Investigation into aspects of the epidemiology of the common HFE gene mutations and their effects on iron loading

Andrew J. Stewart

Submitted for the degree of Doctor of Medicine
in the University of Edinburgh, 2006

Declaration

I, Andrew Stewart, declare that this thesis, submitted for the degree of MD in the University of Edinburgh, is my own work and that the work described in it is also my own. It has not been submitted in candidature for any other degree, postgraduate diploma or professional qualification.

Andrew Stewart
May 2005

Acknowledgements

I would like to acknowledge the support and encouragement given to me by Dr Peter Johnson, Royal Infirmary of Edinburgh, in carrying out the work for this thesis and subsequently in committing it to paper; without this, the thesis would never have been written.

I am also grateful to Professor Christopher Ludlam for his support in allowing me to change the field of my research while I was Clinical lecturer in Haematology at the Royal Infirmary of Edinburgh and to the biomedical scientists in the haematology laboratory at the Royal Infirmary of Edinburgh for their assistance and forebearance.

Dr David Stirling provided invaluable advice and assistance in setting up the PCR system.

Professor J. Porter and his team at University College Hospital in London kindly performed the assays of non-transferrin bound iron.

I would also like to thank most sincerely the following groups of colleagues:

The nursing and medical staff of the Edinburgh Blood Donor Centre, especially Dr Jack Gillon, gave me much appreciated assistance and support in performing the work for Chapter 4 of this thesis;

The staff of the Cardiology Department, Royal Infirmary of Edinburgh for assistance in collecting patient information for Chapter 5; Dr Fraser Witherow for analysing and scoring the coronary angiograms.

The staff of the Scottish Liver Transplant Unit for allowing me access to patients' records and helping to collect blood samples for Chapter 6.

The staff of the Department of Pathology, Edinburgh University for scoring the iron stains of liver biopsies and advice on PCR techniques.

Dr David Mazza provided much needed feedback and encouragement about the questionnaire used in Chapter 8.

My colleagues at the Department of Haematology, Monklands Hospital, Airdrie have been very generous in allowing me study leave to enable me to complete the thesis, and I am very grateful to Mr A Craig, Medical Illustration Department, Monklands Hospital for help in laying out and producing the thesis.

Finally, my wife, Rachel, has provided unstinting support and encouragement during the many periods of time when I nearly gave up.

List of abbreviations

A	adenine
ARMS	amplification refractory mutation system
BSA	bovine serum albumen
BMI	body mass index
C	cytosine
CDC	Centers for Disease Control and Prevention
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ΔHb	change in haemoglobin concentration
Δferr	change in ferritin
EASL	European Association for the study of the Liver
EDTA	ethylenediaminetetraacetic acid
ESR	erythrocyte sedimentation rate
FP1	ferroportin 1
G	guanine
GP	general practitioner
Hb	haemoglobin
HII	hepatic iron index
HLA	human leucocyte antigen
HH	haemochromatosis
IRE	iron responsive element
IRP(1,2)	iron responsive protein (1,2)
LFT	liver function test
MADGE	microplate array diagonal gel electrophoresis
MBG	molecular biology grade
MCV	mean cell volume

MHC	major histocompatibility complex
MI	myocardial infarction
MW	molecular weight
NCH	non classical haemochromatosis
NHS	National Health Service
NTBI	non-transferrin bound iron
OLT	orthotopic liver transplant
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SNBTS	Scottish National Blood Transfusion Service
SQUID	superconducting quantum interference device
T	thymine
TAE	tris-acetate-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TfR (2)	transferrin receptor (2)
TIBC	total iron binding capacity
TS	transferrin saturation
U	uracil
U&E	urea and electrolytes
UIBC	unsaturated iron binding capacity
USF2	upstream stimulatory factor 2
UTR	untranslated region
UV	ultraviolet
WCC	white cell count

Investigation into aspects of the Epidemiology of the common HFE gene mutations and their effects on iron loading

Abstract of thesis

Background: The discovery of mutations in the HFE gene has stimulated a great deal of interest in the epidemiology of hereditary haemochromatosis, its prevalence, questions about population screening, and the possible role of the mutations in conditions other than haemochromatosis. In addition, more and more information is becoming available about the mechanisms controlling iron absorption in normal and pathological states. There is some evidence that haemochromatosis and its associated genetics are not widely understood within the medical community.

Aim: The aim of this project was to investigate some aspects of the role of HFE gene mutations in conditions other than hereditary haemochromatosis in which iron loading might have a pathogenetic role and to examine the hypothesis that heterozygosity for the C282Y mutation offers some protection against iron deficiency. An additional aim was to assess the level of knowledge about haemochromatosis among primary care physicians in the south east of Scotland and the feasibility of using microplate array gel electrophoresis technology to facilitate the laboratory assessment of the mutations.

Methods: DNA extracted from peripheral blood was amplified in a multiplex amplification refractory mutation system (ARMS). The results were analysed using microplate array diagnostic gel electrophoresis technology (MADGE).

A number of separate studies were performed:

1. Haemoglobin concentration, iron stores and HFE genotypes of 366 male blood donors were studied as they donated blood over a period of one year, with the aim of identifying any protection afforded by HFE gene mutations against the development of iron deficiency induced by blood donation.
2. Iron stores and HFE genotypes of 180 recipients of orthotopic liver transplants were assessed and liver biopsy specimens were graded for tissue iron deposition. Correlation was sought between HFE genotypes and iron measurements as well as length of stay in the transplant unit after the initial transplant procedure.
3. HFE genotypes were analysed for a population of 351 individuals identified as iron deficient and compared with genotype results from a control population of 521 individuals with no full blood count evidence of iron deficiency.
4. Correlation between iron stores or HFE genotype and the severity of coronary artery disease was assessed in a population of 286 patients undergoing coronary angiography.
5. A postal questionnaire was sent to 540 general practitioners to assess their level of awareness of hereditary haemochromatosis.

Results: It was demonstrated that it was feasible to use the MADGE system to facilitate rapid HFE genotyping of relatively large numbers of samples.

The prevalence of HFE genotypes in these studies correlated well with that reported for comparable populations in the literature. No evidence was found to support the theory that heterozygosity for the C282Y mutation can protect against the development of iron deficiency. There was no evidence of a correlation between increasing iron stores or HFE genotype and coronary artery disease in the population studied. No influence of HFE genotype on the outcome after orthotopic liver transplantation was identified. The questionnaire to general practitioners revealed a great variation in individuals' awareness of hereditary haemochromatosis.

Table of Contents

Page

Declaration i

Acknowledgements ii

List of abbreviations iii

Abstract of thesis v

Background v

Aim v

Methods v

Results vi

Table of Contents vii

List of Figures and Tables xiii

1 Preface 1

2 Background to thesis and Review of the Literature 3

2.1 Historical Overview 3

2.1.1 Discovery of the "haemochromatosis gene" 4

2.2 Epidemiology - the distribution of HFE gene mutations in different populations 6

2.3 Genetic heterogeneity of haemochromatosis 9

2.3.1 Nomenclature 9

2.3.2 Mutations of HFE apart from C282Y 10

2.3.2.1 The significance of H63D 10

2.3.2.2 Other mutations 11

2.3.3 Non-HFE-linked haemochromatosis 12

2.3.4 The iron status of heterozygotes for hereditary haemochromatosis 15

2.3.4.1 Iron deficiency in heterozygotes 16

2.4 Making the diagnosis of haemochromatosis 16

2.4.1 Assessment of iron overload 16

2.4.1.1 Blood measures of iron load 16

2.4.1.2 Liver biopsy in the diagnosis of haemochromatosis 17

2.4.2 Clinical features 20

2.4.3 Diagnostic algorithm 20

2.5 Prevalence of haemochromatosis and penetrance of the HFE genotypes 20
2.6 Screening for haemochromatosis 25
2.6.1 Case definition of haemochromatosis 26
2.6.2 Education of health-care workers and the public 27
2.6.3 Which initial test to use in screening? 28
2.6.4 Cost-effectiveness of screening 30
2.6.5 Potential harm of screening 33
2.6.6 Which population to screen: case finding or population screening? 35
2.7 Iron metabolism 37
2.7.1 Storage and transport of iron 37
2.7.2 Absorption of iron 38
2.7.3 Iron uptake by somatic cells 41
2.7.4 Regulation of iron absorption 41
2.7.4.1 Iron regulation at the level of the whole organism 41
2.7.4.2 Iron regulatory proteins as iron sensors at the cellular level 42
2.7.4.3 Molecular characterisation of iron regulatory proteins 43
2.7.4.4 The structure and possible function of HFE 44
2.8 Conclusion 45

3 Methods 46
3.1 Extraction of DNA from peripheral blood 46
3.1.1 Materials 46
3.1.2 Preparation of reagents 46
3.1.3 Method 48
3.2 Measurement of DNA concentration 49
3.3 Extraction of DNA from paraffin blocks 49
3.3.1 Removal of paraffin 49
3.3.1.1 Materials 49
3.3.1.2 Methods 49
3.3.2 DNA Extraction method 1 50
3.3.2.1 Materials 50
3.3.2.2 Preparation of reagents 50
3.3.2.3 Method 50
3.3.3 DNA Extraction method 2 51
3.3.3.1 Materials 51
3.3.3.2 Preparation of reagents 51
3.3.3.3 Method 52

3.4 Detection of mutations in the HFE gene by a multiplex amplification refractory mutation system 52
3.4.1 Principle of method 52
3.4.2 Interpretation of PCR results 52
3.4.3 Preparation for and execution of the PCR 55
3.4.3.1 Materials 55
3.4.3.2 Preparation of reagents 56
3.4.3.3 Method 57
3.5 HFE mutation detection by PCR and restriction enzyme digestion 58
3.5.1 Polymerase chain reaction 58
3.5.1.1 Materials 58
3.5.1.2 Method 58
3.5.2 Restriction enzyme digestion 59
3.5.2.1 Materials 59
3.5.2.2 Method 59
3.6 HFE intron 4 polymorphism and inaccuracy in diagnosis 60
3.7 Polyacrylamide and agarose gel electrophoresis 61
3.7.1 Polyacrylamide gel 61
3.7.1.1 Materials 61
3.7.1.2 Preparation of reagents 61
3.7.1.3 Methods 62
3.7.2 Agarose gel 62
3.7.2.1 Materials 62
3.7.2.2 Methods 62
3.8 Microplate array diagonal gel electrophoresis (MADGE) 64
3.8.1 Materials 64
3.8.1.1 Preparation of reagents 64
3.8.1.2 Methods 64
3.9 Optimisation of PCR and electrophoresis techniques 65
3.9.1 Magnesium concentration 67
3.9.2 Number of PCR cycles 67
3.9.3 Primer concentration 67
3.9.4 Q solution 67
3.9.5 DNA concentration 68
3.9.6 Gel composition 68
3.9.7 Gel destaining 68

3.10 Quality Assurance 68
3.11 Use of the MADGE system 69
3.12 Isolation of DNA from paraffin sections 69
3.13 Laboratory measurement of iron stores 69
3.13.1 Serum ferritin 69
3.13.2 Serum iron 69
3.13.3 Total iron binding capacity 70
3.13.4 Transferrin saturation 70
3.13.5 Non-transferrin bound iron 70
3.14 Statistical Analysis 70

4 Investigation of the effect of HFE gene genotype on blood donors who make frequent red cell donations 71
4.1 Introduction 71
4.2 Methods 73
4.2.1 Subject recruitment 73
4.2.2 Sample collection 74
4.2.3 Sample analysis 75
4.2.4 Data set 75
4.2.5 Follow-up of participants 75
4.3 Statistical analysis 76
4.4 Results 76
4.4.1 HFE genotypes 78
4.4.2 Questionnaire 90
4.5 Discussion 90
4.5.1 Genotype frequency 90
4.5.2 Iron deficiency among donors 90
4.5.3 Haemochromatosis-associated genotypes 91
4.5.4 Iron status at study entry 92
4.5.5 Change in haemoglobin and ferritin 92
4.6 Conclusions 92

5 Study of the prevalence of HFE genotypes in iron deficiency 93

5.1 Background 93

5.2 Aim 93

5.3 Methods 93

5.3.1 Iron deficient samples 93

5.3.2 Control samples 94

5.4 Statistical analysis 94

5.5 Results 94

5.6 Discussion 97

5.7 Conclusion 98

6 Haemochromatosis gene mutations and hepatic iron deposition in patients undergoing orthotopic liver transplantation 99

6.1 Background 99

6.2 Methods 100

6.2.1 DNA extraction 101

6.2.2 HFE genotyping 101

6.2.3 Ascertainment of iron status 101

6.2.4 Examination of liver biopsies 102

6.3 Statistical analysis 102

6.4 Results 102

6.5 Discussion 109

6.6 Conclusion 110

7 Iron status and HFE gene mutations in patients with coronary artery disease shown at coronary angiography 111

7.1 Introduction 111

7.2 Aim 114

7.3 Method 114

7.3.1 Subject recruitment 114

7.3.2 Sample collection 114

7.3.3 Data collection and analysis 115

7.3.4 Coronary angiography 115

7.3.5 Assay of non-transferrin bound iron 115

7.4 Statistical analysis 116

7.5 Results 116
7.6 Discussion 121
7.7 Conclusions 122

8 Survey of General Practitioners' attitudes to and experience of haemochromatosis 123
8.1 Introduction 123
8.2 Data collection - theoretical considerations 124
8.3 Method 125
8.4 Results 126
8.5 Comments from GPs 130
8.6 Discussion 132
8.7 Conclusion 135

9 Concluding comments 137

References 140

Presentations of this work to learned societies 174

Appendix 1: Information sheet for donors 175
Appendix 2: Questionnaire for participants in study of blood donors' iron levels 176
Appendix 3: Letter to C282Y heterozygotes 178
Appendix 4: Letter to General Practitioner of C282Y heterozygotes 180
Appendix 5: Letter to participants wild type for C282Y 181
Appendix 6: Information sheet for patients participating in coronary angiography study 182
Appendix 7: The Hardy-Weinberg equilibrium 183
Appendix 8: Questionnaire for GP survey 184
Appendix 9: Data on control and iron deficient subjects 185

List of Figures and Tables

Page

Figure 2.1 Structure of HFE, its association with β_2 microglobulin, and location of the two common mutations 5

Table 2.2 Allele frequencies of HFE mutations in control populations 7

Table 2.3 Frequency of HFE genotypes in haemochromatosis patients 8

Figure 2.4 Algorithm for the diagnosis of hereditary haemochromatosis 22

Table 2.5 Screening for haemochromatosis in specific patient populations 36

Table 2.6 Iron distribution and flux in a 70kg man 39

Figure 3.1 Separation of aqueous and chloroform layers after centrifugation 48

Figure 3.2 ARMS system for amplification of C282Y mutation 53

Table 3.3 Contents of PCR reaction mixtures 57

Table 3.4 Mastermix for PCR for restriction digest analysis 60

Figure 3.5 Photograph of standard polyacrylamide gel electrophoretic pattern after ARMS PCR for HFE gene mutations 63

Figure 3.6 Photograph of MADGE gel showing PCR products after ARMS PCR for HFE gene mutations 66

Table 4.1 Reasons for failure to complete four study blood donations 77

Table 4.2 Characteristics of donors excluded because haemoglobin <125g/L 77

Figure 4.3 Distribution of HFE genotypes 79

Table 4.4 Distribution of HFE genotypes in the study population 80

Table 4.5 Characteristics of subjects homozygous for C282Y 80

Table 4.6 Characteristics of compound heterozygote subjects 80

Table 4.7 Breakdown of initial haemoglobin by genotype 81

Figure 4.8 Scatter plot representation of initial haemoglobin values by genotype 81

Table 4.9 Breakdown of initial ferritin by genotype 82

Figure 4.10 Scatter plot representation of initial ferritin values by genotype 82

Table 4.11 Breakdown of initial transferrin saturation by genotype 83

Figure 4.12 Scatter plot representation of initial transferrin saturation values by genotype 83

Table 4.13 Change in ferritin and haemoglobin between first and last study visits for individuals of different genotypes 84

Figure 4.14 Change in haemoglobin between first and last study visit by genotype 85

Figure 4.15 Change in ferritin between first and last study visit by genotype 86

Figure 4.16 Change in ferritin between first and last study visit by donor weight 87

Figure 4.17 Change in ferritin between first and last study visit by number of occasions red meat eaten per week 88

Figure 4.18 Change in haemoglobin between first and last study visit by donor weight 89

Table 5.1 Characteristics of iron deficient and normal populations 95

Figure 5.2 Distribution of HFE genotypes in an iron deficient and a non-iron deficient population 96

Table 6.1 Distribution of HFE genotypes among liver transplant recipients 104

Table 6.2 Characteristics of liver transplant recipients with genotype HY/HY 104

Table 6.3 Characteristics of liver transplant recipients with genotype HY/DC 105

Table 6.4 Characteristics of patients with severe hepatic iron overload 105

Figure 6.5 Scatter plot of the distribution of serum ferritin according to iron stain grade of hepatic biopsy 106

Figure 6.6 Scatter plot of the distribution of hepatic iron stain grades among patients with the various HFE genotypres 107

Figure 6.7 Scatter plot of the distribution of length of stay in transplant unit according to HFE genotype 108

Table 7.1 Characteristics of the populations with and without coronary artery disease at angiography 117

Table 7.2 Distribution of HFE genotypes between the two study populations 118

Figure 7.3 Scatter plot of the relationship between HFE genotype and non-transferrin-bound iron (NTBI) 119

Figure 7.4 Scatter plot of the distribution of non-tranferrin-bound iron according to angiography scores 120

Table 8.1 Distribution of GP practice size among respondents (Q1) 126

Table 8.2 Number of patients with haemochromatosis identified by responding physicians within their own practice (Q2) 127

Table 8.3 Responses to question 4 regarding laboratory tests 128

Table 8.4 Opinions on the necessity of various clinical signs and symptoms for the diagnosis of haemochromatosis to be made (Q5) 128

Table 8.5 Opinions on the treatment and presentation of haemochromatosis 129

Table 8.6 Physicians' attitudes to screening for haemochromatosis 130

1. Preface

The discovery, in 1996, of the HFE gene mutations associated with hereditary haemochromatosis led to a burgeoning literature covering many aspects of this condition. There was huge interest in the epidemiology of HFE mutations and especially the discovery that most patients of northern European ancestry with hereditary haemochromatosis are homozygous for the C282Y HFE mutation. However, while the C282Y mutation is prevalent in northern European populations, its frequency is much lower in other populations in some of which there are increasing numbers of reports of non-HFE linked haemochromatosis. The maintenance of C282Y at a relatively high frequency in northern European populations has led to speculation that its carriers may be at an evolutionary advantage, possibly through a degree of protection against iron deficiency.

At the same time, knowledge of the cellular mechanisms governing human iron absorption and of the pathways regulating this at both cellular and whole organism levels is steadily advancing as more becomes clear about the possible role of the HFE protein and its interaction with transferrin receptor –1. The possibility that iron might be implicated in the pathogenesis of coronary heart disease has evoked considerable interest, as has the related hypothesis that iron stores (and/or carriage of C282Y) might correlate with an increased incidence of other disease states such as malignancy and venous thrombosis.

With the discovery of a genetic marker for haemochromatosis has come a vigorous debate about screening. Haemochromatosis is easily and effectively treated if diagnosed before irreversible liver damage or diabetes has occurred and some have proposed mass screening programmes to facilitate early detection. Others have argued equally strongly against population screening on the basis of increasingly strong evidence that many individuals with a "haemochromatosis genotype" may not ever develop clinically relevant iron overload.

The aim of this thesis was to examine the effects of iron overload in different patient groups. Patients with coronary artery disease were investigated for any correlation between the extent of their arterial disease and their iron stores and HFE genotypes. None was found, which is an important contribution to the debate about the role of iron in the pathogenesis of coronary artery disease. Because of some evidence that patients with haemochromatosis fare less well after orthotopic liver transplantation (OLT) than other patient groups, a group of patients who had had an OLT were investigated for their iron stores and HFE genotypes. Several patients with previously undiagnosed haemochromatosis were identified.

Because of the possibility that C282Y carriage affords protection against iron deficiency, male blood donors were investigated for any evidence that C282Y heterozygotes could donate blood more frequently than those without this mutation without becoming deficient in iron. This study demonstrated that it is unlikely to be feasible to select donors on the basis of HFE gene mutations to donate blood more frequently - an important negative result at a time when there is intense interest in attempts to augment the blood supply. A further study showed no evidence of an increased prevalence of C282Y in an iron-deficient, compared with a non-iron deficient population.

There is evidence in the literature to suggest that many in the medical profession are unaware of the prevalence, clinical features and diagnosis of haemochromatosis. To investigate this, a questionnaire on haemochromatosis was sent to all general practitioners covering a population of approximately 750,000 in south east Scotland to assess their clinical experience of diagnosing and treating this condition and their general awareness of it. The returns suggested considerable variability in both experience and awareness among the respondents.

The thesis also examined the possibility of using a microplate array system to facilitate the simultaneous determination of the HFE genotype of large numbers of samples using a multiplex amplification refractory mutation system and identified this as a means of determining accurately the presence or absence of both the C282Y mutation and the other common HFE mutation, H63D, simultaneously in a relatively large number of DNA samples.

2. Background to thesis and review of the literature

2.1 Historical Overview

The first recognition of the clinical effects of advanced haemochromatosis was made by Trousseau, when he described the combination of diabetes mellitus and hyper-pigmentation of the skin (Trousseau, 1865). The initial clue to the pathogenesis of the disorder was provided by Troisier's description six years later of "diabète bronzé et cirrhose pigmentaire" (Troisier, 1871). von Recklinghausen who, in 1889, advanced a theory that the pigmentation of the liver he saw at autopsy was due to the accumulation of iron derived from the blood, was the first to use the term haemochromatosis ("Hämochromatose") (von Recklinghausen, 1889). In 1935 Sheldon reviewed the extant literature on more than 300 cases and suggested that the condition could be hereditary. He also drew attention to the age profile (90% over 35 years old) and the sex incidence (over 90% in males) of sufferers (Sheldon, 1935). Twenty years later, Finch and Finch reviewed a further 707 histologically proven cases of haemochromatosis that had appeared in the literature since the appearance of Sheldon's monograph (Finch, 1955). They described haemochromatosis as uncommon and, based on records of hospital admissions and deaths, estimated that there were about 20 000 sufferers in the United States. The most useful clinical clue to alert the physician of the time to a possible diagnosis of haemochromatosis was the "classical tetrad" of skin pigmentation, diabetes, liver disease and heart disease.

Not everyone was initially convinced of the heritable nature of haemochromatosis, however. In the mid 1960s MacDonald concluded that iron overload and haemochromatosis resulted from the consumption of alcoholic beverages (MacDonald, 1964). In 1969, Saddi and Feingold proposed a recessive mode of inheritance (Saddi, 1969). Seven years later, the discovery that haemochromatosis in the population of Brittany was linked to human leukocyte antigen allele A3 (HLA-A3) in the Major Histocompatibility Complex (MHC) on the short (p) arm of chromosome 6 ushered in the modern era of genetic investigation (Simon, 1975). This discovery enabled the use of HLA-typing to track the haemochromatosis allele in families containing a haemochromatosis proband (Milman, 2000).

The linkage to HLA-A3 was confirmed in other populations of northern European extraction, in among other countries, the U.K. (Bomford, 1977), Germany (Dyrszka, 1979) and Sweden (Ritter, 1984).

2.1.1 *Discovery of the "haemochromatosis gene"*

The ability further to characterise the ancestral haplotype of haemochromatosis came with the advent of microsatellite mapping. This permitted the demonstration that the microsatellite marker D6S105 was associated with haemochromatosis (Jazwinska, 1993). In all populations studied using microsatellite mapping, it has been possible to identify the alleles linked with haemochromatosis as: D6S265 –1, HLA-A3 and D6S105-8, although the strength of association of each of these alleles varies somewhat from population to population (Raha-Chowdhury, 1995; Jazwinska, 1996; Gandon, 1996; Camaschella, 1996).

In 1996, Feder and others used linkage disequilibrium studies and high resolution haplotype analysis to build upon this information and identify as a candidate gene for haemochromatosis an HLA class I-like gene. Initially, this was called HLA-H, but it was soon renamed HFE in compliance with the accepted nomenclature for the HLA system (Bodmer, 1997).

Feder et al (1996) endeavoured to identify the probable ancestral mutation that resulted in the haemochromatosis phenotype. A likely candidate for this was a G to A change at nucleotide 845, resulting in a substitution of tyrosine (Y) for cysteine (C) at amino acid 282, a highly conserved residue involved in an intramolecular disulphide bond (see Figure 2.1). In their group of 178 patients, this mutation, referred to from here onwards as C282Y, was found on 85% of all haemochromatosis chromosomes. 3.2% of control chromosomes carried the mutation. All the chromosomes that carried C282Y also contained the ancestral haplotype. However, consideration of the number of heterozygotes revealed an excess over that predicted by the Hardy-Weinberg equilibrium (see Appendix 7), and led to a search for a second mutation. A second mutation was found: a C to G change resulting in a change from histidine to aspartic acid at amino acid 63 (H63D). This mutation was found with a high frequency on control chromosomes and 4% of haemochromatosis patients were compound heterozygotes for C282Y and H63D.

The structure of the molecule encoded by HFE and its potential role in iron metabolism are discussed in section 2.7.4.4. The HFE molecule is illustrated schematically in figure 2.1.

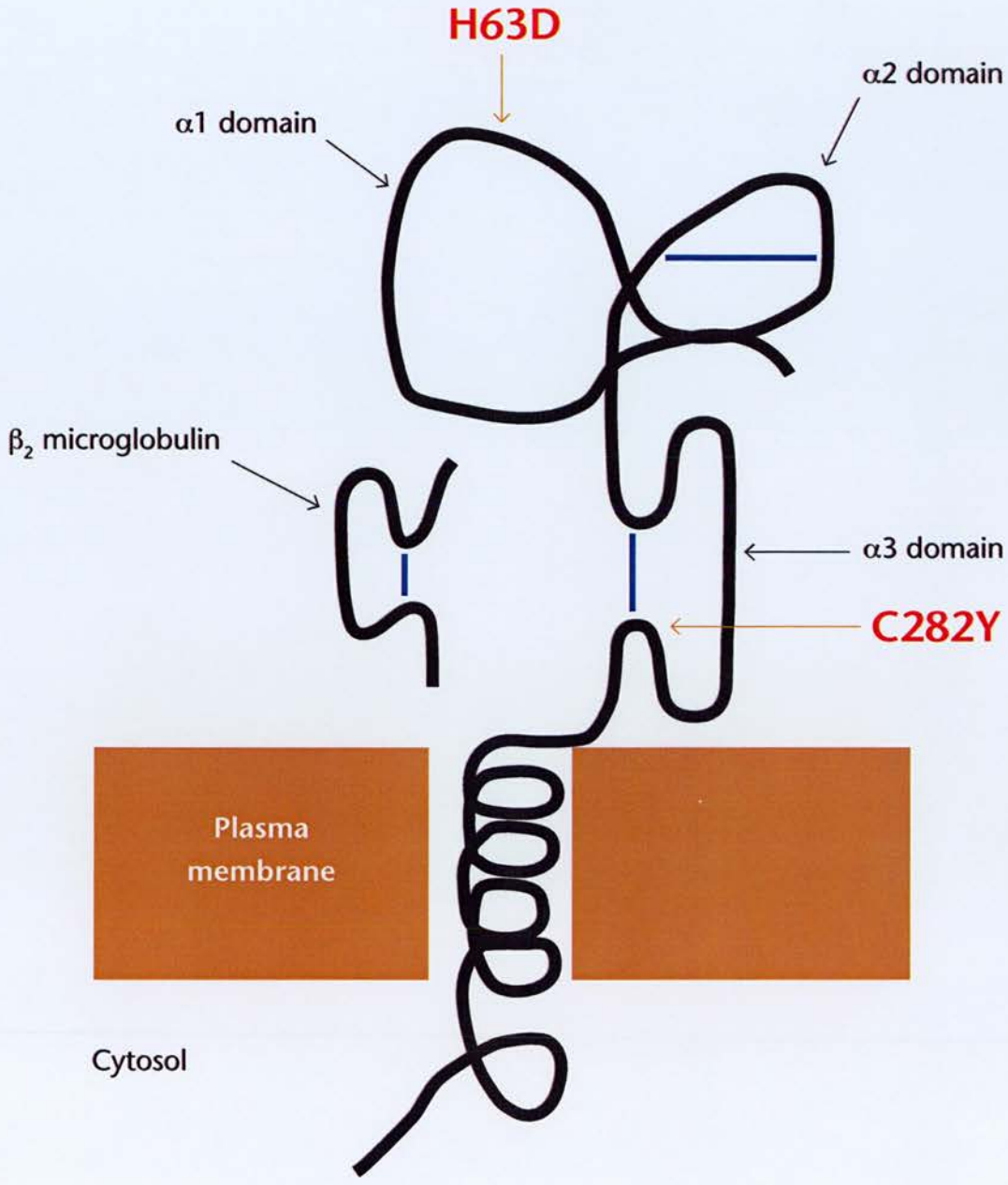


Figure 2.1 Structure of HFE, its association with β_2 microglobulin, and location of the two common mutations (Adapted from Feder, 1996).

The blue lines indicate disulphide bridges.

2.2 Epidemiology - the distribution of HFE gene mutations in different populations

Over two decades ago, Simon proposed that the genetic mutation leading to haemochromatosis might have originally arisen in Celtic populations before their Iron Age migration from central Europe (Simon, 1980). It has also been suggested (Simon, 1987) that the haemochromatosis C282Y mutation occurred in history on a particular chromosome carrying the HLA haplotype A3, B7, which was subsequently modified by genetic recombination. This haplotype has been termed the ancestral haplotype and is about 6 Mb in size.

HLA data have lent support to the hypothesis of a possible Celtic origin for the C282Y mutation. This is supported by the work of Ryan et al which found an allele frequency of 14% (the highest reported) for the C282Y mutation of HFE in an Irish population (Ryan, 1997). Lucotte and colleagues analysed data from other studies and combined results from Celtic populations in south Wales and Brittany to obtain a frequency of 6.88% for C282Y in this composite population (Lucotte, 1998). This was significantly higher than values for composite Nordic, southern European and Russian, but not Anglo-Saxon populations. Smith and colleagues screened employees of the Massachusetts Polaroid Corporation in the U.S.A. and found a significant association of hereditary haemochromatosis with Celtic ancestry (although this was based on analysis of the ancestry of only five individuals with haemochromatosis) (Smith, 1997).

