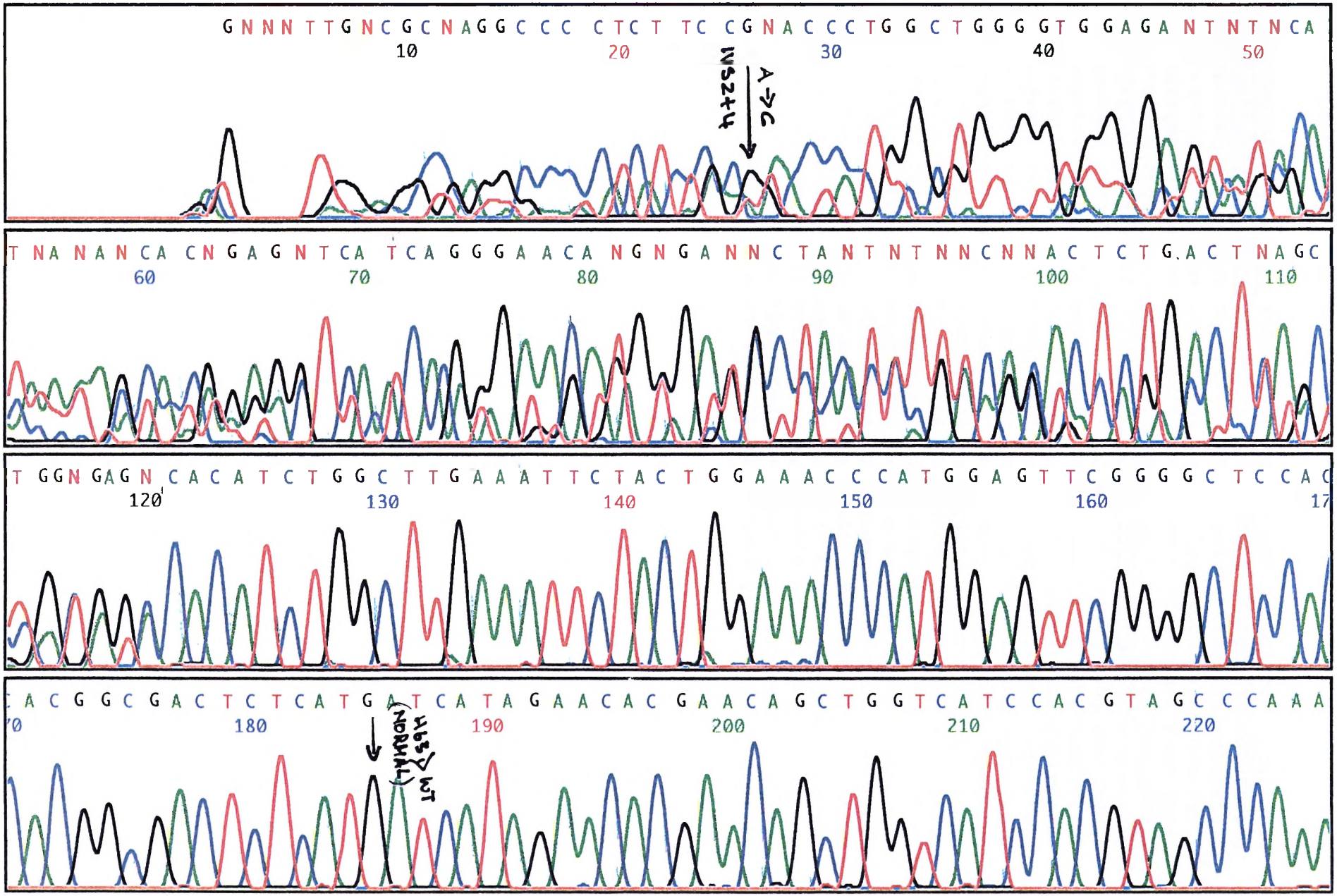
HHA0 39

Model 377
Version 3.4.1
ABI100
Version 3.3.1

24•MEDC 6
MEDC 6
Lane 24

Signal G:2766 A:2768 T:1666 C:2501
DT {BD Set Any-Primer}
dRhod_BigDye
Points 903 to 3850 Pk 1 Loc: 903

Fri, 8 Jun, 2001 8:40 am
Thu, 7 Jun, 2001 4:13 pm
Spacing: 9.75{9.75}



A section of the HFE gene.

```
4561 ctgatggtat gagttgatgc aggtgtgtgg agcctcaaca tcctgctccc ctcctactac
4621 acatggttaa ggcctgttgc tctgtctcca ggttcacact ctctgcacta cctcttcatg
4681 ggtgcctcag agcaggacct tggtctttcc ttgtttgaag ctttgggcta cgtggatgac
4741 cagctgttcg tgttctatga tcatgagagt cgccgtgtgg agccccgaac tccatgggtt
4801 tccagtagaa tttcaagcca gatgtggctg cagctgagtc agagtctgaa agggtgggat
4861 cacatgttca ctgttgactt ctggactatt atggaaaatc acaaccacag caaggtatg
4921 tggagagggg gcctcacctt cctgaggttg tcagagcttt tcatctttc atgcatcttg
4981 aaggaaacag ctggaagtct gaggtcttgt gggagcaggg aagagggaag gaatttgctt
5041 cctgagatca tttggtcctt ggggatggtg gaaataggga cctattcctt tggttgcagt
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5521 gggaagggac tttctcaatc ctagagtctc taccttataa ttgagatgta tgagacagcc
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EXON 2

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 McNamara

CLEARANCE CERTIFICATE PROTOCOL NUMBER M990715

PROJECT The Link Between Hereditary Haemochromatosis, Arthritis And Osteoporosis

INVESTIGATORS Mrs L McNamara

DEPARTMENT Department of Medicine, Johannesburg Hospital

DATE CONSIDERED 990730

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 990806 CHAIRMAN ..... pp (Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof AP McPhail
Dept of Department of Medicine, SAIMR

Works2\lain0015\HumEth97.wdb\M 990715

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 10/8/99 SIGNATURE .....

PROTOCOL NO.: M 990715

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES