

# **ANALYSIS OF HEREDITARY HAEMOCHROMATOSIS AND CLINICAL CORRELATIONS IN THE ELDERLY**

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## **Declaration**

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

## SUMMARY

Hereditary haemochromatosis (HH) is an autosomal recessive iron storage disease where the accumulation of iron in parenchymal organs may lead to diabetes, heart failure, liver cirrhosis, arthropathy, weakness and a variety of other ailments if preventive measures are not taken. HH is often not considered as a cause of these conditions, particularly not in the elderly where the background frequencies of type II diabetes, osteoarthritis and heart failure are generally high. Heterozygosity for C282Y, the HFE-mutation causing HH in approximately 80% of affected individuals worldwide, has been linked to a raised incidence of malignancies of the colon and rectum, stomach and the haematological system. One of the highest carrier-frequencies (1/6) in the world for this mutation has been reported in the South-African Afrikaner population, resulting in C282Y-homozygosity in approximately 1 in every 115 people in this group.

A sample of 197 elderly Afrikaner volunteers was recruited for genotype/phenotype association studies. Their clinical presentation was denoted, biochemical iron-status determined and HFE genotyping performed. Either an increase or decrease in survival, or both, were proposed, depending on possible gender effects. HH has been positively associated with various cancer types, but may also protect against iron-deficiency anaemia which is by far the most frequent cause of anaemia in the older person.

This study has led to the following findings:

1. The carrier frequency of mutation C282Y was found to be 1/8 in the elderly population (similar in males and females), which is slightly lower than the 1/6 reported in younger adults from the same population. Only one C282Y homozygote and two C282Y/H63D compound heterozygotes were detected, all of them female.
2. The prevalence of diabetes, heart disease, arthropathy or a combination of these conditions did not differ significantly in C282Y heterozygotes and the mutation-negative group.
3. Among 24 C282Y heterozygotes only one individual with rectal carcinoma was detected compared with two cases with rectal- and seven with colonic malignancies in 153 mutation-negative individuals. The single female C282Y homozygote identified suffered from both rectal and colon carcinoma and died approximately 6 months ago as a consequence of her colon malignancy.
4. Serum ferritin appears to be a highly unreliable parameter of iron status, particularly in the elderly where a variety of factors that may influence the levels are often present in elderly individuals. This may be due to ageing alone or as a result of multiple co-morbidities.
5. Serum ferritin levels were lower than expected in elderly subjects with mutation C282Y and compound heterozygotes with both C282Y and H63D, which may be related to a variable penetrance of the HFE gene mutations. It is possible that variation in other genes exist that confer protection against iron-loading by gene-gene interaction. The probability that environmental factors (e.g. a low iron diet) are more important in this respect cannot be excluded, although this is considered less likely in the light of the fact that the same trend was observed in all mutation-positive elderly

individuals. It is therefore highly likely that C282Y-positive subjects with significant iron loading have died before reaching their seventies, particularly since none of the males included in this study were homozygous or compound heterozygous for the mutations analysed.

In conclusion, possession of a mutant HFE gene does not appear to confer a survival advantage in old age, neither does it seem that mutation carriers with significant iron-loading are overlooked by the medical fraternity. Further investigations are warranted to shed more light on the contributions of gene-gene and gene-environment interaction in the clinical manifestation of HH, and how these processes can be manipulated to prevent the symptoms of this largely underdiagnosed disease.

## OPSOMMING

Oorerflike hemochromatose (OH) is 'n outosomaal resessiewe yster-oorladings siekte waar akkumulering van yster in parenkimale organe kan lei tot suikersiekte, hartversaking, lewer sirroos, artropatie, moegheid en 'n verskeidenheid van ander probleme indien voorkomende maatreëls nie getref word nie. OH word gewoonlik nie oorweeg as moontlike oorsaak vir hierdie toestande nie, veral nie in ouer mense nie waar die agtergrond-frekwensie van tipe II diabetes, osteoartritis en hartversaking in elk geval hoog is. Heterosigositeit vir die HFE mutasie C282Y, wat OH veroorsaak in ongeveer 80% van geaffekteerde gevalle wêreldwyd, is geassosieer met 'n verhoogde voorkoms van kanker van die kolon, rektum, maag en ook die hematologiese sisteem. Van die hoogste draer frekwensies ter wêreld vir hierdie mutasie (1/6) is gevind in die Afrikaner populasie van Suid-Afrika, wat daarop dui dat 1 uit elke 115 mense in die groep homosigoties vir die C282Y mutasie kan wees.

Eenhonderd sewe-en-negentig bejaarde Afrikaner vrywilligers het aan die studie deelgeneem wat daarop gemik was om genotipe/fenotipe korrelasies uit te voer. Die kliniese beeld van elke individu is gedokumenteer, die yster status biochemies bepaal en HFE genotipering uitgevoer. Die *à priori* veronderstelling was dat oorlewings sou toeneem of afneem, of beide, afhangende van die geslag van die individu. Daar is voorheen 'n verband gevind tussen OH en die ontwikkeling van bogenoemde maligneiteite, maar aan die ander kant kan dit moontlik ook beskerm teen anemie as gevolg van yster gebrek, wat juis die mees algemene oorsaak van anemie in die ouer persoon is.

Hierdie studie het tot die volgende bevindings gelei:

1. Die draer frekwensie van mutasie C282Y was 1/8 in die bejaardes (dieselfde in mans en vrouens), wat effens laer is as die 1/6 wat gerapporteer is in jonger volwassenes. Slegs een C282Y homosigoot en twee C282Y/H63D saamgestelde heterosigote is opgespoor, en al drie was vroulik.
2. Die voorkoms van suikersiekte, hartsiekte, gewrigspyne of 'n kombinasie van hierdie aandoenings het nie betekenisvol verskil tussen die C282Y heterosigote en die mutasie-negatiewe groep nie.
3. Daar was slegs een persoon met rektum karsinoom in die groep van 24 bejaarde C282Y heterosigote, terwyl daar twee gevalle met rektum kanker en sewe gevalle met kolon kanker gevind is onder die 153 mutasie-negatiewe individue. Die enkele vroulike C282Y homosigoot wat opgespoor is het beide rektum- en kolonkanker gehad en is ongeveer 6 maande vóór voltooiing van die tesis oorlede aan haar kolon karsinoom.
4. Dit wil voorkom asof serum ferritien veral in bejaardes 'n hoogs onbetroubare maatstaf is vir yster status, aangesien dit deur 'n verskeidenheid faktore beïnvloed word wat dikwels in bejaardes aanwesig is as gevolg van veroudering of veelvuldige komorbiditeite.
5. Die serum ferritien vlakke was laer as verwag in sowel die bejaarde C282Y-homosigoot as in die twee saamgestelde heterosigote met mutasies C282Y en H63D, wat moontlik die gevolg is van die wisselende graad van penetrasie van HFE mutasies. Dit is moontlik dat variasie in ander gene beskerming bied teen yster-oortolering deur middel van geen-geen interaksie. Die moontlikheid dat omgewingsfaktore (soos 'n lae-yster dieet) 'n belangrike rol speel in hierdie verband kan nie uitgesluit word nie, hoewel dit minder waarskynlik lyk te wees in die lig van die feit dat dieselfde neiging

waargeneem is in alle mutasie-positiewe bejaardes. Die kans is dus redelik groot dat individue met die C282Y mutasie en betekenisvolle yster oorlading oorlede is voordat hulle die sewentiger jare kon bereik, veral omdat geeneen van die mans wat ingesluit is in die studie homosigoot of 'n saamgestelde heterosigoot was vir die mutasies wat geanaliseer is nie.

Opsommend wil dit voorkom asof die teenwoordigheid van 'n mutante HFE geen nie 'n beter oorlewingskans bied op ouer leeftyd nie, en dit blyk ook dat mutasie draers met betekenisvolle ysteroorlading nie deur dokters misgekyk word nie. Verdere navorsing is nodig om meer lig te werp op die bydrae van geen-geen- en geen-omgewing interaksie in die kliniese manifestasie van OH, en ook hoe hierdie prosesse gemanipuleer kan word om die simptome van hierdie onder-gediagnoseerde siekte te voorkom.



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## LIST OF ABBREVIATIONS

AD	Alzheimer's disease
Ca	carcinoma
Ca <sup>++</sup>	calcium ion
CNS	central nervous system
DMT1	divalent metal transporter1
Fe	iron ion
Fer	ferritin
Hb	haemoglobin
HH	hereditary haemochromatosis
HFE	human Fe gene
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
INF-g	interferon-gamma
IRE	iron responsive element
IRP	iron regulatory protein
LIP	labile intracellular pool of iron
mRNA	messenger ribonucleic acid
NO	nitric oxide
NTBI	non-transferrin-bound iron
NRAMP	natural resistance-associated macrophage protein
RBC	red blood cell
RES	reticulo endothelial system

SFer	serum ferritin
Tf	transferrin
TfR	transferrin receptor

***“OUDERDOM IS DIE VERVALDAG VAN DIE  
UITSTEL WAT AAN DIE JEUG GEGEE IS”***

***C. J. Langenhoven***

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# **CHAPTER 1**

## **Introduction**

### **Iron status in old age: An overview**

## 1.1 The role of iron in health and disease

The iron status of an individual is determined by a combination of nutritional, environmental and genetic factors. Normal iron status ensures immediate availability of iron for metabolic activity, including functioning of the immune system, while at the same time impeding uptake by microorganisms and denying growth advantages to tumour cells. How far one can go to either side of the "normal" situation without upsetting this balance is probably the critical factor in understanding how changes in iron status can predispose to a wide variety of disorders, including infectious, inflammatory, neurologic and neoplastic disease. Iron is indispensable for life, mainly because of its role within oxygen-binding and respiratory proteins, but may also lead to the production of free radicals and consequently tissue damage (Bothwell et al. 1979). This may be particularly relevant in the elderly, since many of the age-related diseases (e.g. atherosclerosis-induced disorders, malignancies) are known to be at least partly due to chronic tissue damage related to formation of free radicals.

Little is known regarding iron status in old age. Most of the research on iron status that has been performed in the elderly has incorporated individuals up to 70 or 75 years of age (the "young-old": NHANES-studies), but few studies included elderly subjects between 75-80 years of age (the "old-old"), and even less included subjects over 85 years of age (the "very-old"). Most research into the basic mechanisms was performed on aged mice, rats, pigs or dogs. Although this is probably the best that can be done at present, mechanisms in these animal models are not always equal to those in the human body as species differences exist in the extraction of iron from food, its absorption and excretion (Lynch et

al. 1982). More is known concerning haematological data, but flaws in study design have rendered the data obtained in many of these studies unreliable (Miller 1939; Hobson and Blackburn, 1953; Myers, 1968; Elwood et al. 1971; Smith and Whitelaw, 1971; Milne and Williamson, 1972; Kelly and Munan, 1977; Kalchthaler and Rigor, 1980; Lipschitz et al. 1981; Zaino, 1981; Österlind et al. 1984; Yip et al. 1984). There is still much controversy whether parameters of iron status generally applied in the adult population can be used reliably in the aged (Dybkaer et al. 1981). The current opinion is that for practical purposes haematologic values in healthy elderly men and women are similar to those of young adults (Zauber and Zauber, 1987).

It is therefore clear that “fragmentary knowledge” would be the best term to describe our level of understanding of the processes involved in iron metabolism, particularly in old age. Part of the problem is the lack of insight into the specific mechanisms that underlie iron-absorption, iron-transport, iron-storage and the regulation of all these processes locally in the intestine and between the different organs and systems that utilise iron. In the next sections a summary of the known information will be presented.

## **1.2 The Iron Compartments**

As most physiological processes change with the ageing of the body, either due to wear-and-tear of tissues and organs or as result of depletion of essential building processes necessary to replace lost or damaged structures, so do the elements that make up the iron compartments. In the human body, iron exists in six compartments (Milman et al. 1986):

1) haemoglobin



- 2) a storage compartment consisting of ferritin (present in virtually all cells of the body and tissue fluids) and haemosiderin (in the reticulo-endothelial system (RES))
- 3) myoglobin
- 4) a labile intracellular pool (LIP) where iron is bound to cell membranes and intra-cellular proteins for short periods of time before it is being incorporated into haem or iron-containing proteins; metabolic redox-active forms of iron have also been found here
- 5) a tissue-compartment with the iron located in a variety of enzymes
- 6) the transport compartment, consisting of the transport protein transferrin (Tf). Iron is being turned over at least ten times a day and transferrin is the final common pathway for interchange between all compartments.

### **Haemoglobin**

In adulthood the haemoglobin (Hb) concentration is significantly higher in males than in females and this difference continues to exist in old age. Aged males show a continuous decline in haemoglobin-concentration (figure 1), with the difference best seen when the Hb of the age-group of 60-79 years old is compared with the Hb of those 80-93 years old, but this difference is not statistically significant (Milman et al. 1986). This decline might be related to the physiological decrease in testosterone production well known to occur in males over 80 years of age, as aged females do not show this age-related lowering (figure 1). The other two hormones that have an influence on erythropoiesis (thyroid hormone and erythropoietin) do not significantly decrease in healthy elderly individuals (Denham and Himsworth, 1984; Powers et al. 1989), although the bone-marrow response to these hormones might change with ageing (Lipschitz et al. 1984), as does its reserve capacity.

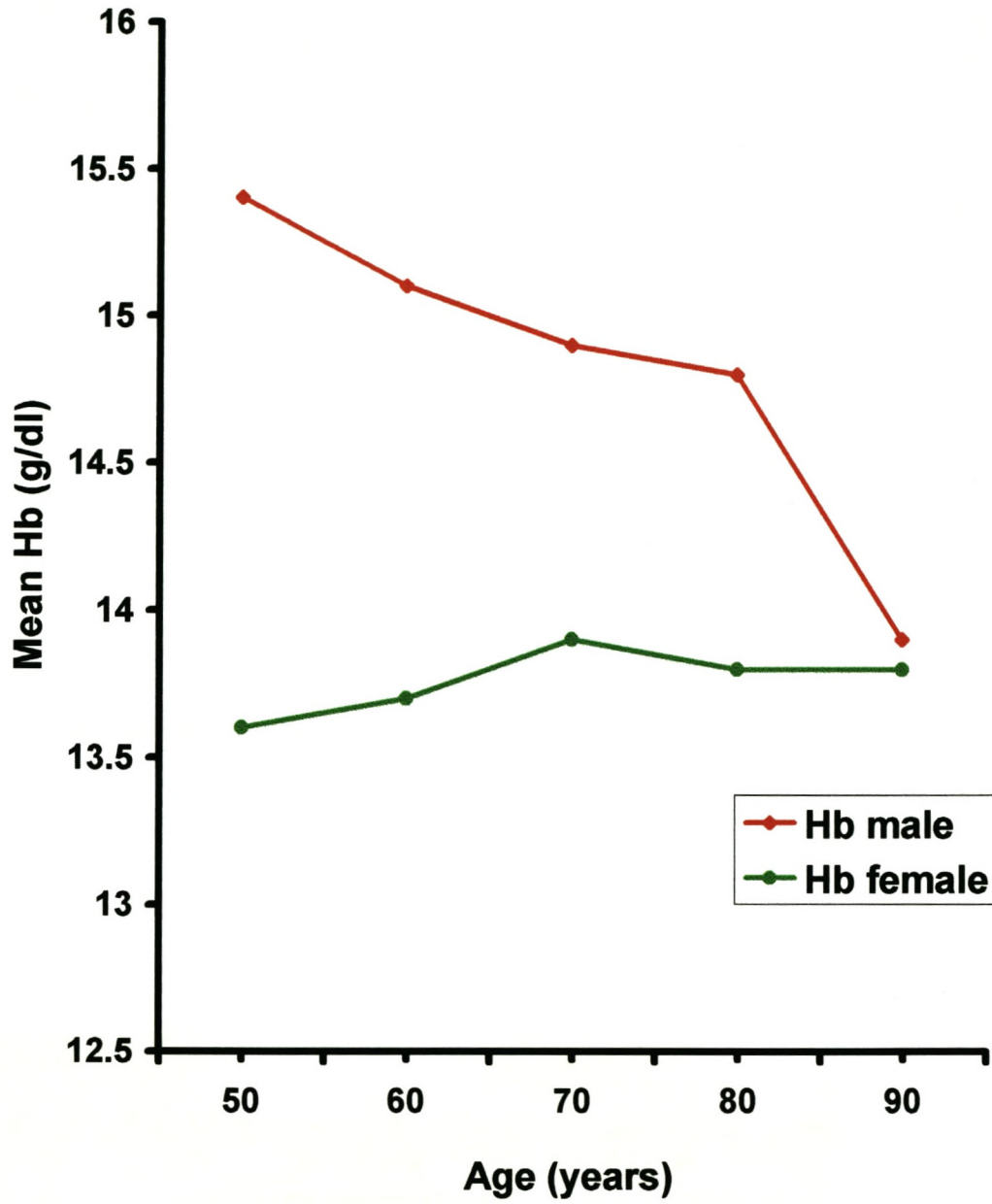


Figure 1. Age-related changes in haemoglobin (Yip et al. 1984, Milman et al. 1986, Kruger, 1987).

## **Ferritin**

Ferritin consists of a hollow protein shell, composed of 24 heavy and light chains forming four-helix bundles that each form a channel, and a mineral core in the middle as a Fe-storage cavity (Lawson et al. 1991). The hollow shell is called apoferritin and ferritin is formed when ferri-hydrate is deposited inside in the mineral core. It is produced intracellularly in virtually all cells of the body. The major function of ferritin is the sequestration of excess intra- and extra-cellular iron and to store this highly reactive ion in a biochemically inert form in order to prevent development of oxygen radicals. The intracellular ferritin is in direct equilibrium with the serum ferritin concentration, which is the small fraction of the body ferritin that circulates in the blood. Approximately 30% of total body iron is bound to ferritin and in healthy adults the serum ferritin is in direct equilibrium with the level of saturation of all the other iron-stores in the body. Men have higher ferritin levels than women and again this difference continues into old age (Milman et al. 1986). Although elderly men have slightly higher levels than young men this does not reach statistical significance, in contrast to elderly women who start accumulating iron after the menopause (figure 2, Milman et al. 1986). Other measures of iron storage, that is hepatic non-haem iron (Charlton et al. 1970; Sturgeon and Shoden, 1971) and bone-marrow iron (Benzie, 1963), have been found to increase with age, particularly in females. However, a variety of factors have been identified that raise the serum ferritin concentration without any relationship to iron status *per se*, as ferritin is an acute phase protein. These include any underlying acute or chronic inflammatory disorder which releases ferritin from affected tissues (Marcus and Freedman, 1985), exercise (Malczewska et al. 2000), alcohol use (Lipschitz et al. 1974), hepatic and ovarian malignancies (Lundin

disorders, which may render serum ferritin an unreliable measure of body iron status in this age group.

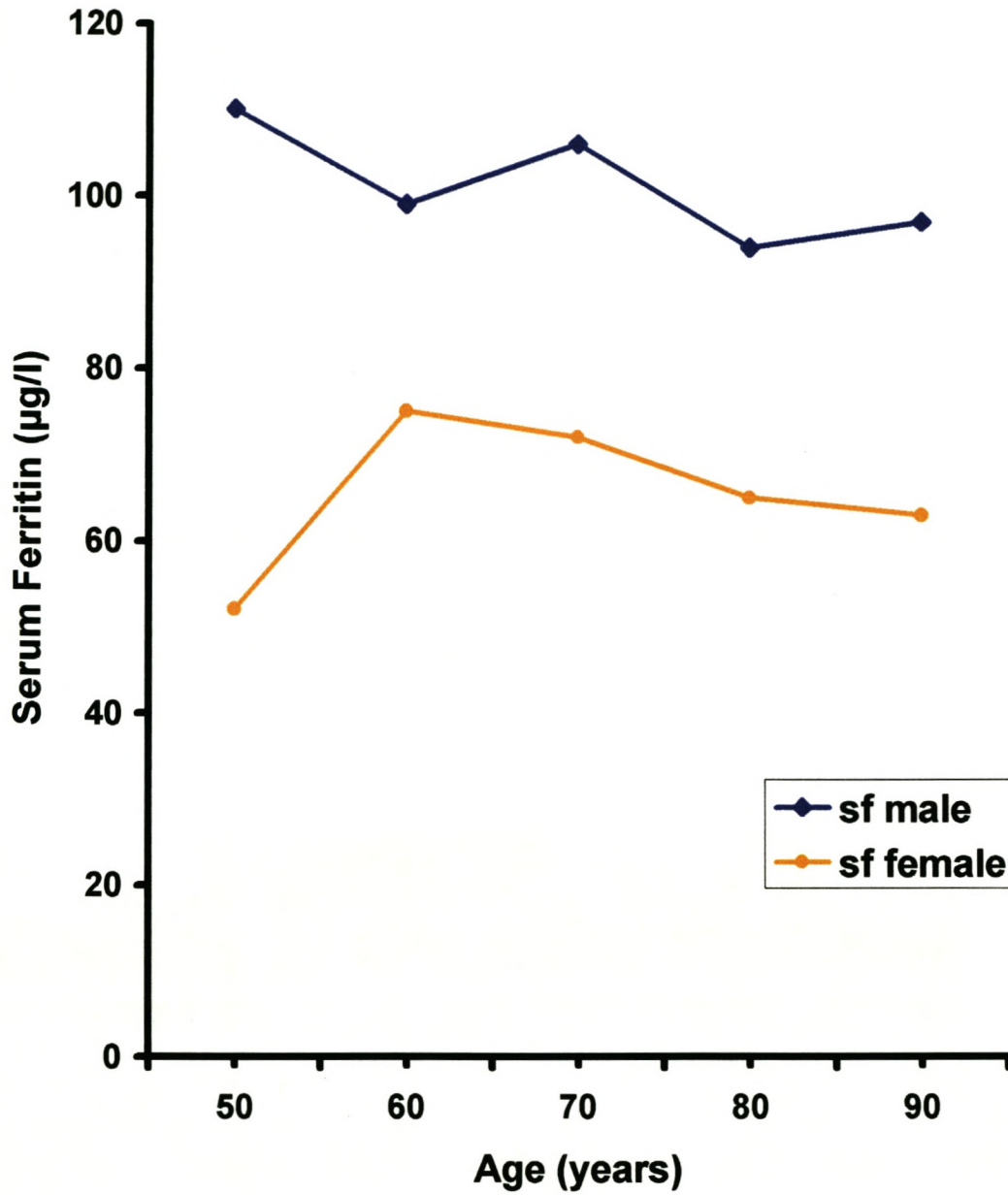


Figure 2. Age-related changes in serum ferritin (sf) (Yip et al. 1984, Milman et al. 1986)

**Haemosiderin**

Haemosiderin is an insoluble protein-iron complex mainly found in the cells of the monocyte-macrophage system, although with bleeding it does accumulate locally in the tissues. It is probably derived from ferritin molecules by partial lysosomal digestion. The effects of ageing on this system and on haemosiderin are basically unknown.

**Myoglobin**

Myoglobin forms the oxygen-reserve of the muscle fiber. With ageing the number of muscle cells decreases, as does the contractile power of the surviving cells. Although the effect of old age on myoglobin or its iron-component has not been studied, it will most likely not be of much significance to the iron status of the individual. In the normal adult male it makes up only 3.5% of the total body iron, compared to 67% in haemoglobin and 27% in the storage-compartment (Marcus and Freedman, 1985).

**Transferrin**

Although the transport protein transferrin (Tf) contains only 0.08% of total body iron, it is the common pathway for communication between all the other compartments and it is readily measurable. In cases with normal liver function, if individuals are not using oestrogens (which raise serum Tf) and if there is no nephrotic syndrome or a chronic illness with a low plasma iron concentration (which both lower serum Tf) (Zilva et al. 1987), no statistically significant change with age or between the sexes has been found (figure 3, Milman et al. 1986, Franzini et al. 2000). The same holds true for the serum iron concentration (Franzini et al. 2000)

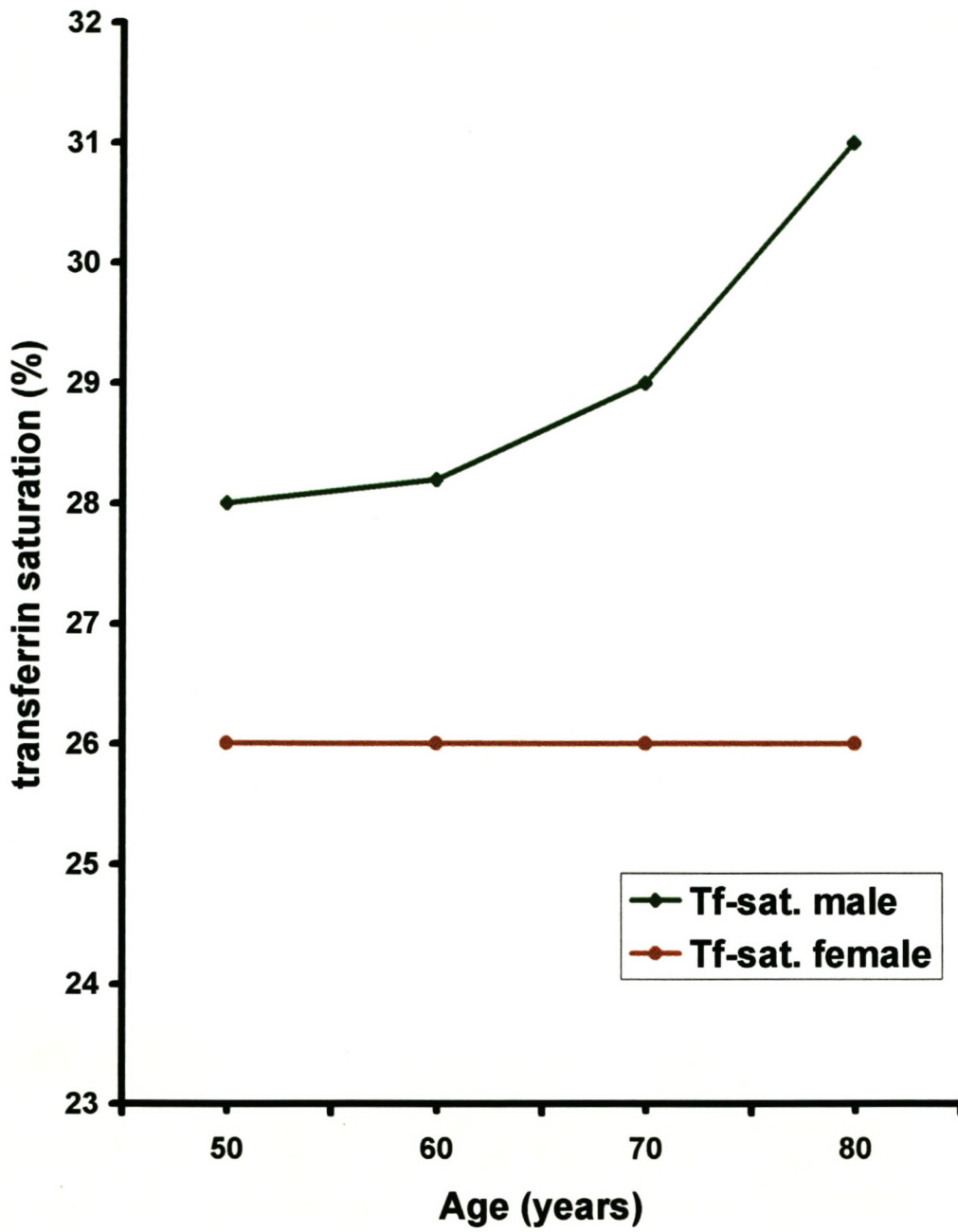


Figure 3. Age-related changes in serum transferrin-saturation (Tf-sat.) (Yip et al. 1984, Milman et al. 1986)

No data could be obtained on any age-related changes in the labile pool of iron or the tissue iron-compartment where the iron is part of proteins and enzymes.

### **1.3 Iron Metabolism**

#### **Iron intake**

Haemoglobin and myoglobin in meat, leafy green vegetables, iron-fortified cereals and egg-yolk contain absorbable iron. Haem-iron can be more readily absorbed than inorganic iron, but elderly people are universally known to eat less meat than younger adults. Iron intake is in direct proportion to the caloric intake of the individual. With ageing both caloric needs and iron-requirements decrease as processes of growth have stopped and much less energy is used in daily life. In spite of the relatively low meat consumption mentioned above and the decreased bio-availability mentioned below, the elderly usually seem to have an adequate iron intake (Lynch et al. 1982; Bunker et al. 1984). As very little iron is lost from the body an older person needs a daily intake of about 1 mg of iron (Marcus and Friedman, 1985).

#### **Bio-availability**

In old age bio-availability of iron is decreased due to a number of factors:

1. As mentioned above, the amount of iron that is available from haem iron declines parallel to meat consumption. When haem iron is taken with food, its absorption is not dependent on the food composition in the same way as non-haem iron, but it has been found that pure haem iron is relatively poorly absorbed. This is probably due to the formation of non-absorbable haem-polymers (Conrad et al. 1966) and the fact that

uptake is markedly increased when either meat or soy-protein is present (Lynch et al. 1985). These other protein-products may preserve haem in the monomeric state. It has repeatedly been found that the elderly consume less meat than adults, which is amongst others due to a decreased ability to chew meat properly and the age-related physiological decrease in saliva production. This will decrease the bio-availability of iron.

2. In the diet of many elderly people most iron is obtained from green vegetables and cereals; these sources contain a variety of iron-chelators (oxalates, fiber, phytates and phosphates, and other inhibitory ligands) that decrease the bio-availability and absorption of non-haem iron.
3. Iron cannot be absorbed in the 3+ form, only as a ferric ion (2+). To transform iron from 3+ to 2+ a reducing agent is needed, and the acid secreted in the stomach fulfills this role in man. In old age this acid production declines significantly (Jacobs and Owen, 1969), again lowering bio-availability.

### **Absorption**

Iron absorption is highest in the proximal part of the duodenum but can take place anywhere in the small intestine. Haem iron is absorbed via a pathway different from non-haem iron, and has been shown to bind to a specific haem receptor (Grasbeck et al. 1979,1982). From here it rapidly enters the intestinal epithelial cell in microendocytic vesicles, where it is split by haem-oxydase into free iron and a tetrapyrrole. This splitting process seems to be the rate-limiting step in haem iron absorption (Wheby and Spyker, 1981). Haem in itself seems to promote the absorption of inorganic iron (Lynch et al. 1982;



Turnlund et al. 1981), although large doses of either iron have been seen to inhibit the uptake of the other (Hallberg et al. 1979). When the amount of iron in the food is progressively increased, a linear increase in the amount of absorbed iron is found as the percentage of iron that is absorbed stays constant (Hallberg et al. 1979; Bezwoda et al. 1983). The mechanism of these processes is unknown, but herein lies the inherent risk for iron overload when the iron content of the food is above physiological levels. Added to this is the fact that inhibition of absorption of haem iron is less pronounced at higher iron-storage levels than the inhibition of non-haem iron (Lynch et al. 1989). Iron overload will therefore more readily occur in societies with a high meat intake, and the aged are less likely to load iron via this pathway. After cleavage the haem iron enters the same intracellular pool as the non-haem iron and is transferred across the basal surface of the mucosal cell to the portal circulation.

With ageing a lowered absorption has been documented (Marcus and Freedman, 1985; Lynch et al. 1982; Bunker et al. 1984). Some speculation exists about the possible mechanisms:

1. Wear-and-tear of the intestinal mucosa with advancing years leads to a decline in the number of villi and villous as well as mucosal atrophy, resulting in lower concentrations of enzymes and a decrease in the available absorptive surface (Knook and Goedhard, 1983).
2. As iron losses are negligible in older persons under normal circumstances, absorption could diminish due to saturation of stores. The intestinal mucosa regulates iron-uptake according to the saturation of the iron-storage compartment via a negative feedback-

loop, and it has repeatedly been found that iron stores increase with age (Benzie, 1963; Sturdeon and Shoden, 1971; Lynch et al. 1982; Lipschitz et al. 1984) thus lowering absorption. How the message is received by the mucosal cells is not known; the mechanism that leads to increased uptake when there is a surplus of iron in the intestinal lumen available in spite of saturated iron stores is also unclear. There appears to be controversy about the change in ferrous iron absorption with age, since this might not change at all (Marx, 1979).

## **Excretion**

Iron is excreted via stool and urine and can be lost with sweating. Excretion does not appear to change with ageing (Lynch, 1982; Bunker et al. 1984; Marcus and Freedman 1985; Milman et al. 1986), but it should be noted that measuring excretion is extremely difficult.

### **1.4 Regulation of iron metabolism**

In the body there are Fe-receptor cells, such as RBC-precursor cells, duplicating and growing cells and hepatocytes, as well as Fe-donor cells (e.g. macrophages, mucosal cells of the duodenum) and also the hepatocytes. In addition, hepatocytes are able to scavenge all forms of non-transferrin-bound iron (NTBI) from plasma and deliver Fe back to the plasma in a form that is easily bound to apoferritin.

When iron is absorbed from food, it is probably transported by the divalent metal transporter (DMT1), also known as the natural resistance-associated macrophage protein-2

(NRAMP2). This transmembrane H<sup>+</sup>-coupled transporter of NTBI is localised in the enterocytes at the apical and lateral aspects of duodenal villi (Wood and Han, 1998; Trinder et al. 2000). Northern blot analysis indicated that mRNA transcripts are expressed at low levels in normal tissue (Vidal et al. 1995). In iron-overload conditions such as HH, DMT1 is found in high concentrations on the plasma membrane of hepatocytes (Trinder et al. 2000) whilst m-RNA concentration may be increased 10-100 times in duodenal villus enterocytes (Han et al. 1999), reflecting the high uptake of NTBI in these states. How the iron is handled intracellularly in the labile intracellular pool of iron (LIP) is not known (Atkinson and Barton, 1999), but as the LIP contains redox-active iron that may cause oxidative stress, most of it should be sequestered by molecules such as ferritin to attenuate this oxidative stress (Ponka, 1999). What is known, however, is that intracellular Fe-homeostasis is influenced by a regulatory system, where two forms of iron regulatory protein (IRP) are found in mammalian cells (Ponka, 1999): IRP1, a cytosolic protein homologous to mitochondrial aconitase and IRP2, also found in the cytosol. These IRP's bind to iron responsive elements (IRE's) on mRNA for proteins that are involved in Fe-uptake, storage and transport, of which the ferritin-mRNA translation and the post-transcriptional regulation of the TfR-mRNA are studied best (Hentze and Kühn, 1996; Ponka, 1999). If intracellular Fe-concentration is low, IRP1 will bind on IRE's in TfR-mRNA to stabilise it, and to IRE's on Fe-mRNA to inhibit its translocation. IRP1 is regulated by Fe-concentration, downregulated by nitric oxide (NO) and upregulated by INF- $\gamma$ ; IRP2 is upregulated by NO<sup>+</sup> and downregulated by INF- $\gamma$  (Hentze and Kühn, 1996). IRP1 has been found to combine reversibly with an iron-sulphur-complex, containing 4 Fe and 4 S atoms, which is called the iron-sulphur-switch. It is a prosthetic

group, indispensable for Fe-containing enzymes in the cell and it plays probably an important role in the binding of IRP1 to IRE's (Hentze and Kühn, 1996). Absence of some of these cluster-proteins has been found in Friedreich's Ataxia and certain mitochondrial myopathies, and it is postulated that the cells that lack these clusters are targets of oxidative destruction in these diseases (Gerlach et al. 1994). The binding of IRP1 to the clusters is influenced by Fe-levels and by hydrogen peroxide: a mediator of oxidative stress (Hentze and Kühn, 1996). Furthermore, some animals have been found to lack IRP2 and they develop progressive neurodegenerative disease due to Fe-accumulation in certain cells in their central nervous system (CNS) (Gerlach et al. 1994).

At the basal membrane of crypt cells in the proximal small intestine, the transferrin-receptor (TfR) is found, which is known to bind transferrin (Tf) extracellularly. When Tf binds to TfR, a signal-cascade is activated, mediated by PI3-kinase, activating RAC-channels (Recceptor Activated Calcium-channels) so that  $Ca^{++}$  enters the cell. Raised intracellular  $Ca^{++}$  promotes secretion of Tf and intracellular Fe into the interstitial space, where the Fe is to be bound to Tf to be transported into the portal circulation (Ponka, 1999). Some iron has also been seen to leave the cells vesicle-bound via exocytosis (possibly ferrous-iron, which is stored in the cell in ferrochelatin, not in ferritin (Wood and Han, 1998). If the TfR binds Fe-loaded-transferrin from serum, the transferrin is internalised, sent to the endosomes where Fe is released and the Tf returns to the basal membrane as apotransferrin to be secreted again (Wood and Han, 1998; Ponka, 1999). Another protein that may influence the efflux of iron from intestinal cells is hephaestin, a glycosylated integral membrane protein, present in duodenal cells, probably at the basal

membrane. It seems to be the intracellular Fe-concentration in the cells of the intestinal mucosa however, that determines apical iron-uptake.

At the TfR-site, the normal HFE-protein binds non-covalently to the receptor, suggesting a role in the uptake of Tf-bound Fe by these cells. The C282Y-mutation, shown to cause HH (Feder et al. 1996) in the majority of affected patients worldwide, disrupts a critical disulfide bond in the  $\alpha 3$  extracellular loop of the protein (Parkkila et al. 1997), which is required for the binding of the HFE protein to the TfR. At the level of the intestinal cell, the abnormal HFE protein acts as a competitive inhibitor of Tf, thus decreasing the affinity of TfR for Tf (Han et al. 1999), while the efflux of iron from the mucosal cell remains identical as do the receptor-distribution and receptor-internalisation. This will lead to an abnormally low intracellular iron concentration and these cells have been found to behave as Fe-deficient, with a resulting up-regulation of DMT1. In monocytes and macrophages, the mutated HFE-protein cannot be presented at the cells' surface as it cannot properly interact with  $\beta 2$ -microglobulin, necessary for surface presentation, and this seems to lead to lack of retention of Tf-bound Fe by these cells (Ponka and Lok, 1999). Regarding the liver in Fe-overload states, the TfR on the hepatocytes is markedly down-regulated but iron is taken up from diferric-saturated transferrin and from NTBI sources (Bonkovsky, 1991). Ceruloplasmin supposedly also plays a role in the efflux of Fe from liver cells. This mechanism, or that whereby haptoglobin (a Hb-binding plasma protein) may assist in hepatic uptake of Hb (Bonkovsky, 1991) has not yet been elucidated.

Cells can also accumulate iron via NTBI (Wood and Han, 1998), although ferric-iron needs to be reduced to ferrous-iron before any transport can take place. NTBI is supposed to arise when there is such an excess of Fe available that the Tf-capacity is exceeded; the NTBI then is deposited in parenchymal cells leading to Fe-toxicity via hydrogen peroxide-mediated damage to membranes inside the cell. There exist a variety of other Fe-transporting proteins such as lactoferrin and the novel melanotransferrin (markedly raised in AD sufferers), which has a high level of sequence homology to both Tf and lactoferrin.

In conclusion, a tremendous improvement in our knowledge regarding the mechanisms of iron uptake, cell-trafficking, transport and storage would be required to understand the processes involved in the handling of this important ion in the human body.

### **1.5 Determination of iron status**

Even though there are a variety of iron-related tests with well-defined reference ranges for age and sex, the fact that each test reflects a different aspect of iron metabolism means that there is no single test that can define iron status or diagnose iron deficiency with a high degree of certainty. Currently, iron deficiency is based on two or more abnormal tests out of three tests utilised. In some cases the term "impaired iron status" is preferred, because the combination of abnormal tests such as low transferrin saturation and elevated protoporphyrin can be the result of metabolic disturbances related to inflammation rather than nutritional iron deficiency. Measurement of the serum iron concentration alone provides little useful clinical information because of the considerable variation from hour to hour and day to day in normal individuals. Low serum ferritin is specific for iron

deficiency as there are no conditions that cause falsely low serum ferritin values. A raised serum ferritin is probably a highly inaccurate parameter of iron status, particularly in the elderly, due to the many co-morbidities that increase the ferritin level. Serum transferrin receptors are detectable in the circulation and appear to reflect the level of bone marrow synthesis; this test may be useful in diagnosing iron deficiency when the individual has not yet developed anaemia, but the determination is assay-dependent and further evaluation of its usefulness is needed (Chua et al. 1999). These aspects emphasise the importance of molecular studies aimed at the development of a comprehensive DNA-based assay for determination of the genetic elements that influence iron-status and consequently the risk-assessment of iron-overload and -deficiency conditions.

## **1.6 Iron status in old age**

The iron status of older persons has fascinated clinicians for almost a century. Anaemia due to iron deficiency - by far the commonest cause of a low haemoglobin in old age - is often the presenting symptom of some major underlying disease. Moreover, a clinical diagnosis is often difficult to make as many elderly only present when their anaemia is already quite severe and symptoms have been wrongly accepted as one of the inevitable evils of getting old. Part of the late presentation can also be explained by adjustments in the oxygen-dissociation curve in anaemia, which causes the 2,3-diphospho-glycerate inside the red cells to increase and thus the oxygen-dissociation curve to shift to the right. The result is an increase in oxygen dissociation at a given oxygen pressure in tissue- capillaries, which retards the development of oxygen-deficit in tissues and thus delays the appearance of symptoms. On the other hand, in the aged anaemia can present in a much more life-

threatening way than in adulthood, e.g. when it precipitates overt heart failure with lung edema or causes delirium in an individual suffering from cognitive decline. During the last two decades of the previous millennium, interest in the iron status of the elderly has risen as a result of the discovery that hereditary haemochromatosis (HH) can present for the first time in individuals over 65 years of age, and that elderly individuals who are homozygous for the most common disease-causing mutation (C282Y) (Feder et al. 1996) do not necessarily have any signs or symptoms of the disease or express their mutation as iron overload (Willis et al. 1999). These findings have led researchers to question the lack of recognition of the disorder by clinicians, the reliability of the tests used in the laboratory to determine iron status and the necessity of population screening for disease-causing mutations. Some of these questions will be dealt with in this thesis.

### **1.7 Iron overload in the elderly**

Iron bio-availability and absorption are decreased in older individuals for various reasons, whereas the stores in liver and bone-marrow are increased. Also, in the elderly iron uptake increases with an increase in supply via food intake, in spite of saturated stores, so that a propensity for iron-overload exists in aged individuals. The mechanisms underlying this phenomenon are unknown.

#### **Role of genetic factors**

Hereditary haemochromatosis (HH) is a common, treatable genetic disease caused by abnormal metabolism of iron. Excess iron is stored in organs and tissues where it causes injuries that may result in a variety of disorders including heart disease, diabetes, arthritis,



cancer, cirrhosis, impotence and sterility. Advancing age is significantly associated with arthritis, cardiac disease and diabetes in patients with HH. Many of the “non-specific” HH symptoms such as fatigue and arthritis are common in the ageing general population without HH, and this may contribute to a lack of recognition of the disease in the elderly.

HH is transmitted as an autosomal recessive disease, wherein affected individuals inherit two copies of the defective gene. The estimated carrier frequency of HH is between 1 in 8 and 1 in 10 in most populations of European descent, resulting in a double dose of the gene in about 1/300 individuals. Previous studies have shown that heterozygosity for HH is associated with an increased risk of colorectal neoplasia, diabetes, haematological malignancies and gastric cancer. Molecular-genetic research has resulted in the identification of a common gene mutation (C282Y) associated with HH in more than 80% of affected patients world-wide (Feder et al. 1996). Studies performed in the South African population have shown that mutation C282Y also occurs at a high frequency (~80%) in affected cases (de Villiers et al. 1999a). The carrier frequency of mutation C282Y was found to be 1 in 6 among South Africans of European descent, implying that approximately 1 in 115 individuals may be homozygous for this mutation. Identification of healthy blood donors with the disease genotype has highlighted the need for further genotype/phenotype correlation studies in South Africa, since probably only a small number of individuals homozygous for the potentially lethal C282Y mutation are presenting clinically. Underdiagnosis of HH can partly be ascribed to the non-specific nature of the presenting features and the wide variety of disorders associated with this disease.

### **Gene-gene interaction**

Little is currently known about the genetic factors that may influence iron stores in humans. Extensive analysis of a large community sample of adult male and female twins recruited from the Australian Twin Registry (Whitfield et al. 2000) has indicated highly significant effects of other as-yet-unidentified genes on iron stores, in addition to the HFE genotype (Figure 4). The remaining differences between monozygotic and dizygotic correlations, after allowing for HFE effects, showed that other genes account for ~45% of the phenotypic variation in serum ferritin and for 30% of the variation in transferrin saturation. Elucidation of the influence of genes affecting iron stores may provide a route to understanding genetic differences in susceptibility to a range of common diseases, since heterozygosity for HH or mutation C282Y in the HFE gene has been implicated as a causative/modifier locus or potential genetic marker for disease susceptibility/ progression in various conditions. These include cancer (Nelson et al. 1995), chronic liver disease (George et al. 1998, 1999), porphyria (Roberts et al. 1997; de Villiers et al. 1999b), inherited anaemias (Yaouanq et al. 1997), cystic fibrosis (Rohlfis et al. 1998), diabetes (Kwan et al. 1998), cardiovascular disease (Tuomainen et al. 1999; Roest et al. 1999), Alzheimer disease (Moalem et al. 2000), and possibly also HIV/AIDS progression (Nielsen et al. 1999). Even in conditions where iron may not have a primary action, its potential secondary role cannot be negated. HH therefore is an important paradigm for medical genetics because it offers an opportunity to explore the complexity of gene-gene and gene-environment interactions. Identification of elderly subjects with genotypes compatible with HH would provide a valuable source of material to identify genes that

phenotypic expression of HH, or may underlie a tendency to iron deficiency in the general population.

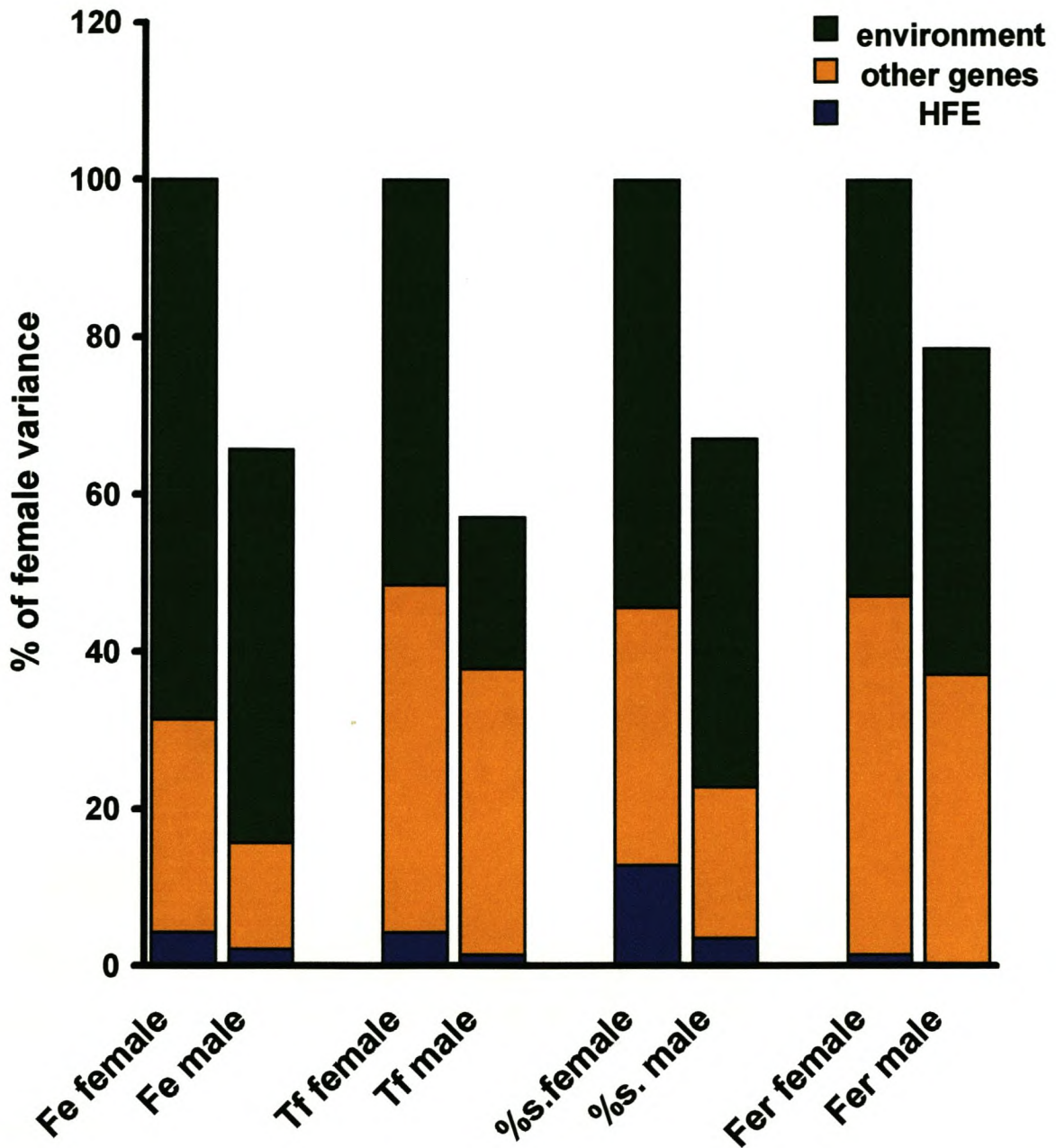


Figure 4. Proportion of variance ascribed to HFE gene variation, the additive effects of other genes and the nonshared environmental effects in men and women. Total variance in women has been standardised to 100 for each variable (adapted from Whitfield et al. 2000).

## 1.8 References

Atkinson PGP, Barton CH. High level expression of Nramp1<sup>G169</sup> in RAW264.7 cell transfectants: analysis of intracellular iron transport. *Immunology* 1999; 96: 656-662.

Benzie RD. The influence of age upon the iron content of bone marrow. *Lancet* 1963; 1: 1074-1075.

Bezwoda WR, Bothwell TH, Charlton RW, Torrance JD, MacPhail AP, Derman DP, Mayet F. The relative dietary importance of haem and non-haem iron. *S Afr Med J* 1983; 64: 552-556.

Birgegard G, Hällgren R, Venge P, Wide L. Serum ferritin during inflammation: a study on myocardial infarction. *Acta Med Scand* 1979; 206: 361-366.

Bonazza M. Ferritinemia in ovarian malignant tumour. *Eur J Gynaecol Oncol* 1983; 4: 18-21.

Bonkovsky H. Iron and the liver. *Am J Med Sci* 1991; 301: 32-43

Bothwell TA, Charlton JD, Cook JD, Finch CA. In: *Iron metabolism in man*. Blackwell Scientific, Oxford, 1979.

Bunker VW, Lawson MS, Clayton BE. Uptake and excretion of iron by healthy elderly subjects. *J Clin Pathol* 1984; 37: 1353-1357.

Charlton RW, Hawkins DM, Mavor WO, Bothwell TH. Hepatic storage iron concentration in different population groups. *Am J Clin Nutr* 1970; 23: 358-371.

Chua E, Clague JE, Sharma AK, Horan MA, Lombard M. Serum transferrin receptor assay in iron deficiency anaemia and anaemia of chronic disease in the elderly. *Q J M* 1999; 92: 587-594.

Conrad ME, Cortell S, Williams HL, Foy AI. Polymerization and intraluminal factors in the absorption of haemoglobin iron. *J Lab Clin Med* 1966; 68: 659-668.

Dandekar T, Stripecke R, Gray NK, Goossen B, Constable A, Johansson HE, Hentze MW. Identification of a novel iron-responsive element in murine and human erythroid delta-aminolevulinic acid synthase mRNA. *EMBO J* 1991; 10: 1903-1909.

Denham MJ, Himsworth RL. Thyroid function tests. In: *Clinical Biochemistry of the Elderly*. 1984, Malcolm Hodgkinson, Churchill Livingstone. London.

de Villiers JNP, Hillermann R, de Jong G, Langenhoven E, Rossouw H, Marx MP, Kotze, MJ. High prevalence of the Cys282Tyr HFE mutation facilitates an improved diagnostic service for hereditary haemochromatosis in South Africa. *S Afr Med J* 1999a; 89: 279-282.

de Villiers JNP, Hillermann R, Loubser L, Kotze MJ. Spectrum of mutations in the HFE gene implicated in hemochromatosis and porphyria. *Hum Mol Genet* 1999b; 8: 1517-1522.

Dybkaer R, Lauritzen M, Krakauer R. Relative reference values for clinical chemical and haematological quantities in "healthy" elderly people. *Acta Med Scand* 1981; 209: 1-9.

Elwood PC, Shinton NK, Wilson CID, Sweetnam P, Frazer AC. Hemoglobin, vitamin B12 and folate levels in the elderly. *Br J Haematol* 1971; 21: 557-563.

Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nature Genet* 1996; 13: 399-408.

Franzini C, Berlusconi A, Favarelli C, Brambilla S. Low frequency of elevated serum transferrin saturation in elderly subjects. *Clin Chim Acta* 2000; 298: 181-186.

George DK, Goldwurm S, Macdonald GA, Cowley LL, Walker NI, Ward PJ, Jazwinska EC, Powell LW. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1998; 114: 311-318.

George DK, Powell LW, Losowsky MS. The haemochromatosis gene: a co-factor for chronic liver diseases? *J Gastroenterol Hepatol* 1999; 14: 745-749.

Gerlach M, Ben-Shachar D, Riederer P, Youdim MBH. Altered brain metabolism of iron as a cause of neurodegenerative diseases? *J Neurochem* 1994; 63: 793-807.

Grail A, Hancock BW, Harrison PM. Serum ferritin in normal individuals and in patients with malignant lymphoma and chronic renal failure measured with seven different commercial immunoassay techniques. *J Clin Pathol* 1982; 35: 1204-1212.

Grasbeck R, Kuovonen I, Lundberg M, Tenhunen R. An intestinal receptor for heme. *Scand J Haematol* 1979; 23: 5-9.

Grasbeck R, Majuri R, Kuovonen I, Tenhunen R. Spectral and other studies on the intestinal haem receptor of the pig. *Biochim Biophys Acta* 1982; 700: 137-142.

Hallberg L, Bjorn-Rasmussen E, Howard L, Rossander L. Dietary haem iron absorption. A discussion of possible mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption. *Scand J Gastroenterol* 1979; 14: 769-779.

Han O, Fleet JC, Wood RJ. Reciprocal regulation of HFE and Nramp2 gene expression by iron in human intestinal cells. *J Nutr* 1999; 129: 98-104

Hentze MW, Kühn LC. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. Review. Proc Natl Acad Sci USA 1996; 93: 8175-8182

Hobson W, Blackburn EK. Haemoglobin levels in a group of elderly persons living at home alone or with spouse. Br Med J 1953; 1: 647-649.

Jacobs AM, Owen GM. The effect of age on iron absorption. J Gerontol 1969; 24: 95-96.

Kalchthaler T, Rigor ME. Anaemia in institutionalised elderly patients. J Am Geriatr Soc 1980; 28: 108-113.

Kelly A, Munan L. Haematologic profile of natural populations: Red cell parameters. Br J Haematol 1977; 35: 153-160.

Knook DL, Goedhard WJA. Voeding van geriatrie patienten—enkele klinische problemen. In: Voeding en de ouder wordende mens, Gerontologische reeks, 1983; The Netherlands, Stafleu, pp. 10.

Kruger A. The limits of normality in elderly patients. Baillieres Clin Haematol. 1987; 1: 271-289.



Kwan T, Leber B, Ahuja S, Carter R, Gerstein HC. Patients with type 2 diabetes have a high frequency of the C282Y mutation of the hemochromatosis gene. *Clin Invest Med* 1998; 21: 251-257.

Lawson DM, Artymiuk PJ, Yewdall SJ, Smith JMA, LivingstoneJC, Treffry A, Luzaggo A, Levi S, Arosio P, Cesareni G, Thomas CD, Shaw WV, Harrison PM. Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts. *Nature* 1991; 349: 541-544.

Lipschitz DA, Cook JD, Finch CA. A clinical evaluation of serum ferritin. *N Engl J Med* 1974; 290: 1213-1215.

Lipschitz DA, Mitchell CO, Thompson C. The anaemia of senescence. *Am J Hematol* 1981; 11: 47-54.

Lipschitz DA, Udupa KB, Milton KY, Thompson CO. Effect of age on haematopoiesis in man. *Blood* 1984; 63: 502-509.

Lundin L, Hällgren R, Birgegård G, Wide L. Serum ferritin in alcoholics and the relation to liver damage, iron state and erythropoietic activity. *Acta Med Scand* 1981; 209:327-331.

Lynch SR, Finch CA, Monsen ER, Cook JD. Iron status of elderly Americans. *Am J Clin Nutr* 1982; 36: 1032-1045.

Lynch SR, Dassenko SA, Morck TA, Beard JL, Cook JD. Soy protein products and heme iron absorption in humans. *Am J Clin Nutr* 1985; 41: 13-20.

Lynch SR, Skikne BS, Cook JD. Food iron absorption in idiopathic haemochromatosis. *Blood* 1989; 74: 2187-2193.

Malczewska J, Blach W, Stupnicki R. The effects of physical exercise on the concentrations of ferritin and transferrin receptor in plasma of female judoists. *Int J Sports Med* 2000; 21: 175-179.

Marcus DL, Freedman ML. Clinical disorders of iron metabolism in the elderly. *Clinics in Geriatric Medicine* 1985; 4: 729-745.

Marx JJM. Normal iron absorption and decreased red cell iron uptake in the aged. *Blood* 1979; 53: 204-211.

Miller I. Normal hematologic standards in the aged. *J Lab Clin Med* 1939; 24: 1172-1176.

Milman N, Andersen HC, Strandberg Pedersen N. Serum ferritin and iron status in "healthy" elderly individuals. *Scand J Clin Lab Invest* 1986; 46: 19-26.

Milne JS, Williamson J. Hemoglobin, hematocrit, leukocyte count and blood grouping in older people. *Geriatrics* 1972; 27: 118-126.

Moalem S, Percy ME, Andrews DF, Kruck TPA, Wong S, Dalton AJ, Mehta P, Fedor B, Warren AC. Are hereditary hemochromatosis mutations involved in Alzheimer disease? *Am J Med Genet* 2000; 93: 58-66.

Myers AM. The hemoglobin level of fit elderly people. *Lancet* 1968; 2: 261-263.

Nelson RL, Davis FG, Persky V, Becker E. Risk of neoplastic and other diseases among people with heterozygosity for hereditary hemochromatosis. *Cancer* 1995; 76: 875-879.

Nielsen P, Degen O, Brummer J, Gabbe EE. Long-term survival in a patient with AIDS and hereditary haemochromatosis. *Eur J Haematol* 1999; 63: 202-204.

Österlind PO, AlaFuzoff I, Löfgren AC, Marklund S, Nystrom L, Sandman PO, Steen B, Winblad BL. Blood components in an elderly population. *Gerontology* 1984; 30: 247-252.

Parkkila S, Waheed A, Britton RS, Bacon BR, Zhou XY, Tomatsu S, Fleming RE, Sly WS. Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary haemochromatosis. *Proc Natl Acad Sci USA* 1997; 94: 13198-13202.

Ponka P. Cellular iron metabolism. *Kidney Int* 1999; 55 suppl 69: S2-S11

Ponka P, Lok CN. The transferrin receptor: role in health and disease. *IJBCB* 1999; 31:1111-1137

Powers JS, Lichtenstein MJ, Collins JC, Krantz SB, Greme HL, Buchhole T. Serum erythropoietin in healthy older persons (letter). *JAGS* 1989; 37: 388-389.

Roberts AG, Whatley SD, Morgan RR, Worwood M, Elder GH, Roth MP, Giraldo P. Increased frequency of the haemochromatosis Cys282Tyr mutation in sporadic porphyria cutanea tarda. *Lancet* 1997; 349: 321-323.

Roest M, van der Schouw YT, de Valk B, Marx JJ, Tempelman MJ, de Groot PG, Sixma JJ, Banga JD. Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation* 1999; 100:1268-1273.

Rohlfes EM, Shaheen NJ, Silverman LM. Is the hemochromatosis gene a modifier locus for cystic fibrosis? *Genet Test* 1998; 2: 85-88.

Smith JS, Whitelaw DM. Hematologic values in aged men. *Can Med Assoc J* 1971; 105: 816-818.

Sturgeon P, Shoden A. Total liver storage iron in normal populations of the USA. *Am J Clin Nutr* 1971; 24: 469-474.

Tuomainen TP, Kontula K, Nyssonen K, Lakka TA, Helio T, Salonen JT. Increased risk of acute myocardial infarction in carriers of the hemochromatosis gene Cys282Tyr mutation: a prospective cohort study in men in eastern Finland. *Circulation* 1999; 100: 1274-1279.

Trinder D, Oates PS, Thomas C, Sadleir J, Morgan EH. Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. *Gut* 2000; 46: 270-276

Turnlund J, Costa F, Margen S. Zinc, copper and iron balance in elderly men. *Am J Clin Nutr* 1981; 34: 2641-2647.

Vidal S, Belouchi AM, Cellier M, Beatty B, Gros P. Cloning and characterization of a second human NRAMP gene on chromosome 12q13. *Mammalian Genome* 1995; 6: 224-230.

Willis G, Wimperis JZ, Smith KC, Fellows IW, Jennings BA. Hemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet* 1999; 354: 221-222.

Wheby MS, Spyker DA. Hemoglobin iron absorption kinetics in the iron deficient dog. *Am J Clin Nutr* 1981; 34: 1686-1693.

Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL, Martin NG. Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000; 66: 1246-1258.

Wood RJ, Han O. Recently identified molecular aspects of intestinal iron absorption. *J Nutr* 1998; 128: 1841-1844.

Yaouanq J, Gorsbois B, Jouanelle AM, Goasguen J, Leblay R. Haemochromatosis Cys282Tyr mutation in pyridoxine-responsive sideroblastic anemia. *Lancet* 1997; 349: 1475-1476.

Yip R, Johnson C, Dallman PR. Age-related changes in laboratory values in the diagnosis of anemia and iron deficiency. *Am J Clin Nutr* 1984; 39: 427-436.

Zaino EC. Blood counts in the nonagenarian. *NY State J Med* 1981; 81: 1199-1200.

Zauber NP, Zauber AG. Hematologic data of healthy very old people. *JAMA* 1987; 257: 2181-2184.

Zilva JF, Pannall PR, Mayne PD. Iron metabolism. From: *Clinical Chemistry in Diagnosis and Treatment*. 1987, Edward Arnold, London.

## **CHAPTER 2**

### **Objectives of the study**

Heterozygosity for the common autosomal recessive disease, hereditary haemochromatosis (HH), is associated with an increased risk of colorectal neoplasia, haematological malignancy and gastric cancer in the general population. The high proportion of patients with HH who are homozygous for mutation C282Y provides us with a potentially powerful tool for population screening. Unlike serum iron and ferritin levels, which have been used previously for screening, the genotype is not affected by factors such as liver disease and inflammation that can modify the levels of serum iron and ferritin both in haemochromatosis and normal subjects. As in the case of other genetic diseases, one would predict that the phenotype of C282Y homozygotes would vary markedly. Some would probably never develop clinical disease. Yet, the treatment of haemochromatosis by phlebotomy is so simple and effective that it would appear prudent to consider a therapeutic phlebotomy program in individuals shown to have the homozygous genotype, and/or to monitor their iron and ferritin levels. When the disease-causing mutation is identified in a family, all relatives can be screened and diagnosed with great accuracy.

We hypothesise that genetic variation in the HFE gene may influence serum iron levels in the elderly and that mutation C282Y, and/or other mutation(s) in the HFE gene, may affect life expectancy adversely due to increased cancer risk. The specific objectives of the study were as follows:



**1. Determine the frequency of the HH mutation C282Y in the elderly for comparison with the frequency found in young adults from the same population group, in order to determine whether this founder-type mutation affects life expectancy adversely.**

In a study of adults of the Afrikaner population-group a relatively high carrier frequency for C282Y (1/6) has been found compared with other populations of Northern European descent (1/8 to 1/10). As males with HH usually become symptomatic in their early fifties and then are either diagnosed and treated, or die as a consequence of organ damage, we were interested to know whether any undiagnosed C282Y homozygotes would survive beyond the age of 70 years.

**2. Compare the clinical features of elderly subjects with an HH genotype with those in younger adults.**

The clinical picture of HH consists of a constellation of one or more non-specific disorders affecting different organs (e.g. type II diabetes mellitus, heart failure, arthritis), which have a high background frequency in the aged population of Northern European descent. Since both ischaemic heart disease and arthritis are highly prevalent in the general Afrikaner population many cases could potentially be missed by unwary physicians. From a preventive perspective it could be highly cost-effective to increase general awareness if the frequency of HH proves to be as high in the elderly as in their younger counterparts.

**3. Perform genotype/phenotype correlation studies to assess whether the C282Y mutation is associated with increased iron levels and/or cancer risk in the elderly.**

In previous studies associations have been reported between possession of one or two C282Y mutations and a raised frequency of colorectal, gastric and haematological malignancies. Most of these study groups have not been randomly selected (e.g. hospital patients, clinic-attenders, diabetics) and selection-bias could have influenced the results. The background frequency for colorectal neoplasms is especially high in the elderly and possible early detection or even prevention by screening for HH might be worthwhile in this age group.

**4. Identify elderly subjects who are homozygous or compound heterozygotes for mutations in the HFE gene for future gene-gene interaction studies, aimed at the possible identification of modifier loci that may cause iron deficiency in the general population.**

Survival of individuals with the HH genotype is dependent on the degree of expression of the relevant mutation(s). It has become clear that some C282Y homozygotes have no iron-loading at all, and therefore the frequency of this genotype in the elderly could be an indication of the frequency of homo- and heterozygote “non-loaders”, based on the assumption that iron-loaders would not survive into old age without being diagnosed. In these individuals, modifier loci might be identified that prevent iron loading via gene-gene interaction.

The project could lead to a better definition of clinical criteria for diagnosis of HH and serve to determine the incidence of clinical/ biochemical misdiagnosis of this common iron overload disease.

## **CHAPTER 3**

### **Results and Discussion**

**HAEMOCHROMATOSIS MUTATION ANALYSIS AND CLINICAL  
CORRELATION IN THE ELDERLY**

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**ABSTRACT**

The frequency of the hereditary haemochromatosis (HH) mutation C282Y has been determined in 197 elderly individuals, in order to determine whether this founder-type mutation affects life expectancy adversely in the South African Afrikaner population of European descent. The carrier frequency of mutation C282Y was approximately 1/8, which is slightly lower than the 1/6 previously found in younger adults from the same population. Subsequent screening of 24 C282Y heterozygotes for mutation H63D (with a relatively mild phenotypic effect), led to the identification of two compound heterozygotes for these mutations. The single C282Y homozygote reported a strong family history of cancer and died of colorectal carcinoma approximately six months prior to completion of the investigation. The serum ferritin concentrations of the elderly subjects with HH genotypes and the C282Y heterozygotes were lower than expected, which raises the possibility that mutation-positive cases with high levels had died at an earlier age due to adverse effects of iron surplus. This was substantiated by the finding that all the individuals with a HH genotype were females, suggesting a negative selection against males. Elderly subjects with the HH genotype in combination with normal iron status can be expected to harbour modifier genes that may lower serum iron levels with concurrent protection against organ damage. This observation warrants further investigation into the role of gene-gene and gene-environment interaction in clinical expression of HH.

## INTRODUCTION

Hereditary haemochromatosis (HH) is an autosomal recessive disease characterised by iron overload, which may damage parenchymal organs such as the liver, pancreas and heart, leading to organ failure. Patients with this condition may also present with arthritis, hypogonadism, pigmentation of the skin, and possibly also neurologic abnormalities (Nielsen et al. 1995). Under-diagnosis of HH can partly be ascribed to the non-specific nature of the presenting features and the variety of disorders associated with this disease. Diagnosis is usually confirmed by measurement of serum transferrin saturation and ferritin levels, but both parameters are influenced by external factors thus decreasing their validity. Treatment of HH entails venesection or iron-chelation therapy. Although normalisation of body iron content may halt disease progression, normal life expectancy would only be possible through early detection prior to the onset of symptoms and regular blood donation.

Recent cloning of the HFE gene underlying HH now enables accurate disease diagnosis (Feder et al. 1996). Screening for disease-related mutations, including mutations H63D and C282Y shown to be responsible for the disease in more than 80% of cases worldwide, is particularly useful in pre-clinical testing. The carrier frequency of mutation C282Y was found to be 1/6 in the South African Afrikaner population (de Villiers et al. 1999a), implying that approximately 1/115 Afrikaners are homozygous for this mutation. This finding emphasised the need for genotype/phenotype correlation studies in South Africa, since not all individuals homozygous for the potentially lethal C282Y mutation are clinically recognisable (Milani and Kotze 1999).

Recently, Willis et al. (1999) reported the detection of several C282Y homozygotes in an elderly male population (4 out of 600 male volunteers). Lack of recognition of HH in the elderly may be due to the fact that many of the conditions (e.g. diabetes) and non-specific features such as fatigue and arthritis that are associated with HH, are also common in the general aged population without HH. In this study the frequency of mutation C282Y was determined in the elderly and compared with that in young adults, in order to assess whether this mutation affects life expectancy and quality of life adversely in the South-African Afrikaner population. Those individuals found to be heterozygous for mutation C282Y were subsequently screened for mutation H63D, in order to determine whether these mutations are associated with an increased risk of malignancy in the elderly. An association with malignancies of the stomach, colon, rectum and the haematological system has previously been reported in HH carriers (Nelson et al. 1995).

## **METHODS**

### **Study population**

The study population consisted of a sample of 197 volunteers (67 males, 130 females) over 70 years of age, from the Afrikaner community of South Africa. All the participants were Afrikaans-speaking with their ancestors having been in South Africa for at least three generations. The Afrikaner gene pool consists of 34.8% Dutch, 33.7% German, 13.2% French, 5.2% British and 13.1% other nationalities (Botha and Beighton 1983).

The study included South African participants from all parts of the country and various social and professional backgrounds. They either stayed alone or with their children, or lived in retirement- or nursing homes. Approximately one-third were healthy elderly living independently, one-third nursing-home residents with all sorts of complaints but not ill as such, and the remaining third consisted of hospitalised elderly with a variety of disorders not directly indicative of possible iron overload (e.g. hip fractures, benign prostate hypertrophy, type II diabetes, strokes, gynaecological problems, myocardial infarctions / angina, psychiatric disorders, peripheral vascular disease, etc.). Of the 201 elderly who were approached, 197 were readily available to take part in this study. All have been recruited, examined and had their specimens collected by the same physician, which largely excludes inter-rater variability. The four subjects who did not want to participate in the study suffered from hip-fractures (two subjects), ischaemic heart disease and a mild stroke. Since these cases comprise less than 2% of the study population no efforts were made to perform follow-up studies. Informed consent was obtained after the possible consequences of a positive test had been explained. A sample of 151 volunteers, including 49 medical students (< 30 years of age) and 102 subjects (<60 years of age) recruited from the Afrikaner population, served as a control group.

### **Biochemical and DNA analysis**

After completion of a questionnaire designed for the study and general physical examination, blood was collected for biochemical iron profile determination and genetic testing. The blood samples of only 178 of the 197 aged volunteers included in the study were suitable for biochemical analysis due to haemolysis of some specimens. Serum iron



was measured with a Beckman CX7 autoanalyser and transferrin with a Beckman Array Nephelometer. Transferrin saturation was subsequently calculated. Genomic DNA was extracted using a standard salting-out method (Miller et al. 1988), and amplified by the polymerase chain reaction (PCR) as previously described (de Villiers et al. 1999). Restriction enzyme analysis was performed to screen for mutations C282Y (*RsaI*) and H63D (*MboI*) (Feder et al. 1996; Roberts et al. 1997). All the samples were screened for mutation C282Y, while only those found to carry this mutation were screened for mutation H63D. Possible mistyping of C282Y homozygosity was excluded by *MseI* restriction enzyme analysis as previously described (de Villiers and Kotze 1999).

### **Statistical analysis**

Allele frequencies were determined by allele counting. Testing for significance of heterogeneity in mutation frequencies among the elderly and control groups was based on the Yates-corrected Chi-square test. For continuous variables groups were compared using the Wilcoxon Rank Sum test for two independent samples with highly unequal variances.

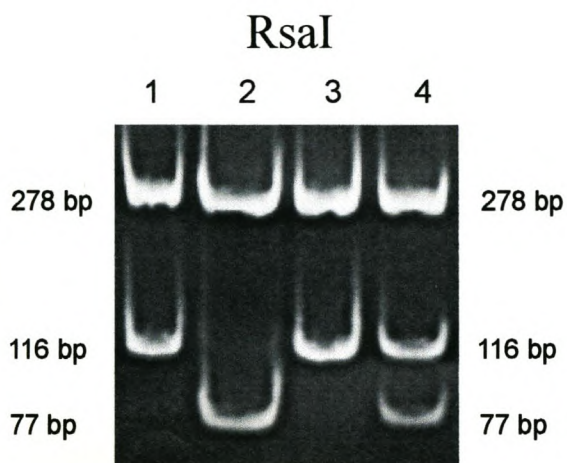
## **RESULTS AND DISCUSSION**

The patient group consisted of 67 males and 130 females, the expected ratio for this age group. *RsaI* restriction enzyme analysis (figure 1) performed in the study population identified one C282Y homozygous female. In table 1 the genotype distribution and allele frequencies of mutation C282Y in the elderly population are compared with those detected in the control group. The carrier frequency in the aged was calculated at approximately 1/8,

compared with 1/6 in those control individuals younger than 30 years of age. These figures were similar to those found in the general Afrikaner population previously studied by de Villiers et al. (1999a), and therefore the data obtained in the two control groups were pooled. There was no statistically significant difference in mutation frequencies between the elderly and control groups (table 1), or between males and females (data not shown). Subsequent screening of C282Y heterozygotes for mutation H63D identified two compound heterozygotes.

The characteristics of 24 C282Y heterozygotes and 1 homozygote identified in the elderly population are summarised in table 2. The 73-year old woman shown to be homozygous for mutation C282Y had a ferritin level of 249 ng/ml, which is well above the upper limit (119 ng/ml) for females, although much lower than that usually encountered (>600 ng/ml) in patients with clinically manifested HH. Two female H63D/C282Y compound heterozygotes presented with serum ferritin levels of 162 ng/ml and 242 ng/ml respectively, which are also above the reference range for females. The ferritin levels in the C282Y heterozygous group varied between 32 and 1136 ng/ml, with 14 (58%) having a normal level. In the C282Y-negative group the ferritin levels ranged from 10 to 3942 ng/ml, and 94 individuals (61.4%) had a normal level. The difference between the groups was not statistically significant and the wide range of serum ferritin levels within each group implies that this parameter of iron status represents an unreliable indicator of iron-stores in the body. It is well known that a variety of factors may influence serum ferritin levels, including any acute or chronic inflammatory disorder which will release ferritin from affected tissues (Marcus and Freedman 1985), exercise (Lipschitz et al. 1974;

Malczewska et al. 2000), alcohol use (Lundin et al. 1981), chronic renal failure (Grail et al. 1982), hepatic and ovarian malignancies (Bonazza, 1983) and myocardial infarction (Birgegard et al. 1979). Since most elderly subjects have multiple age-associated co-morbidities, serum ferritin may be considered an unreliable measure of body iron-status particularly in this age group.



**Figure 1.** Identification of mutation C282Y in elderly individuals using RsaI restriction enzyme analysis. Lanes: (1) DNA of a normal control individual, (2) DNA of a C282Y homozygote, (3) DNA of a C282Y-negative subject and (4) DNA of a C282Y heterozygote.

**Table 1.** Comparison of genotype distribution and allele frequencies of mutation C282Y in the elderly and control groups

	Controls* (n=151)	Elderly (n=197)
<i>Genotype</i>		
C282Y++	3 (2%)	1 (0.5%)
C282Y+-	22 (15%)#	26 (13%)#
C282Y--	126 (83%)	170 (86%)
<i>Allele</i>		
282-G (normal)	0.91	0.93
282-A (mutant)	0.09	0.07
Carrier frequency	16.9%	13%

\*102 controls from de Villiers et al. (1999)

#two subjects within each group were compound heterozygotes for mutations C282Y and H63D

In the control group younger than 30 years of age, the serum ferritin levels ranged from 3.9 to 330.4 ng/ml, all within normal limits. Transferrin saturation ranged from 9.5% (mutation-negative female) to 55.8% (carrier male). The serum ferritin levels in C282Y heterozygote males (mean 259.27 ng/ml) were significantly higher ( $P < 0.01$ ) than in the mutation-negative male controls (mean 94.89 ng/ml). This finding suggests that young males may be prone to iron loading in the heterozygous state. All female controls, including a 24-year old homozygote (24.7 ng/ml) and the four C282Y-heterozygotes (12.8-16.1 ng/ml) had relatively low ferritin levels. The transferrin saturation levels ranged between 11.9-45% in the C282Y heterozygotes, while the female homozygote control presented with a level of 41.5%.

**Table 2.** Characteristics of 1 C282Y homozygote and 24 C282Y heterozygotes identified in the elderly population

Age (years)	Gender	Serum Ferritin (F: 12-119ng/ml) (M: 29-396ng/ml)	Transferrin sat (F: 15-50%) (M: 20-50%)	Clinical features
<i>C282Y homozygote</i>				
73	F	249	ND	Type II DM, IHD Colorectal Ca
<i>C282Y heterozygotes</i>				
86	F	89	15.7	IHD, A, Dementia
74	F	323	16.3	DM, IHD, FHx:A
90	F	107	8.8	A
71	F	41	33.7	DM, A, FHx:DM,A
78	F	149	18.1	IHD, A, RCa
79	M	217	57.7	Dementia
76	F	238	30.0	FHx:DM,IHD,A
74	M	143	9.7	IHD, FHx:DM,IHD
81*	F	242	100	Dementia
86*	F	162	ND	IHD, A
71	F	143	9.8	IHD, A, FHx:IHD,Ca
70	F	32	ND	IHD, A, FHx:IHD
75	F	52	17.4	A, FHx:A
79	F	78	15.1	DM, IHD, FHx:IHD,Ca
81	F	319	16.3	DM, IHD, FHx:DM, IHD,A
76	F	44	11.3	IHD, A, FHx:IHD,Ca,A
72	F	231	9.6	IHD, Ca, FHx:DM,IHD,Ca
73	M	88	23.0	DM, IHD, A, prostCa, FHx:IHD,A,Ca
86	M	1136	63.6	A, FHx:IHD
71	F	157	15.7	IHD, A, FHx:Ca,IHD
71	F	84	36.5	DM, IHD, A, Ca FHx:DM,IHD,A,Ca
77	M	384	25.1	IHD, prostCa, A FHx:IHD, Ca
76	M	39	10.8	IHD, FHx:IHD, Ca
70	M	35	38.7	DM, IHD, A, FHx:IHD, Ca

\*compound heterozygotes for mutations C282Y and H63D

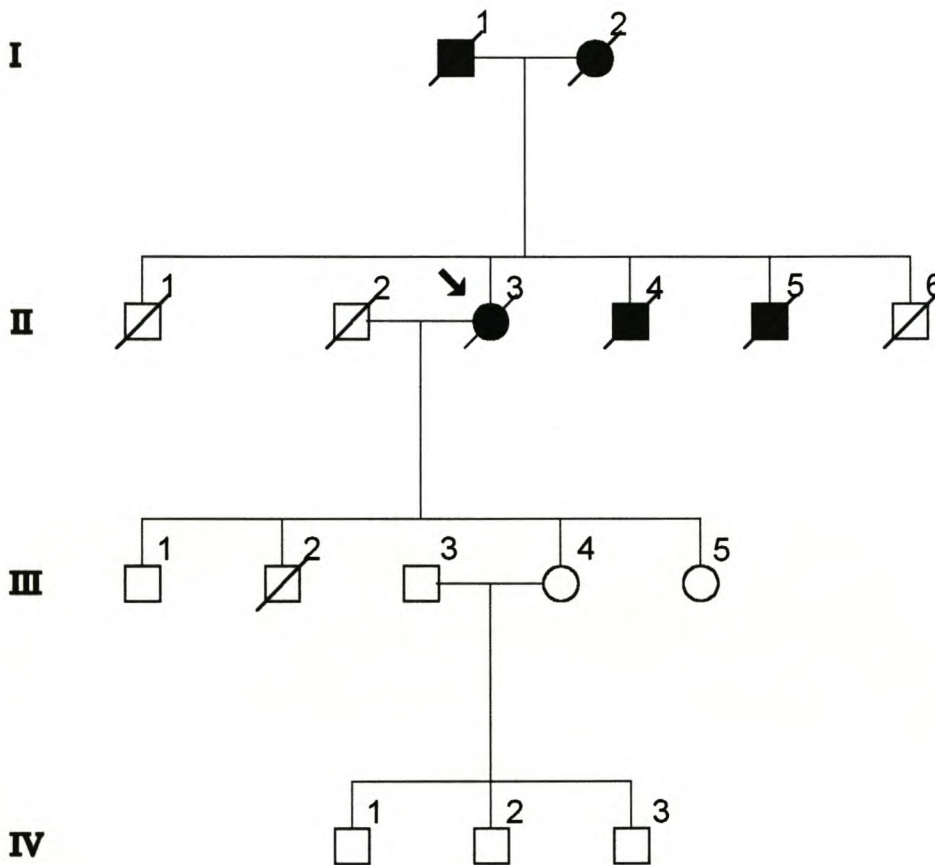
Reference ranges given in brackets for males (M) and females (F)

ND, not determined; IHD, ischaemic heart disease; DM, diabetes mellitus Type II; A, arthritis; FHx, family history; Ca, cancer (of any type)

The C282Y homozygote presented with type II diabetes mellitus, ischaemic heart disease and osteoporosis. Family follow-up is under way to determine whether the strong family history of malignancies (figure 2) reported by the index case (arrow) may be associated with HH. The C282Y homozygote died of colon cancer approximately six months prior to completion of the investigation; therefore a repeat sample could not be obtained to determine a full iron profile of this subject. The two females found to be compound heterozygotes for mutations C282Y and H63D suffer from ischaemic heart disease and dementia, respectively. Notably, Moalem et al. (2000) reported a possible role of HFE gene mutations in dementia. Studies are underway to screen all the mutation carriers for other known mutations in the HFE gene for further genotype/phenotype association studies, using a recently-developed reverse-hybridisation teststrip assay (Oberkanins et al. 2000). This assay includes a novel mutation E168Q identified in one of our elderly Afrikaner individuals with mutation H63D.

The clinical features observed in C282Y-positive (heterozygotes) and C282Y-negative elderly subjects are compared in table 3. The group of 24 heterozygotes comprised 7 males and 17 females between 70 and 90 years old. Differences in iron parameters between C282Y heterozygotes and mutation-negative subjects were not statistically significant. In the C282Y heterozygous group there were 12 individuals (3 men and 9 women), with a combination of any two out of the three clinical “give-aways”: diabetes, heart failure and osteoarthritis; we found only mildly raised serum ferritin levels in three of the women. The finding that all other iron parameters in these 12 individuals were normal provides an indication of the non-specificity of these clinical features in relation to HH, particularly in

the elderly. Among the 33 C282Y-negative individuals that developed a malignancy on follow-up, 7 (4.6% of the total) were found to have a colon malignancy and 2 an adenocarcinoma of the rectum (1.3%). A family history of cancer was denoted in 77 persons (50.3%, including the 33 subjects without mutation C282Y), 14 having a relative with colon carcinoma (9.2%) but none with adenocarcinoma of the rectum. Although the incidence of diabetes tended to be higher in subjects with mutation C282Y (29% versus 17%), this difference did not reach statistical significance ( $p=0.2$ ).



**Figure 2.** Pedigree of the elderly individual found to be homozygous for mutation C282Y. Dark-shaded symbols indicate family members who died of colon cancer (I-1, II-3, II-4, II-5) or liver cancer (I-2). Subjects II-1 and II-6 suffered from cardiovascular disease and asthma, respectively. Subjects III-1 and III-5 are regular blood donors.

**Table 3.** Comparison of clinical features observed in C282Y-positive (heterozygotes) and C282Y-negative elderly subjects

	C282Y heterozygotes			C282Y negatives		
	Total (n=24)	M (n=7)	F (n=17)	Total (n=153)	M (n=53)	F (n=100)
Type II Diabetes	29%	2 (29)	5 (29)	17%	8 (15)	18 (18)
FHx	25%	1	5	32%	9	40
Ischaemic Heart Dis	71%	5 (71)	12 (71)	61%	34 (64)	60 (60)
FHx	100%	5	12	77%	37	81
Arthritis	62.5%	4 (57)	11 (64)	66%	28 (53)	73 (73)
FHx	33%	1	7	41%	16	47
Malignancy on F/U	21%	2 (29)	3 (18)	22%	15 (28)	18 (18)
FHx	42%	4	6	50%	20	57
		1 rectum		2 rectum		
		2 prostate		7 colon		
		1 tongue carcinoma				
		1 myeloma				
Serum ferritin range	32 - 1136			10 - 3942 (ng/ml)		
% with normal level	58%			61%		

F, female; M, male; FHx, family history

In the C282Y-negative group, 74 people took either acetyl salicylic acid (ASA), warfarin or some non-steroidal anti-inflammatory drug (NSAID), potentially causing iron loss due to gastrointestinal bleeding. Normal ferritin levels were present in 53 (71.6%) of these subjects, versus 51 out of 79 non-takers (64.6%). Of the 21 individuals whose levels were raised, 10 (47.6%) had levels ~double the normal, in 5 it was ~3 times the normal and in one person the level was elevated to 5 times above normal parameters. As discussed above, factors such as liver disease, malignancy, haemolysis and a host of inflammatory conditions may cause elevation of the serum ferritin concentration, but the levels of this



acute phase protein does apparently not change significantly in the case of probable blood loss. Blood loss is much more frequent in elderly subjects using ASA, warfarin or a NSAID than in the younger population taking the same medications. The usefulness of serum ferritin determination is thus probably limited to the follow-up of individuals with established disease. The group of C282Y-negatives that developed colon carcinoma consisted of 5 women and 2 men, all with serum ferritin levels within the normal range; only 2 took ASA. The two patients with rectal carcinoma also had normal levels and took no ASA.

It is noteworthy that none of the males included in this study were homozygous for mutation C282Y, while Willis et al. (1999) detected four homozygous males in their group of 600 elderly men studied. Besides the possible selection bias reported in this test group as a consequence of possible haemochromatosis-related diseases, it is possible that some of these subjects were mistyped as a consequence of an *MseI* polymorphism in intron 4 of the HFE gene, as described for one of the elderly Afrikaner subjects initially diagnosed with putative HH by de Villiers and Kotze (1999). The detection of a single female C282Y homozygote in our study population may also be related to early death in Afrikaner men with the HH genotype. The daily diet of most Afrikaners (particularly males) contains a considerable amount of red meat and relatively few green vegetables, thus promoting iron-loading. Garry et al. (1997) were also unable to identify any C282Y homozygotes among 287 healthy elderly volunteers, although seven subjects were compound heterozygotes for mutations H63D and C282Y (2.4%). We noticed the similarity in C282Y heterozygote frequency (11.8%) and compound heterozygote frequency (1.1%) in our study population,

but the lack of H63D-homozygosity probably reflects the influence of genes from Hispanic origins in this population of New Mexico. It has been well described that in these populations the frequency of H63D is much higher than in people of Celtic descent (Sánchez et al. 1998). The absence of C282Y homozygotes could be due to selection bias as this group consisted of healthy volunteers, but one can also presume that those with high iron stores had an increased mortality rate and the findings may therefore be the result of a natural selection process.

In conclusion, we have demonstrated that the C282Y mutation occurs in aged Afrikaners albeit at a slightly lower frequency than in the younger population from the same gene pool. It therefore appears to be unlikely that one or two mutant alleles for HH confer a survival advantage for the aged, which may be envisaged for multiparous women as a means of keeping up their iron-stores (Datz et al. 1998). This study underscores the difficulties of clinical diagnosis in the absence of a reliable laboratory assay, as the general clinical picture in older individuals is characterised by non-specific symptoms associated with HH and a high background frequency of those diseases that can cause the symptom-complex specific for HH (type II diabetes, heart failure, osteoarthritis). As multiple factors are known to influence both the serum iron and ferritin values, these biochemical tests are probably of limited value in HH diagnosis, particularly in the elderly. In our random sample of elderly subjects no relationship was found between possession of mutation C282Y and the development of colorectal malignancies, although the single female C282Y homozygote died of colorectal cancer. Failure to indicate a significant association with cancer may be due to the relatively high incidence of colorectal neoplasia in this age group,

and/or the likelihood that C282Y homozygotes with relatively high serum iron levels died before reaching their seventies. A prospective study of the cohort of young individuals included in the study might in future provide valuable information in this respect. Individuals with normal iron status despite a genetic predisposition for HH (Rhodes et al. 1997; Adams, 2000) who survive into old age can be expected to harbour modifier genes (Rhodes et al. 1997; Whitfield et al. 2000) that guard them from iron overload.

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**REFERENCES**

Adams PC. Nonexpressing homozygotes for C282Y hemochromatosis: minority or majority of cases? *Mol Genet Metab* 2000; 71: 81-86.

Birgegard G, Hällgren R, Venge P, Wide L. Serum ferritin during inflammation: a study on myocardial infarction. *Acta Med Scand* 1979; 206: 361-366.

Bonazza M. Ferritinemia in ovarian malignant tumour. *Eur J Gynaecol Oncol* 1983; 4: 18-21.

Botha MC, Beighton P. Inherited disorders in the Afrikaner population of southern Africa. Part I. Historical and demographic background, cardiovascular, neurological, metabolic and intestinal conditions. *S Afr Med J* 1983; 64, 609-612.

Datz C, Haas T, Rinner H, Sandhofer F, Patch W, Paulweber B. Heterozygosity for the C282Y mutation in the hemochromatosis gene is associated with increased serum iron, transferrin saturation, and hemoglobin in young women: a protective role against iron deficiency? *Clin Chem* 1998; 44: 2429-2432.

de Villiers JNP, Hillermann R, de Jong G, Langenhoven E, Rossouw H, Marx MP, Kotze, MJ. High prevalence of the Cys282Tyr HFE mutation facilitates an improved diagnostic service for hereditary haemochromatosis in South Africa. *S Afr Med J* 1999; 89: 279-282.

de Villiers JNP, Kotze MJ Significance of linkage disequilibrium between mutation C282Y and a MseI polymorphism in population screening and DNA diagnosis of hemochromatosis. *Blood Cells Mol Dis* 1999; 15: 250-252.

Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class-I like gene is mutated in patients with hereditary haemochromatosis. *Nature Genet* 1996; 13: 399-408.

Garry, PJ, Montoya GD, Baumgartner RN, Liang HC, Williams TM, Brodie SG. Impact of HLA-H mutations on iron stores in healthy elderly men and women. *Blood Cells Mol Dis* 1997; 23: 277-287.

Grail A, Hancock BW, Harrison PM. Serum ferritin in normal individuals and in patients with malignant lymphoma and chronic renal failure measured with seven different commercial immunoassay techniques. *J Clin Pathol* 1982; 35: 1204-1212.

Lipschitz DA, Cook JD, Finch CA. A clinical evaluation of serum ferritin. *N Engl J Med* 1974; 290: 1213-1215.

Lundin L, Hällgren R, Birgegård G, Wide L. Serum ferritin in alcoholics and the relation to liver damage, iron state and erythropoietic activity. *Acta Med Scand* 1981; 209:327-331.

Malczewska J, Blach W, Stupnicki R. The effects of physical exercise on the concentrations of ferritin and transferrin receptor in plasma of female judoists. *Int J Sports Med* 2000; 21: 175-179.

Marcus DL, Freedman ML. Clinical disorders of iron metabolism in the elderly. *Clinics in Geriatric Medicine* 1985; 4: 729-745.

Milani MY, Kotze MJ. Molecular diagnosis of hereditary haemochromatosis: identify an affected person and save a family. *S Afr Med J* 1999; 89: 263-264.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 1988; 16: 1215.

Moalem S, Percy ME, Andrews DF, Kruck TPA, Wong S, Dalton AJ, Mehta P, Fedor B, Warren AC. Are hereditary hemochromatosis mutations involved in Alzheimer disease? *Am J Med Genet* 2000; 93: 58-66.

Nelson RL, Davis FG, Persky V, Becker E. Risk of neoplastic and other diseases among people with heterozygosity for hereditary hemochromatosis. *Cancer* 1995; 76: 875-879.

Nielsen JE, Neerup L, Jensen K, Krabbe K. Hereditary haemochromatosis: a case of iron accumulation in the basal ganglia associated with a parkinsonian syndrome. *J Neurol Neurosurg Psychiatry* 1995; 59: 318-321.

Oberkanins C, Moritz A, de Villiers JNP, Kotze MJ, Kury F. A reverse-hybridization assay for the rapid and simultaneous detection of nine HFE gene mutations. *Genet Testing* 2000; 4: 121-124.

Roberts AG, Whatley SD, Morgan RR, Worwood M, Elder GH, Roth MP, Giraldo P. Increased frequency of the haemochromatosis Cys282Tyr mutation in sporadic porphyria cutanea tarda. *Lancet* 1997; 349: 321-323.

Rhodes DA, Raha-Chowdhury R, Cox TM, Trowsdale J. Homozygosity for the predominant Cys282Tyr mutation and absence of disease expression in hereditary haemochromatosis. *J Med Genet* 1997; 34: 761-764.

Sánchez M, Bruguera M, Bosch J, Rodés J, Ballesta F, Oliva R. Prevalence of the Cys282Tyr and His63Asp HFE gene mutations in Spanish patients with hereditary hemochromatosis and controls. *J Hepatol* 1998; 29: 725-728.

Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL, Martin NG. Effects of the HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000; 66: 1246-1258.

Willis G, Wimperis JZ, Smith KC, Fellows IW, Jennings BA. Hemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet* 1999; 354: 221-222.



## **CHAPTER 4**

### **Conclusions and Future Prospects**

## 4.1 Conclusions

In this study a sample of elderly Afrikaner citizens has been tested for the presence of the most common mutation (C282Y) underlying hereditary haemochromatosis (HH) in association with possible iron overload. The clinical picture associated with HH is non-specific in nature, and analysis of the elderly emphasised the difficulties of clinical diagnosis of this common autosomal recessive disease. Attempts to identify HH in patients presenting with type II diabetes mellitus did not indicate a higher rate of detection (Sampson et al. 2000; Turnbull et al. 1997), although in a group of individuals presenting with arthropathy the prevalence was found to be 5 times the population prevalence at 1.5% (Olynyk et al. 1994). However, in a document produced by the European Association for the Study of the Liver (EASL) International Consensus Conference on HH, it was clearly stated that "both diabetes and rheumatological diseases are so frequent in the general population that these conditions can easily be over-interpreted as belonging to the phenotypic expression of haemochromatosis". Large-scale epidemiological studies are therefore needed to determine the sensitivity and specificity of the components of the clinical picture in predicting the likelihood of underlying HH genotypes.

This study has shown that the discrepancy between the genotype findings on the one hand and serum iron concentration and phenotype on the other, which has been described previously in adults (Adams et al. 1997; Edwards et al. 1998; Olynyk et al. 1999; Whitfield et al. 2000), does persist in our aged population. Generally however, iron levels are lower than expected, either because those with raised iron stores have died before reaching their

seventies, or they belong to a group of individuals who do not express homo- or heterozygosity for mutation C282Y (Adams, 2000). Reasons for this non-expression are as yet speculative but could be related to environmental factors such as a low-iron diet and/or gene-gene interaction resulting in incomplete penetrance of a HH mutation due to protection against iron loading.

With regard to the biochemical tests of iron status, serum ferritin was found to be a rather non-specific measure of body iron stores and this parameter is therefore not recommended as a marker in this age group. Raised serum ferritin in the elderly may therefore be a confounder, i.e. an indicator of an underlying disease such as an inflammatory process or atherosclerosis, rather than of iron loading. Moreover, metabolic disorders have been described that manifest with significant iron overload but with a normal transferrin saturation and the wild type HFE gene (Moirand et al. 1997). These patients were significantly older than those with HH and had one or more of the following conditions: obesity, hyperlipidaemia, abnormal glucose metabolism and hypertension.

As serum iron has also been found to be unreliable (Nodtvedt et al. 2000), the total iron binding capacity is probably the most reliable parameter for HH screening in the elderly, as has also been advocated for younger adults (Hickman et al. 2000). Even in the group of heterozygotes identified in this study only two individuals with a serum transferrin saturation above the upper limit of 50% were detected. This finding confirms a report by Franzini et al. (2000), indicating that transferrin saturation is lower in the aged and that those with significant iron loading have probably already died. However, one of the

compound heterozygotes with both mutations H63D and C282Y presented with a serum transferrin saturation concentration of 100%. Unfortunately the serum transferrin saturation could not be determined in the second compound heterozygote or the C282Y homozygote for comparative analysis. The serum ferritin values of these individuals with HH genotypes were above the reference value (396 ng/ml, range 162-249 ng/ml), although lower than expected in HH (usually >600 ng/ml). Investigations are underway to perform follow-up studies in family members of the C282Y homozygote, to determine whether the strong family history of cancer in this family can be ascribed to iron overload due to the presence of mutation C282Y.

Our increasing understanding of the molecular basis of genetic disorders of iron metabolism in mammalian species is providing new insight into genes involved in mediating intestinal iron absorption. It has become clear that the control of iron metabolism is interconnected with other cellular pathways, and further analysis of these aspects are likely to provide a better understanding of pathological processes (such as radical-mediated tissue damage or neurodegenerative disease) in which iron may participate. Over the past 3-4 years new technology enabled the identification of an increasing number of iron-related genes (by positional cloning strategies) from humans or animals with known genetic defects in intestinal iron absorption. Genome research and bioinformatics undoubtedly offer significant promises towards elucidating the genetics of molecularly uncharacterised conditions that may be caused by a disturbance in iron metabolism. It also provides the necessary tools to identify many different genes underlying a well-defined phenotype, previously considered to be caused by a single gene.

## 4.2 Future Prospects

Genetic analysis of patients with HFE-associated HH has revealed a complex spectrum of phenotypic expression. Although there is no doubt that the C282Y mutation in HFE is a disease-causing mutation, there is a small subgroup of C282Y homozygotes who does not develop clinically significant iron overload. On the other hand, some C282Y homozygotes develop severe iron overload early in life, and some C282Y heterozygotes develop haemochromatosis, indistinguishable from the picture of the homozygotes. This variability cannot be explained by environmental factors alone (Whitfield et al. 2000), and therefore the existence of other modifier genes that influence the HH disease phenotype is the most probable alternative. There are likely to be a number of genes with individual small and additive effects. Location of such genes may be achieved through association studies of candidate genes or it may require a genome-wide search.

Screening of healthy C282Y homozygotes for genes that may interact with the HFE gene, thereby resulting in possible non-expression of the HH phenotype, may lead to the identification of genes underlying iron deficiency and/or protect against iron overload/organ damage. One of the genes that are likely to interact with the HFE gene is the human divalent metal transporter (DMT1), identified as a possible cause of inherited iron deficiency anaemia (Fleming et al. 1997; Vulpe and Gitschier, 1997). This gene was previously known as the natural resistance-associated macrophage protein-2 (NRAMP2) gene (Vidal et al. 1995). Only one of the elderly individuals with mutation C282Y tested positive for a novel splice site mutation recently identified in the DMT1 gene (R

Hillermann and JNP de Villiers, unpublished results). Although the heterozygote frequency of this variant IVS2+6t→g was similar in the elderly and younger adult groups, further studies are warranted to investigate the likelihood that this mutation affects normal splicing of the DMT1 gene, thereby affecting serum iron status in the general population.

The human NRAMP1 gene that shows a high degree of sequence homology to DMT1/NRAMP2, represents another candidate gene that may affect iron homeostasis in humans. This gene is involved in the regulation of iron and is in turn also regulated by iron. Interestingly, we have shown that the allelic distribution of the Z-DNA forming repeat length polymorphism in the promoter region of the NRAMP1 gene differs significantly between the elderly Afrikaner population compared with younger controls (Kotze et al. submitted/Appendix B). This finding confirms the previous speculation (Searle and Blackwell 1998) that certain detrimental NRAMP1 alleles related to autoimmune disease susceptibility, may be maintained in the population because they improve survival to reproductive age following infectious disease challenge. This may imply that NRAMP1 alleles found to predominate in the elderly may confer protection against infection, iron overload and/or oxidative processes implicated in aging. Iron deficiency anaemia was evident in the two elderly males (ferritin 12 µg/l and 39 µg/l, transferrin saturation 10.8% and 16.5%) with this genotype, while higher levels were measured in the elderly female (ferritin 273 µg/l, transferrin saturation 20%) with alleles 3 and 5 of NRAMP1. It is noteworthy that the male with the lowest transferrin saturation level was heterozygous for mutation C282Y.

Animal models provide another means of identifying genes that may modify the haemochromatosis phenotype, although this should subsequently be verified in humans. Levy et al. (2000) have bred HFE knockout mice with strains carrying other mutations that impair normal iron metabolism. Compound mutant mice lacking both HFE and its interacting protein,  $\beta$ 2 microglobulin, deposited more tissue iron than mice lacking HFE only. HFE knockout mice carrying mutations in the iron transporter DMT1 failed to load iron, indicating that haemochromatosis involves iron flux through DMT1. Similarly, compound mutants deficient in HFE and hephaestin showed less iron loading than do HFE knockout mice alone, suggesting that iron absorption in haemochromatosis involves the function of this gene as well. Finally, compound mutants lacking HFE and the transferrin receptor accumulated more tissue iron than did mice lacking HFE alone, consistent with the idea that interaction between these two proteins contributes to the control of normal iron absorption. In addition to providing insight into the pathogenesis of HH, these results suggest that many different genes might influence iron homeostasis in humans.

Elderly subjects from the genetically homogeneous Afrikaner population provide a valuable source of material for a study aimed at the delineation of gene-gene and/or gene-environment interaction in HH. The 74-year old Afrikaner female identified in this study with two copies of mutation C282Y, can be expected to harbour gene(s) that protect against iron overload. Elderly subjects with haemochromatosis genotypes (Willis et al. 1999) will in future be subjected to extensive molecular analysis to identify genes/mutations that may underlie iron deficiency/protection against iron overload within the South African context. Determination of the incidence of clinical / biochemical

misdiagnoses in HH may lead to a better definition of clinical criteria for diagnosis of HH, and ultimately, the development of a comprehensive DNA-based test for accurate assessment of iron status in South African subjects. Mutation analysis in the elderly subjects recruited for the current study will in future be extended to screening of the TFR2 gene, recently implicated as a second locus for adult-onset haemochromatosis (Camaschella et al. 2000).

Since mutation accumulation late in life can only be identified by studying the elderly, future studies aimed at the identification of iron-related genes that exert both an age-dependent negative and a positive effect on survival may increase our understanding of the role of iron in health and disease. We indeed have much to learn from our elders.

## **REFERENCES**

Adams PC, Campion ML, Gandon G, Legall JY, David V, Jouanolle AM. Clinical and family studies in genetic haemochromatosis: microsatellite and HFE studies in five atypical families. *Hepatology* 1997; 26: 986-990.

Adams PC. Nonexpressing Homozygotes for C282Y Hemochromatosis: minority or majority of cases? *Mol Genet Metab* 2000;71:81-86.



Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. *Nature Genet* 2000; 25: 14-15.

Edwards CQ, Griffen LM, Ajioka RS, Kushner JP. Screening for hemochromatosis: Phenotype versus Genotype. *Seminars Hematol* 1998; 35: 72-76.

Fleming MD, Trenor CC 3rd, Su MA, Foernzler D, Beier DR, Dietrich WF, Andrews NC. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat Genet* 1997; 16: 383-386.

Franzini C, Berlusconi A, Favarelli C, Brambilla S. Low frequency of elevated serum transferrin saturation in elderly subjects. *Clin Chim Acta* 2000; 298: 181-186.

Hickman PE, Hourigan LF, Powell LW, Cordingley F, Dimeski G, Ormiston B, Shaw J, Ferguson W, Johnson M, Ascough J, McDonell K, Pink A, Crawford DH. Automated measurement of unsaturated iron binding capacity is an effective screening strategy for C282Y homozygous haemochromatosis. *Gut* 2000; 46: 405-409.

Levy JE, Montross LK, Andrews NC. Genes that modify the hemochromatosis phenotype in mice. *J Clin Invest* 2000; 105: 1209-1216.

Moirand R, Mortaji AM, Lóreal O, Paillard F, Brissot P, Deugnier Y. A new syndrome of liver iron overload with normal transferrin saturation. *Lancet* 1997; 349: 95-97.

Nodtvedt GM, Lovli OC, Asberg A. Diagnostikk av tomme jernlagre—ikke bruk s-jern. *Tidsskr Nor Laegeforen* 2000; 120: 1639-1641.

Olynyk J, Hall P, Ahern M, Kwiatek R, Mackinnon M. Screening for genetic haemochromatosis in a rheumatology clinic. *Aust NZ J Med* 1994; 24: 22-25.

Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population-based study of the clinical expression of the haemochromatosis gene. *N Engl J Med* 1999; 341: 718-724.

Sampson MJ, Williams T, Heyburn PJ, Greenwood RH, Temple RC, Wimperis JZ, Jennings BA, Willis GA. Prevalence of HFE mutations in unselected patients with type 2 diabetes. *J Lab Clin Med* 2000; 135: 170-173.

Searle S, Blackwell JM. Evidence for a functional repeat polymorphism in the promoter of the human NRAMP1 gene that correlates with autoimmune versus infectious disease susceptibility. *J Med Genet* 1998; 36: 295-299.

Turnbull AJ, Mitchison HC, Peaston RTP, Lai LC, Bennett MK, Taylor R, Bassendine MF. The prevalence of hereditary haemochromatosis in a diabetic population. *Q J Med* 1997; 90: 271-275.

Vidal S, Belouchi AM, Cellier M, Beatty B, Gros P. Cloning and characterization of a second human NRAMP gene on chromosome 12q13. *Mammalian Genome* 1995; 6: 224-230.

Vulpe C, Gitschier J. Ironing out anaemia. *Nat Genet* 1997; 16: 319-320.

Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL, Martin NG. Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000; 66: 1246-1258.

Willis G, Wimperis JZ, Smith KC, Fellows IW, Jennings BA. Hemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet* 1999; 354: 221-222.

# **APPENDIX A**

## **LABORATORY PROCEDURES**

The laboratory procedures used for this work consisted of four different phases:

1. Genomic DNA-extraction
2. Polymerase Chain Reaction
3. Restriction Enzyme Digestion
4. Gel Electrophoresis

### **1.1 GENOMIC DNA-EXTRACTION**

General guidelines:

- EDTA-preserved blood is needed (10ml)
- Always use your own and fresh reagents
- Beware of tissue contamination

Protocol:

1. After proper shaking, the EDTA-blood is placed in a 50ml Falcon tube, the EDTA-tubes are kept for back-up and the Falcon tubes are numbered (top and side).
2. Add cold lysis-buffer, up to a volume of 45 ml and shake well ( solution will become turbid).
3. Put the tubes in the fridge for about 10 minutes until the solution is translucent.
4. Centrifuge at 1500 rpm for 15 minutes, then carefully remove the supernatant so that the pellet with white blood cells (WBC) remains in the Falcon tube.
5. Rinse the pellet carefully with 10 ml phosphate buffered saline (PBS) from the fridge and carefully remove supernatant. Do not disturb WBC pellet.

6. Add 3 ml of nuclei lysis buffer, 30  $\mu$ l proteinase-K and 300  $\mu$ l of a 10% sodium dodecyl sulphate (SDS)-solution and mix by vortexing.
7. Incubate the tubes in a 55°C waterbath, overnight.
8. Add 1ml of 6 M NaCl the next morning and shake vigorously for about one minute.
9. Centrifuge for 20 minutes at 2500 rpm to pellet the cellular debris and pour the supernatant off immediately.
10. Add 2 volumes 100% Ethanol from the freezer to precipitate the DNA and proteins.
11. Carefully remove the DNA and wash in 1.5ml 70% ethanol.
12. Centrifuge for 30s at 13000 rpm and remove the supernatant.
13. To vaporise the ethanol, leave the pellet in the tube for 10 minutes, exposed to the air to dry.
14. Reconstitute the DNA pellet in 1ml SABAX sterile water and shake on mechanical shaker until the whole pellet is dissolved.

## 1.2 POLYMERASE CHAIN REACTION

### General Guidelines:

- First clean the whole area, gloves and all the racks with hibitane
- You always need to have a positive control, a negative control and a placebo-control to ensure that the polymerase chain reaction (PCR) has worked and was not contaminated.

**Protocol:**

For each PCR a 25 µl reaction volume is used containing the DNA, Taq Polymerase, buffer, dNTPs, forward and reverse PCR primers and sterile water. The mixture has to be mixed on the Vortex for a few seconds. The oligonucleotide primers are 18-22 base-pairs long and homologous to the known DNA sequence. Taq Polymerase is stored in glycerol to prevent freezing and so will form a little “cloud” upon adding to the cocktail. Its optimal temperature is 72° C, but will survive 95° C, with some reactivity at a low 20° C.

The PCR program consists of repeated cycles: denaturation, where the double-stranded DNA is separated into two single strands by heating at 95° C., then annealing of the primers on the strands by lowering the temperature to 55°C (the annealing temperature of a primer depends on the GATC-content:  $T_m = (4x [G+C]) + (2x [A+T])$ ), and finally extension. For the primers used in this study the optimal annealing temperature is 65°-68°C. The extension time depends on the length of the template.

**PCR-Program for exon 4 of the HFE gene:**

Incubate at 95°C for 2 min, then start the 1st cycle:

95°C –10 seconds	}	
65°C –10 seconds	}	FOR 10 CYCLES
72°C –30 seconds	}	
95°C –for 10 seconds	}	
60°C –for 10 seconds	}	FOR 25 TO 30 CYCLES.
72°C –for 30 seconds	}	

### 1.3 RESTRICTION ENZYME DIGESTION OF PCR PRODUCTS

For digestion with restriction endonucleases a cocktail is prepared:

- 1) 10µl DNA (too much DNA will hamper analysis)
- 2) 2 µl of 10X buffer
- 3) 7.5µl. sterile water
- 4) 0.5µl of enzyme-solution (containing 5-10 Units of enzyme to ensure full digestion; this must be less than 10% of the total volume, as the glycerol in the enzyme-solution may inhibit digestion).

RsaI has a specific recognition site (GT/AC) and cuts the normal 394-bp exon 4 into 278bp- and 116-bp fragments. In the case of the C282Y mutation a new cutting site is created by this G to A mutation at nucleotide position 845, where GTGC changes to GTAC. This leads to the digestion of the smaller 116-bp fragment into two fragments. In the case of a C282Y heterozygote three DNA fragments will be visible on the gel (278bp, 116bp and 77bp), and in the case of a homozygote only the 278bp and 77bp fragments will be observed.

The mixture can either be left overnight or for 2 ½ hours in a 37° C water bath to undergo optimum digestion. Check temperature with two thermometers (mercury and alcohol).

### 1.4 POLYACRYLAMIDE GEL-ELECTROPHORESIS

To assemble the Hoefer SE600 gel-apparatus:

1. Place four glass plates alongside one another and in a horizontal position on the table.
2. Clean the glass plates and the spacers with wet tissues and dry.



3. Grease the spacers on one side to prevent current-leakage.
4. Assemble the plates and spacers, ensuring that the upper borders are at the same level, and secure the structure by applying clamps. Tighten it using the clamps.
5. Place assembled glass plates in gel pourer container.
6. Melt agarose (2%) for 3 min at full power in microwave. Pour enough agarose in the container so that a small (1cm) plug is formed at the bottom between the glass plates. Tighten plates by using tightening knobs.

GELMIX (20 ml in total for a gel of 15 x 15 cm.):

- 1) 7 ml polyacrylamide (3.4% cross link, 30% stock)
- 2) 4 ml 5 X TBE-buffer (tris-boric acid and EDTA)
- 3) 9 ml sterile water
- 4) 160  $\mu$ l. APS (ammonium persulphate)
- 5) 80  $\mu$ l TEMED (tetra-methyl-ethylene-diamine or N,N,N',N')

The concentration of the gel will determine its power to separate fragments of different size. The gel mix is poured in the 0.75 mm cavity between the glass plates. The agarose prevents the gel mix from leaking by forming a gel barrier. Care must be taken that no bubbles are formed, as this will affect the results and DNA mobility. A comb is inserted at the top of the gel between the plates so that after polymerisation and removal of the comb, cavities are created for loading the DNA samples. Upon removal of the comb, rinse the cavities with 1X TBE buffer to clear out all possible debris. Prepare the DNA samples by adding 5  $\mu$ l of blue loading buffer, the reasoning for this being:

DNA is weighed down so that when loaded into the cavities, it does not float out. The blue stain acts as a marker that migrates with known DNA fragment sizes. Load 15µl of the sample in the formed cavities together with an uncut control sample and DNA marker.

Assemble gel apparatus by adding and fastening the top buffer chamber and add 1X running buffer up to a maximum level and apply current (250 V for 2 hours). Run gel until only the light blue band is visible.

Remove glass plates from apparatus and separate so that the 0.75 mm gel is exposed. Stain gel for 10 minutes in a 1X TBE solution containing ethidium bromide. Destain for 2 minutes in distilled water to remove excess ethidium bromide that may cause background staining. The gel is placed on a trans illuminator and exposed to UV light for 2 seconds upon which a photograph is taken using a CCD camera. The photograph is printed and the gel analysed by comparing the resulting band sizes with controls loaded on the same gel.

## **APPENDIX B**

**MANUSCRIPT ACCEPTED FOR PUBLICATION:**

**BLOOD CELLS, MOLECULES AND DISEASES**

**Analysis of the NRAMP1 gene implicated in iron transport: association with multiple sclerosis and age effects**

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**ABSTRACT**

Multiple sclerosis (MS) is believed to be an autoimmune process occurring in genetically susceptible individuals after an appropriate environmental exposure. We have exploited the homogeneous Afrikaner population of European ancestry to investigate the likelihood that iron dysregulation, in association with infectious and/or autoimmune disease susceptibility, may underlie the MS phenotype in a subgroup of patients. The functional Z-DNA forming repeat polymorphism of the natural resistance-associated macrophage protein-1 (NRAMP1) gene was analyzed in 104 patients diagnosed with MS and 522 Caucasian controls. A family-based control group consisting of 32 parental alleles not transmitted to MS offspring was additionally studied to exclude the likelihood of population substructures. Statistically significant differences in allelic distribution were observed between the patient and control samples drawn from the same population ( $P < 0.01$ ). Evidence is furthermore provided that alleles considered to be detrimental in relation to autoimmune disease susceptibility may be maintained in the population as a consequence of improved survival to reproductive age following infectious disease challenge. Although it remains to be determined whether the disease phenotype in MS patients with allele 5 of the NRAMP1 promoter polymorphism is directly related to dysregulation of iron or modified susceptibility to viral infection and/or autoimmunity, a combination of these processes most likely underlie the disease phenotype in these patients. In view of the emerging role of polymorphic variants in complex diseases and minimizing of possible confounding factors in this association study, we conclude that allelic variation in the NRAMP1 promoter may contribute significantly to MS susceptibility in the South African Caucasian population.

## INTRODUCTION

The cause of multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS), remains unknown after more than a century of study. Current knowledge suggests that MS is associated with autoimmunity, with genetic susceptibility and infectious agents as important risk factors (1,2). The overall lifetime risk for siblings of MS patients to manifest the disease is estimated at 4-5%, compared to a population prevalence of approximately 0.1%. Although data on family studies have excluded all but a polygenic mode of inheritance, and an autosomal dominant or recessive single locus with low penetrance, most evidence suggests that several genes control disease susceptibility (3). Autopsy studies in a cohort of MS patients indicated four fundamentally different patterns of demyelination, suggesting pathogenetic heterogeneity with important implications for the diagnosis and treatment of the disease (4).

Previous genome-wide gene searches and association studies suggest that the genetic susceptibility factors of MS may vary between different populations (3). The white Afrikaner population of South Africa represents an ideal group (5) for the identification of genes involved in this complex disease, since the prevalence of MS is relatively high in the European populations that contributed to the gene pool of this genetic isolate. Earlier studies indicated a relatively low incidence ( $\sim 0.4/100\ 000$ ) of the disease in the Caucasian population of South Africa (6), but more recently Dean (7) reported that the prevalence of MS is on the increase in the Afrikaner population. Although the Afrikaner population is mainly of Dutch, German and French origin, settlers from the United Kingdom with a relatively high prevalence ( $12/100\ 000$ ) of MS amongst Scottish individuals (8), have also

made a considerable contribution (~5%) to the gene pool (9). The unique genetic background of the Afrikaner population therefore offers the advantage to study the genetics of MS on the basis of an expected limited number of disease-associated genes/mutations introduced from Europe. Genetic analysis in the African context furthermore provides the opportunity to assess the relevance of potentially important sequence changes in indigenous population groups, where MS is considered to be extremely rare (10).

The importance of genetic factors underlying population/ethnic differences in disease risk was illustrated by the finding that the hereditary hemochromatosis (HH) mutation C282Y in the HFE gene (11), associated with this common iron overload disease in the majority (70-100%) of affected Caucasians worldwide, is virtually absent in the South African Black population (12). Of relevance to this study is the apparent absence of clinically manifested HH among 1700 patients with MS encountered by Valberg et al. (13), despite the higher serum ferritin levels detected in patients compared with controls and the fact that MS and HH affect the same ethnic group. Similar findings were reported in an Afrikaner family where two sisters with MS were found to be homozygous for the common HH mutation C282Y (14), which largely excluded the likelihood of a linkage disequilibrium effect with a putative MS-related gene on chromosome 6. These findings raised the possibility that MS and HH may be mutually exclusive due to gene-gene interaction in certain populations.

There are several lines of support for a role of iron dysregulation in the pathogenesis of MS (15-20). This pertains to the fact that iron overload may predispose an individual to virus infection, whilst on the contrary, inadequate supply of iron may impair immune function. To further investigate the potential role of iron metabolism in

association with infectious and/or autoimmune disease susceptibility in MS, we genotyped 104 South African MS patients and 532 control individuals from the same population for the functional Z-DNA forming repeat polymorphism of the natural resistance-associated macrophage protein-1 (NRAMP1) gene. This gene that regulates iron, and is also regulated by cellular iron levels (21), has been linked to many infectious and autoimmune diseases (22-25). Although these findings may be related to any one of the multiple pleiotropic effects associated with macrophage activation, it seems highly likely that regulation of iron transport via NRAMP1 may contribute directly to disease susceptibility (23). Some of the diseases associated with the NRAMP1 gene may pathogenetically be related to MS (26-30) and apparently also to abnormalities in iron absorption (31,32), that may be linked to defective iron supply for erythropoiesis (33). The anemia associated with autoimmune disease may be related to the proposed role of NRAMP1 in scavenging iron from senescent red cells via splenic macrophage phagosomes for transport back to the blood.

## **MATERIALS AND METHODS**

**Study population.** Relevant information was obtained from 281 South African patients, following the distribution of a questionnaire that was published in the South African National Multiple Sclerosis Society newsletter. Blood samples were obtained, after informed consent, from 156 South African respondents who completed the questionnaire and 197 of their close relatives. The patients' neurologists were subsequently contacted to verify the MS diagnoses, based on clinical features (34), lumbar puncture, magnetic resonance imaging, evoked potentials and/or exclusion of other diseases known to manifest



similarly. Uncertain cases included in the study were re-examined by J.C. Non-Caucasian South African MS patients, recent immigrants, and respondents without a confirmed diagnosis of MS were excluded from the study, leaving 104 index cases diagnosed with definite MS for genetic analysis.

DNA samples of 329 South African individuals of European descent (mainly German, Dutch, French and British) below the age of 70 years were included as unrelated controls. The individuals consisted of 231 laboratory personnel and medical students (68 males and 163 females), and 98 spouses of patients with MS (16 males and 13 females) or familial hypercholesterolemia (34 males and 35 females). Additionally, 193 elderly individuals above the age of 70 years (67 males and 126 females) and a family-based control group consisting of 32 parental alleles not transmitted to MS offspring were included for comparison within and between subgroups. Inclusion and exclusion criteria were similar in the experimental and control groups. MS patients and controls found to be homozygous for the HH mutation C282Y were excluded from this study. Data obtained on laboratory personnel, medical students and spouses of index cases were pooled with the "affected-family-based control" (AFBAC) group (35) after verification of similar allele frequencies in the different groups. DNA samples from 448 non-Caucasians (278 Xhosas, 50 KhoiSan and 120 subjects of Mixed Ancestry), 37 healthy Scottish individuals and 143 Germans were included as controls for population studies. Matched case-control groups are particularly important in diseases such as MS where environmental triggers may be involved. The study protocol has been approved by the Ethics Review Committee of the University of Stellenbosch.

**Hematological and Biochemical analysis.** Hemoglobin, serum iron, transferrin and ferritin concentrations were determined using standard methods. Serum iron was measured with a Beckman CX7 autoanalyser, and transferrin with a Beckman Array Nephelometer. Transferrin saturation was subsequently calculated. None of the patients analysed experienced episodes of MS at the time iron concentrations were determined.

**DNA analysis.** Genomic DNA was isolated from whole blood using a standard salting-out method (36). The PCR primers used to amplify a 483-bp fragment of the NRAMP1 promoter were 5'-GGGGTCTTGGA ACTCCAGAT-3' (forward) and 5'-GGGCAGCTCCTCAGCCTGCAC-3' (reverse). The PCR conditions were as follows: 200 ng of genomic DNA, 0.2  $\mu$ M of each dGTP, dCTP, dATP, dTTP, 20 pmol of each primer, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris.HCl, 50 mM KCl and 1U *Taq* DNA Polymerase. PCR cycle conditions included an initial denaturing step at 95°C for 2 minutes, followed by 35 cycles at 95°C for 30 seconds, 65°C for 45 seconds, and 72°C for 30 seconds. Screening of PCR-amplified DNA for potential disease-related mutations was performed using a combined HEX-SSCP method according to Kotze et al. (37). PCR products corresponding to aberrant bands were sequenced using an automated system (ABI 310, Perkin Elmer Applied Biosystems). For better discrimination between different alleles of the (gt)<sub>n</sub> repeat, *RsaI* restriction enzyme analysis was performed according to Graham et al. (25). Following the amplification of a shorter 116-bp fragment using a new reverse primer 5'-TACCCCATGACCACACCC-3', PCR products were digested to completion with *RsaI*, subjected to polyacrylamide (PAA) gel-electrophoresis (12% PAA, 5% C), stained with ethidium bromide and visualized by ultraviolet light.

**Statistical methods.** NRAMP1 allele frequencies were analysed by STATISTICA for Windows (38), which offers different statistical methods to determine significant associations. The Chi-square ( $\chi^2$ ) test and/or Fisher-exact test were performed to assess significant associations with NRAMP1 in the study population. Due to the low frequency of certain variants in the study population, the Yates' correction was applied to improve the approximation of the  $\chi^2$  test, thereby providing a more conservative estimation. The Arlequin program (39) was used to test for various associations of polymorphic variants in the disease and control populations. This program assesses the likelihood that false associations may be obtained due to population substructures. For continuous variables groups were compared using unpaired t tests. The Wilcoxon (Kruskal-Wallis) and median tests were used for variables (ferritin) with skew distributions.

## **RESULTS**

### **Serum iron parameters**

Iron parameters were determined in 34 South African MS patients with relapsing-remitting (RR) MS, 10 with secondary progressive (SP) MS and 9 with primary progressive (PP) MS. Similar to that of a control group including 30 Caucasian individuals, the hemoglobin, serum iron, transferrin, transferrin saturation and ferritin values were within the normal range in the majority of cases. The serum ferritin concentration was significantly lower in the RR group than in the PP group ( $p < 0.05$ ), but none of the other parameters tested differed between the three patient groups (Table 1). When males are excluded, the statistical significance disappears even though the trend remains the same (RR 71.6 $\mu$ g/l,

SP 88.0  $\mu\text{g/l}$ , PP 109.6  $\mu\text{g/l}$ ). The patients with the progressive illness showed greater variability in ferritin levels. Patients with a RR disease course were the youngest on average ( $P<0.08$ ). The age at onset was also the youngest in the RR MS group (29.5, SD 9.6), followed by the SP (34.3, SD 11.1) and PP (35.3, SD 6.8) groups.

### **Analysis of the functional Z-DNA forming NRAMP1 repeat polymorphism**

Many variants have been described in the NRAMP1 gene, one of which is a functional Z-DNA forming repeat promoter polymorphism affecting NRAMP1 expression (22). The different alleles detected within the amplified DNA fragment spanning this polymorphic region are shown in Table 2, together with the allelic distribution observed in South African Caucasian MS patients and controls drawn from the same population. The allelic distribution of the Z-DNA forming dinucleotide polymorphism differed significantly between the MS patients and population-matched controls ( $P<0.01$ ), including 329 individuals from the general population within the same age group, a family-based control (AFBAC) group including 32 parental alleles not transmitted to MS offspring, and 193 elderly Afrikaner individuals above the age of 70 years. Although the sample sizes of the different subgroups analysed are relatively small, no population substructures were evident upon application of the Ewens-Watterson test of selective neutrality and the Chakraborty's test for population amalgamation (39). The genotype distribution and allele frequencies of the NRAMP1 promoter variant were in Hardy-Weinberg equilibrium in the populations tested.

Genotyping of the 193 elderly Afrikaner individuals from the general population also indicated a significant deviation from the younger control group ( $P<0.01$ ) (Table 2).

This finding suggests that alleles considered to be detrimental in relation to autoimmune disease susceptibility may be maintained in the population because they improve survival to reproductive age following infectious disease challenge (22), a phenomenon that may be of particular relevance in the African context. This may imply that alleles 3 and 5 found at a higher frequency in the elderly group compared to younger controls might confer protection against infection, iron overload and/or oxidative processes implicated in aging. Iron deficiency anemia was evident in the two elderly males (ferritin 12  $\mu\text{g/l}$  and 39  $\mu\text{g/l}$ , transferrin saturation 10.8% and 16.5%) with this genotype, whilst higher levels (ferritin 273  $\mu\text{g/l}$ , transferrin saturation 20%) were measured in the elderly female with alleles 3 and 5 of NRAMP1.

The clinical characteristics of the index MS patients with allele 5 are summarized in Table 3, together with that of two affected female relatives with this allele who were also diagnosed with definite MS. Extended clinical and family follow up are underway to clarify the potential role of NRAMP1 in the MS phenotype.

### **Population studies**

NRAMP1 allele 5 was not detected in Africans following DNA screening of 278 Xhosas, 50 KhoiSan and 120 subjects of Mixed Ancestry. This finding is in accordance with the potential significance of allele 5 in MS, since this disease is extremely rare in Africans (10). Population and haplotype studies using intragenic NRAMP1 polymorphisms are in progress to trace the origin of NRAMP1 allele 5 in European populations that contributed to the Afrikaner gene pool. Preliminary data obtained in DNA samples of healthy Scottish individuals indicated a significantly higher frequency (2/37, 5.4%) of NRAMP1 allele 5 in

this population compared with the general Afrikaner population (0.3%) ( $P < 0.01$ , 2 d.f.  $\chi^2 = 14.33$ ), while an intermediate frequency was observed in the German population (3/143, 2.1%). These findings raise the possibility that NRAMP1 allele 5 may be one of the factors underlying the high prevalence (8) of MS in Scotland. Approximately 15% of the 104 South African MS patients (1/7 with allele 5) included in this study reported that at least one of their ancestors was of Scottish descent.

## **DISCUSSION**

Iron is an essential nutritional element for normal cellular functioning of a large number of enzymes, including those involved in myelin formation, neurotransmitter synthesis and degradation, oxidative phosphorylation and heme biosynthesis (40-42). Hemoproteins are involved in electron transport, which could be critical to axonal as well as to myelin integrity. The human NRAMP1 gene was analysed in this study as a first candidate to investigate the hypothesis that intermittent iron deficiency (19) may underlie the MS phenotype in a subgroup of patients. The potential value of iron concentration determination in the cerebrospinal fluid (CSF) in distinguishing between MS patients with different disease courses has been demonstrated (16,17), and this may also apply to determination of serum iron parameters (20). Preliminary data furthermore indicated a significantly higher mean serum ferritin concentration in the South African patient group compared to controls (data not shown), which is in accordance with previous findings (13). This difference remained statistically significant when only females were compared, and in this group mean transferrin saturation was lower in patients compared with controls,

although the values were within the normal range in both groups. The statistically significant differences in NRAMP1 allelic distribution observed between MS patients and control individuals drawn from the same population ( $P < 0.01$ ) are in accordance with possible involvement of iron metabolism in the pathogenesis of MS. Notably, different cellular iron levels in Nramp1 wildtype (low) versus mutant (high) macrophages in mice may affect mRNA stability for MHC class II molecules (23), which highlights the link between iron metabolism and cell-mediated immunity (43). The enhanced responsiveness of Nramp1 wildtype macrophages to a range of biological stimuli (44), including bacterial lipopolysaccharide (LPS), interferon- $\gamma$ , glucocorticoids such as dexamethasone or cortisone (frequently applied in the treatment of MS), may relate to the enhancer activity of the Z-DNA forming dinucleotide repeat (22) analysed in this study.

The functional significance of the NRAMP1 promoter polymorphism analysed argues against the likelihood that the increased frequency of the potentially functional Z-DNA variant in South African MS patients is due to association with another mutation in the NRAMP1 gene or a nearby gene. Sequence changes which directly affect the gene product are more likely to demonstrate genetic and phenotypic differences between patient and control samples than indirect markers, particularly if it is present in only a small proportion of the study population as demonstrated in this study. This may explain why previous investigators failed to identify human chromosome 2q as a potentially important region in whole genome screens for MS and highlights the potential involvement of separate candidate loci in different populations (3). Association studies appear to be of greater power than linkage analysis in genetic studies of complex human diseases (45), since associated (disease-predisposing) polymorphisms are not necessarily linked to a

disease. A mathematical explanation for this phenomenon has been provided by Greenberg (46), which emphasizes the fact that the detection of certain alleles (usually at a lower frequency) in a control population does not exclude the likelihood that the sequence change may cause or contribute to the disease phenotype. MS is considered to be a heterogeneous disease (4, 47) where many different genes may be defective. Consistent linkage to the HLA region on chromosome 6 (47) in different genomic data sets and different populations may be related to possible polygenic control of MS, where disease susceptibility in conjunction with environmental factors is determined by non-MHC genes and disease severity/course by modulation of T cell responses to particular antigens, much like the situation described in mice (48). Interestingly, Jacobsen et al. (49) have recently demonstrated an association between MS and the gene (PTPRC) encoding protein-tyrosine phosphatase, receptor-type C (CD45), known to be essential for the activation of T and B cells.

The many cellular functions dependent on iron and other metal ions as cofactors may explain the complex role of NRAMP1 in infectious and autoimmune disease. Future studies may reveal whether the frequent co-existence of MS and inflammatory bowel disease (26,27), which share some common features (28), may be explained by the involvement of the NRAMP1 gene in both conditions (24). The increased frequency of allele 5 of the Z-DNA forming polymorphism detected in patients with primary biliary cirrhosis (PBC) studied by Graham et al. (25), further supports the view that PBC and MS may be related by a similar autoimmune process (29). The likelihood that the relatively high frequency of allele 5 detected in the South African MS cohort is caused by co-



existence of PBC, was largely excluded by previous studies performed by Reich et al. (50), recently extended to our study population.

None of the South African MS patients with NRAMP1 allele 5 were anemic, which highlights the fact that standard parameters of iron status may not necessarily reflect abnormalities in iron-related genes (51,52). Expression of iron-related genes is furthermore modulated by environmental (e.g. diet, the menstrual cycle, multiple pregnancies, regular blood donation) and genetic factors (53), as demonstrated by frequent detection of clinically unaffected subjects with the HH genotype, even in the elderly population (54). It is well known that a significant inflammatory process can cause interference of iron delivery for heme synthesis resulting in altered iron levels, such as raised ferritin levels and decreased transferrin saturation. Two index cases with a family history of MS and their two female relatives diagnosed with definite MS reported porphyria-like symptoms similar to those described by Rooney et al. (19), supporting the hypothesis that a disturbance of the heme biosynthesis pathway may be involved in the etiology of MS in a subgroup of patients. The likelihood that the allelic association described here may only be due to the atypical porphyria-like symptoms and is unrelated to MS *per se*, was largely excluded by the fact that only two of the five remaining allele 5-positive index cases without a family history of MS reported similar symptoms.

The data presented in this study are consistent with pathogenic roles for iron-related gene-environment interactions in MS and the previous speculation (22) that certain detrimental NRAMP1 alleles related to autoimmune disease susceptibility, may be associated with improved survival. Although it remains to be determined whether the disease phenotype in patients with NRAMP1 allele 5 is directly related to dysregulation of

iron or modified susceptibility to viral infections and/or autoimmunity, a combination of these processes most likely underlie the disease phenotype in a subgroup of South African MS patients with the potential disease-predisposing promoter variant. In view of the emerging role of polymorphisms in complex diseases (55), the functional significance of the Z-DNA forming NRAMP1 dinucleotide repeat in autoimmune and infectious disease susceptibility linked to iron regulation, and minimizing of possible confounding factors in this association study, we conclude that allelic variation in the NRAMP1 promoter may contribute significantly to MS susceptibility in the South African population of European descent. A prominent role of iron in the etiology of many neurological diseases may be forthcoming via its role as a co-factor of heme proteins which, when dysfunctional, may give rise to deficiencies in energy production, axonal degeneration and porphyric neuropathy (56-58).

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## REFERENCES

1. Ebers, G.C., and Dyment, D.A. (1998) Genetics of multiple sclerosis. *Semin. Neurol.* **18**, 295-299.
2. Noseworthy, J.H. (1999) Progress in determining the causes and treatment of multiple sclerosis. *Nature* **399** (Suppl), A40-47.
3. Bell, J.I., and Lathrop, M. (1996) Multiple loci for multiple sclerosis. *Nature Genet.* **13**, 377-378.
4. Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (2000) Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. *Ann. Neurol.* **47**, 707-717.
5. Groenewald, J.Z., Liebenberg, J., Groenewald, I., and Warnich, L. (1998) Linkage disequilibrium analysis in a recently founded population: Evaluation of the variegated porphyria founder in South African Afrikaners. *Am. J. Hum. Genet.* **62**, 1254-1258.
6. Dean, G. (1967) Annual incidence, prevalence, and mortality of multiple sclerosis in white South-African-born and in white immigrants to South Africa. *Brit. Med. J.* **2**, 724-730.
7. Dean, G. (1985) A new multiple sclerosis epidemic? *S. Afr. Med. J.* **68**, 630.
8. Rothwell, P.M. and Charlton, D. (1998) High incidence and prevalence of multiple sclerosis in south east Scotland: Evidence of a genetic predisposition. *J. Neurol. Neurosurg. Psychiatry* **64**, 730-735.
9. Botha, M.C., and Beighton, P. (1983) Inherited disorders in the Afrikaner population of southern Africa. Part I. Historical and demographic background, cardiovascular, neurological, metabolic and intestinal conditions. *S. Afr. Med. J.* **64**, 609-612.
10. Dean, G. Bhigjee, A.I., Bill, P.L., Fritz, V., Chikanza, I.C., Thomas, J.E., Levy, L.F., and Saffer, D. (1994) Multiple sclerosis in black South Africans and Zimbabweans. *J. Neurol. Neurosurg. Psychiatry* **57**, 1064-1069.
11. Feder, J.N., Gnirke, A., Thomas, W., *et al.* (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nature Genet.* **13**, 399-408.
12. De Villiers, J.N.P., Hillermann, R., Loubser, L., and Kotze, M.J. (1999) Spectrum of mutations in the HFE gene implicated in haemochromatosis and porphyria. *Hum. Mol. Genet.* **8**, 1517-1522.

13. Valberg, L.S., Flanagan, P.R., Kertesz, A., and Ebers, G.C. (1989) Abnormalities in iron metabolism in multiple sclerosis. *Can. J. Neurol. Sci.* **16**, 184-186.
14. Kotze, M.J., de Villiers, J.N.P., Carr, J., Schmidt, S., Warnich, L., and Hillermann, R. (1999) Lack of clinical expression of mutation C282Y implicated in haemochromatosis and porphyria in two sisters diagnosed with multiple sclerosis. *Eur. J. Hum. Genet.* **7** (Suppl), 59.
15. Schiffer, R.B., Wetkamp, L.R., Ford, C., and Hall, W.J. (1994) A genetic marker and family history study of the upstate New York multiple sclerosis cluster. *Neurology* **44**, 329-333.
16. LeVine, S.M., Lynch, S.G., Ou, C.N., Wulser, M.J., Tam, E., and Boo, N. (1999) Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients. *Brain Res.* **821**, 511-515.
17. Weller, M., List, U., Schabet, M., Melms, A., and Dichgans, J. (1999) Elevated CSF lactoferrin in superficial siderosis of the central nervous system. *J. Neurol.* **246**, 943-945.
18. Hulet, S.W., Powers, S., and Connor, J.R. (1999) Distribution of transferrin and ferritin binding in normal and multiple sclerosis human brains. *J. Neurol. Sci.* **165**, 48-55.
19. Rooney, R.N., Kotze, M.J., de Villiers, J.N.P., Hillermann, R., and Cohen, J.A. (1999) Multiple sclerosis, porphyria-like symptoms and a history of iron deficiency anemia in a family of Scottish descent. *Am. J. Med. Genet.* **86**, 194-196.
20. Zeman, D., Adam, P., Kalistova, H, Sobek, O., Kelbich, P., Andel, J., and Andel, M. (2000) Transferrin in patients with multiple sclerosis: a comparison among various subgroups of multiple sclerosis patients. *Acta. Neurol. Scand.* **101**, 89-94.
21. **Atkinson, P.G.P., and Barton, C.H. (1998) Ectopic expression of Nramp1 in COS-1 cells modulates iron accumulation. FEBS Lett. 425, 239-242.**
22. Searle, S., and Blackwell, J.M. (1998) Evidence for a functional repeat polymorphism in the promoter of the human NRAMP1 gene that correlates with autoimmune versus infectious disease susceptibility. *J. Med. Genet.* **36**, 295-299.
23. Blackwell, J.M., and Searle, S. (1999) Genetic regulation of macrophage activation: Understanding the function of Nramp1 (=Ity/Lsh/Bcg). *Immunol. Lett.* **65**, 73-80.
24. Hofmeister, A., Neibergs, H.L., Pokorny, R.M., and Galandiuk, S. (1997) The natural resistance-associated macrophage protein gene is associated with Crohn's disease. *Surgery* **122**, 173-178.

25. Graham, A.M., Dollinger, M.M., Howie, S.E.M., and Harrison, D.J. (2000) Identification of novel alleles at a polymorphic microsatellite repeat region in the human NRAMP1 gene promoter: Analysis of allele frequencies in primary biliary cirrhosis. *J. Med. Genet.* **37**, 150-152.
26. Minuk, G.Y., and Lewkonja, R.M. (1986) Possible familial association of multiple sclerosis and inflammatory bowel disease. *N. Engl. J. Med.* **314**, 586.
27. Agranof, D., and Schon, F. (1995) Are focal white matter lesions in patients with inflammatory bowel disease linked to multiple sclerosis? *Lancet* **346**, 190-191.
28. Lossos, A., River, Y., Eliakim, A., and Steiner, I. (1995) Neurological aspects of inflammatory bowel disease. *Neurology* **45**, 416-421.
29. Pontecorvo, M.J., Levinson, J.D., and Roth, J.A. (1992) A patient with primary biliary cirrhosis and multiple sclerosis. *Am. J. Med.* **92**, 433-436.
30. Bergsteinsdottir, K., Yang, H.T., Pettersson, U., and Holmdahl, R. (2000) Evidence for common autoimmune disease genes controlling onset, severity and chronicity based on experimental models for multiple sclerosis and rheumatoid arthritis. *J. Immunol.* **164**, 1564-1568.
31. Weber, J., Werre, J.M., Julius, H.W., and Marx, J.J. (1988) Decreased iron absorption in patients with active rheumatoid arthritis, with and without iron deficiency. *Ann. Rheum. Dis.* **47**, 404-409.
32. Nielsen, O.J., Andersen, L.S., Hansen, N.E., and Hansen T.M. (1994) Serum transferrin receptor levels in anaemic patients with rheumatoid arthritis. *Scand. J. Clin. Lab. Invest.* **54**, 75-82.
33. Cazzola, M., Ponchio L., De Benedetti, F., *et al.* (1996) Defective iron supply for erythropoiesis and adequate endogenous erythropoietin production in the anemia associated with systemic-onset juvenile chronic arthritis. *Blood* **87**, 4824-4830.
34. Poser, C.M., Paty, D.W., Scheinberg, L., *et al.* (1983) New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann. Neurol.* **13**, 227-231.
35. Thomson, G. (1995) Mapping disease genes: family-based association studies. *Am. J. Hum. Genet.* **57**, 487-498.
36. Miller, S.A., Dykes, D.D., and Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl. Acids Res.* **16**, 1215.
37. Kotze, M.J., Theart, L., Callis, M., Peeters, A.V., Thiart, R., and Langenhoven, E. (1995) Nonradioactive multiplex PCR screening strategy for the simultaneous

- detection of multiple low-density lipoprotein receptor gene mutations. *PCR Methods Appl.* **4**, 352-356.
38. StatSoft, Inc. (1995). STATISTICA for Windows [Computer program manual]. Tulsa, OK: StatSoft, Inc., e-mail: [info@statsoft.com](mailto:info@statsoft.com), WEB: <http://www.statsoft.com>.
  39. Schneider, S., Roessli, D., and Excofier, L. (2000) Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept, of Anthropology, University of Geneva.
  40. Aisen, P. (1994) Iron Metabolism in Health and Disease. *In* Iron metabolism: An evolutionary perspective. (Brock, J.H., Halliday, J.W., Pippard, M.J., and Powell, L.W. Eds) pp. 1-30. Saunders Co. Ltd. London.
  41. Roskams, A.J.I., and Connor, J.R. (1994) Iron, transferrin, and ferritin in the rat brain during development and aging. *J. Neurochem.* **63**, 709-716.
  42. Ponka, P., and Lok, C.N. (1999) The transferrin receptor: Role in health and disease. *Int. J. Biochem. Cell. Biol.* **31**, 1111-1137.
  43. Weiss, G., Wachter, H., and Fuchs, D. (1995) Linkage of cell-mediated immunity to iron metabolism. *Immunol. Today* **16**, 495-500.
  44. Blackwell, J.M. (1996) Structure and function of the natural resistance-associated macrophage protein (NRAMP1), a candidate protein for infectious and autoimmune disease susceptibility. *Mol. Med. Today* **2**, 205-211.
  45. Risch, N., and Merikangas, K. (1996) The future of genetic studies of complex human diseases. *Science* **273**, 1516-1517.
  46. Greenberg, D.A. (1992) Linkage disequilibrium of "necessary" disease loci versus "susceptibility" loci. *Am. J. Hum. Genet.* **52**, 135-143.
  47. Haines, J.L. Terwedow, H.A., Burgess, K., *et al.* (1998) Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. *Hum. Mol. Genet.* **7**, 1229-1234.
  48. Blackwell, J., Freeman, J., and Bradley, D. (1980) Influence of H-2 complex on acquired resistance to *Leishmania donovani* infection in mice. *Nature* **283**, 72-74.
  49. Jacobson, M., Schewe, D., Ziegler, A., *et al.* (2000) A point mutation in PTPRC is associated with the development of multiple sclerosis. *Nature Genet.* **26**, 495-499.
  50. Reich, B., Ehlers, I., Will, H., Heesen, C., de Villiers, J.N.P., Rooney, R.N., and Kotze, M.J. (1998) Autoantibodies against nucleolar structures correlate

- significantly with an unusual association between multiple sclerosis and porphyria symptoms. *J. Neuroimmunol.* **90** (Suppl), 77.
51. Sampietro, M., Piperno, A., Lupica, L., *et al.* (1998) High prevalence of the His63Asp HFE mutation in Italian patients with porphyria cutanea tarda. *Hepatology* **27**, 181-184.
  52. Levy, J.E., Jin, O., Fujiwara, Y., Kuo, F., and Andrews, N.C. (1999) Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nature Genet.* **21**, 396-399.
  53. Whitfield, J.B., Cullen, L.M., Jazwinska, E.C., *et al.* (2000) Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am. J. Hum. Genet.* **66**, 1246-1258.
  54. Willis, G., Wimperis, J.Z., Smith, K.C., Fellows, I.W., and Jennings, B.A. (2000) Haemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet* **354**, 221-222.
  55. Bennet, S.T. (1995) Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nature Genet.* **9**, 284-292.
  56. Meyer, U.A., Schuurmans, M.M., and Lindberg, R.L.P. (1998) Acute porphyrias: Pathogenesis of neurological manifestations. *Sem. Liver Disease.* **18**, 43-52.
  57. Lindberg, R.L., Martini, R., Baumgartner, M., *et al.* (1999) Motor neuropathy in porphobilinogen deaminase-deficient mice imitates the peripheral neuropathy of human acute porphyria. *J. Clin. Invest.* **103**, 1127-1134.
  58. Heales, S.J., Bolanos, J.P., Stewart, V.C., Brookes, P.S., Land, J.M., and Clark, J.B. (1999) Nitric oxide, mitochondria and neurological disease. *Biochim. Biophys. Acta*, **1410**, 215-228.



**Table 1.** Biochemical iron status parameters measured in the serum of 53 South African MS patients classified according to relapsing-remitting, secondary progressive and primary progressive disease types.

Variable	Relapsing-remitting (n=34)		Secondary progressive (n=10)		Primary progressive (n=9)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	40.3	10.9	46.4	12.1	47.8	7.1
S-iron ( $\mu\text{mol/l}$ )	17.0	6.8	17.8	6.8	16.6	6.4
Transferrin (g/l)	2.8	0.4	2.9	0.4	2.6	0.4
Transf sat (%)	25.1	10.6	25.4	12.0	25.4	10.0
Ferritin ( $\mu\text{g/l}$ )	77.9	63.5	117.0	83.1	171.8*	135.7

\*Relapsing-remitting vs primary progressive MS: P=0.04

**Table 2.** Comparison of allelic distribution between South African MS patients (22 males, 82 females) and controls. The controls (1076 alleles) are grouped according to "affected-family based control" (AFBAC), general population-matched controls (118 males, 211 females) and elderly subjects (67 males, 126 females).

Alleles	MS Patients	Controls			
		<i>General</i>	<i>AFBAC</i>	<i>Elderly</i>	<i>Total</i>
2 (30%)	41 (20%)	223 (34%)	11 (34%)	86 (22%)	320
3 (70%)	160 (77%)	434 (66%)	21 (66%)	297 (77%)	752
5 (0.4%)*	7 (3%)	1 (0.1%)	0	3 (0.8%) <sup>#</sup>	4
<b>Total</b>	<b>208</b>	<b>658</b>	<b>32</b>	<b>386</b>	<b>1076</b>

*NRAMP1* alleles are numbered according to Ref 25.

\*MS vs total control group:  $P < 0.01$ , 2 d.f.,  $\chi^2 = 35.2$ ; MS vs general control group:  $P < 0.01$ , 2 d.f.,  $\chi^2 = 30.8$ ; MS (16 patients) vs AFBAC (32 parental alleles):  $P < 0.01$ , 2 d.f.,  $\chi^2 = 43.79$ ; MS vs elderly control group:  $P = 0.05$ , 2 d.f.,  $\chi^2 = 5.7$

<sup>#</sup>General control group vs elderly subjects:  $P < 0.01$ , 2 d.f.,  $\chi^2 = 16.6$

**Table 3.** Clinical characteristics of South African MS patients with NRAMP1 allele 5.

	Index Patients (n=7)							Relatives with definite MS (n=2)	
Patient numbers	43	58	67	70	73	81	90	Sister of 43	Aunt of 81
Gender	F	F	F	F	M	F	F	F	F
Age (years)	48	38	27	35	45	27	29	39	50
Age of onset (years)	38	24	20	32	41	23	15	15	23
Age at diagnosis ( years)	41	30	24	33	42	23	23	25	46
Disease course	RR	RR	RR	RR	SP	RR	RR	RR	SP
<i>Iron parameters*</i>									
Hemoglobin (11.5-16.5 g/dl)	13.5	15.0	14.4	12.6	14.0	12.0	13.7	ND	13.6
Serum iron (6-32 µmol/l)	11.0	14.2	23.3	21.5	16.3	29.7	16.1	ND	17.1
Transferrin (1.8-3.8 g/l)	3.0	2.4	3.0	3.1	2.3	2.5	3.0	ND	2.4
Transferrin saturation (15-50 %)	15	24	31	30.3	28	52.7	21	ND	31
Ferritin (12-119 µg/l)	33.5	88.8	70.2	157.9	329.5	216.6	14.5	51.2	77.3

F, female; M, male; RR, relapsing-remitting; SP, secondary progressive; ND, not determined

\*Reference values for males: hemoglobin 12.5-17.5 g/dl, serum iron 7-35 µmol/l, transferrin 1.8-3.8 g/l, saturation 20-50%, ferritin 29-396 µg/l