Studies on populations in south east Asia have suggested that the C282Y and H63D mutations might have arisen more than once, independently, in different populations. (Rochette, 1999). Subsequently, by a series of genetic recombinations, the haemochromatosis allele was introduced onto chromosomes having different HLA haplotypes, thus accounting for the association of haemochromatosis with HLA-A3, B7 in, for example, Germany (Dyrszka, 1979) and Canada (Lloyd, 1978) and with HLA-A3, B14 in England (Bomford, 1977). H63D, appears to be an older mutation, occurring on a wider variety of haplotypes in a greater diversity of population groups (Rochette, 1999; Merryweather-Clarke, 1997).

After the discovery of the HFE gene mutations, it was possible to screen both normal populations and patients already diagnosed with haemochromatosis for the genetic defect. Some of the results of these studies are summarised in tables 2.2 and 2.3. Merryweather-Clarke and others (1997) have analysed chromosomes from populations around the world.

Table 2.2 Allele frequencies of HFE mutations in control populations

			Allele Frequency (%) & 95% confidence intervals[a]	
Reference	**No of subjects**	**Country**	**C282Y**	**H63D**
Willis, 1997	200	England	8.5 (5.8-11.2)	0.16
Miedzybrodzka, 1999	188	Scotland	8.4 (6-12)	15.7 (12-19)
Merryweather-Clarke, 1998	411	Jersey	8.3 ± 1.9	15± 2.5
UK Haemochromatosis Consortium, 1997	101	UK	5.4	15.8
Burt, 1998	1064	New Zealand	7	14.4
Jackson, 2001	10500 (blood donors)	Wales	8.23	15.2
Ryan, 1998	109	Ireland	14	16
Olynyk, 1999	3011	Australia	6.5	ND[b]
McDonnell, 1999a	1459	USA	6.1	16.6
Distante, 2000	1900 (inpatients)	Norway	6.6	ND
Cardoso, 1998	117	Sweden	3.8	12.4
Brissot, 1999	278	France	2.9	16.5
Carella, 1997	50	Italy	1	10
Papanikolaou, 2000	158	Greece	0.6	14.5

Notes

[a] confidence intervals quoted where given in article

[b] ND indicates insufficient or no data in article

Table 2.3 Frequency of HFE genotypes in haemochromatosis patients

			Genotype[a]					
Reference	n	Country	HY/HY n (%)	HY/HC n (%)	HC/HC n (%)	HC/DC n (%)	DC/DC n (%)	HY/DC n (%)
Jazwinska, 1996	112	Australia	112 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
UK haemochromatosis consortium (1998)	115	UK	100 (91)	1 (1)	5 (4)	0 (0)	1 (1)	3 (3)
Brissot, 1999	217	France	209 (96)	2 (3)	0 (0)	1 (0.5)	1 (0.5)	4 (2)
Willis, 1997	18	England	18 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Miedzybrodzka, 1999	54	Scotland	49 (91)	0 (0)	2 (4)	0 (0)	0 (0)	3 (6)
Ryan, 1998	78	Ireland	70 (90)	2 (3)	2 (3)	1 (1)	0 (0)	3 (3)
Cardoso, 1998	87	Sweden	80 (92)	1 (1)	1 (1)	1 (1)	1 (1)	3 (3)
Feder, 1998	178	USA	148 (83)	9 (5)	12 (7)	0 (0)	1 (0.5)	8 (4.5)
Papanikolaou, 2000	10	Greece	3 (30)	0 (0)	5 (50)	0 (0)	0 (0)	2 (20)
Carella, 1997	75	Italy	48 (64)	2 (2.7)	16 (21)	3 (4)	1 (1.3)	5 (7)
Piperno, 1998	158	Nth Italy	69%	6.3%	11.3%	7.6%	1.3%	4.4%
	30	Sth/central Italy	33.3%	3.3%	36.7%	13.3%	3.3%	10%

Notes:

[a] genotypes notated as follows:

HY/HY homozygous for C282Y

HY/HC heterozygous for C282Y

HC/HC homozygous wild-type

HC/DC heterozygous for H63D

DC/DC homozygous for H63D

HY/DC heterozygous for both C282Y and H63D

They found worldwide allele frequencies of 1.9% for C282Y and 8.1% for H63D. Their results were consistent with those of others (see table 2.2) who have shown the highest prevalence of C282Y in populations of northern European origin. The mutation is rare or absent in non-Caucasian populations (Merryweather-Clarke, 1997; Cullen, 1998). Broadly speaking, the frequency of C282Y in Europe decreases in north-to-south and west-to-east directions, away from the areas of north eastern Europe that were inhabited by early Celtic populations, again providing evidence in favour of an origin for this mutation in that population (Lucotte, 2001). H63D was more widely distributed with the highest frequency being found among the Basques (Merryweather-Clarke, 1997).

Other studies have demonstrated significant variations in the prevalence of HFE gene mutations even within the same countries (Rivard, 2000; Cardoso, 2001).

Among patients already diagnosed with haemochromatosis, it is clear that the vast majority in northern European populations is homozygous for C282Y (see table 2.3). However, the proportion in the south of Europe (for example, in Greece and Italy) is much lower, suggesting genetic heterogeneity of haemochromatosis in these populations. (Carella, 1997; Piperno, 1998; Papanikolaou, 2000).

2.3 Genetic heterogeneity of haemochromatosis

As discussed below, there appears to be no simple relationship between homozygosity for C282Y and clinical expression of iron overload (Shaheen, 1998). This fact, in conjunction with the existence of a significant minority of haemochromatosis patients who are wild-type for C282Y and H63D, has led investigators to search for other mutations of HFE. In addition, the occurrence of iron overload in HFE mutation-negative patients has indicated the existence of other genetic modifiers of iron metabolism.

2.3.1 *Nomenclature*

As more information has come to light about the genetic heterogeneity of haemochromatosis, so the potential for confusion in nomenclature has increased. HFE-linked haemochromatosis (C282Y homozygosity, H63D/C282Y or clinically milder forms linked with S65C) is often referred to as "classical" hereditary haemochromatosis. All references to haemochromatosis in this thesis may be taken to refer to this condition, unless otherwise specified.

2.3.2 *Mutations of HFE apart from C282Y*

2.3.2.1 The significance of H63D

The precise role of H63D in hereditary haemochromatosis remains uncertain. In the large amount of data published after the discovery of HFE (Feder, 1996), it appeared clear that no chromosome ever carried both C282Y and H63D. Such was the weight of evidence that some authors stated categorically that the two never occurred in "cis" (Brissot, 1999). Recent case reports suggest that this assumption is not valid as this occurrence has been recorded in three, geographically widely separated, individuals. Two were homozygous for C282Y and heterozygous for H63D (Thorstensen, 2000; Best, 2001) and one was heterozygous for C282Y and homozygous for H63D (Lucotte, 2001). The genotype in all three cases was confirmed by more than one method including DNA sequencing. This is significant in having potential misdiagnosis of compound heterozygosity as a consequence. However, given that upwards of 60 000 individuals have been genotyped (Best, 2001; Jackson, 2001; Beutler, 2002a) and only these three have been described who were homozygous for one mutation and heterozygous for the other, it seems likely to be a very rare phenomenon.

In their original report in 1996, Feder et al found 4.5% of the haemochromatosis probands studied were compound heterozygotes for C282Y and H63D. Some researchers (Carella, 1997) have suggested that H63D is a polymorphic change, without clinical significance, but this is disputed by others (Beutler, 1997; Aguilar Martinez, 1997). It was noted by Feder and colleagues (Feder, 1996) that the H63D allele was over-represented among their iron overloaded patients compared with the control group. Cell transfection studies have provided an insight into the possible functional significance of H63D (Feder, 1998)

Beutler (1997) analysed all the published data then available on the relationship between the presence or absence of the H63D mutation and iron overload in C282Y heterozygotes. He concluded that there was a relationship between iron overload and H63D in these patients and very low probability that this association occurred by chance ($p < 10^{-10}$). The point must be made, however, that he assumed that only C282Y non-bearing chromosomes were at risk for H63D. On the basis of data collated from a number of studies, the clinical penetrance of the compound heterozygous genotype has been estimated at 1-2% (Bacon, 1999b). McDonnell and colleagues found a relative risk of 9 for haemochromatosis for homozygotes for H63D, although the confidence limits were wide (McDonnell, 1999). Sham and co-workers found H63D homozygosity

in three out of 61 patients who had already been diagnosed with haemochromatosis by "classical" criteria, including liver biopsy (Sham, 1997). Beutler et al reported raised iron indices in carriers of H63D among over 10 000 screened individuals (Beutler, 2000). Porto et al reported strong linkage disequilibrium between the H63D mutation and HLA allele A29. The H63D/HLA-A29 haplotype appeared to be associated with what the authors termed non-classical haemochromatosis. The patients with non-classical haemochromatosis (NCH) did not fulfil the diagnostic criteria for classical haemochromatosis studies either because they had other clinical conditions which can be associated with iron overload or their total body iron stores were lower (<5g) than those typically found in classical haemochromatosis. Seven of the 25 patients with NCH had hepatic iron overload, but normal transferrin saturation (Porto, 1998). Some of these may overlap with the dysmetabolic iron overload syndrome described by Moirand et al (1997b), but the clinical data given in the paper of Porto and colleagues are insufficient to be certain of this.

The frequency of H63D and its effect on iron stores in 2531 inhabitants of an Australian town were recently determined (Gochee, 2002). No influence of H63D genotype on serum ferritin levels was reported, but both homozygotes and heterozygotes had significantly increased transferrin saturation compared with wild-types. Homozygotes for H63D were not at increased risk of clinically significant iron overload. The authors concluded that H63D in the absence of C282Y is not clinically significant.

2.3.2.2 *Other Mutations*

Since the discovery of HFE in 1996, several polymorphisms in the gene have been reported (Totaro, 1997; Douabin, 1997.) Many of these are located in intron sequences and are of no clinical significance.

While in northern Europe, the vast majority of cases with the hereditary haemochromatosis phenotype are either homozygous for C282Y or compound heterozygous for C282Y and H63D, this is not the case in many parts of the south of the continent. Genetic heterogeneity is a particular feature of haemochromatosis in the Italian population (Carella, 1997; Piperno, 1998; Pietrangelo, 1999). Some patients in whom at least one chromosome lacks one of the "classical" HFE mutations have other mutations in HFE (Piperno, 2000).

Additionally, a number of low-frequency "private" mutations have been discovered to contribute to the iron-loaded phenotype in some patients (Wallace, 1999; Beutler 2002b; Douabin, 1997).

One polymorphism, 193A→T, leads to a serine to cysteine substitution (S65C) in exon 2 of the gene and may be quite frequent in the general population. (Arya, 1999; Beutler, 2000; Beckman, 2001). Mura and co-workers analysed HFE mutations among 711 haemochromatosis probands from Brittany. As expected, the majority (80%) of probands were homozygous for C282Y. Taken together, the C282Y and H63D mutations accounted for 90.8% of hereditary haemochromatosis alleles. S65C was present with significantly greater frequency (7.2% vs. 2.49%) on the non-C282Y or H63D bearing chromosomes of haemochromatosis probands than of controls. 16% of C282Y heterozygotes and 7.4% of H63D heterozygotes from among the probands carried S65C, leading the authors to postulate that S65C could contribute to iron overload in mildly affected haemochromatosis patients (Mura, 1999). An association between S65C carriage and mild to moderate hepatic iron overload was recently confirmed in a Swedish population (Holström, 2002), but its clinical significance is still uncertain as S65C was not associated with an increased transferrin saturation in a study of healthy blood donors (Arya, 1999).

2.3.3 *Non-HFE-linked haemochromatosis*

It is clear that not all patients with inherited iron overload carry mutations in the HFE gene. It was noted soon after the identification of HFE that some patients lacking HFE mutations nonetheless suffered from typical haemochromatosis with severe iron overload (Shaheen, 1998). Additionally, there were identifiable geographic areas, particularly southern Europe, with a higher proportion of patients lacking HFE mutations than other areas (Piperno, 1998).

In recent years, there has been tremendous progress in the identification of the genetic mutations implicated in the causation of primary iron overload other than those linked to HFE. Some of these (transferrin receptor 2-associated haemochromatosis and juvenile haemochromatosis) share many features in common with HFE linked haemochromatosis, while ferroportin-associated iron overload has quite different epidemiology, clinical presentation and natural history (Pietrangelo, 2004a).

The term "juvenile haemochromatosis" has been used to refer to an iron overload syndrome with a similar phenotype to adult haemochromatosis, but typically presenting much earlier in life. Typically, affected individuals present with hypogonadism in the second decade of life, while cirrhosis of the liver develops only later (de Gobbi, 2002). It is now clear that this condition is genetically heterogeneous. There is evidence for linkage to a gene on chromosome 1q (Roetto, 1999; Roetto 2000) and a candidate

gene originally named HFE 2 and now HJV has been cloned in this region. The gene product of HJV, hemojuvelin, may modulate expression of hepcidin (Papanikolaou, 2004). It is also possible that mutations of the HAMP gene on chromosome 19, affecting the production or function of hepcidin, may result in a similar phenotype (Pietrangelo, 2004a). There is increasing evidence to suggest that HAMP may act as a modifying gene to increase the phenotypic expression of the HFE mutation C282Y (Jacolot, 2004; Merryweather-Clarke, 2003). This may effect the phenotype of both heterozygotes and homozygotes for C282Y (see also section 2.3.4)

After the discovery that the HFE protein interacts with the transferrin receptor (discussed in detail in section 2.7.4.4), attention turned to the gene for the transferrin receptor (TfR) as a possible site for mutation. Complementary DNA (cDNA) sequences of 21 patients with hereditary haemochromatosis were sequenced, and no likely mutation was found (Tsuchihashi, 1998). In 1999, a second transferrin receptor molecule, transferrin receptor 2 (TfR2) was cloned (Kawabata, 1999). A number of mutations in TfR2 have been discovered (Camaschella, 2000; Girelli, 2002; Mattman, 2002; Roetto, 2002) in Italian patients with iron overload, who lack HFE gene mutations. Examination of liver histology in two patients and of biochemical markers of iron status in heterozygotes led to the suggestion that haemochromatosis in these patients may behave clinically very similarly to classical haemochromatosis (Girelli, 2002).

A further type of haemochromatosis has been described in a number of patients in many different countries with mutations in the SLC40A1 gene (previously called SLC11A3) on chromosome 2q, which encodes the iron transport protein ferroportin (Njajou, 2001; Montosi, 2001). Whereas the inheritance of classical haemochromatosis follows an autosomal recessive pattern, these patients displayed autosomal dominant inheritance (Pietrangelo, 1999; Montosi, 2001). Additionally, the histological pattern of hepatic iron overload was distinct from that seen in classical haemochromatosis in that reticuloendothelial rather than hepatocellular iron deposition predominated. Patients were often anaemic early in life with a normal transferrin saturation but a raised ferritin and demonstrated low tolerance to phlebotomy (Montosi, 2001). A valine deletion at position 162, possibly resulting in haploinsufficiency of ferroportin 1 and impairment of iron export from macrophages (Fleming, 2001), is a frequent finding in this form of iron overload (Montosi, 2001; Roetto, 2002; Devalia, 2002; Wallace, 2002)

One family with another form of autosomal dominantly inherited iron overload has been identified in Japan. The genetic basis of this is a mutation in the gene encoding the H sub-unit of ferritin (Kato, 2001)

Some of these non-HFE mutations such as that in the H subunit of ferritin, which has been reported in one family worldwide, seem at present to be rare. The prevalence of the mutations in the genes for transferrin receptor-2 and ferroportin is not yet certain. Transferrin receptor 2 mutations were not responsible for iron overload in French patients (Aguilar-Martinez, 2001), white, Asian or African Americans (Lee, 2001a), Caucasian patients from Alabama or Caucasian (including individuals of Italian descent) or African-American controls (Barton, 2001). Lee et al sequenced the coding and flanking regions of the transferrin receptor-1, ferroportin, caeruloplasmin, ferritin light and heavy chains, iron regulatory proteins-1 and -2 and hepcidin in a relatively small number of white, Asian and African American normal or iron-overloaded individuals (Lee, 2001b). They found no significant association between iron stores and any of a number of polymorphisms in the genes studied. Ferroportin mutations have, however, been reported in Dutch, French, French Canadian and Asian families (Pietrangelo, 2004a).

It had been thought that the iron overload seen in sub-Saharan Africa was due to environmental factors, especially excess dietary intake of iron. However, there is now some evidence of a non-HLA genetic linkage (Moyo, 1998). This also is unrelated to classical HFE-linked haemochromatosis.

Primary iron overload is also a feature of congenital atransferrinaemia (Hamill, 1991) and caeruloplasmin deficiency (Morita, 1995)

Non-transfusional secondary iron overload occurs in a number of unrelated conditions, among which are:

- β-thalassaemia major and some other haemoglobinopathies in which only part of the iron overload is attributable to transfusion therapy (Bottomley, 1998).
- Congenital dyserythropoietic anaemias types I and II, which may present with iron overload (Halpern, 1985)
- Hereditary sideroblastic anaemia (Cazzola, 1983)
- Porphyria cutanea tarda (Lundvall, 1970)
- Hereditary haemolytic anaemias – in some cases, iron overload may result from co-inheritance of haemochromatosis alleles (Edwards, 1982; O'Mahony, 1987).

These will not be discussed further here.

2.3.4 *The iron status of heterozygotes for hereditary haemochromatosis*

A large study was conducted in Utah, USA, examining clinical and biochemical abnormalities in heterozygotes for haemochromatosis (Bulaj, 1996) defined by HLA-linkage studies. The analysis of serum iron, transferrin saturation and ferritin was broken down by age and sex of the subjects. In all age groups, the mean serum iron concentration and transferrin saturation were higher in heterozygotes than in normal subjects. The mean serum ferritin was higher in all groups of heterozygotes apart from the youngest group of females. However, statistical significance was reached only in the group of male subjects aged 31 to 60 years and in women of 31 to 60 and 61 to 90 years of age. In total, 18 percent of male heterozygotes and 11 percent of female heterozygotes had transferrin saturation values more than 2 standard deviations above the mean values for normal subjects. Four percent of male heterozygotes had an initial transferrin saturation greater than 62 percent, taken by the study's authors to be the threshold for homozygous haemochromatosis in men. However, all those in whom re-testing while fasting was possible had transferrin saturation values of 62 percent or less. Of the 44 of 553 female heterozygotes (8 percent) with an initial transferrin saturation of greater than 50 percent (taken as the equivalent threshold for women), 2 subjects had a value of more than 50% on re-testing. Three percent of women in the normal control population had transferrin saturation values in excess of 50 percent (although it is not clear whether these individuals were subjected to re-testing). The authors concluded that heterozygotes for haemochromatosis do have an increased body iron burden, but that this seems to have few if any clinical consequences. Comparable conclusions had been previously reached by Adams et al in a study of 255 heterozygotes, again identified by HLA typing (Adams, 1994).

Crawford and colleagues examined the phenotypes and HFE genotypes of 300 subjects of whom 115 met the clinical diagnostic criteria for haemochromatosis. Of 8 (4.8%) iron-overloaded subjects heterozygous for C282Y, 7 were compound heterozygotes with H63D. (Crawford, 1998). A study of over 1000 randomly selected individuals in New Zealand confirmed that heterozygotes for C282Y or H63D had higher values of transferrin saturation than those lacking such mutations (Burt, 1998).

The awareness that heterozygotes for C282Y may have larger iron stores than those without this mutation has stimulated research into whether these increased iron stores are potentially harmful. The risk of organ damage in patients with hereditary haemochromatosis is clear, but whether carriers are also at some risk is not yet known. Chapters 6 and 7 of this thesis present data to examine this question, with reference to patients with coronary artery disease and patients undergoing orthotopic liver transplantation, respectively.

2.3.4.1 *Iron Deficiency in Heterozygotes*

The Utah study (Bulaj, 1996) showed that female heterozygotes for haemochromatosis had a lower incidence of iron deficiency than normal females. Datz and co-workers, too, have suggested that heterozygosity for C282Y protects women from the development of iron deficiency (Datz, 1998), but their study did not make a distinction between C282Y/wildtype heterozygotes and C282Y/H63D compound heterozygotes, making their data more difficult to interpret. This distinction was made in a cross-sectional analysis of 1327 women in Australia (Rossi, 2000), which showed no influence of the C282Y/wildtype genotype on iron stores. However, further support for a possible protective effect against iron deficiency was made in a study of patients with coeliac disease (Butterworth, 2002). This study made the interesting observation that C282Y in the coeliac patients studied, in contrast to the control patients, was in linkage disequilibrium with HLA-A*01 and B*08.

The issue of the persisting high prevalence of C282Y in northern European populations, as discussed in section 2.2 above, merits further study. It has been suggested that the putative protection against iron deficiency afforded by carriage of C282Y may have offered heterozygotes an evolutionary advantage, so ensuring that the mutation was not gradually selected out. If the theory of protection against iron deficiency is correct, a corollary should be that C282Y is under-represented in iron deficient populations compared with non-iron deficient populations. This hypothesis is examined further in chapter 5 of this work. Another consequence could be that carriers of C282Y might be protected to some degree against not only "natural" iron deficiency but also that induced iatrogenically, for example by blood donation. Chapter 4 of this thesis examines a population of male blood donors who gave blood regularly over a period of up to one year, to investigate whether this is indeed the case.

2.4 Making the Diagnosis of Haemochromatosis

2.4.1 *Assessment of Iron Overload*

2.4.1.1 *Blood measures of iron load*

In iron overload, serum iron is typically increased, with a reduced serum transferrin, a reduced iron-binding capacity and a raised transferrin saturation (Zilva, 1998). A raised transferrin saturation provides an early indication of iron overload at which stage serum ferritin may still be normal (Edwards, 1993). A recent study examined the correlation

between total body iron stores, as calculated from the volume of blood removed in phlebotomy and serum ferritin, serum iron, unsaturated iron binding capacity and transferrin saturation, measured before commencement of phlebotomy (Beutler, 2002b). The correlation between all the measurements and total iron burden was greater in patients homozygous for C282Y than in other patients, and overall ferritin was found to be the best predictor of body iron stores. Unfortunately, there are many causes other than iron overload of both a raised transferrin saturation and an elevated serum ferritin. Despite evidence that serum ferritin does correlate with body iron stores in normal individuals and those with uncomplicated iron overload (Jacobs, 1972; Lipschitz, 1974; Halliday, 1977), elevated serum ferritin values must be interpreted in the light of clinical information. A study of the causes of serum ferritin values in excess of 1000 ng/mL demonstrated that even extremely elevated ferritin concentrations may not be a good guide to the presence of tissue iron overload in patients with, for example liver disease, renal disease, infection and malignant disease (Lee, 1996). Specific disease states, for example, adult onset Still's Disease (Schwarz-Eywill, 1992) or the recently characterised syndrome of hyperferritinaemia and cataracts (Girelli, 1995) are also characteristically associated with high serum ferritin values. Liver disease may make the interpretation of serum ferritin values particularly difficult (Chapman, 1982; Di Bisceglie, 1992; Bell, 1994). The choice of test in screening for haemochromatosis is discussed in more detail in section 2.6.3.

Because of these concerns about the validity of blood tests for iron overload, histological examination of liver tissue has been considered an essential step both in confirming the diagnosis of haemochromatosis and assessing the degree of liver damage present (Witte, 1996). The benefits of histological evaluation have been felt to outweigh the (relatively small) risks of liver biopsy (Witte, 1996).

2.4.1.2 *Liver biopsy in the diagnosis of haemochromatosis*

Intracellular iron is normally stored as ferritin. As iron accumulates, ferritin molecules eventually coalesce to form haemosiderin, and it is in this form that most tissue iron is found in haemochromatosis. Haemosiderin is an amorphous deposit of iron and peptide that probably consists of degraded ferritin protein and ferric hydroxide polymers or cores of varying size (Ward 1989). It can be visualised using Perls' acid ferrocyanide technique, considered the most sensitive and specific means of staining for iron (Searle, 1994). Hepatic iron deposition in haemochromatosis follows a characteristic pattern. Typically, iron deposition is observed initially in periportal (zone 1) hepatocytes with hepatocytes in zones 2 (midzone) and 3 (centrilobular) becoming

iron loaded as the disease progresses. This gradient in iron deposition across lobules is typically maintained throughout the course of the disease. The relative paucity of iron in hepatic reticuloendothelial cells in the early stages of iron overload is an important feature of hereditary haemochromatosis (Searle, 1994; McLaren, 1989). This contrasts with the situation in secondary iron overload, such as may be seen in patients with haemoglobinopathies or other forms of chronic haemolysis. In these conditions, iron accumulation occurs principally in Kupffer cells. A reticuloendothelial pattern of iron deposition has been found, in a study of 103 liver biopsies from patients with various liver diseases, to predict reliably the absence of C282Y homozygosity. In the same study, however, the converse was shown not to be true: a hepatocellular pattern with zonal gradient did not correlate well with haemochromatosis (42 out of 72 biopsies showing this pattern were C282Y homozygotes (Brunt, 2000)). This indicates that the hepatocellular pattern of iron deposition should not be used as a surrogate marker for haemochromatosis.

A variety of histochemical grading systems are in use for the recording of hepatic iron load. One of the most widely used is that of Scheuer in which iron deposition is assigned one of four grades, with grade 1 the least and grade 4 the most intense staining for hepatic iron (Scheuer, 1962). Only hepatocellular iron is graded. Scheuer's paper gives no written description of the criteria he used; the grading is illustrated merely by means of four photomicrographs and is thus, perhaps, open to somewhat subjective interpretation. Although other systems are available (described, for example, by Brissot (Brissot, 1981)), the practice guideline for haemochromatosis developed by the College of American Pathologists (Witte, 1996) recommends the use of a modification of Scheuer's system.

Quantitation of hepatic iron by chemical means can also provide useful information. The hepatic iron concentration can be measured by atomic absorption spectrophotometry. As the iron content of the liver rises with increasing age, the iron concentration may be adjusted to take account of age by calculation of the hepatic iron index (HII) (Bassett, 1986). This is achieved by dividing hepatic iron concentration (μmol iron/g dry weight) by the age in years of the individual. The HII has been considered of particular use in discriminating iron overload associated with alcoholism from that of haemochromatosis. Witte quotes the following "reference" values (Witte, 1996):

- Normal individuals: HII ≤ 1.1
- Alcoholic liver disease: HII ≤ 1.7
- Haemochromatosis heterozygotes: HII ≤ 1.9
- Haemochromatosis homozygotes: HII ≥ 1.9

A number of reports have suggested that the HII may be less specific in the presence of cirrhosis which may, regardless of the underlying liver disease, be associated with an HII greater than 1.9 (Deugnier, 1997; Ludwig, 1997; Press, 1998).

The importance of histological examination of the liver lies also in its utility in detecting fibrosis and cirrhosis which have prognostic implications for patients with haemochromatosis.

The possibility of non-invasive quantification of hepatic iron using magnetic resonance imaging has been investigated. The use of gradient-recalled-echo sequences has produced encouraging results (Bonkovsky, 1999). However, this means of assessment of hepatic iron overload is not widely available in the UK at present and would probably not eliminate the need for liver biopsy entirely as a means to assess hepatic fibrosis or cirrhosis and the presence of iron-free foci, which may be pre-malignant (Deugnier, 1992). Magnetic susceptibility (SQUID) allows accurate quantification of hepatic iron (Brittenham, 1988), but access to this technology is extremely limited worldwide (British Committee on Standards in Haematology, 2000).

The advent of HFE genotyping has led to a change in the role of liver biopsy in the diagnosis of hereditary haemochromatosis. A study of 197 French patients, all homozygous for C282Y, revealed that severe fibrosis was absent in all of 96 patients who had a serum ferritin ≤ 1000 ng/ml, a normal value for aspartate aminotransferase and no hepatomegaly (Guyader, 1998). Current guidelines (British Committee on Standards in Haematology, 2000; EASL, 2000; Powell, 2000) suggest that liver biopsy is unnecessary in C282Y homozygous patients meeting these criteria. However, in those who do not, and perhaps in those aged over 46 with an alcohol intake of more than 60g per day (Powell, 2000), biopsy still has an important role, although in prognostication rather than making the diagnosis. There is less agreement over the need for liver biopsy in patients compound heterozygous for C282Y and H63D. The Australian Broadsheet on hereditary haemochromatosis (Powell, 2000) suggests that these patients should be biopsied, in contrast to the British guidelines (British Committee on Standards in Haematology, 2000). Advice in this area seems likely to be subject to further modification as knowledge increases. A recent paper from the USA has suggested that biopsy may be unnecessary in haemochromatosis patients with serum ferritin levels less than 1000 µg/L, regardless of age or serum liver enzymes (Morrison, 2003)

Finally, there are miscellaneous other methods of estimating of body iron stores. The amount of iron removed in a phlebotomy programme can be calculated if it is assumed that one unit of blood contains approximately 225 mg iron (Powell, 2000). In the

majority of patients with haemochromatosis, more than 4-5g of iron can be removed before iron deficiency is precipitated. The use of circulating markers of hepatic fibrosis in haemochromatosis has also been investigated. A study of 42 patients with hereditary haemochromatosis and 19 controls showed that a serum concentration of type IV collagen of >115 ng/ml was 100% sensitive and 69% specific in detecting the presence of severe fibrosis or cirrhosis (George, 1999).

2.4.2 *Clinical Features*

The classical clinical features of hereditary haemochromatosis, diabetes, increased skin pigmentation and hepatic cirrhosis are nowadays unusual presenting features of the disease. More frequently, patients present (often in early middle age) with non-specific symptoms of fatigue, abdominal or joint pain. A significant number may have no symptoms at all, a high ferritin having been discovered incidentally during investigation of an unrelated complaint. When symptomatic organ involvement does occur, liver disease usually predominates, but iron overload may also affect the endocrine organs (causing diabetes, thyroid dysfunction and hypogonadal hypogonadism), the heart (cardiac failure and dysrhythmias) and the joints (arthritis). Although the prognosis of untreated haemochromatosis is poor, institution of therapeutic phlebotomy and successful reduction of tissue iron load results in a normal life expectancy for those patients without hepatic cirrhosis or diabetes mellitus (Niederau, 1996).

2.4.3 *Diagnostic algorithm*

A possible algorithm for the diagnosis of hereditary haemochromatosis is given in figure 2.4. The exact order in which tests are performed may vary depending on setting. For example, it may be considered preferable or convenient to perform HFE genotyping at the same time as the assessment of fasting TS. Subjects who have been identified as a result of family screening will, as a rule, be genotyped as part of their initial testing.

2.5 Prevalence of Haemochromatosis and Penetrance of the HFE Genotypes

The data given in table 2.2 and discussed above indicate an allele frequency for C282Y in populations of northern European extraction of between 6 and 8.5%. This equates to a frequency for homozygotes in these populations of about 1 in 200, although in some countries this may be an underestimate (Ryan, 1997). As discussed in detail below, it is incorrect to think of haemochromatosis as an uncommon disorder. However, there is a gap between the prevalence of C282Y homozygosity (and compound heterozygosity

with H63D) and that of phenotypic haemochromatosis, expressed as iron overload (Ho, 2001). This is illustrated by data showing that in Jersey in 1998, 18 patients were receiving treatment for haemochromatosis (ca 0.02% of the population). According to the population frequency of C282Y homozygosity (ca 0.7%), however, there are approximately 586 homozygotes among the Jersey population (Merryweather-Clarke, 1998).

In 1955, Finch and Finch stated that haemochromatosis was recognised in 20 000 hospital admissions in the United States, and once in 7000 hospital deaths (Finch, 1955). However, even as early as the 1960s, there was there some evidence that this was might be an underestimate in Scotland (MacSween, 1966). A clinico-pathological review of 520 cirrhotic patients who had died in Glasgow between 1900 and 1969 revealed a diagnosis of haemochromatosis in 7.5% (MacSween, 1973) although it is difficult to infer a population frequency from that report as only cirrhotic livers were examined. Two Swedish studies suggested a prevalence of between 0.5% (Olsson, 1983) and 0.1% (Lindmark, 1985) in different regions of that country. Other studies have suggested a population prevalence of iron overload of between 0.34% and 0.45% (Edwards, 1988; Bradley, 1996b)

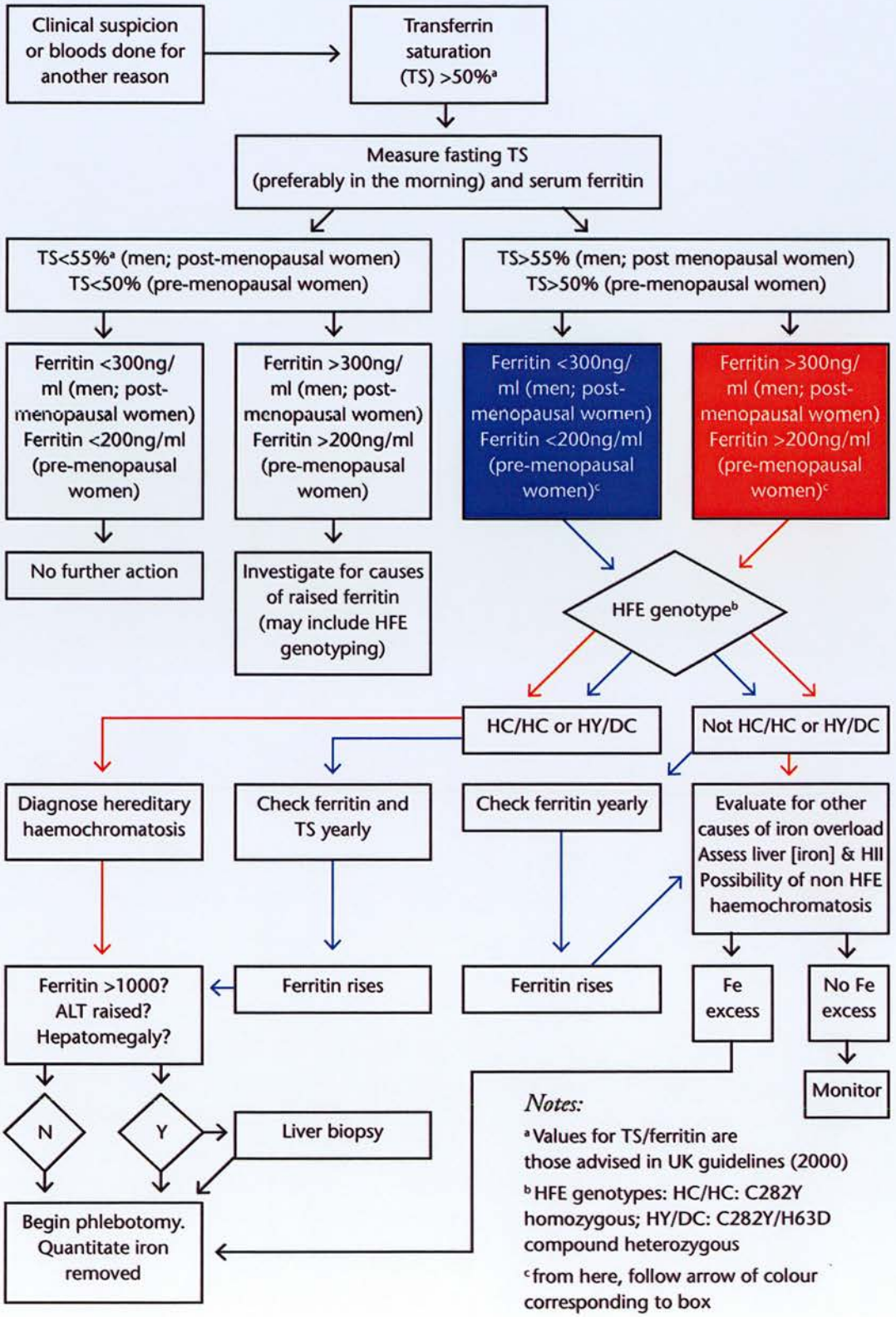
The reason for this disparity seems likely to be due either to under-diagnosis of haemochromatosis or to a low penetrance of the C282Y genotype. As discussed below, there seems to be little doubt that cases of haemochromatosis do go undetected, for a variety of reasons. However, it is very difficult to assess the magnitude of this effect.

In the United States, the National Hospital Discharge Survey (1979, 1983-1987) indicated an estimated hospitalisation rate for haemochromatosis as the first-listed diagnosis of 13.4 per 100 000 hospitalisations (0.01%) (Gayle, 1992). The age-adjusted rate of haemochromatosis-associated death in the US was 1.2 per million in 1979 and 1.8 per million in 1992 (Yang, 1998). The apparent increase in the rate of death due to haemochromatosis (which was evident also before 1972 (Yang, 1998)) might be best explained in terms of increasing recognition of the disease.

In the years immediately following the cloning of the HFE gene the opinion was expressed by some researchers that most individuals homozygous for C282Y would eventually become symptomatic of iron-overload (Edwards, 1998; EASL, 2000). There is now increasingly convincing evidence to suggest that this may not be so. Olynyk and colleagues studied 3011 randomly selected individuals from the town of Busselton, Australia. They found that 0.3% (95% confidence intervals 0.1-0.6%) were homozygous for C282Y. Eleven of these 16 individuals underwent a liver biopsy for assessment of hepatic iron load. Fifteen of them had a transferrin saturation greater

Figure 2.4 Alogrithm for the diagnosis of haemochromatosis

See text for details



than 55%. The one who did not had a raised serum ferritin concentration. In all the biopsied subjects, hepatic iron levels were above normal, although three had a hepatic iron index of less than 1.9 μmol/g/year. Eight of the 16 homozygotes had clinical features of haemochromatosis and four had hepatic cirrhosis or fibrosis. There was, however, no assessment of symptoms and signs in non-homozygous controls, which may make questionable the implicit attribution of the symptoms of these eight subjects to iron overload (Olynyk, 1999). A similar figure was obtained by Bradley et al who analysed 5 published series in which family studies in haemochromatosis had been performed by HLA-typing. They concluded that 48% of homozygous family members had at least one clinical manifestation of haemochromatosis (Bradley, 1996a).

There is evidence that the rate of non-expression of iron overload in individuals homozygous for C282Y may be higher in women than in men. In the Busselton study, referred to above (Olynyk,1999), four homozygotes (all women aged 30-45) had had normal serum ferritin concentrations for years previously and these remained normal when re-measured at the time of the study. Similar results, indicating that around 30% of women who are homozygous for C282Y are not iron overloaded were obtained in an examination of 300 Australian subjects. The same study also found non-expression of iron overload in 6.7% of C282Y homozygous men (Crawford, 1998). In their examination of iron levels and HFE genotypes in 10 500 Welsh blood donors, Jackson et al found 72 (0.7%) to be homozygous for C282Y. Twenty-five of 29 male homozygotes and 18 of 41 female homozygotes had TS >50%, suggesting that screening using a TS cut-off of 50% would fail to identify up to 20% of men and over 50% of women (Jackson, 2001). None of 63 homozygotes who were interviewed had symptoms or signs of iron overload. In the United States, 152 C282Y homozygotes and 616 compound heterozygotes for C282Y and H63D were identified among 41 038 individuals attending a health appraisal clinic (Beutler, 2002c). Seventy-five percent of male homozygotes (76% after exclusion of frequent blood donors) and 40% of females (41%) had TS >50%. The majority of homozygotes (76% of men and 54% of women) had elevated serum ferritin levels. There was no significant increase among homozygotes or compound heterozygotes in the prevalence of symptoms commonly associated with haemochromatosis, compared with the rest of the study population. One homozygous male had probable "fully manifest" haemochromatosis, with diabetes, heart failure and possible hyperpigmentation of the skin. The results led the author to estimate that fewer than 1% of homozygotes develop frank clinical haemochromatosis. McCune and colleagues identified all patients currently being treated in two health authority areas in South Wales and found a maximum of 76 homozygotes (it was not possible to genotype all the patients because of the unavailability of some medical records). They calculated

the expected number of homozygotes in the population of 1.3 million resident in these health authority areas, based on previously published allele frequencies for the C282Y mutation in that population. Based on these data, 6292 individuals would be expected to be homozygous for C282Y. Only 1.2% had been diagnosed and were receiving treatment, leading the authors to conclude that the majority of C282Y homozygotes will remain healthy and not express a phenotype leading to a diagnosis of hereditary haemochromatosis (McCune, 2002). A Danish group examined the rate of progression of iron loading in 23 C282Y homozygotes, identified by genotyping participants in the Copenhagen City Heart Study, over a 25 year period (Andersen, 2004). None of these individuals developed what the authors termed "clinically overt" haemochromatosis, although these individuals did have higher serum ferritins than individuals with non-mutated HFE. They found that C282Y homozygotes only accumulated iron at a "modest" rate, probably no greater than that seen in individuals not homozygous for C282Y. However, clinical evaluation was incomplete, and it is difficult to be certain that some of the individuals identified (3 had a serum ferritin in excess of 1000 μg/L) would not have benefitted from phlebotomy.

Beutler et al also made the observation that, if homozygous individuals died prematurely, the prevalence of homozygotes in the population should fall, and the number of homozygotes detected should be lower than that predicted by the Hardy-Weinberg equilibrium (see Appendix 7). This was not the case in their study population (Beutler, 2002c). Willis and colleagues genotyped blood from 600 elderly men, 500 of whom were over 80 years old. Although making the point that the samples investigated came from patients already under medical care, and were therefore possibly biased towards patients with haemochromatosis, they did attempt to exclude samples from patients known to have the condition. They found no under-representation of C282Y homozygosity in the samples they studied, suggesting that many remain free of life-threatening haemochromatosis-related disease well into old age (Willis, 1999). Further support for this is provided by the work of Coppine and colleagues who found no under-representation of either heterozygotes or homozygotes for C282Y in a group of 492 French centenarians (Coppine, 2003).

A study of Irish patients with haemochromatosis and C282Y homozygotes detected by family screening led Ryan and colleagues to conclude that under-diagnosis of haemochromatosis resulted from the non-specific nature of early symptoms rather than a low penetrance of C282Y (Ryan 2002). However, their assumption that screening asymptomatic relatives of patients with haemochromatosis is a satisfactory surrogate for population screening is questionable, as it is possible that other unidentified heritable modifiers of C282Y expression may be operating in these families.

The presence of other genetic modifiers of HFE that might modulate the expression of C282Y-associated hereditary haemochromatosis has been suggested to explain the wide variation in phenotypic expression of C282Y homozygosity (Piperno, 1996). A study (Pratiwi, 1999) using linkage disequilibrium analysis in Australian haemochromatosis patients has shown that iron stores in C282Y homozygotes might be influenced by a modifier in the region of D6S105 on chromosome 6p. Specifically, male patients homozygous for D6S105 allele 8 had significantly greater liver iron stores than patients heterozygous for or lacking this allele. Experiments with HFE knockout mice carrying other mutations that impair normal iron metabolism have lent weight to the theory that the activity of genes other than HFE is important in modifying the human haemochromatosis phenotype (Levy, 2000).

2.6 Screening for Haemochromatosis

Criteria laid down by the United States Preventive Services Task Force (U.S Preventive Services Task Force, 1996) state that for a condition to be suitable for a screening programme, it must fulfil the conditions listed below:

1. Prevalence: it must be "relatively common".
2. Burden of suffering: it must be a condition of "major clinical importance", that is, it must be the cause of significant morbidity and/or mortality in the general population
3. Effectiveness of screening test: the screening intervention must detect cases earlier than they would have been detected without screening and with sufficient sensitivity and specificity to avoid producing large numbers of false positive and false negative results.
4. Effectiveness of treatment: detection by screening and subsequent treatment should improve the likelihood of a favourable health-outcome (reduced disease-specific morbidity or mortality) compared to treatment initiated when subjects present with signs or symptoms of the disease.
5. Population benefit and adverse events: potential benefits of the screening intervention to the population as a whole should outweigh possible adverse effects of screening
6. Cost-effectiveness: the screening intervention should be cost-effective.

Some authors are convinced that hereditary haemochromatosis fulfils these and other, similar criteria (Adams, 1999a). Others, however, recommend against screening because of persisting uncertainty about the magnitude of the burden of suffering caused by haemochromatosis and unanswered questions about its natural history (Haddow,

1999; Cogswell, 1999; Jackson, 2001; McCullen, 2002). This has been discussed in some detail in section 2.5. There are a number of other possible factors to explain the continuing debate about whether widespread population screening for this disease is warranted. First and most important is the continued lack of a consensus diagnosis of what constitutes hereditary haemochromatosis. The discovery of the HFE gene (Feder, 1996) and the subsequent development of a widely available genetic test for haemochromatosis has added to difficulties of definition in recent years. The second factor is lack of knowledge about haemochromatosis among both the medical community and the general population. This may have led to misconceptions about the prevalence of haemochromatosis among medical staff and a failure of the condition to catch the imagination of the public as a potential health problem. Finally, the evidence for the cost effectiveness of screening may not have been effectively presented to developers of Public Health Policy.

2.6.1 *Case Definition of Haemochromatosis*

Four groups of patients who may be considered to have haemochromatosis can be distinguished. First, those who present with the classic triad of diabetes, hepatic cirrhosis and skin pigmentation or other evidence of iron-related organ damage. The diagnosis in this group is not controversial, but such florid presentations are rare. However, perhaps reflecting what was until relatively recently taught to medical students (Cartwright, 1974; Bothwell, 1982), the equation of haemochromatosis with these clinical features may contribute to the persistence within the medical community of the notion that haemochromatosis is uncommon (Edwards, 1993; McDonnell, 1998a; Adams, 2000a). The second group comprises those who are found to have biochemical evidence of iron overload. This group may also contain individuals with organ damage as a result of iron accumulation, particularly when screening is performed in disease-specific clinics (O'Brien, 1990; Singh 1992, Olynyk, 1994). Often, however, persons in this group are asymptomatic or have non-specific symptoms such as tiredness, weakness or abdominal pain. (Witte, 1996; Niederau, 1996; Adams, 1997). The third group is those individuals who were identified as having haemochromatosis linked with HLA serotype A3 after this association was recognised in the mid 1970s (Simon, 1975; Lipinski, 1978; Doran 1981). The final group comprises those found to have the mutations in the HFE gene associated with haemochromatosis (homozygosity for the C282Y mutation or, less frequently, dual heterozygosity for both that mutation and the H63D mutation).

The problem that this poses for screening is that it has led to considerable variability in the definition of haemochromatosis used by different workers. (Wetterhall, 1998; McDonnell, 1998b; Cogswell, 1998; Franks, 1999, Adams, 1999a; Edwards, 2000). This question has been considered by the British Society for Haematology (British Committee for Standards in Haematology, 2000), the College of American Pathologists (Witte, 1996), the Royal College of Pathologists of Australasia (Powell, 2000) and others (European Association for the Study of the Liver (EASL), 2000). There is a measure of agreement that the case definition should include a combination of biochemical markers of iron overload and the presence of homozygosity for the C282Y mutation of HFE or compound heterozygosity for the C282Y and H63D mutations (see also the discussion in Section 2.5, above).

2.6.2 *Education of health-care workers and the public*

As mentioned above, there is evidence of lack of awareness among the medical community about the prevalence and clinical presentation of haemochromatosis. The need for strategies to heighten health care providers' awareness about haemochromatosis is recognised in the current recommendations of the United States Centers for Disease Control on screening for iron overload (Reyes, 2002) and also emphasised elsewhere (Bradley, 1996b; Cogswell, 1999; Rosenberg, 1999).

Barton and colleagues conducted an education programme about haemochromatosis and iron overload among health care professionals and the public in Birmingham, Alabama (Barton, 1997). They performed 15 slide and lecture presentations to various groups of health-care workers. After the education programme, each of 55 physicians who had never previously diagnosed a case of haemochromatosis identified at least one case.

The programme to increase awareness among the public consisted in interviews or presentations for radio, television and newspapers, community information programmes and patient support groups. As a result, 22 people presented themselves for evaluation, and three were found to have haemochromatosis.

McDonnell et al emphasise the need for better education about haemochromatosis from medical school onwards and the imperative to disseminate as effectively as possible information about haemochromatosis case-detection among doctors (McDonnell, 1998a). In addition, they demonstrated the benefits of physician education in the context of four different screening programmes (McDonnell, 1998b).

There has been very little published about the state of knowledge of haemochromatosis among healthcare workers in the United Kingdom. The rationale behind McDonnell's studies applies equally well in the UK, and chapter 8 of this thesis presents the results of a survey of awareness of haemochromatosis among general practitioners working in the southeast of Scotland.

2.6.3 *Which initial test to use in screening?*

The use of differing case definitions (see section 2.6.1) can make the comparison of data from screening studies complicated. However, the move towards a genetic definition fuels the debate about whether screening programmes should be phenotypic (using biochemical tests to detect iron overload) or genotypic (looking for HFE gene mutations as the initial step).

Phenotypic screening involves the detection by biochemical tests of evidence of iron overload. The transferrin saturation (TS, derived in most studies as the ratio of serum iron to total iron binding capacity) is the preferred initial test (Bassett, 1988; Edwards, 1993; Witte, 1996; British Committee on Standards in Haematology, 2000). The threshold used affects the sensitivity and specificity of the test. Looker and Johnson examined the prevalence of raised TS among over 15 000 American adults using a variety of threshold values (Looker, 1998). The results ranged from 6.3% (both sexes combined) with a cut-off of >45% to 0.9% with a threshold of >60%. At each threshold, the prevalence of elevated TS was 1.3 to 1.8 times greater in men than women. Blood samples were drawn once only from each subject in this study and no conclusions could therefore be drawn about the prevalence in the population of persistently elevated values of TS. Single values may be affected by the presence of inflammatory conditions (Zilva, 1988), by dietary iron intake (Crosby, 1984) and by diurnal variation (Hamilton, 1950; Statland, 1976) (although diurnal variation in haemochromatosis homozygotes may be minimal (Edwards, 1989)). The use of two different phlebotomy times and variable periods of pre-test fasting is acknowledged by the authors of Looker's paper as a potential weakness. In order to control for such factors, many studies using TS have required that subjects with an initial TS above a determined threshold return for a second, fasting determination of TS.

McLaren and colleagues screened 1652 asymptomatic Australian adults using an initial TS cut-off of 45% (McLaren, 1998). Those with a TS above this value were re-tested. If the repeat value was also >45% and the serum ferritin was elevated as well, the diagnosis was confirmed with a liver biopsy. Those whose ferritin was not raised had a TS checked

a third time, under fasting conditions. In addition, TS values in a group of 485 subjects known to be heterozygous or homozygous for haemochromatosis were measured. The authors found, by means of mixture modelling analysis, that using an initial TS cut-off of 45% would obviate unnecessary testing in unaffected subjects and allow detection of 98% of expressing homozygotes. Other studies have set the threshold for further evaluation at 55% (Bassett, 1988; Phatak, 1998), 55% for men and 45% for women (Bradley, 1996b), 60% for men and 50% for women (Adams, 1995), 60% (Bhavnani, 2000), 62% (Baer, 1995, Edwards, 1988). Recent UK guidelines (British Committee for Standards in Haematology, 2000) have suggested that 55% for males and 50% for females be accepted as the threshold for an abnormal TS "until an appropriate threshold has been determined for the UK". This demonstrates the difficulty in agreeing upon a threshold TS that will achieve an acceptable balance of sensitivity and specificity. It should also be noted that the TS value appropriate in studies on screening blood donors, such as those by Edwards (Edwards, 1988) and Adams (Adams, 1995) is not necessarily identical to that most suitable for a general population in which most individuals have not undergone any iron-depleting procedure such as blood donation.

Adams and colleagues (Adams, 2000) have suggested that the unsaturated iron-binding capacity (UIBC) performs better in detecting haemochromatosis homozygotes and is more cost-effective than TS. This strategy was used also by Witte (Witte, 1997). Immunochemical measurement of transferrin may be preferable to derivation of TS from iron-binding capacity (Crawford, 2000). However, this method has not been standardised. Although reference methods have been proposed for the measurement of TIBC (International Committee for Standardization in Haematology, 1978; National Committee for Clinical Laboratory Standards, 1990), these have not gained widespread international currency. This has led to concerns being raised (McDonnell, 1998) about possible variability of derived TS values resulting from inadequate standardisation and quality control in the measurement of TIBC. Such concerns apply equally to estimation of UIBC and are given weight by evidence of an imperfect correlation between chemically measured TIBC and immunologically measured transferrin concentrations (Gambino, 1997).

Transferrin saturation alone has been used as the initial test in many screening studies (for example: Edwards, 1988; Edwards, 1993; Phatak, 1998; McDonnell, 1998; McDonnell, 1999a; Leggett, 1990). Those who are recalled for further assessment on the basis of an elevated TS usually have their serum ferritin measured. Niederau and co-workers (Niederau, 1998) suggest that both ferritin and TS should be performed initially as this facilitates detection of iron deficiency and, potentially, other disorders.

This is opening up a new question of whether screening for haemochromatosis should be combined with screening for iron deficiency. If the detection of iron deficiency is to be a declared aim of a screening programme, then it merits separate consideration and the case is not made for inclusion of serum ferritin as a first-line test. However, any phenotypic screening programme for iron overload will detect a number of individuals who might be iron deficient and a decision must be taken *ab initio* how these individuals will be dealt with. This might have significant implications for cost-effectiveness.

After the linkage between haemochromatosis and HLA-A3 was discovered in the mid 1970s (Simon, 1975), many subjects suspected of having haemochromatosis were tissue typed. After the discovery of HFE, this is no longer appropriate. The precise role of genetic testing in screening remains controversial.

Voices have been raised in support of the use of first-line genetic testing (Allen, 1999; Beutler, 1996). However, these papers assumed a high penetrance of C282Y, which, as has been pointed out in response to Allen (Willis, 2000), may not be a valid assumption. The choice between phenotypic and genotypic screening may be influenced by the population being screened: universal screening of neonates would of necessity involve genetic testing only, and has been advocated by some (Allen, 1999; Olynyk, 1999)

An expert panel was convened in 1997 by the United States Centers for Disease Control and Prevention and the National Human Genome Research Institute specifically to evaluate the role of genetic testing in screening for hereditary haemochromatosis (Burke, 1998). The consensus reached was that genetic testing should not be used in population screening for hereditary haemochromatosis as it offered no advantage over phenotypic testing and could introduce unnecessary risk. Others share the view that the uncertainty about the penetrance of the C282Y mutation and inability to provide all detected homozygotes with a reliable estimate of the risk that they will develop clinical haemochromatosis are of themselves sufficient to recommend against the use of first-line genetic testing (Burke, 1998; Worwood, 2000; Crawford and Hickman, 2000; Asberg, 2001). This is also the recommendation of the current UK guidelines (British Committee on Standards in Haematology, 2000)

2.6.4 *Cost-effectiveness of screening*

There have been many evaluations of the cost-effectiveness of putative screening programmes for hereditary haemochromatosis, dating from both before and after the discovery of HFE. This discovery has had significant implications for the cost

of screening, as it has led to a re-evaluation and gradual lessening of the role of liver biopsy in confirming the diagnosis, as discussed above. Studies dating from before the availability of a genetic test on the whole reflected the established dogma that liver biopsy was essential in confirming the diagnosis of haemochromatosis (Sallie, 1991; Powell, 1996, Witte, 1996). Liver biopsy, while retaining a place in the evaluation of iron overload, is no longer considered imperative in all patients (British Committee on Standards in Haematology, 2000; Powell, 2000)

Cost-effectiveness studies may have limited relevance outside the particular health-care system in which they are set and there has been no study looking at this issue in the context of the U.K. National Health System. However, studies from the US, Canada and Australia all lend weight to the evidence that screening may be beneficial and there is general agreement that detecting and initiating treatment of haemochromatosis before the development of serious complications is cheaper than treating those complications. Baer et al performed TS on 3977 men aged 30 years or older when they attended for routine health checks. Subjects having an initial TS ≥62% underwent a second, fasting measurement of TS and ferritin. If TS was still ≥62% and ferritin was ≥500 ng/ml, subjects were referred for liver biopsy. The personal physician of all individuals with a TS <15% was alerted. A year after the completion of the initial examinations, a review of all subjects' medical records took place. Initial testing identified 40 subjects with raised TS of whom 36 attended for follow-up. All were asymptomatic. Fourteen patients were subsequently referred for liver biopsy and 12 underwent this procedure. Eight of these were identified as having haemochromatosis. Screening the relatives of subjects with haemochromatosis identified three additional affected persons. Costs were those applicable in northern California. The cost per case of haemochromatosis identified (including screening of relatives) was estimated at US$17 000. Had they limited screening to white men only, they estimated that the costs would have been approximately halved (Baer, 1995).

In a larger study, Balan and co-workers screened 12 258 patients at the Mayo Clinic. Fasting serum iron was the initial test. TS and ferritin were determined in those who had a serum iron concentration of >180 mg/dl. TS >62% and ferritin >500 ng/ml was found in 44 patients and was unexplained in eight. Of these eight, six were found to have evidence of haemochromatosis on liver biopsy. The cost per case discovered was US$5261 (Balan, 1994).

Buffone and Beck performed a detailed analysis combining a standard decision-tree and a Markov cycle tree, which allowed them to simulate the various health states that sufferers from haemochromatosis could occupy during the course of their illness. Their analysis was limited to men aged ≥25 years and compared three initial choices: treatment of the entire population with lifelong phlebotomy without prior testing, phenotypic

screening (initial TS, followed by fasting TS, ferritin and, if both were raised, liver biopsy) and no intervention until the development of clinical symptoms. The Markov model used allowed six possible outcomes: no health problem; haemochromatosis under treatment with phlebotomy; haemochromatosis not under treatment; haemochromatosis with disease-related complications; death from a non haemochromatosis-related cause; death from haemochromatosis. The authors acknowledge that the costs they have used for the treatment of haemochromatosis are estimates because of uncertainties around the incidence of possible complications of haemochromatosis and the probability of disease occurring in homozygotes. They concluded that there was a reasonable likelihood that screening would be cost-effective (Buffone, 1994).

Phatak et al used decision-tree analysis to examine screening in hypothetical cohorts of 30 year old white men, compared with initiating treatment only at the appearance of symptoms. The assumption was made that undetected homozygotes become symptomatic at age 50. The screening strategy involved an initial TS. The threshold was set at 55% and subjects recalled underwent a fasting TS and ferritin. If the repeat TS was ≥55% and serum ferritin was >200 ng/ml, a liver biopsy was offered. It was assumed that 50% of patients would decline biopsy and these would be offered hepatic iron quantification by magnetic resonance imaging (MRI). Assumptions made about the prevalence of haemochromatosis, the proportion of homozygotes who develop organ dysfunction, the costs of the initial screening tests and the discount rate (a measure of the present value of future benefits) were of major importance in the outcome of the model. Screening was found to be cost-effective provided that haemochromatosis had a prevalence of at least 3 per 1000, that at least 40% of homozygotes developed symptomatic disease, that the TS cost $12 or less and that the discount rate was less than 3% (Phatak, 1994).

Bassett and colleagues estimated the cost of a hypothetical Australian screening programme, and evaluated eight different strategies:

1. Non-fasting TS followed by liver biopsy if TS ≥45%;
2. Non-fasting TS. If TS ≥45%, TS and ferritin repeated, fasting. Liver biopsy advised for those with persistently elevated TS and elevated ferritin.
3. As for (1) above, but with TS threshold of 55%.
4. As for (2) above, but with TS threshold of 55%.
5. Non-fasting TS. If TS ≥45%, genetic test performed.
6. Non-fasting TS. If TS >45%, fasting TS and ferritin assessed. Genetic test for those with persistently elevated TS and raised ferritin.
7. As for (5) above, but with TS threshold of 55%.
8. As for (6) above, but with TS threshold of 55%.

The study revealed that strategies involving a genetic test (priced at US$120) were cheaper than those in which liver biopsy was recommended. Strategy 8 gave a cost per genetic haemochromatosis case detected (including family screening) of US$2457, while the cost of strategy 4 was US$3245 per case. The costs for strategies 3 and 7 were US$3598 and US$2484 respectively. The authors made the point that these costs compare very favourably with those reported for screening for breast cancer, prostrate cancer, colon cancer and cystic fibrosis (Bassett, 1997).

Adams and Valberg used decision analysis techniques to compare screening using genotypic or phenotypic screening in a hypothetical cohort of 10 000 blood donors and 50 siblings of the identified homozygotes. They set the threshold for TS at 50% for women and 60% for men. The phenotypic strategy involved performing a liver biopsy on those subjects with persistently raised TS and elevated ferritin. Genotypic screening comprised first-line testing for the C282Y mutation of HFE. Homozygotes were further assessed by measurement of ferritin. Those with elevated values were treated, while those with normal values were followed up with 5 yearly re-evaluation of serum ferritin. Heterozygotes were not followed up and it was acknowledged that compound heterozygotes, who might be iron overloaded, would have been missed. An assumption was made that 43% of men and 28% of women would develop life-threatening manifestations of haemochromatosis if not detected by screening. The authors concluded that screening programmes have the potential to save money and that phenotyping is cheaper than genotyping unless the cost of the genetic test falls below $28 (Adams, 1999b). Commenting on this, Tavill concurs that issues of cost-effectiveness may be paramount in determining whether or not screening programmes are established, but reiterates that other issues such as uncertainty about penetrance are important factors in determining how screening should be carried out (Tavill, 1999).

2.6.5 *Potential harm of screening*

Although cost-effectiveness is clearly of great importance in the evaluation of potential screening programmes, there are other issues which, while harder to quantify, are especially pertinent to genetic testing. These were highlighted by an expert panel convened in the United States in 1997 to evaluate the role of genetic testing in screening (Burke, 1998). The problem of identifying homozygotes for the C282Y mutation of HFE as having a disease is one potential problem. Many such individuals would be entirely healthy when they were detected (the proportion would be likely to depend on the age at which screening was carried out as well as on the penetrance of the homozygous state). They would need to be followed up with repeated phenotypic

testing. Concerns have been raised about possible stigmatisation, diminished self-worth and intra-familial strife (Burke, 1998). In the United States, there have been calls for scrutiny of genetic tests because of these worries (Task Force on Genetic Testing of the NIH-DOE Working Group on Ethical, Legal and Social Implications of Human Genome Research, 1997) and similar calls have been made within the United Kingdom too (Advisory Committee on Genetic Testing, 1998). Zimmern has argued that there is confusion between what he terms "gene testing" (i.e. tests on DNA, RNA or chromosomes) and "genetic testing" (any type of test that indicates that an individual is likely to have a genetic or familial disorder) (Zimmern, 1999). He contends that if this distinction is observed, there is no need for special treatment of testing involving nucleic acids or chromosomes and that the concerns outlined should be taken to apply to any genetic test, as defined by him. With this line of argument, testing for haemochromatosis using biochemical markers of iron overload could be considered "genetic". However, his analysis fails to take into account the fact that screening tests for iron overload are not specific to genetic haemochromatosis, and I would argue that the testing only becomes "genetic" when confirmatory testing with either a liver biopsy or with HFE mutation analysis (the "gene" test) is performed. Thus in the case of screening for haemochromatosis there may indeed be an ethical distinction between first-line testing using iron indices and testing using DNA-based tests, in contradistinction to Zimmern's contention.

Power and Adams were unable to identify any adverse psychological or social effects of genetic testing for haemochromatosis. They studied homozygotes for C282Y, discovered through a screening programme of blood donors and also a group of patients referred to a specialist haemochromatosis clinic by their family physicians. The latter group was referred because of suspicious symptoms, family history or abnormal iron tests. Study participants completed two widely used health questionnaires before and a year after learning of the result of their genetic test. The investigators found no significant deleterious psychological effects of testing on anxiety or on mental or physical health status. Moreover, anxiety decreased significantly in homozygotes and heterozygotes for C282Y after testing, while remaining constant in C282Y-negative individuals. Two patients reported feelings of guilt engendered by the effect of testing on their families (no further details given) and none reported adverse effects on work or insurance (Power, 2001).

There is evidence from the United States of insurance discrimination against persons found to be homozygous for haemochromatosis (Alper, 1994; Barash, 2000). The likelihood of this happening or not will depend on the existence of national agreements

or legislation to prevent it. Such an agreement appears to exist in Australia (Delatycki, 2002) and the researcher was given a similar written assurance by the British Association of Insurers during preparation of the work presented in this thesis. More recently, the British government has imposed a moratorium for five years on the use of genetic testing to determine insurance premiums (Adam, 2001).

2.6.6 *Which population to screen: case finding or population screening?*

The appropriate population to target for screening also requires further clarification. World wide, there are marked differences in the prevalence of HFE-associated haemochromatosis and it is rare in Asia, the Middle East and parts of Africa (Merryweather-Clarke, 1997). Blood donors at their first attendance to give blood have been suggested (Adams, 1995), but only a small fraction of the population in any country is likely to be a blood donor. Women express haemochromatosis at a lower frequency than men but can have full phenotypic expression of the disease (Adams, 1997; Garry, 1997; Moirand, 1997a). There is, therefore, no evidence to suggest that it is justifiable to exclude women from screening programmes (Adams, 1995; Moirand, 1997a). Others have suggested that screening be directed only at first degree relatives of haemochromatosis sufferers or at patient groups with symptoms that might be caused by iron-overload (Cogswell, 1998). The cost-effectiveness of genetic testing of first degree relatives of affected patients has been demonstrated (El-Sareg, 2000). Krawczak and co-workers calculated that up to 40% of individuals at-risk for hereditary haemochromatosis would be identifiable by screening first- to third-degree relatives of known sufferers from the condition. They contended that the efficiency of this method of screening may be 50 times greater than that of full population screening under conditions obtaining in 2001 (Krawczak, 2001).

There have been a number of studies performed to examine the frequency with which haemochromatosis can be detected among patients already attending other specialist disease clinics. These have used both phenotypic and (since 1997) genotypic screening strategies. Unfortunately, many of these studies have lacked an appropriate control group, hindering the interpretation of their findings. Their conclusions have been varied, and no consensus has emerged about, for example, the benefits of screening a rheumatology (Olynyk, 1994; Willis, 2002) or a diabetic (George, 1995; Braun, 1998) clinic population. However, this case-finding approach is advocated by the Centers for Disease Control (CDC) in the United States (Reyes, 2002). The CDC recommends that

...persons experiencing the unexplained symptoms compatible with hemochromatosis (these symptoms include severe weakness or fatigue; unexplained joint or abdominal pain; signs of liver disease, diabetes or heart problems; impotence; infertility; and loss of menstrual periods) should ...be tested.

Table 2.5 summarises some papers that have examined the case-finding approach to screening for haemochromatosis in various different patient populations.

Table 2.5 Screening for haemochromatosis in specific patient populations

Disease	N[a]	Country	Cont[b]	Screening method	Conclusion	Reference
Liver disease	132	U.S.A	No	TS/ferritin/ gene[c]/liver biopsy	6% C282Y homozygous	Bacon, 1999a
Chronic hepatitis	108	U.S.A.	No	TS/ferritin/ Liver biopsy	2.5% HC[d] (HII[e]>2)	Di Bisceglie, 1992
Liver disease	427	England	No	TS/ferritin/ gene[c]	3% C282Y homozygous	Moodie, 2002
Rheumatology clinic	339	Australia	No	TS/ferritin /liver biopsy	1.5% HC (hepatic iron[f])	Olynyk, 1994
Inflammatory arthritis	1000	England	Yes	Gene[g]	0.5% C282Y homozygous	Willis, 2002
Diabetes mellitus	1194	Scotland	No	TS/liver biopsy /HLA type	0.5% HC (hepatic iron)	George, 1995
Diabetes mellitus	406	England	No	TS/HLA type	0.5% HC	Singh, 1992
Type II diabetes mellitus	401	Germany	Yes	Gene[c]	0.5% C282Y homozygous	Braun, 1998
Type I diabetes mellitus, onset >30 y	716	Denmark	Yes	TS/gene[c]	1.26% C282Y homozygous	Ellervik, 2001
Type II diabetes mellitus	238	England	Yes	Gene[g]	0.4% C282Y homozygous	Frayling, 1998

Notes

[a] N = number of subjects in study

[b] cont indicates the presence (yes) or absence (no) of a control group

[c] testing for both the C282Y and H63D mutations was performed

[d] HC = hereditary haemochromatosis

[e] HII = hepatic iron index

[f] hepatic iron content raised

[g] testing for the C282Y mutation alone was performed

There is one aspect in which case-finding within such patient-groups performs much less well than population screening: by definition, the individuals with haemochromatosis are not being detected at an early stage. The majority of diabetic haemochromatosis sufferers are already cirrhotic (Strohmeyer, 2000), and many others may have other irreversible complications, which lead to a reduced life expectancy even with iron-depleting therapy. This has led to renewed calls for the search for haemochromatosis in the presence of clinical disease to be abandoned in favour of population screening (Niederau and Strohmeyer, 2002).

2.7 Iron Metabolism

Iron plays an indispensable role in many metabolic processes, but in excess can be toxic (see section 7.1). Under physiological conditions, iron loss from the male human body is minimal (approximately 1 mg per day in the sloughing of cells from skin and gut, with additional small amounts in sweat, bile and faeces). Menstruating females lose on average an extra milligram daily from menstruation, and about 500 mg with each pregnancy. Because of the limitations on iron loss, control over iron absorption appears crucial in maintaining iron homoeostasis and preventing the development of iron deficiency or overload. Although much has become known in recent years about the mechanisms involved in this process and their perturbation in states of iron overload, many uncertainties remain.

Healthy adults have a body iron content of 3 to 5 grams, the great majority of which is incorporated into haemoglobin. Of the remainder, most is in the form of storage proteins (ferritin and haemosiderin) in reticulo-endothelial cells in the liver, spleen and bone marrow. The flow of iron between these various compartments, and the major postulated sites of regulation is illustrated in figure 2.6.

2.7.1 *Storage and transport of iron*

The majority of storage iron in normal individuals is present in ferritin. Human ferritin consists of 24 subunits of two immunologically distinct types, H and L, coded for by genes on chromosomes 11 and 19 respectively. A spherical apopoprotein shell encloses up to 4000 iron atoms. The proportion of L and H subunits varies from tissue to tissue, with L-rich ferritin predominating in the spleen, liver, placenta and serum and H-rich ferritin in heart and red cells. Circulating ferritin is about 60% glycosylated.

Haemosiderin is a water-soluble protein-iron complex, with an amorphous structure. It may be formed by the partial digestion of ferritin aggregates by lysosomal enzymes (Pippard, 1999). In conditions of iron overload, the proportion of iron stored as haemosiderin rises. Normally, haemosiderin is located mainly in reticulo-endothelial cells, but in iron overload it is found in increased quantities in hepatocytes, too.

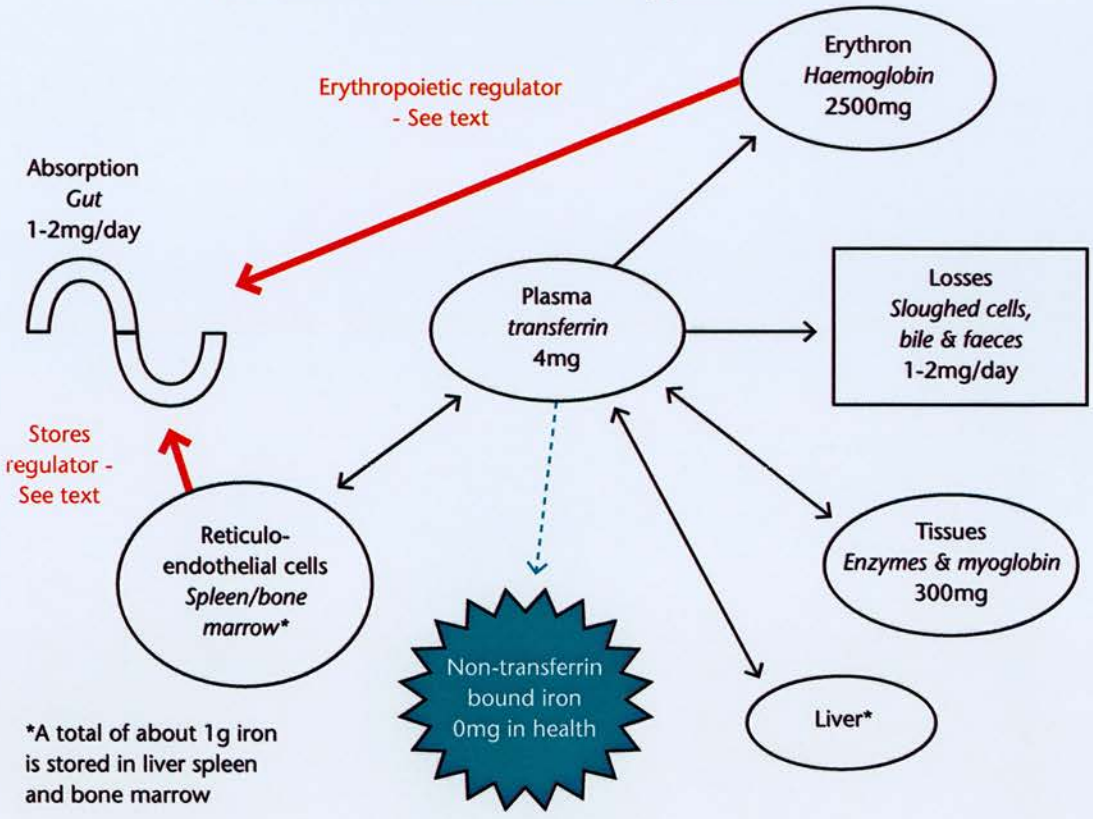
Under normal conditions, iron is transported in the plasma bound to transferrin, a single chain glycoprotein synthesised mainly in the liver. Synthesis is inversely related to iron stores. Each molecule can bind two atoms of ferric iron. Transferrin uptake into cells depends on the presence of transferrin receptors on the cell surface. Two forms of transferrin receptor have been discovered. The type 1 transferrin receptor consists of two monomers joined by a disulphide bridge, each subunit able to bind one transferrin molecule. It is encoded by a gene on chromosome 3 and synthesised mainly in the liver (Pippard, 1999). Like transferrin receptor type 1 receptors, transferrin receptor type 2 also binds diferric transferrin (Kawabata, 1999). However, transferrin receptor 2 is expressed mainly in the liver (Fleming, 2000) and appears unable to bind HFE (West, 2000) in contrast to transferrin receptor type 1. Its function remains to be further characterized.

2.7.2 *Absorption of Iron*

Only a small fraction of the 15 mg iron provided daily by a normal western diet is absorbed across the intestinal mucosa into plasma (Pippard, 1999): about half is solubilised in the gut lumen, approximately 3 mg is taken up by mucosal cells and only 1 mg reaches the portal blood. The uptake of iron derived from haemoglobin or myoglobin in red meat (haem iron) is less affected by luminal and mucosal factors than is that of non-haem dietary iron which predominates in the diet of most populations in the world. Non-haem iron absorption is inhibited by alkaline conditions (see below), and by binding to phosphates and phytates in the diet. Iron absorption requires the iron to be in a solubilised form, and dietary components which promote this under the acidic conditions that prevail in the proximal small intestine facilitate absorption of iron. Certain amino acids, sugars and ascorbic acid thus favour iron absorption by the formation of low molecular weight soluble chelates (Conrad, 2000).

The mechanisms of absorption of haem and non-haem iron across the luminal membrane of enterocytes differ. Haem iron binds to specific membrane receptors and is then taken up into the cell in vesicles. Once within the cell, iron is released from the porphyrin ring by the action of mucosal haem oxygenase. (Pippard, 1999).

Figure 2.6 Iron distribution and flux in a 70kg man (adapted from Conrad, 2000; Pippard, 1999)



The existence of a pathway for the direct absorption of ferric (Fe^{3+}) non-haem iron has been suggested by one group (Umbreit, 1998). They postulate that ferric iron is chelated at the intestinal mucosal surface by mucin. Transmembrane transport of iron is mediated by a β_3-integrin, and once in the cytoplasm, iron may be bound to a 56 kDa protein called mobilferrin, either in its monomeric form or as part of paraferritin, a 520 kDa multipolypeptide complex. However, questions remain about the evidence in support of this theory (Wolf, 1994) and it is more generally accepted that the absorption of non-haem iron from the intestinal lumen requires its reduction to the ferrous (Fe^{2+}) form (Fleming, 2002; Philpott, 2002; Pietrangelo, 2002).

Antibodies against duodenal cytochrome b (Dcytb) are able to block iron reduction at the enterocyte brush border, and this molecule is likely responsible for the ferric reductase activity at this site (McKie, 2001). After reduction, transport of iron across the cell membrane is the role of divalent metal transporter 1 (DMT1, also called DCT1 and Nramp2). Gunshin and co-workers identified DMT1 as responsible for the ability of rat duodenum mRNA to stimulate iron uptake when injected into Xenopus oocytes (Gunshin, 1997) and Fleming and colleagues found that a mutation in DMT1 was

responsible for the defective intestinal iron uptake that occurs in the microcytic anaemia (mk/mk) mouse (Fleming, 1997). In situ hybridisation in rats has demonstrated that the expression of DMT1 mRNA in the crypt and villus enterocytes falls in iron overloaded animals, compared with control and iron deficient ones. DMT1 protein, on the other hand, was localised at the luminal membrane of villus enterocytes in iron deficient rats and entirely in the cytoplasm of these cells in iron overload (Trinder, 2000). These findings lend further support to a role for DMT1 in the uptake of iron from the intestinal lumen.

Once within the cell, iron may be incorporated into ferritin for storage or it may cross the basolateral cell membrane to enter the circulation. Using positional cloning techniques, Donovan and colleagues identified the gene responsible for the hypochromic anaemia of the Zebrafish mutant weissherbst. They named the gene ferroportin 1 (FP1) and found that it encoded a transmembrane protein which could function as an iron exporter when expressed in Xenopus oocytes (Donovan, 2000). Two other independent groups (McKie, 2000; Abboud, 2000) also identified the gene using different techniques, accounting for its various designations as Ireg1 and MTP1 in addition to ferroportin. Mutations of the ferroportin1 gene have been implicated in the pathophysiology of a form of non-HFE linked haemochromatosis (Njajou, 2001; Montosi, 2001) (see section 2.3.3).

Once across the basolateral cell membrane, ferrous iron must be re-oxidised to ferric in order to bind to circulating transferrin (Philpott, 2002). A candidate ferroxidase has been identified by studies on the sex-linked anaemia (sla) mouse (Vulpe, 1999). Mice carrying the sla mutation develop moderate to severe microcytic anaemia resulting from a failure of iron taken up from the intestinal lumen to enter the circulation. A novel gene, Heph, was found which was mutant in these mice and which was highly expressed in villus enterocytes. The gene encoded a transmembrane protein termed hephaestin, which is a homologue of caeruloplasmin, a copper-binding protein with ferroxidase activity. As mentioned above (section 2.3.3), deficiency of caeruloplasmin is associated with iron overload.

2.7.3 *Iron uptake by somatic cells*

Diferric transferrin binds to type 1 transferrin receptors on the cell surface and the iron-transferrin-receptor complex localises to clathrin-coated pits, whence they enter the cell by endocytosis. At the acidic pH within the endosome, iron is released from transferrin and transferrin and the transferrin receptor are recycled to the cell surface (Pietrangelo, 2002). Before it can be transported across the endosomal membrane, ferric iron is reduced to ferrous by an unidentified reductase (Philpott, 2002). It is likely that the transmembrane transport is effected by DMT1, as this DMT1 occurs in various tissues and has been detected in endosomes (Trinder, 2000; Gruenheid, 1999)

2.7.4 *Regulation of iron absorption*

The site of regulation of iron absorption is the villus enterocyte. This regulation occurs at two levels: iron homoeostasis at the level of the individual cells is controlled at least in part by a system of iron regulatory proteins responding to changes in the cellular labile iron pool; at the level of the whole organism, humoral factors are thought to act as regulators, transmitting messages about iron balance from reticulo-endothelial stores and the erythron to the enterocyte (Roy, 2000).

Both of these systems are examined in greater detail below.

2.7.4.1 *Iron regulation at the level of the whole organism*

The so-called stores regulator (figure 2.6) increases or decreases iron uptake at the level of the enterocyte in conditions of iron depletion or excess, respectively. The variation in iron absorption brought about by the stores regulator is limited in extent to approximately 1 mg per day (Pietrangelo, 2002). Hepcidin, a possible candidate for the humoral factor that acts as stores regulator, has recently been discovered. Its expression is predominantly hepatic (Park, 2001). Targeted disruption of the gene encoding upstream stimulatory factor 2 (USF2) in mice results in the failure of expression of hepcidin and the development of an iron-loading phenotype resembling that of human haemochromatosis (Nicolas, 2001). Pigeon and colleagues showed that hepcidin mRNA is increased both with dietary iron loading and in β_2-microglobulin knockout mice (Pigeon, 2001). These observations and the fact that mutations in the HAMP gene may be associated with a haemochromatosis phenotype (section 2.3.3) support a role for hepcidin in iron homoeostasis, but whether it does indeed function as the stores regulator remains to be proved.

The existence is postulated of a second humoral regulator, the erythropoietic regulator, which responds to the erythropoietic needs of the organism. As marrow iron requirements increase, iron absorption may be upregulated even in the presence of normal or increased iron stores. Iron absorption can be substantially increased in anaemic individuals who are treated with therapeutic doses of iron (Finch, 1994), indicating that the variation induced by the demands of erythropoiesis in iron absorption is much greater than that resulting from the action of the stores regulator. This difference may indicate that the two factors act on different iron absorption pathways. The soluble transferrin receptor has been proposed as one possible candidate for erythropoietic regulator (Cazzola, 1999).

2.7.4.2 *Iron regulatory proteins as iron sensors at the cellular level*

The sensing of iron needs at the cellular level and the post-transcriptional control of the expression of many of the proteins involved in iron homoeostasis are coordinated by a system of iron regulatory proteins. Initial evidence that the translation of ferritin mRNA varied in response to cellular iron balance led to the discovery of a stem-loop structure in the 5' untranslated region (UTR) of ferritin mRNA, termed the iron responsive element (IRE). This structure was found to interact with a cytoplasmic protein called iron regulatory protein (IRP). Binding of IRP occurs in conditions of low cellular iron availability and inhibits the translation of the mRNA. Conversely, when cellular iron supply is high, IRP does not bind mRNA and translation is able to proceed, resulting in increased ferritin synthesis. Transferrin receptor 1 synthesis is also regulated by the IRE/IRP system, but in the opposite direction from ferritin, so that receptor synthesis increases when iron is scarce and falls in conditions of plentiful iron. In transferrin receptor 1 mRNA, 5 IREs have been found in the 3' UTR. As described in the case of ferritin, IRPs are active and can bind to an IRE when cellular iron is low. However, in the case of transferrin receptor 1 mRNA, the effect of this binding is to stabilise the mRNA, probably by sterically hindering degradation by an endonuclease. This has the effect of allowing iron deplete cells to synthesise and present at the cell surface increased amounts of transferrin receptor 1 and so to internalise increased amounts of transferrin with bound iron (Kühn, 1998). Transferrin receptor 2 mRNA, on the other hand, does not contain an IRE (Kawabata, 2001) and is not regulated by iron at the cellular level.

Ferroportin1 mRNA has an IRE at the 5' UTR (Abboud, 2000) suggesting upregulation in conditions of high availability as is the case with ferritin. DMT1 exists in two isoforms, one lacking an IRE and the other, contrary to ferroportin, with an IRE in its 3' UTR (Lee, 1999), suggesting that the mRNA would be stabilised and protein synthesis increased when iron availability is low. However, analysis of DMT1 and ferroportin1 expression in human duodenal biopsy specimens reveal that mRNA and protein levels of both of them are increased in iron deficiency (Zoller, 2001). This could be explained by IRP/IRE interaction for DMT1, but is the opposite from what would be predicted by the IRP system for ferroportin1. Furthermore, doubts have been expressed about the functionality of the IRE on DMT1 mRNA (Zoller, 2001). It seems likely, therefore that other regulatory factors are also involved (Philpott, 2002).

2.7.4.3 *Molecular characterisation of iron regulatory proteins*

There are two distinct IRPs, IRP-1 and IRP-2, both of which are activated to be able to bind mRNA in the presence of low levels of intracellular iron. The proteins are homologous in structure, but while IRP-1 appears ubiquitous, the tissue distribution of IRP-2 is more limited (Kühn, 1998).

Under conditions of adequate normal iron availability, IRP-1 functions as a cytoplasmic aconitase – an enzyme responsible for the conversion of citrate to iso-citrate. Its fulfilment of this role depends on its having incorporated into its structure a 4Fe-4S cluster. The holo-protein, containing the iron-sulphur cluster cannot bind IREs. When cellular iron levels fall, however, a cytoplasmic apoprotein lacking the iron-sulphur cluster is found, which has no aconitase activity, but which can bind to IREs.

IRP-2, on the other hand, does not contain an iron-sulphur cluster and has no aconitase activity. It is not found in the cytoplasm other than under conditions of restricted iron availability. Once iron becomes available, IRP-2 is probably subject to proteosomal degradation (Kühn, 1998)

2.7.4.4 *The structure and possible function of HFE*

The HFE gene encodes a 343-residue transmembrane protein, which is homologous to class I MHC molecules and associates with the class I light chain, β_2-microglobulin. It has six domains: a 22-amino acid signal peptide, three extracellular domains (designated α1, α2 and α3), a transmembrane region and an intracellular domain (figure 2.1) (Feder, 1996). The C282Y mutation in the HFE gene results in the substitution of a tyrosine for a cysteine residue. This eliminates a disulphide bond in the α3 domain and prevents the association between HFE protein and β_2-microglobulin, which is essential for expression of HFE at the cell surface (Feder, 1997; Waheed, 1997). The H63D mutation, resulting in an aspartate for histidine substitution in the α1 domain has no effect on either the association of HFE with β_2-microglobulin or cell-surface expression. Support for the contention that HFE is involved in the regulation of iron homoeostasis was provided by the discovery that HFE knockout mice rapidly developed iron overload while their HFE normal littermates did not (Zhou, 1998).

Feder and colleagues provided an important insight into the possible function of HFE when they found that wild type HFE protein and H63D protein form stable complexes with the transferrin receptor. The C282Y mutation nearly completely prevents this association (Feder, 1998). Crystallographic studies with high concentrations of HFE

and transferrin receptor 1 have further characterised the nature of the interaction and have suggested a stoichiometry of 2:2 (HFE:transferrin receptor) under equilibrium conditions (Bennett, 2000), which would probably prevent binding of iron-loaded transferrin to transferrin receptor. Studies with lower concentrations of HFE and transferrin receptor in solution have suggested the formation of a ternary complex of HFE, transferrin receptor and iron-loaded transferrin with a stoichiometry of 1:2:1 (Lebrón, 1999). It is much less clear whether or not transferrin receptor 2 interacts directly with HFE (Pietrangelo, 2004a).

An increase in the duodenal expression of DMT1 and ferroportin1 in mouse models of haemochromatosis and haemochromatosis patients has been reported by several groups (Fleming, 1999; Griffiths, 2001; Zoller, 2001). In addition, duodenal ferritin mRNA expression is low in at least a high proportion of patients with hereditary haemochromatosis, with a high spontaneous activity of duodenal iron related protein (Pietrangelo, 1995).

Duodenal crypt cells migrate towards the tip of the villi, maturing into absorptive enterocytes as they go. The distribution of HFE and other proteins involved in iron metabolism in crypt and villus enterocytes is different: HFE is expressed strongly in crypt cells, but to a far lesser extent in villus cells (Parkkila, 1997; Waheed, 1999). Conversely, other proteins involved in iron homoeostasis, Dcytb, DMT1 and ferroportin1 are expressed strongly in villus but not crypt cells (McKie, 2001; Griffiths, 2000; Donovan, 2000). These findings have suggested that crypt cells may act as the site for sensing of iron balance, both on a cellular and whole body level, on the basis of which the level of iron absorption is set and maintained as the cells mature and travel up the villi.

Recent work (Waheed, 2002) has suggested that over-expression of HFE without β_2-microglobulin in a Chinese hamster ovary cell line results in a decrease in transferrin receptor mediated iron uptake. However, when both HFE and β_2-microglobulin were over-expressed, the opposite effect, an increase in iron uptake, was found. This is contrary to the results of earlier work by other groups (eg Feder, 1998) which found that the association of HFE and transferrin receptor resulted in a lowered affinity of transferrin receptor for iron-laden transferrin.

Although the details of the role of HFE in iron homoeostasis are still unclear, the information summarised has allowed the formulation of a model to explain how dietary iron absorption might be regulated (Philpott, 2002). Duodenal crypt cells sense the body's requirements for iron via humoral factors such as the stores and erythropoietic regulators discussed above. They are informed about the availability of transferrin-

bound iron via the transferrin receptor/transferrin receptor 2 system. The signals received by the cell and the intracellular iron pool act together, in part via the IRP/IRE system to set the level of transcription of Dcytb, DMT1, ferroportin 1 and ferritin. This level of activity is then maintained as the maturing cell migrates up the villus and develops the ability to absorb iron from the gut lumen. In the absence of functional HFE, uptake and sensing via the transferrin receptor is impaired. This results in the translational activity of DMT1 and ferroportin 1 being set at an inappropriately high level, and an inappropriately low level of ferritin synthesis. In turn, this leads to the absorption of amounts of dietary iron in excess of requirements and, in time, the development of the iron overloaded phenotype.

The discovery of hepcidin and the emergence of evidence of its possibly central role in iron homoeostasis (Pietrangelo, 2004b) has led to a challenge to the crypt-programming

2.8 Conclusion

The diversity of current research into genetic iron overload and iron metabolism can be appreciated from the survey of the literature presented in this chapter. In the remainder of this thesis, the author seeks, in chapters 4 to 7, to examine in more detail the iron status of various groups of individuals and, in chapter 8, to assess awareness of hereditary haemochromatosis among primary care physicians.

There is a considerable body of published literature speculating about the reasons for the continued high prevalence of the C282Y mutation of HFE in northern European populations. The possibility that it may offer carriers some protection from the development of iron deficiency led to the work presented in chapters 4 and 5 of this work. Chapter 4 looks at the influence of HFE genotype on the development of iron deficiency in male blood donors, and chapter 5 at the relative prevalence of different genotypes in patients with and without iron deficiency.

Chapter 6 examines HFE genotypes and iron stores in patients who have undergone orthotopic liver transplantation, with the aim of identifying both the prevalence of undiagnosed haemochromatosis in this group and also any influence of HFE genotype on clinical outcome after this procedure. Chapter 7 re-examines the suggestion that iron stores have a bearing upon the development of coronary artery disease. Strong arguments have been advanced in favour of this hypothesis, but the published evidence is not conclusive.

3. Methods

This chapter contains details of all the experimental methods used in the work that comprises this thesis.

3.1 Extraction of DNA from peripheral blood

DNA was extracted from whole blood anticoagulated in EDTA by an in-house method (Royal Infirmary of Edinburgh, 2000).

3.1.1 *Materials*

Reagent A
Reagent B
5M sodium perchlorate (BDH, Poole, UK)
Chloroform (BDH, Poole, UK) – ice-cold
Absolute ethanol (BDH, Poole, UK) – ice-cold
Tris-EDTA buffer, pH 7.6
Rotatory mixer
Denly AutoBench centrifuge
Water bath to provide water at 65° C
15 ml centrifuge tubes (Helena Bioscience)
Disposable pastettes (Alpha laboratories)
Glass Pasteur pipettes

3.1.2 *Preparation of reagents*

1M tris (hydroxymethylmethylamine) (Tris)
In each litre:
121.14 g Tris

The Tris was dissolved in 1 litre distilled water and the pH adjusted to 7.4 with hydrochloric acid. The solution was autoclaved at 121° C.

0.5M disodium EDTA
In each litre:
186.12 g disodium EDTA
20 g sodium hydroxide

The pH was adjusted to 8 using 1M sodium hydroxide.

Reagent A
In each litre:
10 ml 1M tris (hydroxymethyl) methylamine (BDH, Poole, UK)
109.54 g sucrose (BDH, Poole, UK)
0.47 g magnesium chloride (BDH, Poole, UK)
10 ml Triton X-100 (Sigma-Aldrich, Poole, UK)

The above components were dissolved in distilled water and the pH adjusted to 8 with sodium hydroxide. The volume was made up to 1 litre with distilled water and the solution was autoclaved at 121° C.

Reagent B
In each litre:
400 ml 1M Tris (BDH)
120 ml 0.5M EDTA (pH 8)
8.76 g sodium chloride (BDH)
10 g sodium dodecylsulphate (SDS) (BDH, Poole, UK)

The components except the SDS were dissolved in distilled water and the pH was adjusted to 8. The volume was made up to 1 litre with distilled water and the solution was autoclaved at 121° C. After autoclaving, while the solution was still warm (to facilitate dissolution), the SDS was added.

5M sodium perchlorate
In each litre:
700 g sodium perchlorate

Tris-EDTA buffer (TE)
In each litre:
10 ml 1M Tris, pH 7.4
1 ml 0.5M EDTA, pH 8

The total volume was made up to 1 litre in each case with distilled water and the solutions were autoclaved at 121° C.

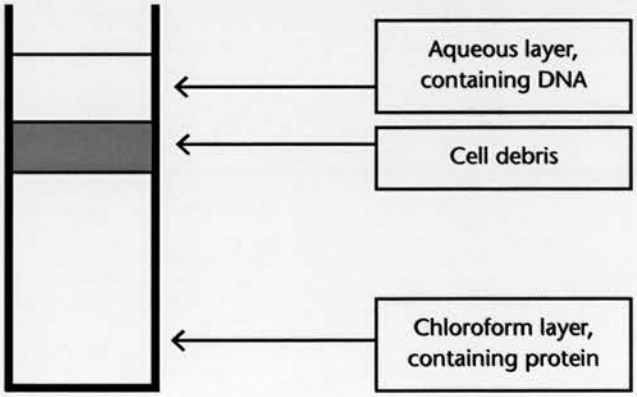
3.1.3 *Method*

The principle of the method is lysis of red cells using reagent A, white cell membrane and nuclear lysis and protein digestion by reagent B and sodium perchlorate, deproteination with chloroform and precipitation of DNA using ethanol.

In detail:

3 to 5 ml whole blood was placed in a 15 ml polypropylene centrifuge tube and diluted to 12 to 15 ml with reagent A (approximately 1 volume blood to 3 volumes reagent A.) After mixing for 4 minutes at room temperature, the tube was centrifuged at 1500g for 5 minutes. The resultant supernatant was decanted off without disturbing the cell pellet. 1 ml reagent B was then pipetted into the tube and the cell pellet was re-suspended by vortexing. After addition to the tube of 250 µl sodium perchlorate, the tube was sealed and mixed thoroughly by inversion. The tube was then incubated for 20 minutes at 65° C. 2 ml ice-cold chloroform was pipetted into the tube and a rotatory mixer was used to mix for 30 to 60 minutes to emulsify the phases. The tube was then centrifuged at 1300g for 2 minutes. This resulted in separation of the layers as illustrated (figure 3.1):

Figure 3.1 Separation of aqueous and chloroform layers after centrifugation



The aqueous phase was then decanted into a fresh tube using a sterile pastette taking care not to disturb the cell debris layer. To the fresh tube was added 2.5 ml ice-cold ethanol and the tube was inverted until a precipitate of DNA was visible. The tip of a glass Pasteur pipette was heated in a flame until the end was sealed and a small hook formed. When cool, this was used to transfer out the precipitated DNA to an Eppendorf tube containing 100 µl TE buffer. The DNA-containing tube was stored at 4° C until use, and then frozen for long term storage at minus 80° C.

3.2 Measurement of DNA concentration

DNA concentration was not measured routinely, for logistic reasons relating to the number of samples. However, it was performed on a number of samples during the process of optimisation of the ARMS reaction.

Samples were diluted 100 fold in distilled water to a volume of 1 ml. A spectrophotometer with an integral ultra-violet lamp was calibrated against a blank of distilled water and the absorbance at a wavelength of 260nm (A_{260}) of the diluted DNA sample was measured. The concentration of the DNA ([DNA]) could be calculated from the equation (Maniatis, 1982):

[DNA] ng/µl = A_{260} x 50 x dilution factor (i.e.100)

This enabled solutions of DNA with a standardised concentration (100 ng/µl) to be made up.

3.3 Extraction of DNA from paraffin blocks

Although it proved impossible to extract DNA from paraffin blocks of sufficient quality to amplify by PCR, the methods used to attempt this are included here for completeness.

3.3.1 *Removal of paraffin*

3.3.1.1 Materials

Xylene (BDH, Poole, UK)
Absolute ethanol
Sterile microfuge tubes
Microcentrifuge (MSE microcentaur)

3.3.1.2 Methods

A 15 µm paraffin section was placed in a microfuge tube, to which 600 µl xylene was added to remove the paraffin. The tube was left to stand for 5 minutes and then spun at 20000g for 10 minutes. The supernatant was discarded and the xylene addition and centrifugation repeated. 600 µl absolute ethanol was then added to the tube to remove

excess xylene. The tube was spun at 20000g for 10 minutes. The supernatant was poured off and the addition of alcohol and centrifugation repeated. The microfuge tube was inverted on a clean paper towel to dry.

3.3.2 *DNA extraction method 1*

QIAamp® Tissue Kit (Qiagen GmbH, Germany)

3.3.2.1 Materials

The kit, designed for 50 extractions, included the following:

Lysis buffer ATL	Elution buffer AE
Buffer AL	Proteinase K (lyophilised)
Wash buffers AW1 and AW2	QIAamp spin columns
Absolute ethanol	

Eppendorf tubes
Microcentrifuge (MSE Microcentaur)
Water baths to provide water at 55 and 70° C

3.3.2.2 Preparation of reagents

Proteinase K was reconstituted in 1.4 ml distilled water.

Buffer AL was prepared by decanting all of reagent AL1 into reagent AL2 and shaking to mix.

Buffers AW1 and 2: 40 ml absolute alcohol was added to buffer AW1 and AW2 concentrate.

3.3.2.3 Method

20 µl proteinase K was added to each sample, mixed thoroughly by vortexing and incubated at 56° C until the tissue was completely lysed. Lysis was aided by regular vortexing during the incubation period, which was often several hours. 200 µl buffer AL was then added, vortexed to mix and incubated at 70° C for 10 minutes. Next, 200 µl ethanol was added and mixed. The mixture thus obtained was placed in a QIAamp spin column, within a 2 ml collection tube, and centrifuged at 6000g for 1 minute. The spin column was placed in a clean collection tube and 500 µl buffer AW1 was added.

After centrifugation at 6000g for 1 minute, the spin column was again placed in a clean collection tube and 500 µl buffer AW2 was added followed by centrifugation at 20000g for 3 minutes. The spin column was then placed within a 1.5 ml microcentrifuge tube and 200 µl buffer AE was added. A 5 minute incubation at room temperature was allowed before centrifugation at 6000g for 1 minute. The elution step with buffer AE was repeated twice more and the eluate of DNA in AE was stored at 4° C until used.

3.3.3 *DNA extraction method 2*

In-house method

Because attempts to amplify DNA extracted by the QIAamp method (method 1, above) were unsuccessful, DNA was extracted from some samples by a second, in-house, method (University of Edinburgh, 1995), described here:

3.3.3.1 Materials

Proteinase K
Lysis buffer
Waterbaths to provide water at 50° C and 100° C
Microcentrifuge
Microfuge tubes

3.3.3.2 Preparation of reagents

Lysis buffer
In each litre:
0.3728 g potassium chloride
0.1211 g Tris
0.0238 g magnesium chloride
1.01 g gelatin
IGEPAL
0.45 ml Tween 20

The above components were dissolved in distilled water to a total volume of 1 litre, and the pH was adjusted to 8.3

Proteinase K
10 mg proteinase K was added to 1 ml distilled water.

3.3.3.3 *Method*

200 µl of proteinase K solution was added to 1 ml of lysis buffer. 200 µl of this solution was added to the microfuge tube containing the tissue sample from which the paraffin had been removed (see above). The microfuge tube was incubated for 2 hours in a waterbath at 50° C, and vortexed once after 1 hour. The tube was then centrifuged at 20000g for 10 seconds. Next, the tube was boiled in a waterbath to inactivate the proteinase K. The tube was spun at 20000g for 10 seconds, cooled on ice and stored at 4° C until use.

3.4 Detection of mutations in the HFE gene by a multiplex amplification refractory mutation system *(ARMS)*

3.4.1 *Principle of method*

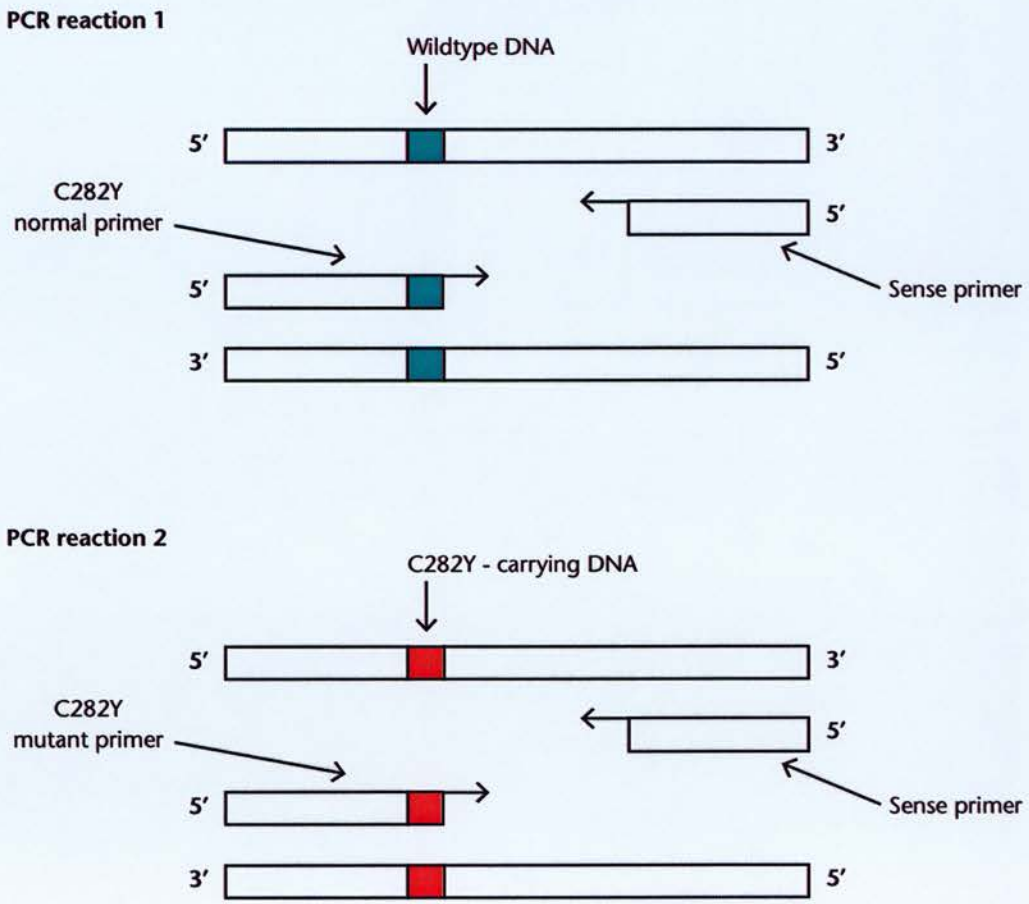
The presence or absence C282Y and H63D mutations in the HFE gene was analysed using a slight modification of the multiplex amplification refractory mutation system (ARMS) described by Baty and colleagues (Baty 1997). The principle of ARMS is a combination of two polymerase chain reactions (PCR) using the same substrate DNA. The ARMS assay for a given mutation uses three primers: along with a sense primer, two other primers (referred to below as "normal" and "mutant", differing only at the 3′ nucleotide) are used. Each of these two primers is specific for one of the allelic variants being studied. This specificity is conferred by the 3′ nucleotide of the primer which complements only one of the two alleles and by the absence of 3′ to 5′ proof-reading activity in Taq DNA polymerase.. Therefore, in the present study, three primers were used to detect C282Y and three to detect H63D. To safeguard against false negative results, a pair of internal control primers (in this case for the human growth hormone (HGH) gene) that amplify a different area of the genome is included in the reaction mixture. The two possible PCR reactions are illustrated for C282Y in figure 3.2.

The alleles present in the reaction mixture can be determined by gel electrophoresis and ethidium bromide staining.

3.4.2 *Interpretation of PCR results*

For each sample of DNA, PCR reactions were performed in 2 tubes, labelled A and B. The composition of each reaction mixture was as stated in table 3.3. PCR product bands were identified initially by comparison with a molecular weight marker, although

Figure 3.2 Amplification refractory mutation system *(ARMS)* for amplification of C282Y mutation



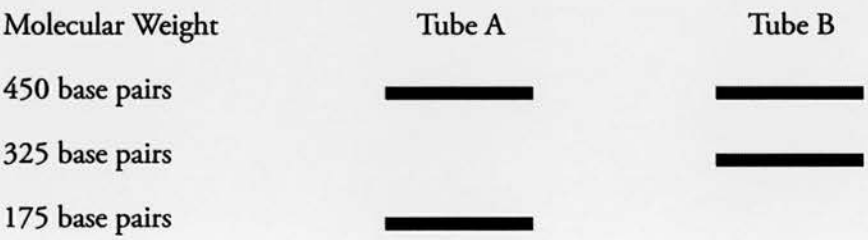
If the DNA is all wildtype, only PCR reaction 1 occurs with amplification of the wildtype product; if C282Y is carried, reaction 2 occurs and if both are present (ie in the heterozygous state) both reactions occur.

this was not feasible when the MADGE system was employed. When MADGE was employed, PCR bands were identified by pattern recognition. This approach was validated by the duplicate analysis of many samples in a reference laboratory and comparison of the results.

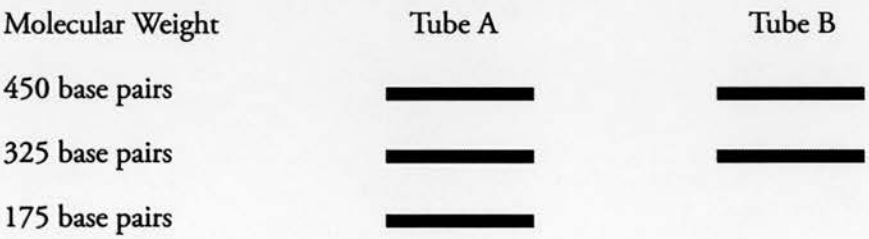
For each sample, PCR fragments corresponding to the C282Y mutation were observed as a band of approximately 325 base pairs in the lane containing reaction mixture A. If C282Y was absent, a 325 base pair band appeared in the lane containing reaction mixture B. PCR fragments corresponding to the H63D mutation appeared as a band of about 175 base pairs in the lane containing reaction mixture B. Fragments corresponding to an allele lacking H63D were observed as a band of 175 base pairs in the lane containing reaction mixture B.

The following patterns were observed:

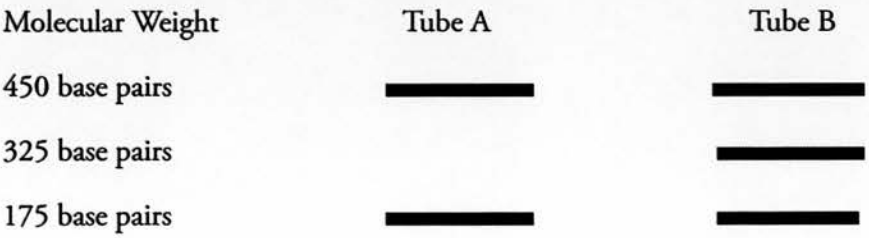
Pattern 1 – wildtype C282Y and H63D



Pattern 2 – C282Y heterozygote; H63D wildtype



Pattern 3 – C282Y wildtype; H63D heterozygote



Pattern 4 – C282Y homozygote; H63D wildtype

Molecular Weight	Tube A	Tube B
450 base pairs		
325 base pairs		
175 base pairs		

Pattern 5 – C282Y wildtype; H63D homozygote

Molecular Weight	Tube A	Tube B
450 base pairs		
325 base pairs		
175 base pairs		

Pattern 6 – C282Y homozygote; H63D homozygote

Molecular Weight	Tube A	Tube B
450 base pairs		
325 base pairs		
175 base pairs		

3.4.3 *Preparation for and execution of the PCR*

3.4.3.1 *Materials*

Peltier Thermal Cycler (PTC-225)
HotStar*Taq* DNA polymerase (Qiagen GmbH, Germany)
10x PCR buffer (Qiagen GmbH, Germany)
Deoxynucleotide triphosphates: 100mM solution of each of : dATP, dCTP, dGTP, dTTP (Promega, Southampton, UK)
Oligonucleotide primers (MWG Biotech AG, Germany). (See below)
Distilled water

Oligonucleotide primers

The following primers were used (all supplied by MWG-Biotech AG, Germany):

1. Sense primers as described by Feder and colleagues (Feder 1996)
   C282Y: 5' – TGGCAAGGGTAAACAGATCC - 3'
   H63D: 5' – ACATGGTTAAGGCCTGTTGC – 3'

2. Antisense ARMS primers (Baty 1997)
   C282Y-normal: 5' – CTGATCCAGGCCTGGGTGCTCCACCTGCC – 3'
   C282Y-mutant: 5' - CTGATCCAGGCCTGGGTGCTCCACCTGCT – 3'
   H63D-normal: 5' – AGTTCGGGGCTCCACACGGCGACTCTCAAG – 3'
   H63D-mutant: 5' – AGTTCGGGGCTCCACACGGCGACTCTCAAC – 3'

3. Human growth hormone (HGH) primers used as internal amplification controls (Kirschbaum 1995)
   HGH-forward: 5' – TGCCTTCCCAACCATTCCCTTA – 3'
   HGH-reverse: 5' – CCACTCACGGATTTCTGTTGTGTTTC – 3'

For each substrate DNA, two separate 25 µl reaction mixtures were set up, referred to below as "A" and "B" respectively. Each reaction mixture contained one of the pair of primers specific for the allelic variant at each of the two possible mutation sites.

3.4.3.2 *Preparation of Reagents*

The four dNTPs were mixed together in equal volumes to a concentration of 25mM. The mixture was further diluted 12.5 times to the desired concentration of 2 mM.

Oligonucleotide primers were diluted to the following concentrations:

HGH primers:	0.05 µM
C282Y primers:	0.25 µM
H63D primers:	1.0 µM

Every PCR mixture contained the components listed in table 3.3. For each batch of reactions, a "master-mix" was made up, in which all the volumes given were multiplied by (N+k), where N = number of (samples + controls + blanks) and k provides extra volume to allow for wastage (for 1-20 samples, k = 1; 21-40 samples, k = 2; 41-60 samples, k = 3; 61 –80 samples, k = 4; 81 –100 samples, k = 5.)

3.4.3.3 *Method*

The *Taq* polymerase was activated by an initial incubation of the reaction mixtures at 95° C for 15 minutes. DNA amplification was then achieved by 30 cycles of the following steps:
60 seconds at 94° C denaturation step;
60 seconds at 59° C annealing step;
60 seconds at 72° C extension step.

The product was then subject to electrophoresis on either polyacrylamide gel (PAGE) or agarose gel (see section 3.7) to separate DNA fragments.

Table 3.3 Contents of PCR reaction mixtures

Reagent	Mixture A	Mixture B
HotStarTaq polymerase	0.2 µl	0.2 µl
10x buffer	2.5 µl	2.5 µl
dNTP	2.5 µl	2.5 µl
HGH – forward	0.625 µl	0.625 µl
HGH – reverse	0.625 µl	0.625 µl
C282Y – sense	0.625 µl	0.625 µl
C282Y – normal		0.625 µl
C282Y – mutant	0.625 µl	
H63D – sense	2.5 µl	2.5 µl
H63D – normal	2.5 µl	
H63D – mutant		2.5 µl
MBG water	11.3 µl	11.3 µl
Solution of DNA	1 µl	1 µl

3.5 HFE mutation detection by PCR and restriction enzyme digestion

Because it proved impssible to determine HFE gene mutations on DNA from tissue samples using ARMS (above), a further attempt to do this using restriction enzyme digestion of amplified DNA was made, as described by Feder and colleagues (Feder, 1996).

3.5.1 *Polymerase chain reaction*

3.5.1.1 Materials

Peltier Thermal Cycler PTC-225
Vertical polyacrylamide gel electrophoresis system (Bio-Rad, Hemel Hempstead, UK)
Oligonucleotide primers (MWG Biotech GmbH, Germany)
Platinum Taq polymerase (Gibco BRL)
10x PCR buffer (Gibco BRL)
Deoxynucleoside triphosphates (dNTP) – concentration 2mM (see section 3.4.3.2)
Magnesium chloride 25 mM (BDH, Poole, UK)
Distilled water

Oligonucleotide primers

For C282Y mutation:
HH1: 5' – CTCAGGCACTCCTCTCAACC – 3'
HH2: 5' – TGGCAAGGGTAAACACATCC - 3'

For H63D mutation:
H631: 5' – ACATGGTTAAGGCCTGTTGC - 3'
H632: 5' – GCCACATCTGGCTTGAAATT - 3'

3.5.1.2 Method

This method employs a simple PCR amplification of each mutation separately, followed by restriction enzyme digestion of the PCR products and gel electrophoresis to display the resultant DNA fragments.

Table 3.4 shows the composition of the mastermix used in the PCR. To each reaction tube was added 0.5 µl DNA. DNA amplification was then attempted with the following thermal cycle: an initial step of 95° C for 5 minutes was followed by denaturation at 94° C for 20 seconds, annealing at 59° C for 25 seconds and extension at 72° C for 30 seconds to a total of 32 cycles. There was a final incubation step at 72° C for 5 minutes.

3.5.2 *Restriction enzyme digestion*

3.5.2.1 Materials

Bovine serum albumin (BSA) 20 mg/ml
10x buffer (Gibco BRL)
Bcl 1 restriction enzyme (BRL)
Rsa 1 restriction enzyme (BRL)
Water baths to provide water at 50° C and 37° C

3.5.2.2 Method

C282Y mutation

For each sample tested, the restriction digest mix consisted of:
BSA 0.5 µl
10x buffer 2 µl
Bcl 1 1.5 µl

3 µl of this mix was added to a PCR tube containing 8 µl PCR product (see section 3.5.1.2) and incubated at 50° C for 4 hours. The product was then subject to polyacrylamide gel electrophoresis (PAGE) (see section 3.7.1) to separate DNA fragments.

H63D mutation

For each sample tested, the restriction digest mix consisted of:
BSA 0.5 µl
10x buffer 2 µl

3 µl of this mix was added to a PCR tube containing 8 µl PCR product (section 3.5.2.1) and incubated at 37° C for 4 hours. DNA fragments were then separated using PAGE (section 3.7.1).

Table 3.4 Mastermix for PCR for restriction digest analysis

Component	Volume per sample (µl)
10x buffer	2.5
dNTP (2mM)	1.25
Magnesium chloride (25mM)	1.25
Primer 1	0.25
Primer 2	0.25
Distilled water	18.5
Taq polymerase	0.1

A separate mastermix was made for each mutation, containing primers HH1 and HH2 for the C282Y mutation and H631 and H632 for the H63D mutation.

3.6 HFE intron 4 polymorphism and inaccuracy in diagnosis

There has been a suggestion that the presence of a common single nucleotide polymorphism (5569G/A) could cause inaccuracies in the detection of homozygotes for C282Y (Jeffrey, 1999b). The polymorphism is located in the binding site of the antisense primer originally described by Feder *et al* (Feder, 1996) and concern was expressed that its presence could lead to the misdiagnosis of heterozygotes for C282Y as homozygotes (Jeffrey, 1999b) by preventing amplification of the wild type allele in heterozygous individuals. It has, however, been shown that under suitable PCR conditions, this does not occur (European Haemochromatosis Consortium, 1999), although the selection of other primers when possible may be ideal. The primers used in this study were those of Feder *et al*, but analysis of duplicate samples and comparison with results obtained by the methods described in this chapter revealed no evidence of over-diagnosis of C282Y homozygosity.

3.7 Polyacrylamide and agarose gel electrophoresis

3.7.1 *Polyacrylamide gel*

3.7.1.1 Materials

Power pack (Gibco BRL)
Vertical electrophoresis tank
16 x 18 cm glass plates
1.5 mm spacers
15 well combs
40% acrylamide solution (BDH, Poole, UK)
2% N,N' – methylene-bisacrylamide (BDH, Poole, UK)
Ammonium persulphate powder (BDH, Poole, UK) made up to10% solution (1g powder in 10 ml distilled water)
20x Tris-acetate-EDTA (TAE) buffer
N,N,N',N' – tetramethylethylenediamine (TEMED) (Sigma-Aldrich, Poole, UK)
Ethidium bromide (10 mg/ml) (Sigma-Aldrich, Poole, UK)
Distilled water
Loading buffer
Gel Doc 1000 ultra-violet light box with integral camera (Bio-Rad, Hemel Hempstead, UK)

3.7.1.2 Preparation of reagents

20x Tris-acetate-EDTA buffer
In each litre:
96.8 g Tris (hydroxymethyl) methylamine
22.8 ml glacial acetic acid
500 ml 0.5M disodium EDTA (pH 8)

Loading buffer
1.5 g Ficoll 400 dissolved in 10 ml distilled water. To this was added:
25 mg bromophenol blue
25 mg xylene cyanol

Used in dilution of 1 part buffer to 6 parts PCR product for electrophoresis.

3.7.1.3 *Methods*

In this project, the separation of DNA fragments produced by ARMS reactions relied heavily on the use of polyacrylamide gel electrophoresis (PAGE). 8% gels were used.

For the full-sized gels, 16 x 18 cm glass plates were separated by 1.5 mm-thick spacers. 10 ml 40% acrylamide was mixed with 10 ml 2% bisacrylamide and 2.5 ml 20x TAE. The volume was made up to 50 ml with distilled water. Immediately before pouring, 500 µl ammonium persulphate and 20 µl TEMED was added to initiate polymerisation. Gels were left to polymerise for approximately 60 minutes before use. Running buffer (1x TAE) was made by diluting 50 ml 20x TAE in 900 ml distilled water and 50 ml tap water. Approximately 2.5 litres of buffer was placed in the lower tank and 500 ml in the upper tank. The reaction products to be separated, mixed with loading buffer, were loaded into the wells in the gel and electrophoresis was performed at a constant voltage of 250 V for 90 minutes.

After they had been stained with ethidium bromide (0.5 mg/l) for 10 minutes, (no de-staining step was performed) the gels were viewed under UV light (see figure 3.5)

3.7.2 *Agarose gel*

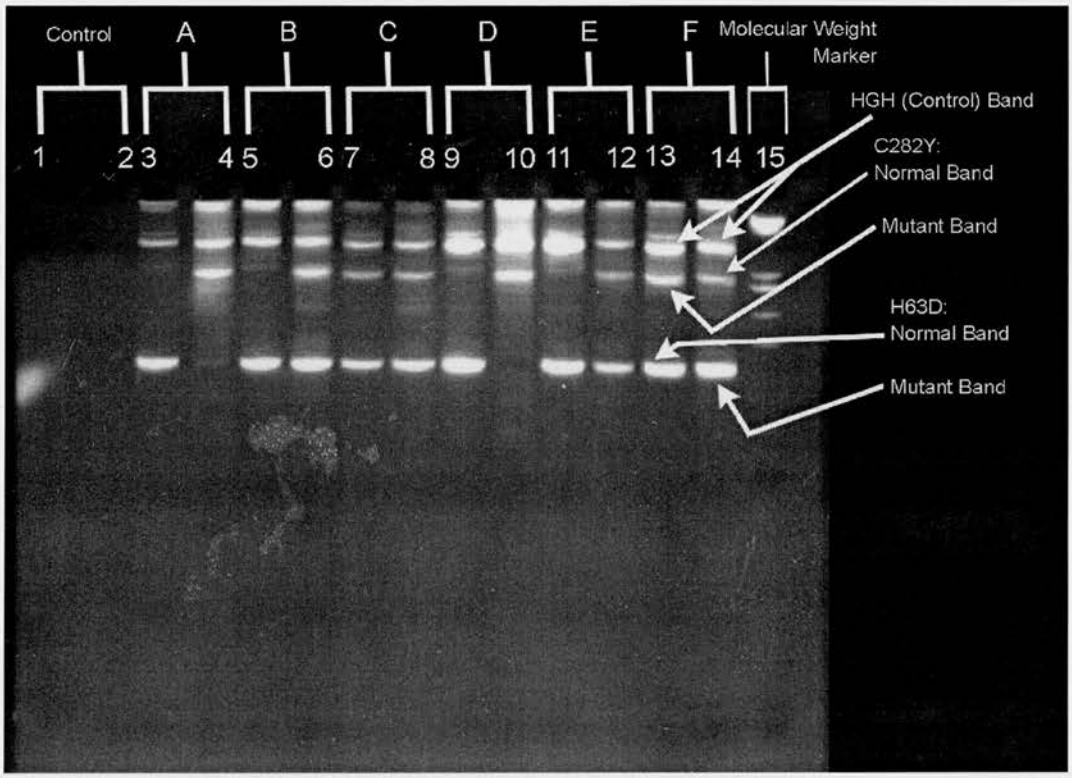
3.7.2.1 *Materials*

Power Pack (Gibco BRL)
Electrophoresis tank
35 well comb
MBG agarose powder (Technocomp Ltd, Hong Kong) or NuSieve 3:1 agarose gel powder (Flowgen, Lichfield, UK)
Distilled water
Horizontal electrophoresis tank (Bio-Rad, Hemel Hempstead, UK)
Ethidium bromide (10 mg/ml)
Gel Doc 1000 ultra-violet light box with digital camera

3.7.2.2 *Methods*

Agarose gels were used only in the initial stages of optimisation of the PCR. A 3% gel was made by dissolving 3 g agarose in 100 ml distilled water. Dissolution was aided by heating in a microwave oven. The gel was poured onto the platform of a horizontal

Figure 3.5 Photograph of standard polyacrylamide gel electrophoretic pattern after ARMS PCR for HFE gene (see text for details)



Key

Each pair of adjacent lanes shows the PCR products from a single sample (details in text)

Control:	no DNA
Sample A:	wild type for both C282Y and H63D
Sample B:	wild type for C282Y; heterozygous for H63D
Sample C:	heterozygous for both C282Y and H63D
Sample D:	wild type for C282Y andH68D
Sample E:	wild type for C282Y; heterozygous for H63D
Sample F:	heterozygous for both C282Y and H63D

electrophoresis tank to a thickness of about 0.5 cm. A 35 well comb was inserted and the gel left for 60 minutes to set. Samples were loaded and electrophoresis was performed for at 140 V for 90 minutes in 1x TAE buffer.

3.8 Microplate array diagonal gel elctrophoresis (MADGE)

3.8.1 *Materials*

FlowMADGE 96 well gel former (Flowgen, Lichfield, UK)
Glass plate to fit gel former
Power Pack
Electrophoresis tank
Gel Slick (AT Biochem)
Sticky silane
Ethidium bromide (10 mg/ml)

3.8.1.1 Preparation of reagents

Sticky silane
0.5 % v/v γ-methacryloxypropyltrimethoxysilane (silane) (Sigma-Aldrich, Poole, UK)
absolute alcohol
0.5% glacial acetic acid

5 µl silane was dissolved in 1 ml absolute alcohol. Immediately before use, 30 µl acetic acid was added.

3.8.1.2 Method

A glass plate of an appropriate size to fit neatly inside the rim of the MADGE gel former was siliconised on one side by coating it with Gel Slick using a tissue. After five minutes' drying, the coated surface was wiped with a damp paper towel and then dried with a dry paper towel. 1 ml of sticky silane was then poured onto the siliconised surface and spread out evenly using the edge of another glass plate. After three minutes, the excess liquid was wiped off with a dry paper towel. The surface was wiped with a damp tissue prior to use.

8% polyacrylamide gel was mixed using 2 ml 40% acrylamide, 2 ml 2% bisacrylamide, 0.5 ml 20x TAE. The total volume was made up to 10ml by the addition of distilled

water. The 96 well gel former was placed on a flat surface in preparation for pouring of the gel. Just before pouring, 100 µl 10% ammonium persulphate and 8 µl TEMED was added to initiate polymerisation. The gel mixture was then poured into the gel former and the prepared glass plate laid with the treated face downwards on top of the gel former so that the plate was in contact with the gel, ensuring that no air bubbles were trapped beneath it. An empty glass bottle was placed on top of the glass plate during the solidification of the gel to ensure good contact between the plate and the gel. The gel was allowed 30 minutes to set, after which the glass plate was prised away from the gel former.

The gel, with its array of 96 micro-wells, was now stuck on the glass plate and was placed in a water bath containing ethidium bromide (0.5 mg/l) on a horizontal rotatory mixer for 20 minutes for pre-staining. ARMS products mixed 6 parts to 1 with loading buffer were then loaded into the micro-wells. Thereafter, the gel, still adhering to the plate, was placed in an electrophoresis tank and subject to 150 V for 40 minutes. The gel was then visualised under UV light (see figure 3.6).

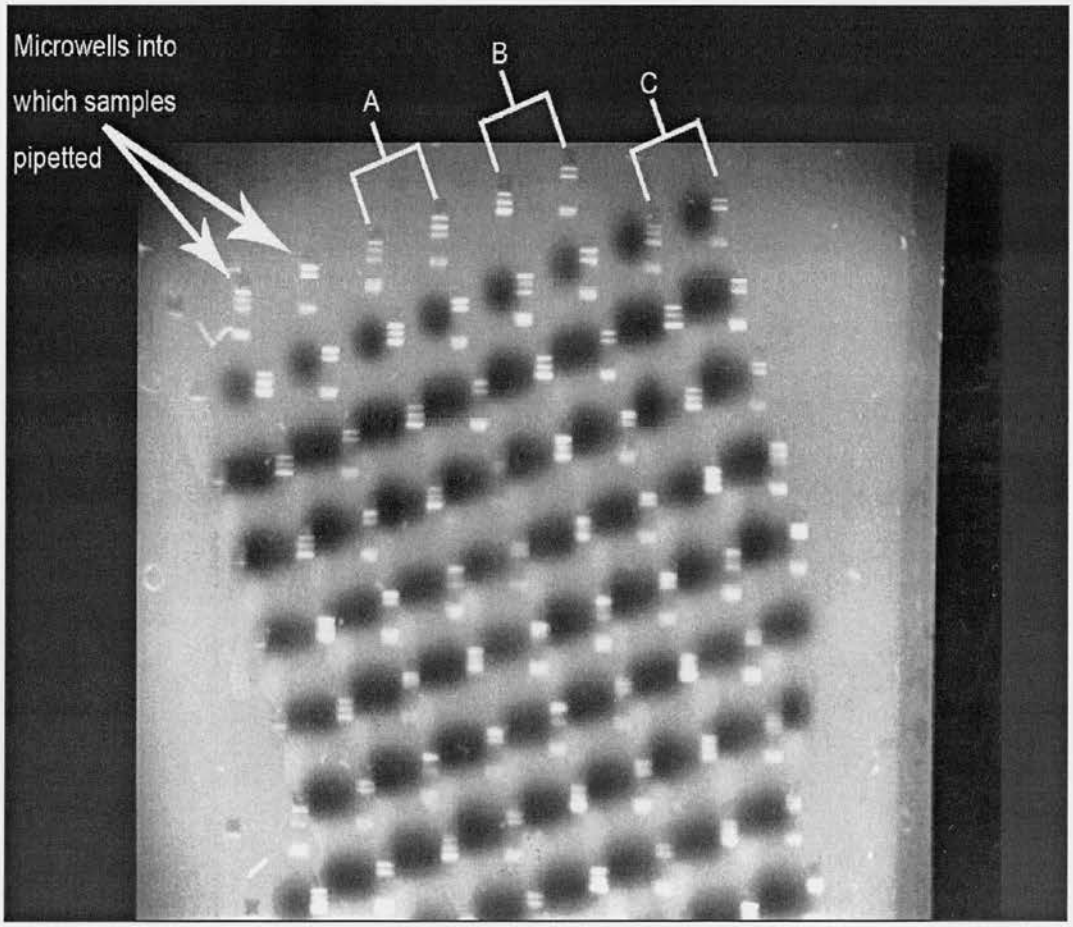
3.9 Optimisation of PCR and electrophoresis techniques

Although a published method for mutation detection was used (Baty, 1996), difficulty was experienced in attaining clear separation of DNA fragments of the desired sizes. Attempts were therefore made to optimise both the reaction conditions for the PCR and also the electrophoresis technique.

Initial attempts using published PCR conditions, and electrophoresis on 0.8% agarose gel resulted in severe smearing of bands after staining. The following factors were investigated in an attempt to achieve clearer separation of bands:

1. Magnesium concentration ([Mg]) in the PCR mix
2. Number of PCR cycles
3. Primer concentrations
4. Presence or absence of Q solution in PCR mix
5. DNA concentration
6. Gel composition
7. Destaining of gel

Figure 3.6 Photograph of MADGE gel showing PCR products after ARMS PCR for HFE gene mutations (see text for details)



Key

Paired samples were pipetted into adjacent wells

Sample A: heterozygous for H63D, heterozygous for C282Y

Sample B: wild type for C282Y and H63D

Sample C: heterozygous for H63D, wild type for C282Y

Note: the orientation of each pair of samples is opposite to that in figure 3.5.

3.9.1 *Magnesium concentration*

Mg^{2+} ions affect the sensitivity and specificity of PCR reactions. They are essential for incorporation of dNTPs and increase the melting temperature of the double-stranded DNA and the primer/template interaction (Newton, 1997). In an attempt to improve the yield of the reaction, [Mg] was increased in 0.5 mM steps between 1.5 and 5 mM. As expected, at the higher [Mg] (>4 mM) there was a suggestion of loss of specificity but overall, variation of [Mg] did not result in increased band clarity.

3.9.2 *Number of PCR cycles*

Reducing the number of thermal cycles had the effect of reducing the PCR yield significantly when the cycle number was reduced from 30 to 24. There was no improvement in the sharpness of the bands.

3.9.3 *Primer concentration*

The method described by Baty and colleagues (Baty, 1996) uses H63D primers in the PCR mastermix at twice the concentration of the C282Y and HGH primers. For reasons of economy, the present study began with lower concentrations of all the primers (H63D, 0.0125 μM, C282Y and HGH, 0.003125 μM). On agarose gel, the problems with streaking, outlined above, were encountered. On polyacrylamide gel (see below), adequate amplification and sharp bands were achieved with these lower concentrations. However, band intensity was consistently lower for H63D primer products and higher for products of the HGH primers than for the C282Y primers. This was dealt with by further alteration in the primer concentrations, such that the final concentrations in the mastermix were:

HGH primers: 0.1 μM

C282Y primers: 0.00625 μM

HGH primers: 0.00125 μM

3.9.4 *Q solution*

Q solution (Qiagen GmbH, Germany) modifies the melting behaviour of DNA (Qiagen, 1999) and can aid amplification. The presence or absence of Q in the PCRs in the present study had no significant effect on the yield of the reaction and it was not used.

3.9.5 *DNA concentration*

During the optimisation process, standardisation of the DNA concentration (see section 3.2) of a number of test samples was performed. The specificity and yield of the PCR was not significantly better in the samples with standardised DNA concentrations than in those in which this procedure had not been performed. It was, therefore, not carried out routinely in this project.

3.9.6 *Gel composition*

A range of agarose gel concentrations (0.8%, 1.5%, 3% and 4%) was tried. With each, the definition of the DNA fragment bands was equally poor, regardless of which of the manoeuvres described above was tried in addition. The use of polyacrylamide gel instead of agarose was the most effective factor in the eventual attainment of a sharp and easily interpretable band pattern.

3.9.7 *Gel destaining*

Destaining the gel in distilled water after the initial ethidium bromide application was not effective in promoting band clarity on agarose or polyacrylamide and was, therefore, not performed routinely.

3.10 *Quality assurance*

During optimisation of the ARMS as above, samples of known HFE genotype were used. These had already been processed within our department, using PCR followed by restriction enzyme digestion (section 3.5). Comparison of the results achieved with ARMS with those obtained with the established technique provided assurance of reliability. 100% concordance was achieved before analysis of unknown samples was begun.

A blank (i.e. PCR reaction without DNA) and a wild-type and double heterozygote control were included in each batch of PCR reactions performed during the study. If the blank showed any evidence of DNA contamination, the batch was repeated after replacement of all reagents. If either control failed to give the expected results, the batch concerned was repeated. Any sample that yielded dubious results was repeated until unequivocal results were obtained on two occasions. In addition, throughout the project a number of samples were sent for repeat testing by the Scottish Molecular Genetics Consortium, Ninewells Hospital, Dundee. There was full agreement between results obtained by the Consortium and the results for the present study.

A molecular weight (MW) marker (ϕX-174) was run routinely with the test samples when vertical PAGE was used to confirm that bands of appropriate molecular weight were being visualised. In fact, the band pattern obtained in the ARMS PCRs, confirmed in each batch by the controls, was sufficiently characteristic to render this unnecessary. It was technically difficult to include a MW marker in the MADGE system, and for these reasons, the MW marker was omitted when MADGE was used (see below).

3.11 *Use of the MADGE system*

The volume of samples to be tested rendered the routine use of vertical PAGE impractical. The possibility of using the MADGE system to visualise the ARMS products was therefore investigated. It proved possible to achieve adequate band separation for reliable interpretation of results, and this method was used routinely during the project for the visualisation of ARMS products. On the whole, clearer bands were obtained by pre-staining the gel before the samples were loaded, than by staining after electrophoresis, and pre-staining was performed for most of the gels.

3.12 *Isolation of DNA from paraffin sections*

The initial intention was to genotype DNA isolated from paraffin blocks obtained from biopsies of the recipient's explanted liver in patients undergoing orthotopic liver transplantation. It proved impossible, despite the use of various different DNA extraction and amplification techniques (see above) to obtain amplification of DNA of sufficient quality for genotyping.

3.13 *Laboratory Measurement of Iron Stores*

All measurements were performed according to the standard operating procedures of the clinical chemistry laboratory of the Royal Infirmary of Edinburgh.

3.13.1 *Serum ferritin*

A heterogeneous sandwich magnetic separation assay was performed on the Technicon Immuno 1 system (Bayer Diagnostics, Newbury, UK).

3.13.2 *Serum Iron*

Assayed using Vitros Fe slides (Johnson & Johnson Clinical Diagnostics, Inc, Rochester, USA) and a Vitros 250 analyser (Ortho-Clinical Diagnostics, Amersham, UK)

3.13.3 *Total iron binding capacity*

Assayed using the alumina column method of Starr (Starr, 1980) with reagents supplied from Vitros (Johnson & Johnson Clinical Diagnostics, Amersham, UK) and a Vitros 250 analyser (Ortho-Clinical Diagnostics, Amersham, UK).

3.13.4 *Transferrin saturation*

This was calculated as: (serum iron ÷ TIBC) x 100%.

3.13.5 *Non-transferrin bound iron*

This was assayed in the laboratory of Professor J B Porter at King's College, London (see also section 7.3.6).

3.14 Statistical Analysis

Statistical analysis was performed with the aid of the Statistics Package for the Social Sciences (SPSS), Chicago, Illinois. Relevant details are provided in each section of the text.

4. Investigation of the effect of HFE genotype on blood donors who make frequent red cell donations

4.1 Introduction

The donation of one unit of red cells results in the loss of 236 mg iron from male donors (Garry, 1995). Regular blood donation, therefore, causes iron depletion in a significant proportion of donors (Finch, 1977; Garry, 1995; Pederson, 1978). Alvarez-Ossario and colleagues found an inverse relationship between donors' serum ferritin concentration and the number of units of blood donated per year (Alvarez-Ossario, 2000). In a study of otherwise healthy elderly blood donors, 10% of males and 15% of females became iron deficient within one year of starting to donate blood and 7% of the females developed iron deficiency anaemia (Garry, 1991). It is not clear whether sub-clinical iron deficiency not associated with anaemia is harmful in adults. Nonetheless, blood donors in the UK are currently permitted to donate red cells with a maximum frequency of one unit every 12 weeks in an attempt to protect them from the development of iron deficiency. Early in the development of iron deficiency, laboratory values may all be normal. Scoog (1993) describes 3 phases: latent iron deficiency in which iron stores, although depleted, are adequate for haemoglobin production; early iron deficiency associated with a low normal to reduced mean cell volume (MCV) and haemoglobin concentration; and established deficiency, when both MCV and haemoglobin concentration are below normal. It is clear that early iron deficiency in blood donors is often not accompanied by a fall in haemoglobin concentration (Finch, 1977). A recent very large study showed that 0.9% of male blood donors and 8% of female donors had a serum ferritin of less than 15 μg/L (Jackson, 2001). As the copper sulphate pre-donation screening test used in the UK (Phillips, 1977) is dependent on haemoglobin concentration rather than any direct measure of iron status, it is likely that many iron deficient donors are able to continue donating (Jaime, 1988).

In Scotland, only five percent of the eligible population donates blood, so there is a constant difficulty in maintaining supplies in the face of rising demand. It has been suggested that using blood drawn from haemochromatosis patients could usefully augment the blood supply (Worwood, 1991).

However, the extent to which this would be so in practice has been fiercely debated. Attempts to estimate the impact of permitting the use of the blood drawn from haemochromatosis on the blood supply in the United States have produced widely differing results (Jeffrey, 1999a; Conry-Cantilena, 2000). A study of Canadian patients with haemochromatosis (Levstik, 1998) estimated that 67% of the units withdrawn during iron-depletion therapy and 88% of units withdrawn during maintenance treatment were potentially available for allogeneic transfusion. In practice, however, only a very small proportion of those eligible to donate were able to do so. In the majority of cases, this was found to be due to the diagnosis of haemochomatosis itself, probably because of a lack of awareness at blood donor centres of the elibibility of haemochomatosis sufferers to donate blood.

The main impediment to the use of blood from haemochromatosis patients for allogeneic transfusion is the concern that this blood might not be safe as blood derived from other donors. In particular, maintenance of the principle of altruism has been considered of vital importance in ensuring the virological safety of the blood supply (Tan, 1999). The American Medical Association argued strongly (Tan, 1999) that the acceptance of patients with haemochromatosis as donors would compromise this principle. Haemochromatosis patients attending for phlebotomy may be motivated by a concern for their own health or a wish to avoid the cost of phlebotomy (where relevant) as well as by altruism (Sanchez, 2001). It has been shown that even non-financial incentives to donate are accompanied by an increase in the rate of positive virological serology and medical deferral (Read, 1993).

Other important issues are the possibility of an increased risk of bacterial contamination, particularly with organisms such as Yersinia enterocolitica, which depend on a ready availability of iron in their micro-environment. Additionally, the greater load of free iron in blood derived from haemochromatosis patients could theoretically be harmful (Conry-Cantilena, 2000). On the other hand, blood drawn during the iron depletion phase of treatment should in theory contain an increased percentage of young red blood cells compared with blood derived from patients not undergoing phlebotomy, which may prolong the inter-transfusion interval in certain recipient groups (Anonymous, 1979).

These merits and demerits are all hypothetical and impossible to prove without a clinical trial, which is highly unlikely ever to be possible to perform. Furthermore, there are almost certainly many patients with undiagnosed haemochromatosis already contributing to the blood supply (Anía, 1999). McDonnell reported that 37% of a sample of more than 2000 haemochromatosis patients had been voluntary blood donors before diagnosis (McDonnell, 1999b). In addition, blood from haemochromatosis patients has been used for transfusion for a number of years in Australia, Norway, Sweden, South Africa and Canada without any reports of adverse effects (Jeffrey, 1999a).

The UK transfusion services have recently changed their guidelines to allow the use of blood from patients with haemochromatosis once they have been depleted of iron (Guidelines for the Blood Transfusion Services in the UK, 2002).

Heterozygosity for haemochromatosis gene mutations may afford some protection against iron deficiency, as discussed in section 2.3.4.1, above. This led to the hypothesis that male heterozygotes might constitute a population of blood donors who are able to donate blood with an increased frequency without becoming iron deficient and so augment the blood supply, while avoiding the controversies surrounding the use of blood from patients with clinical haemochromatosis. This study was designed to test this hypothesis.

4.2 Methods

4.2.1 *Subject recruitment*

366 male blood donors were recruited during their attendance to give blood at the Scottish National Blood Transfusion Service's (SNBTS) donor centre in central Edinburgh. Recruitment took place between July and October 1999. Sequential donors who had given at least one unit of blood in the Edinburgh Blood Donor Centre in the previous 12 months were approached personally by the researcher or one of the Donor Centre nursing or medical staff, as they waited to donate. First time donors were not included because of ethical concerns about their ability to consent to participate in a study involving repeated blood donation before they had fully experienced this procedure for the first time. Donors who had not given within the previous year were excluded in an attempt to increase compliance with a study in which participants were asked to return to donate every 12 weeks (the minimum interval presently allowed by SNBTS regulations). Donors were excluded also if donor centre records revealed deferral of donation within the previous two years owing to failure of the copper sulphate gravimetric assessment of haemoglobin concentration, inability to complete a previous donation because of difficulties with venous access, or if they were unable to undertake to donate blood every 12 weeks for the next year.

A verbal description of the study was given, supplemented by a written information sheet (Appendix 1). Once the donation was completed, the donors were approached again and, if they were willing to participate, written consent was obtained. They were included in the study only if they returned for their next donation, as requested, within approximately 14 weeks (98 days) of giving consent. In order to facilitate

attendance at appropriate intervals, donors participating in the study were offered a specific appointment (date and time) for their subsequent visit, although there was no compulsion to accept this offer.

Donor centre medical and nursing staff and participating donors were blinded to the results of iron studies and HFE gene analysis until the end of the study.

Donors' General Practitioners were informed of their participation in the study.

The study was approved by the Lothian Research Ethics Committee.

4.2.2 *Sample collection*

Study samples were taken during participants' first and subsequent visits for donation after they had given consent. The initial intention was to exclude donors whose inter-donation interval exceeded 98 days during the study period. Practical difficulties in determining this for Donor Centre clerical staff led to some subjects' continuing in the study despite a longer interval between donations. At each visit during the study, participants were subject to the normal pre-donation screening procedure, and all the standard criteria laid down by the UK blood transfusion services for deferral of donation applied, including the requirement for a pre-donation haemoglobin concentration in excess of 125 g/L laid down by the Scottish National Blood Transfusion Service during the study period. Any donor whose donation was deferred for any reason (including low haemoglobin concentraton) was excluded from further participation in the study. After completion of the pre-donation questionnaire, the capillary haemoglobin concentration was determined, and a sample of venous blood was taken from each donor. On the first study visit, serum iron, total iron binding capacity (TIBC) and serum ferritin were determined from the venous blood sample and 3.5 ml blood was taken into ethylene-diamine-tetra-acetic acid (EDTA) for later extraction of DNA and HFE gene mutation analysis. On the second and subsequent visits, only haemoglobin concentration and serum ferritin were measured. Samples for determination of ferritin, iron and TIBC were dealt with under standard laboratory conditions in the clinical chemistry laboratory of the Royal Infirmary of Edinburgh. Samples for DNA extraction were stored at 4°C until DNA extraction could be performed (within fourteen days).

In June 2000, a postal questionnaire was sent to all donors remaining in the study, requesting information about participants' weight, red meat consumption and the use of iron supplements. This is reproduced as Appendix 2.

4.2.3 *Sample analysis*

Ferritin, iron and TIBC were assayed as described in sections 3.13.1 – 3.13.3. DNA extraction and HFE gene analysis were performed as set out in Chapter 2.

Capillary haemoglobin concentration was measured using a HemoCue machine (HemoCue, Sheffield, UK).

4.2.4 *Data set*

After consent was given, a record was made of each participant's date of birth. Information was also collected on the number of red cell donations made by the donor in the previous two years. However, this information was not used in the analysis because it proved impossible to be certain which previous donations had been of whole blood and which of non-red cell components such as platelets or plasma. No other demographic data were collected until the questionnaire referred to above was administered.

4.2.5 *Follow-up of participants*

Study participants who were found to be homozygous for the C282Y mutation of the HFE gene were contacted individually and offered an appointment with either the researcher or a consultant in transfusion medicine. When appropriate, further investigation was planned and arrangements for family screening made.

Participants who were heterozygous for both the C282Y and H63D mutations were offered individual appointments for counselling and, if appropriate, further investigation.

Those with a significantly raised (>60%) non-fasting transferrin saturation were offered an appointment for further investigation.

Heterozygotes for C282Y were sent written information (Appendix 3) about the mutation and its consequences. They were also sent information to pass on to their General Practitioner, if they wished to do so (Appendix 4).

Other participants were informed by letter that no significant abnormality was discovered (Appendix 5).

4.3 Statistical Analysis

The distribution of initial haemoglobin concentration, transferrin saturation and ferritin among the genotypes was examined by one-way analysis of variance, for the three commonest genotypes only (because the numbers of donors with the three least common genotypes were considered too small for meaningful analysis). In addition, non-parametric analysis was performed using the Kruskal Wallis test. The correlation of change in haemoglobin concentration or ferritin during the study with donors' weight or donors' red meat consumption was examined by both parametric (Pearson's coefficient of correlation, r) and non-parametric (Spearman's rank correlation coefficient, ρ) methods. It was felt that normal distribution of the variables concerned could not be assumed, and if significance determination by the two methods differed, the result using non-parametric tests was accepted.

4.4 Results

366 donors gave their consent to participate in the study. Of these, 297 (81%) were eligible to begin blood donation within the study. The mean age of these donors was 41.6 years (range 18-63). Eligibility was determined by the return of the donor within a pre-specified time (approximately 98 days) to give his first on-study donation. No data were collected from the 69 donors who did not attend for their first study donation and these individuals were not included in the subsequent analysis. 238 (65%) donors attended for the second donation, 200 (55%) for the third and 159 (43%) for the fourth and final study donation.

No attempt was made to contact donors who did not attend to donate on schedule. Data on the reasons for dropping out are, therefore, incomplete. Insofar as they were ascertainable, the reasons for failure to complete the four study donations are given in table 4.1. Among the 37 donors deferred for medical reasons during the study period were eight whose deferral resulted from a low pre-donation haemoglobin concentration. Of these all had a serum ferritin less than 11 µg/L at the start of the study. Seven had a normal HFE genotype. The remaining subject was a carrier of H63D. See table 4.2 for more details.

Table 4.1 Reasons for failure to complete four study blood donations

Reason	Number (%)* of subjects
Failure to attend within 98 days from previous donation	162 (44.3)
Medical deferral of donation**	37 (10.1)
Incomplete donation obtained	1 (0.3)
Inability to obtain venous blood samples	5 (1.4)
Started taking iron during the study	2 (0.5)
Uncertain	10 (2.7)
Total	217 (59.3)

*percentage of the total number (n=366) of donors initially enrolled.

**In addition to general medical reasons, includes travel to malarial region (3 donors); recent tattoo (1 donor); syncopal episode after donation (1 donor); low Hb (8 donors) – see text for details.

Table 4.2 Characteristics of donors excluded because haemoglobin concentration <125g/L

Donor	Age (y)	Haemoglobin concentration (g/L) at each indicated study visit				Ferritin (µg/L) at each indicated study visit				TS (%)	HFE
		1	2	3	4	1	2	3	4		
1	48	135	132	134	124	11	11	13	8	31	HC/HC
2	28	124	exc	exc	exc	6	exc	exc	exc	10	HC/HC
3	48	130	127	116	exc	5	<5	<5	exc	11	HC/DC
4	44	125	125	126	116	11	7	9	6	25	HC/HC
5	42	129	128	126	119	7	6	<5	5	11	HC/HC
6	36	135	137	132	123	9	9	7	7	12	HC/HC
7	51	135	129	118	exc	10	8	7	exc	12	HC/HC
8	44	n.a.	150	118	exc	10	8	8	exc	24	HC/HC

Legend: HFE = HFE genoptype
TS = transferrin saturation
exc = donor excluded because of previous low haemoglobin concentration

Full laboratory data were obtained from133 of 159 subjects who completed four donations in the study. The reasons for incomplete data collection were: failure to record HemoCue result (22 occasions); missing ferritin result (eight occasions); missing transferrin saturation (five occasions); failure to extract DNA from blood for HFE gene analysis (five occasions) (more than one item was incomplete for some individuals).

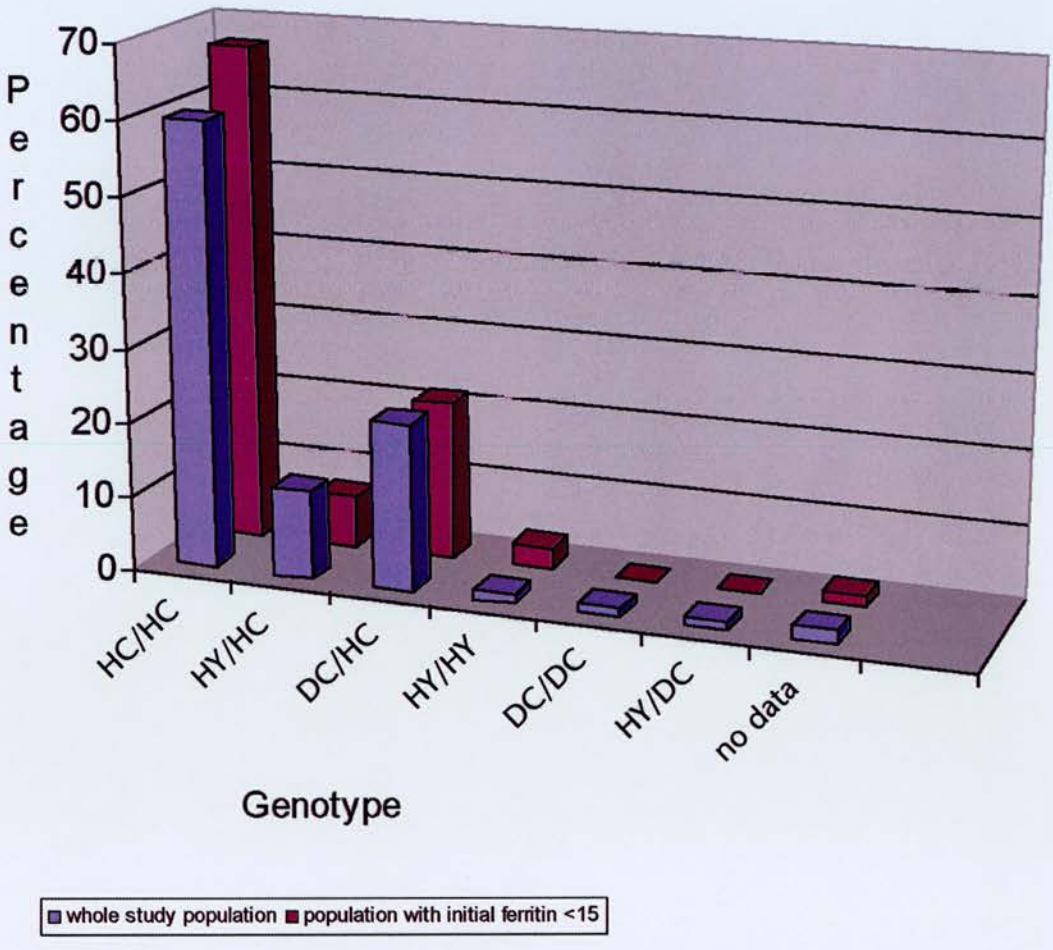
4.4.1 *HFE genotypes*

The distribution of HFE genotypes in the study population is illustrated in figure 4.3 and table 4.4. The allele frequencies were 8.9% (95% confidence interval 7.1-10.6) for C282Y and 14.8% (10.7-18.8) for H63D. A total of 70 donors (23.6% of the study population) had a ferritin of less than 15 µg/L at their first study visit. The genotype of one of these is not known. Of the remaining 69, 47 (68.1%) were of wild type genotype, 15 (21.7%) were H63D carriers, 5 (7.2%) were C282Y carriers and 2 (2.9%) were C282Y homozygotes.

A total of seven donors had the HFE genotypes most commonly associated with the development of significant iron overload: four were homozygous for the C282Y mutation, and three were compound heterozygous for C282Y and H63D. The characteristics of these individuals are summarised in tables 4.5 and 4.6.

Tables 4.7, 4.9 and 4.11 and the accompanying scatter plots (figures 4.8, 4.10 and 4.12) give a breakdown of mean ferritin, mean haemoglobin concentration at first visit and mean transferrin saturation by genotype. Because of the small numbers concerned, meaningful statistical analysis was not possible for genotypes HY/HY, DC/DC or HY/DC. For the remaining three genotypes, there was no significant difference in initial ferritin or haemoglobin concentration, but initial transferrin saturation was significantly greater in genotype HY/HC than in HC/HC or DC/HC.

Figure 4.3 Distribution of HFE genotypes



Legend: HC/HC = wildtype
HY/HC = C282Y carrier
DC/HC = H63D carrier
HY/HY = C282Y homozygote
DC/DC = H63D homozygote
HY/DC = compound heterozygote

Table 4.4 Distribution of HFE genotypes in the study population

Genotype	Number (%* [95% confidence intervals]) of donors (total population)	Number (%* [95% CI]) of donors (initial ferritin <15)
Wild type (HC/HC)	178 (60.8 [55.2-66.3])	47 (68.1 [55.8-78.8])
C282Y carrier (HY/HC)	36 (12.3 [8.5-16.0])	5 (7.2 [2.4-16.1])
H63D carrier (DC/HC)	67 (22.9 [18.1-27.7])	15 (21.7 [12.7-33.3])
C282Y homozygote (HY/HY)	4 (1.4 [0.4-3.5])	2 (2.9 [0.3-10.1])
H63D homozygote (DC/DC)	5 (1.7 [0.6-3.9])	0
Compound heterozygote (HY/DC)	3 (1.0 [0.2-3.0])	0
No data	5	1

*percentages of population for which there is data (n=293)

Table 4.5 Characteristics of subjects homozygous for C282Y

Donor	Age (years)	TS* (%)	Initial haemoglobin concentration (g/L)	Initial ferritin (µg/L)
1	59	22	155	12
2	34	24	131	11
3	32	84	147	73
4	49	71	145	326

*TS = transferrin saturation

Table 4.6 Characteristics of compound heterozygote subjects

Donor	Age (years)	TS* (%)	Initial haemoglobin concentration (g/L)	Initial ferritin (µg/L)
1	32	51	142	55
2	21	50	140	50
3	28	40	139	40

* TS = transferrin saturation

Table 4.7 Breakdown of initial haemoglobin concentration (Hb1) by genotype

Genotype	Mean Hb1 (g/L)	N	Standard deviation
HC/HC (1)	145.68	174	8.89
HY/HC (2)	147.50	34	6.57
DC/HC (3)	146.33	67	9.52
HY/HY (4)	144.50	4	9.98
DC/DC (5)	148.25	4	8.66
HY/DC (6)	142.33	3	2.52

For the 3 genotypes with the most data, there was no significant difference in Hb1 ($p = 0.5$, analysis of variance)

Figure 4.8 Scatter plot representation of initial haemoglobin concentration values (HB1 expressed in g/L) by genotype.
The key to the genotype numbers can be found in table 4.7 (above)

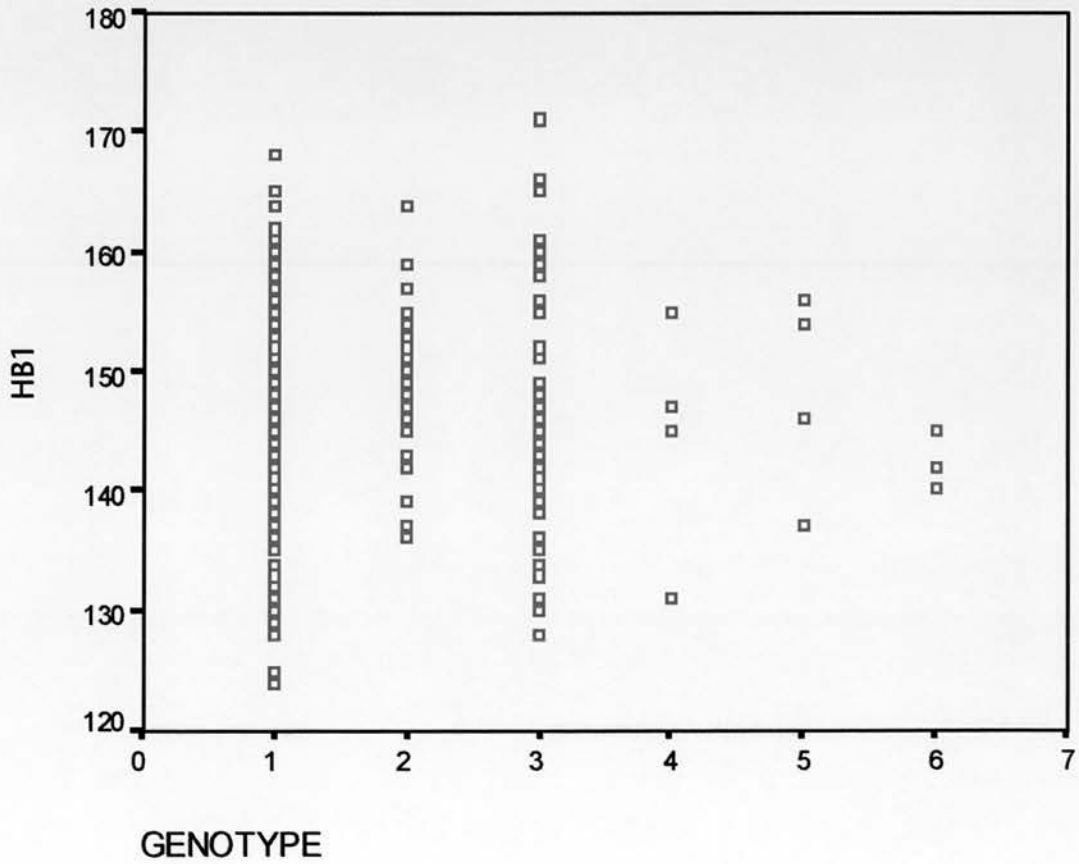


Table 4.9 Breakdown of initial ferritin (ferr) by genotype

Genotype	mean ferr1 (µg/L)	N	Standard deviation
HC/HC (1)	29.32	178	24.29
HY/HC (2)	30.33	36	15.78
DC/HC (3)	29.64	67	20.15
HY/HY (4)	115.25	4	154.44
DC/DC (5)	40.25	4	16.07
HY/DC (6)	56.33	3	7.09

For the 3 genotypes with the most data, there was no significant difference in ferr1 ($p = 0.97$, analysis of variance).

Figure 4.10 Scatter plot representation of initial ferritin values (Ferr1 expressed in µ/L) by genotype.
The key to the genotype numbers can be found in table 4.9 (above)

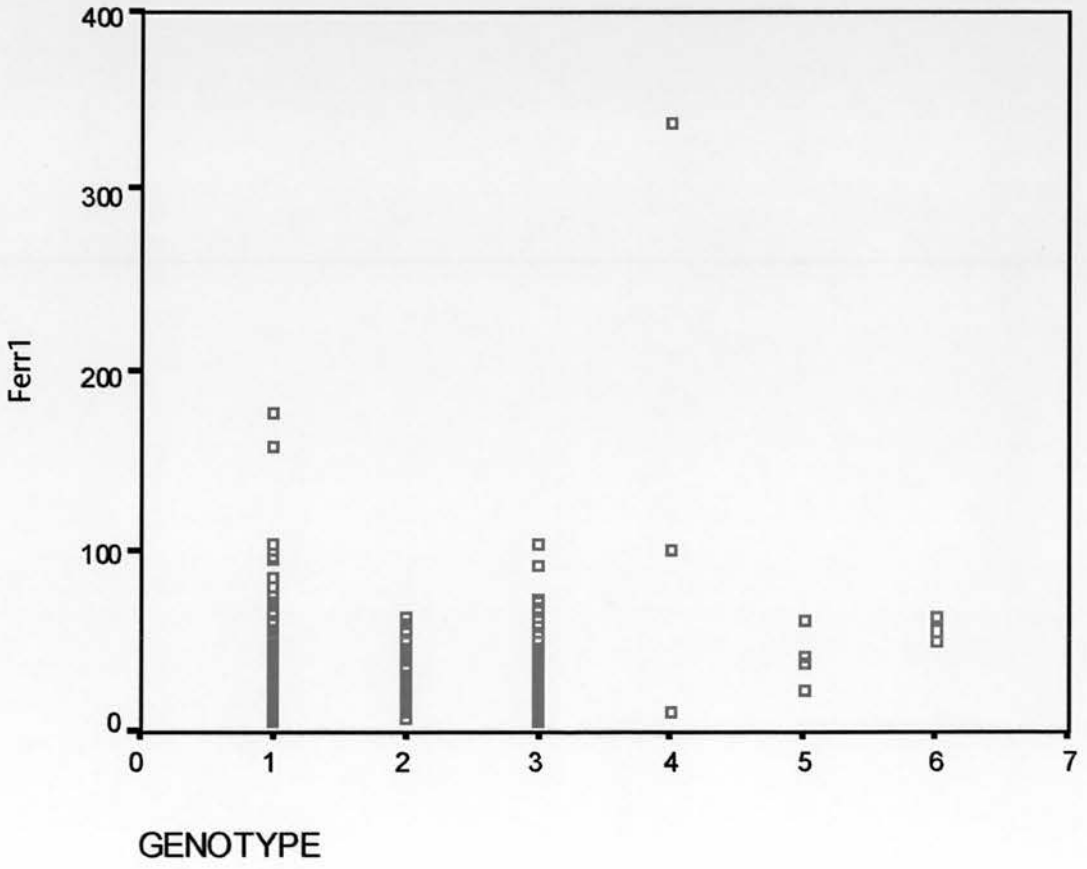
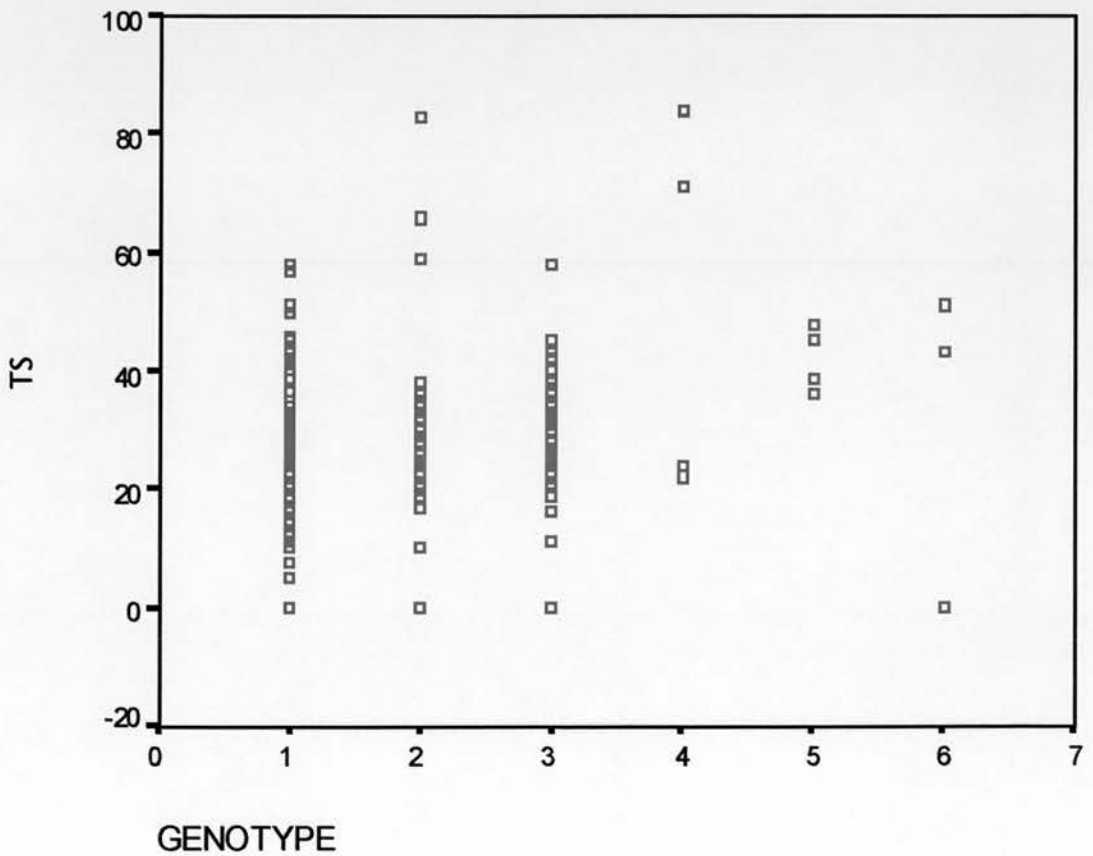


Table 4.11 Breakdown of initial transferrin saturation (ferr) by genotype

Genotype	Mean TS (%)	N	Standard deviation
HC/HC (1)	26.23	178	9.91
HY/HC (2)	30.44	36	14.64
DC/HC (3)	28.99	67	9.35
HY/HY (4)	50.25	4	31.92
DC/DC (5)	42.00	4	5.48
HY/DC (6)	31.33	3	27.43

For the 3 genotypes with the most data, TS was significantly greater in genotype HY/HC than in the other 2 genotypes (HC/HC and DC/HC) ($p < 0.05$, analysis of variance).

Figure 4.12 Scatter plot representation of initial transferrin saturation values (TS expressed as percentage) by genotype.
The key to the genotype numbers can be found in table 4.11 (above)



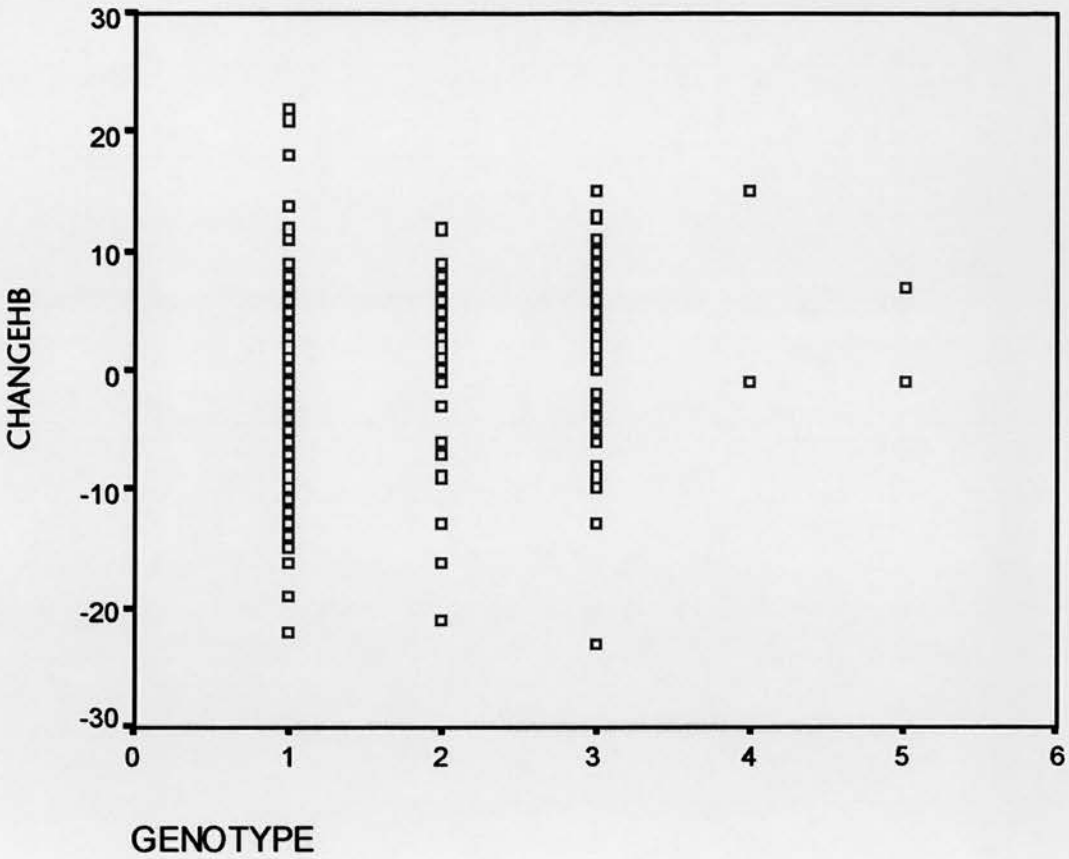
For those donors who completed all four planned study donations, the presence of any correlation between the change in haemoglobin concentration (ΔHb) or ferritin (Δferr) between the first and final study visit and genotype, donor weight or red meat consumption was sought. The results are presented in table 4.13 and graphically in figures 4.14- 4.18. (There was no similar analysis for transferrin saturation because this was measured only at visit 1). There was no correlation between ΔHb or Δferr and genotype. When ΔHb or Δferr were examined with respect to donor weight or donor weekly red meat consumption, there was a negative correlation between red meat consumption and Δferr ($r = -0.212$, $p<0.05$), but not between Δferr and donor weight ($r = 0.041$) or between either variable and ΔHb ($r = 0.114$ for weight; $r = -0.19$ for red meat consumption). Only the scatter plot for ΔHb and donor weight is reproduced (as figure 4.18).

Table 4.13 Change in ferritin and haemoglobin between first and last study visits for individuals of different genotypes

Genotype	Change in haemoglobin concentration		Change in Ferritin	
	Number	Mean (S.D.)	Number	Mean (S.D.)
HC/HC (1)	85	-1.74 (9.17)	89	-7.70 (10.10)
HY/HC (2)	23	-1.00 (8.14)	23	-9.43 (9.83)
DC/HC (3)	34	0.41 (8.12)	34	-6.53 (8.20)
HY/HY (4)	2	7.00 (11.31)	2	-26.00 (38.18)
DC/DC (5)	2	3.00 (5.66)	2	-17.00 (16.97)

Full data for either ferritin or haemoglobin concentration were not available for any of the individuals with genotype HY/DC (genotype 6).

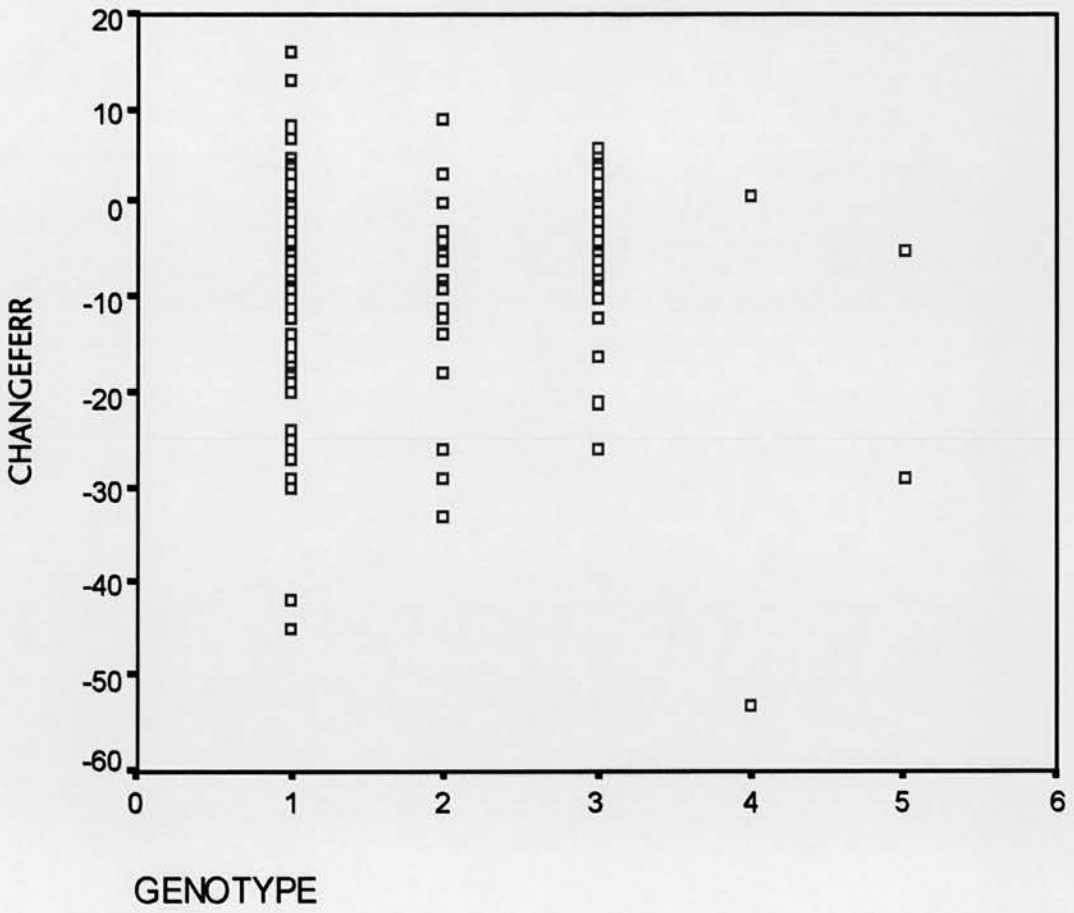
Figure 4.14 Change in haemoglobin concentration between first and last study visit (CHANGEHB expressed in g/L) by genotype



Key to genotypes:

1. HC/HC
2. HY/HC
3. DC/HC
4. HY/HY
5. DC/DC
6. HY/DC

Figure 4.15 Change in ferritin between first and last study visit (CHANGEFERR expressed in μg/L) by genotype



Key to genotypes:

1. HC/HC
2. HY/HC
3. DC/HC
4. HY/HY
5. HY/DC
6. DC/DC

Figure 4.16 Change in ferritin (CHANGEFERR expressed in μg/L) between first and last study visit by donor weight

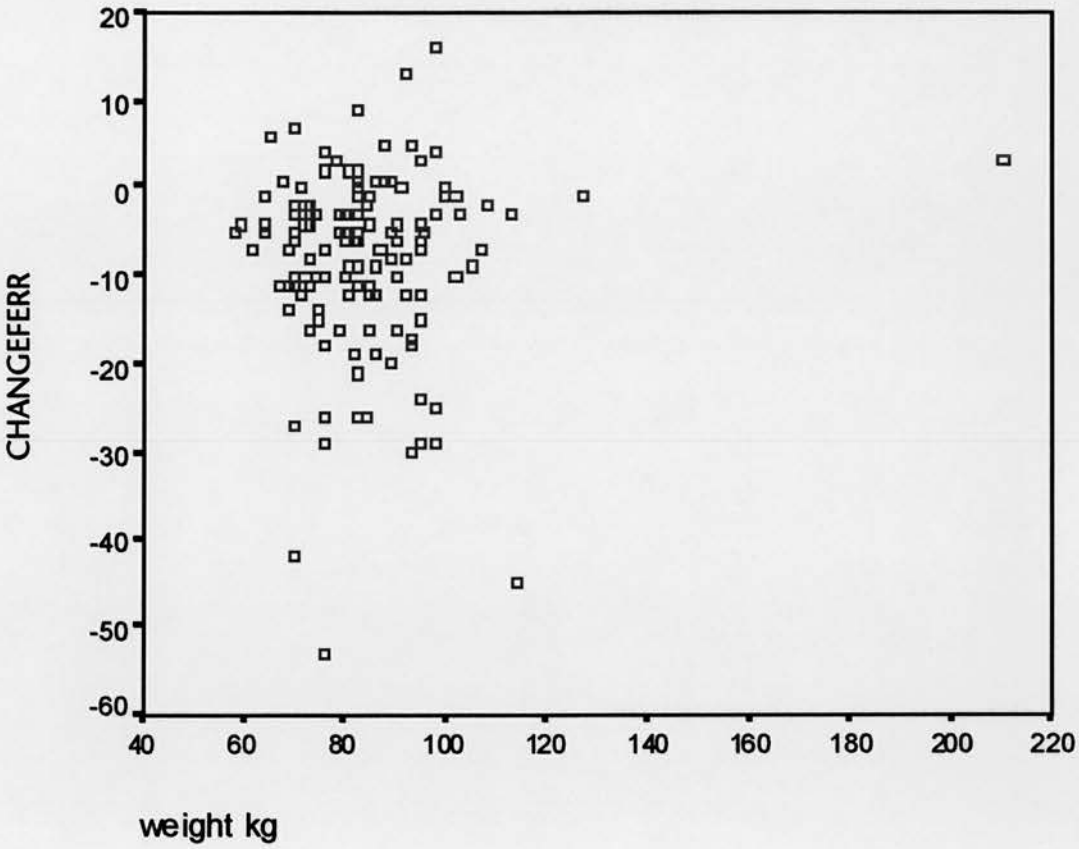


Figure 4.17 Change in ferritin between first and last study visit (CHANGEFERR expressed in μg/L) by number of occasions red meat eaten per week

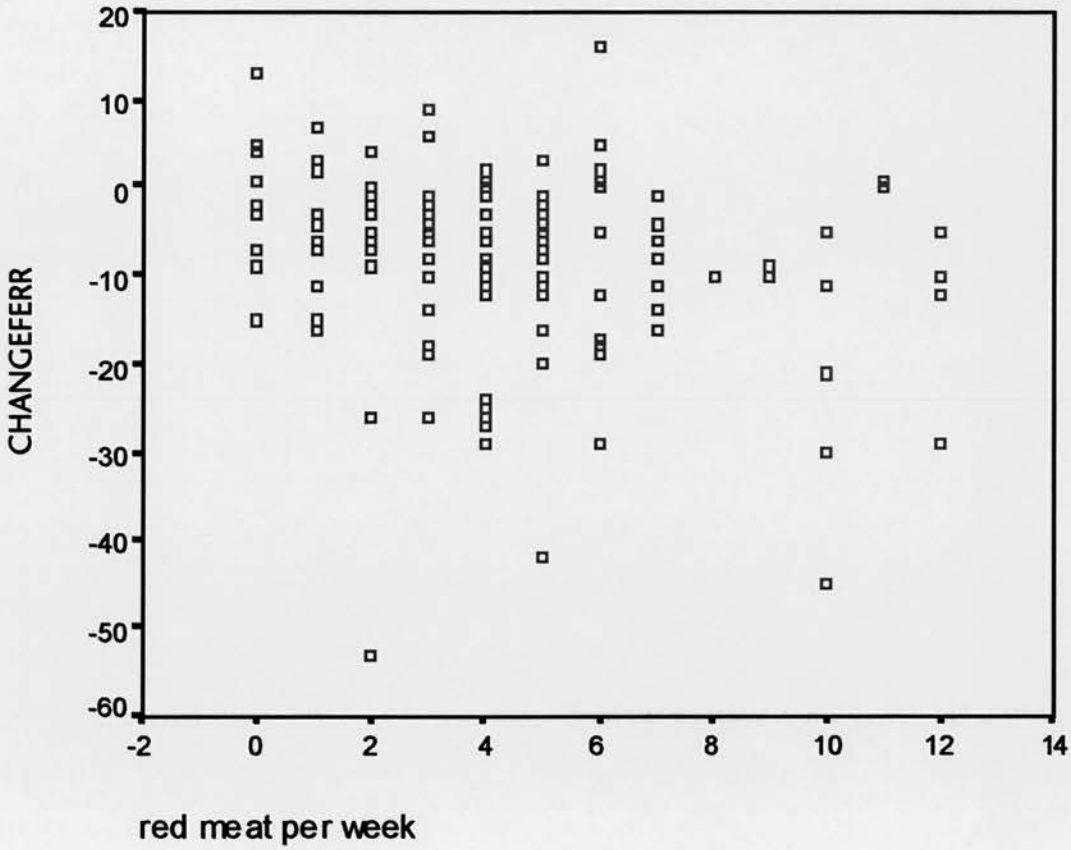
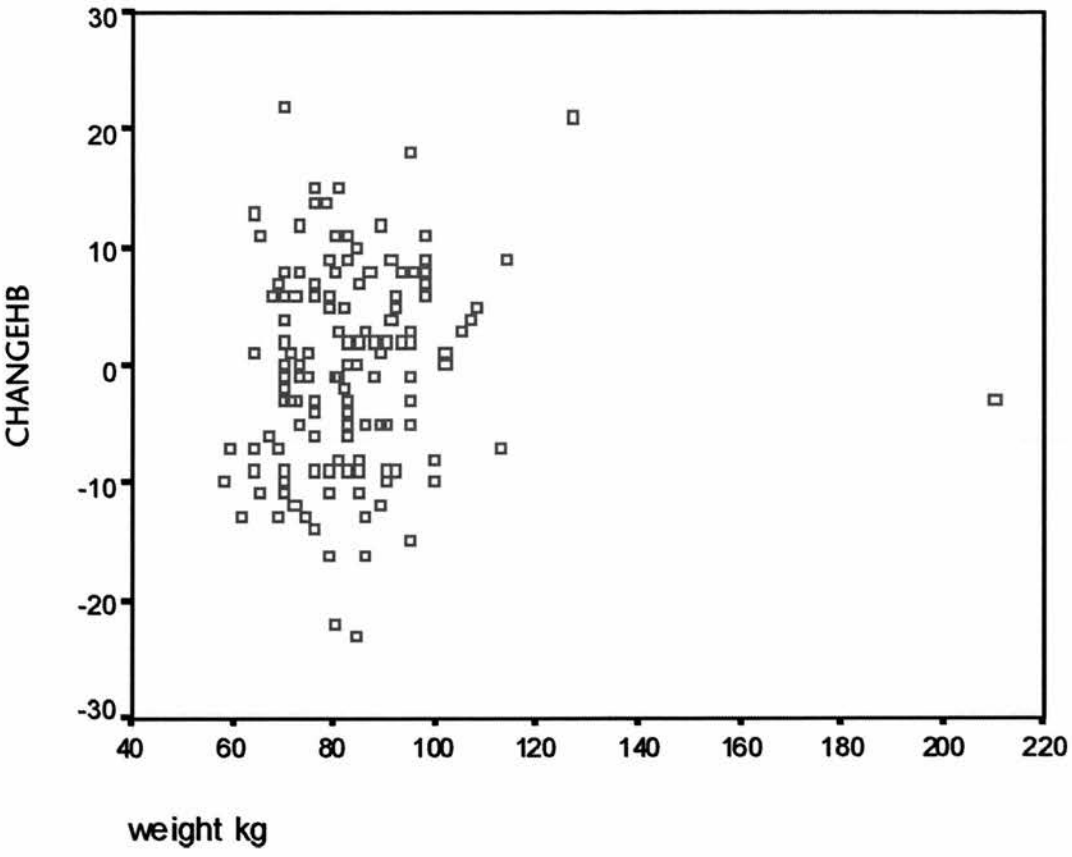


Figure 4.18 Change in haemoglobin concentration between first and last study visit (CHANGEHB expressed in g/L) by donor weight



4.4.2 *Questionnaire*

A total of 218 completed questionnaires were returned, a response rate of approximately 73%, assuming that all non-returned questionnaires reached their intended destination. Donors provided information on their weight, their average weekly consumption of meat (number of times per week that red meat is eaten) and on whether they were presently using iron or vitamin supplements or whether they had done so within the last two years. A total of 11 donors (5.0% of respondents) were presently taking proprietary dietary supplements, which included iron and one additional donor had previously done so. Five donors (2.3%) reported taking non-iron containing dietary supplements at the time of the study. 39 donors (17.9%) had taken non-iron containing supplements in the previous two years.

Red meat was not consumed at all by 19 donors (8.6% of respondents). 145 donors (66.5%) consumed it 1-5 times per week, 42 (19.2%) 6-10 times per week and 12 (5.5%) on more than 10 occasions per week.

4.5 Discussion

4.5.1 *Genotype frequency*

This was a small study in which some data were incomplete. The allele frequencies for C282Y and H63D were similar to those obtained in other UK studies (Boulton, 2000; Jackson, 2001; Miedzybrodska, 1999).

4.5.2 *Iron deficiency among donors*

Our results confirm that a significant proportion (23.6%) of male blood donors are iron deficient, when this is defined as a ferritin of less than 15 μg/L. Why this is so much greater than that found by Jackson and colleagues (Jackson, 2001) in their much larger study is not certain. Although the inclusion criteria differed in that Jackson included only donors who had given a minimum of 2 units of blood, while the criteria used in the present study specified that one unit must have been donated in the previous year, this is unlikely to account for the disparity in the number of iron deficient donors. It is possible that the requirement in the present study for donors to commit to trying to give blood every three months for a year biased inclusion in favour of donors who were already donating frequently and therefore who were more likely than less frequent donors to be iron deficient.

Consumption of iron supplements by donors could influence data such as those presented here. Only a small minority of donors (5.5%) reported having taken supplementary iron at any time in the previous two years. It is difficult to analyse statistically whether or not this had any effect, but the numbers are sufficiently small that any major influence on the results is unlikely. However, it is recognised that the reliability of the figure for the number of donors who had taken iron may be open to question because both of donors' inaccuracy in remembering which supplements they had taken in the previous two years and inability to be certain which supplements did contain iron. (Information provided by donors was taken at face value; no attempt was made to verify this independently).

There was no significant difference in the HFE genotypes represented among the iron deficient group and the whole study population although again the limitations imposed by small numbers are recognised.

4.5.3 *Haemochromatosis-associated genotypes*

The point has been made in Chapter 2 that many individuals with the typical haemochromatosis genotype do not express the iron-overloaded phenotype. Of the four donors who were homozygous for C282Y in the present study, only two had evidence of iron overload in terms of elevated transferrin saturation. Only the elder of the two (aged 49) had a serum ferritin above normal (326 μg/L), and it is possible that both of them had been protected from more severe iron accumulation by blood donation. The remaining two C282Y homozygotes did not appear to express the haemochromatosis phenotype and both had serum ferritin values in the iron deficient range. Both were of an age (34 and 59 years) at which evidence of iron overload might have been expected in expressing individuals. While none of the three individuals heterozygous for both C282Y and H63D had elevated ferritins, two had transferrin saturations equal to or greater than 50%. While it is difficult to draw conclusions from a single elevated transferrin saturation result for the reasons explained in section 2.6.3 above, individuals with this genotype are at risk of developing iron overload (Beutler, 1997).

4.5.4 *Iron status at study entry*

Donors heterozygous for the C282Y mutation did not have a higher initial ferritin or transferrin saturation than those with a wild type genotype or heterozygotes for H63D, in contrast to what has been found in other studies of either blood donors (Jackson, 2001; Boulton, 2000) or the general population (Adams, 1994; Beutler, 2000). By contrast, transferrin saturation at study entry was significantly higher in heterozygotes for C282Y than in individuals with a normal genotype or heterozygotes for H63D.

4.5.5 *Change in haemoglobin concentration and ferritin*

Genotype alone had no influence on the change in haemoglobin concentration or ferritin between the first and last study visits in donors who completed all four visits. However, ferritin did fall by a smaller amount in those donors who consumed more red meat. It has been suggested that heterozygosity for C282Y might afford protection from iron deficiency (Datz, 1998). These data do not suggest that donors with this genotype were protected to a greater extent than those with the wild type genotype against iron depletion by blood donation. It is therefore unlikely that heterozygous men comprise a group who could donate blood more frequently than others without an increased risk of iron deficiency.

4.6 Conclusions

It was not possible to identify a sub-population of male blood donors who, on the basis of their HFE genotype, may be particularly resistant to the development of iron deficiency. This is an important negative result in view of the current debate about potential ways to augment the blood supply. It does not address the question of any potential evolutionary benefit of heterozygosity for C282Y such as protection from the development of iron deficiency. However, it does suggest that any such protection is limited and, if it does exist, cannot significantly mitigate the negative effects of frequent blood donation on individuals' iron balance. The issue of the influence of heterozygosity for C282Y on iron balance is discussed further in section 5.7 of this thesis.

5. Study of the prevalence HFE genotypes in iron deficiency

5.1 Background

In order to explain the persisting high allele frequency of the C282Y mutation of HFE in northern European populations, it has been proposed that possession of this mutation may offer some evolutionary survival advantage in terms of relative protection against iron deficiency.

This theory was advanced recently by Datz and colleagues (Datz, 1998). They studied full blood counts, serum iron, transferrin and transferrin saturation in 468 female healthcare workers aged 18-40. All individuals had their HFE C282Y genotype determined. They found a significantly higher haemoglobin concentration, serum iron and serum transferrin saturation in women who were heterozygous for C282Y. Further support for a protective role for C282Y against iron deficiency was provided by the work of Butterworth *et al* (Butterworth, 2002) who studied haemoglobin concentrations and fasting serum iron levels as well as C282Y genotypes in 145 patients with coeliac disease. Patients with the C282Y mutation had higher mean haemoglobin concentrations and serum iron levels than those with the wild type genotype.

5.2 Aim

The aim of this study was to compare the distribution of HFE genotypes in iron deficient individuals with those in a control population drawn from the same geographic area.

5.3 Methods

Samples were collected over period of several weeks in 2000. No distinction was made between samples received from general practitioners or those from the within the hospital, and this information was not recorded.

5.3.1 *Iron deficient samples*

Full blood count samples sent to the haematology department of the Western General Hospital, Edinburgh, which were found to be iron deficient, were selected for DNA extraction and HFE genotyping. The protocol followed by the laboratory entailed measurement of serum ferritin on any full blood count sample with a mean cell volume below the normal range or with blood film features suggestive of iron deficiency. Iron

deficiency was defined as a plasma ferritin <15 μg/L. A random selection of each week's iron deficient samples was tested for this study. The gender and age of the individual from whom the samples were taken and the haemoglobin concentration and ferritin from the sample were recorded. After selection, the samples were rendered anonymous before DNA extraction was performed.

5.3.2 *Control samples*

Control samples were obtained from the department of haematology at the Royal Infirmary of Edinburgh. Samples were taken randomly from those received in the laboratory which yielded normal full blood count results on standard laboratory analysers. Normal results were defined as a haemoglobin concentration greater than 130 g/L for males or 115 g/L for females, a total white cell count of 4-11 x 10^9/L, a platelet count of 150-400 x 10^9/L and a mean cell volume (MCV) of 76-100 fL. Samples were rendered anonymous before DNA was extracted.

The raw data from subjects of this study (full blood counts and serum ferritin concentration) are presented in Appendix 9.

5.4 Statistical analysis

The iron deficient and non-iron deficient populations were compared by means of the χ^2 test. A p-value of ≤ 0.05 was taken as significant.

5.5 Results

Samples were analysed from 520 iron deficient and 351 non-iron deficient individuals. The characteristics of the two populations are presented in table 5.1. The HFE genotype was ascertained in 474 (91.2%) of the iron deficient subjects and 335 (95.4%) of the non-iron deficient subjects. The failure to ascertain a genotype resulted in all cases from the inability to extract DNA. The distribution of genotypes in the iron deficient and non-iron deficient populations is shown graphically in figure 5.2.

The iron deficient population contained 8.3% (95% confidence intervals 2.3% to 14.3%) more women than the non-iron deficient population (p=0.006). The iron deficient population was older than the non-iron deficient (p<0.0001), while the iron deficient population had a lower mean haemoglobin concentration than the non-iron deficient population for both mean and women (p<0.0001 in each case).

Table 5.1 Characteristics of iron deficient and normal populations

		Normal population	Iron deficient population	
Total number		351	520	
	male	106 (30.2%)	114 (21.9%)	
	female	245 (69.8%)	406 (78.1%)	p=0.006
Age in years (SD)		50.2 (18.9)	55.8 (21.0)	p<0.0001
Ferritin (µg/L)		Not measured	7 (5.6)	
Hb in g/L (SD)	male	144.7 (8.8)	98.4 (20.2)	p<0.0001
	female	130.6 (9.3)	98.3 (16.8)	p<0.0001
Genotype *(The figures in square brackets are 95% confidence intervals)**	HC/HC	212 (63.3% [58.1,68.4])	301 (63.5% [59.2,67.8])	ns
	HY/HC	33 (9.9%[6.9,13.6])	44 (9.3%[6.7,11.9])	ns
	DC/HC	74 (22.1% [17.6,26.5])	109 (23%[19.2,26.8])	ns
	HY/HY	1 (0.3% [0, 1.6])	0 (0%[0,0.8])	ns
	DC/DC	10 (3.0%[1.4,5.4])	9 (1.9%[0.9,3.6])	ns
	HY/DC	5 (1.5%[0.5, 3.4])	11 (2.3%[1.2,4.2])	ns
No genotype ascertained**		16 (4.6%)	46 (8.8%)	

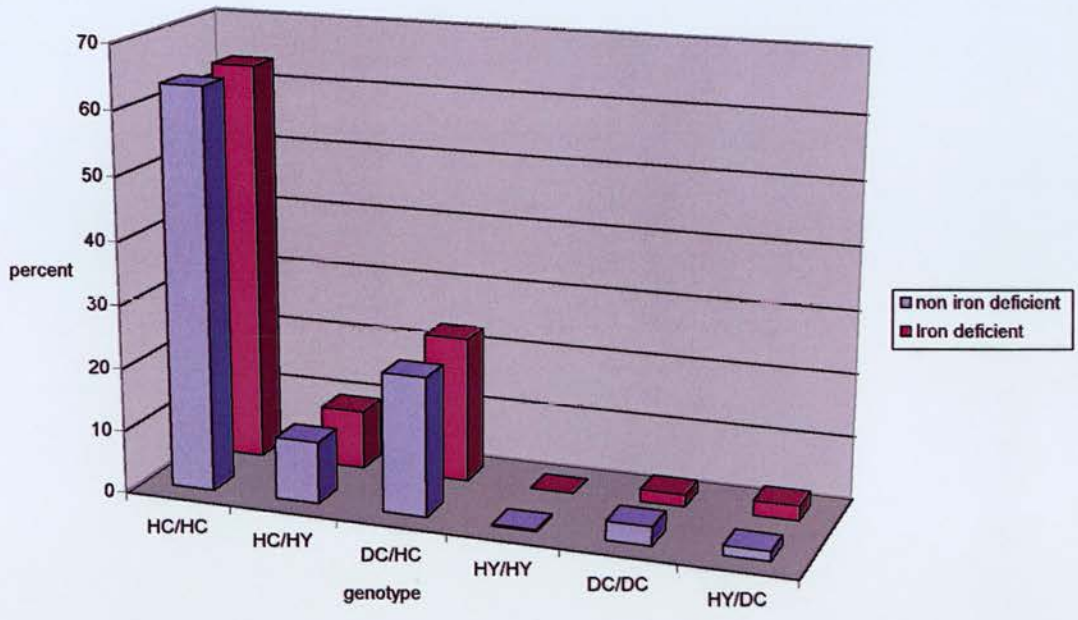
SD = standard deviation
Hb = haemoglobin concentration
ns= not significant

Values for age, ferritin and haemoglobin concentration are expressed as the mean.

* Percentages in this section of the table refer to the total number of samples in each population for which a genotype was ascertained.

** The reason for genotype not being ascertained was either failure of DNA extraction or failure of PCR. Percentages in this row refer to the total number of samples analysed.

Figure 5.2 Distribution of HFE genotypes
in an iron deficient and a non-iron deficient population



9.3% (44/474) iron deficient individuals were heterozygous for the C282Y mutation, compared with 9.9% (33/335) in the non-iron deficient group. This difference was not significant (p=0.66). Of all the study subjects only one, a male in the non-iron deficient population, was homzygous for C282Y. None of the other HFE genotypes differed significantly in its distribution between the two populations.

5.6 Discussion

The proportion of individuals in the two populations with each of the six HFE genotypes is consistent both with those found in other studies within this thesis and also with those reported for a Scottish population (Miedzybrodska, 1999). In that there is no difference in the proportion of C282Y carriers between the iron deficient and non-iron deficient population, these results do not provide support for the theory that the C282Y mutation protects against iron deficiency.

The study is open to a number of criticisms. No attempt was made to obtain a homogenous population of iron deficient patients and no demographic data were collected other then age, sex and details of full blood count and ferritin results. There must have been, therefore, a range of underlying pathologies contributing to the iron deficiency; the efficacy of any protection afforded by C282Y against iron deficiency may depend at least in part on the cause of that deficiency. Additionally, some patients were anaemic while others were not and it is possible that these two groups should constitute two separate populations. No attempt was made to match the iron deficient and non-iron deficient populations for age or sex, and there were in fact significant differences between them. The definition of iron deficiency as a plasma ferritin of <15 µg/L was arbitrary and took no account of the difference between the lower end of the normal range for males and females, so that some iron deficient males were probably missed. In addition, some of the iron deficient samples were likely to have been detected on the basis of a plasma ferritin assay triggered by a low mean cell volume or other morphological features of iron deficiency. This may have introduced some further bias because of an under-representation of iron deficient individuals with a normal MCV. However, the distribution of the HFE genotypes within each population was similar to that expected from other surveys of populations of similar ethnicity. The control population was by definition not "normal" in that it was composed of individuals who had, for some health reason had a full blood count checked. Additionally, as this population was screened for iron deficiency only on the basis of a normal full blood count, it is likely that some iron deficient individuals were included.

Given, however, the similarity in the proportion of C282Y carriers in each population, it is unlikely that correction even of all these imperfections would have affected the conclusion of the study.

5.7 Conclusion

Notwithstanding the criticisms that may be levelled at the methodology of this study, the finding that the prevalence of C282Y amongst patients with iron deficiency does not seem to be lower than that in a control population provides additional evidence that possession of C282Y does not protect against iron deficiency. This is in agreement with results presented elsewhere in this thesis that show that heterozygosity for C282Y does not protect male blood donors from the development of iron deficiency (see sections 4.4 and 4.5). This contrasts with the conclusions reached by the smaller studies of Datz (Datz 1998) and Butterworth (Butterworth 2002). They analysed their data differently however, comparing mean ferritin and transferrin saturations in individuals with different HFE genotypes. Data presented in the present work (section 4.4) showed that male blood donors who were heterozygous for C282Y had a higher mean transferrin saturation than those lacking this mutation, but there was no significant difference in mean ferritin values.

6. HFE gene mutations and hepatic iron depositon in patients undergoing orthoptic liver transplantation

6.1 Background

As a result of the portal blood circulation, the liver is the first target organ in haemochromatosis (Brissot, 2000). Unchecked iron accumulation in the liver may lead ultimately to the development of hepatic fibrosis, cirrhosis and hepatocellular carcinoma. However, individuals with haemochromatosis are now increasingly identified before the development of evidence of hepatic damage. Many of these may be asymptomatic (Leggett, 1990; Adams, 1991).

A common symptom at presentation with haemochromatosis is abdominal pain (Niederau, 1996), the precise aetiology of which remains uncertain. In particular, it is unclear whether this is related to hepatic pathology. Among physical signs at diagnosis, hepatomegaly is one of the most prevalent. In a series of 251 German patients with haemochromatosis, 70% of those without and 89% of those with cirrhosis had hepatomegaly (Niederau, 1996). A survey of over 1000 iron overloaded patients in Brittany, France, also demonstrated the common occurrence of hepatomegaly, albeit at a lower prevalence than that found in the German study (Moirand, 1999).

Individuals with hepatic cirrhosis and/or hepatocellular carcinoma as a result of haemochromatosis may be treated with orthotopic liver transplantation (OLT). A number of investigators have reported that patients with haemochromatosis fare less well after OLT than patients with chronic liver disease of other aetiologies (Farrell, 1994; Kowdley, 1995; Poulos, 1996; Brandhagen, 2000, Crawford, 2004). In addition, there is evidence that in a considerable number of transplant recipients, haemochromatosis has been undetected at the time of operation (Farrell, 1994; Kowdley, 1995; Brandhagen, 2000). Whether this is changing with the ready availability of HFE mutation analysis in recent years, is uncertain.

Interpretation of serum measures of iron overload in patients with liver disease may be difficult, because many liver pathologies are associated with elevated values of blood iron studies in the absence of haemochromatosis (Di Bisceglie, 1992). Even the finding of increased hepatocellular iron on histological examination of liver tissue is

not specific for haemochromatosis (Deugnier, 1997). Because of this, the hepatic iron index (HII) which is the ratio of the hepatic iron concentration to the patient's age has often been used to try to identify cases of hepatic siderosis due to haemochromatosis rather than other causes. An HII of greater than 1.9 has been considered indicative of haemochromatosis. However, it has become clear that this value cannot be used as an absolute: some patients, especially those with cirrhosis, may have HII values in excess of 1.9 without having haemochromatosis, others with a definite diagnosis of haemochromatosis have an HII of less than 1.9 (Ludwig, 1997; Kowdley, 1997).

It has been shown in previous studies that patients coming to liver transplantation may have very significant hepatic iron deposition in the absence of HFE linked haemochromatosis (Farrell, 1994; Deugnier, 1997). In an American study of patients who underwent liver transplantation at the Mount Sinai Medical Center, 19 out of 918 had hepatic iron deposition graded 3 or 4 (Fiel, 1999). The investigators showed a poor correlation of pre-transplant phenotypic diagnosis of haemochromatosis with HFE genotype. Nine of the 19 patients had wild type HFE genotypes, three were homozygous for C282Y, one was heterozygous for C282Y and six were H63D heterozygotes. It was clear that serum measures of iron status in these patients did not accurately reflect hepatic iron overload.

The aim of the present study was to determine the HFE genotype of all patients transplanted on the Edinburgh liver transplant programme since its inception in 1992. Furthermore, it was intended to determine the number of transplant recipients with unsuspected haemochromatosis, and to ascertain whether any conclusions could be drawn in this patient population about the effect of HFE genotype on survival after OLT.

6.2 Methods

Ascertainment of the HFE genotype and iron status of liver transplant recipients under follow-up at the Scottish Liver Transplant Unit, Royal Infirmary of Edinburgh was achieved by means of case note review. The transplant physicians were of the opinion that it was clinically necessary to perform HFE genotyping on all their liver transplant recipients and gave permission for this analysis to be undertaken.

6.2.1 *DNA extraction*

DNA was extracted from the peripheral blood of patients attending the liver transplant follow-up clinic.

Where available, paraffin-embedded biopsy material from the explanted livers of patients who had died was obtained from the University of Edinburgh Department of Pathology and was subject to DNA extraction.

6.2.2 *HFE genotyping*

If the HFE genotype was documented in a patient's case notes, this was recorded. For all patients in whom this was not the case, genotyping for the C282Y and H63D mutations of HFE was performed. Because the results on the living patients were being obtained for clinical purposes, it was considered important that the results entered into the patients' notes were verified by a laboratory in which appropriate quality assurance was operational. All DNA samples were therefore sent for repeat analysis to the Scottish Molecular Genetics Consortium at the University of Dundee. The reports from this source were forwarded to the transplant physicians. There was 100% concordance between the study results and those from the Molecular Genetics Consortium.

6.2.3 *Ascertainment of iron status*

For the living recipients, post-transplant iron status (serum ferritin and transferrin saturation) was recorded when this information was already documented in the notes. If it was not recorded, blood was taken for the relevant analyses at the following clinic visit. The case notes of all clinic attendees over a period of approximately nine months were reviewed, and data were obtained on the majority of the surviving transplant population. No attempt was made to try to standardise the time interval between the transplant procedure and measurement of iron status.

Details of patients who had died following transplantation were obtained from the database of the Scottish Liver Transplant Unit.

6.2.4 *Examination of liver biopsies*

In those cases in which a Perls' stain for iron had been performed on a biopsy of the explanted liver, this was examined by an independent pathologist who was unaware of the ferritin or genotype results. Hepatocyte iron deposition was assessed on a scale of 1 to 4+ according to the criteria of Searle *et al.* (1994). In this method, grade 0 is absence of granules or barely visualised granules at x 400; grade 1 is granules visible at x 250 and easily seen at x 400; grades 2 - 4 are granules seen at x 100, x 25 and x 10 (or with the naked eye) respectively.

6.3 Statistical Analysis

The distribution of HFE genotypes among patients with different Perls' stain grades was examined by Fisher's exact test, as 86.7% of cells in a 2 x 2 table for χ^2 analysis had an expected count of less than 5. The distribution of hepatic iron grade, ferritin values and length of stay in the transplant unit could not be assumed to be parametric, so correlation of ferritin with hepatic iron deposition and HFE genotype and length of stay was examined by calculation of Spearman's rank correlation coefficient (ρ).

6.4 Results

The Scottish Liver Transplant unit performed 307 orthotopic liver transplants on 271 recipients between its inception in 1992 and May 2000 when this study was performed. Of these, 62 transplant recipients had died. An HFE genotype was obtained on 180 of the 209 living recipients (86%). A serum ferritin value was obtained for 183 (88%) individuals and a transferrin saturation for 173 (83%). Failure to obtain blood for testing for these parameters resulted in nearly every case from the failure of the patient to attend successive review appointments at the transplant clinic. No effort was made to pursue patients to obtain specimens. Some of these patients may have been under only infrequent review at the transplant unit because, for example, of the distance they lived away from Edinburgh.

A total of 135 Prussian Blue stained liver biopsy slides of samples taken from the explanted livers of 126 living transplant recipients were available for grading of iron deposition. Of these, 102 (75.6%) stained at grade 0, 13 (9.6%) at grade 1, 11 (8.1%) at grade 2, 6 (4.4%) at grade 3 and 3 (2.2%) at grade 4. For one specimen, the pathologist was unable to grade it definitely as 3 or 4. This has been arbitrarily included as grade 3.

The distribution of HFE genotypes among the liver transplant patients is shown in table 6.1. Three transplant recipients were homozygous for C282Y – this was not known before the transplant in any of the cases. The characteristics of these recipients are summarised in table 6.2.

Table 6.3 shows the characteristics of the seven patients who were compound heterozygous for the C282Y and H63D genotypes.

Nine patients had evidence of severe hepatic iron deposition (defined as Perls' stain grade 3 or 4) on their liver biopsy specimens. These patients are presented in table 6.4. The distribution of serum ferritin values in patients with differing grades of hepatic iron deposition is illustrated graphically in figure 6.5. No correlation between serum ferritin and hepatic iron deposition was found (Spearman's $\rho = 0.139$; 2-tailed $p = 0.15$).

Figure 6.6 illustrates the distribution of hepatic iron distribution according to the patient's HFE genotype. No correlation either was found between these two variables ($p = 0.161$, Fisher's exact test).

Figure 6.7 shows the effect of genotype on length of stay in the Transplant Unit. There was no correlation identified between these variables (Spearman's $\rho = 0.037$; 2-tailed $p = 0.661$)

It proved impossible, despite the use of various techniques (discussed in the general Methods chapter), to extract DNA from the paraffin sections of liver biopsy. There are therefore no data on HFE genotype for those patients who had died before the present study began.

Table 6.1 Distibution of HFE genotypes among liver transplant recipients

Genotype	Number	Percentage	95% CI
HC/HC	108	60.0	52.8-67.2
HY/HC	21	11.7	7.0-16.4
DC/HC	38	21.1	15.1-27.1
HY/HY	3	1.7	0.3-4.8
DC/DC	3	1.7	0.3-4.8
HY/DC	7	3.9	1.6-7.8

CI = confidence intervals

Data were unavailable on 46 patients out of a total of 226. Percentages quoted are calculated on the basis of a total of 180 patients for whom there were data.

Table 6.2 Characteristics of liver transplant recipients with genotype HY/HY

Patient number	Ferritin (µg/L)	TS (%)	Perls' stain grade
1	8	<10	0
2	832	14	0
3	32	27	0

Table 6.3 Characteristics of liver transplant recipients with genotype HY/DC

Patient number	Ferritin (µg/L)	TS (%)	Perls' stain grade
1	350	25	3
2	429	44	0
3	11	31	0
4	870	73	0
5	518	50	0
6	23	28	Not known
7	157	31	0

Table 6.4 Characteristics of patients with severe hepatic iron overload (grade 3 or 4)

Patient	Perls' stain grade	Genotype	Ferritin (µg/L)	TS (%)
1	3	HY/DC	350	25
2	3	HC/HC	42	13
3	3	HC/HC	179	26
4	3	nd	nd	nd
5	3	nd	nd	nd
6	3	nd	nd	nd
7	4	DC/DC	157	22
8	4	DC/HC	219	33
9	4	nd	nd	nd

nd = no data available

Figure 6.5 Scatter plot of the distribution of serum ferritin (in μg/L) according to iron stain grade of hepatic biopsy

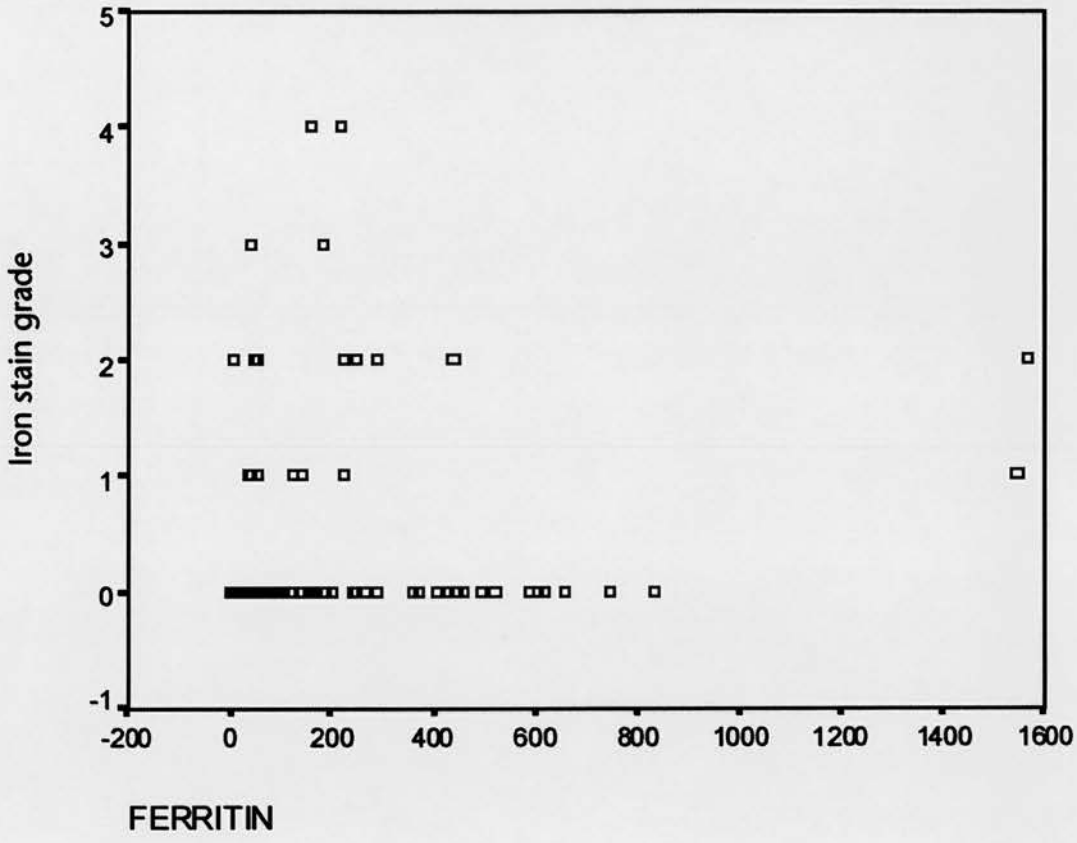
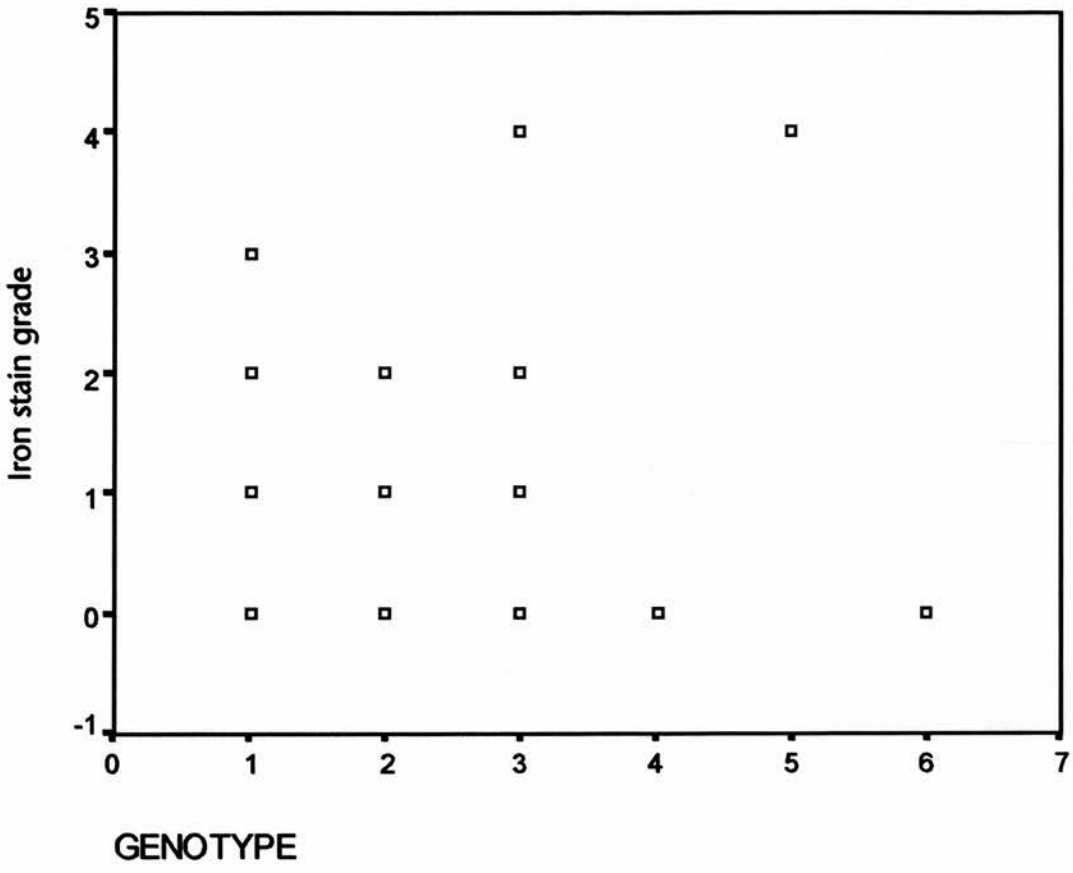


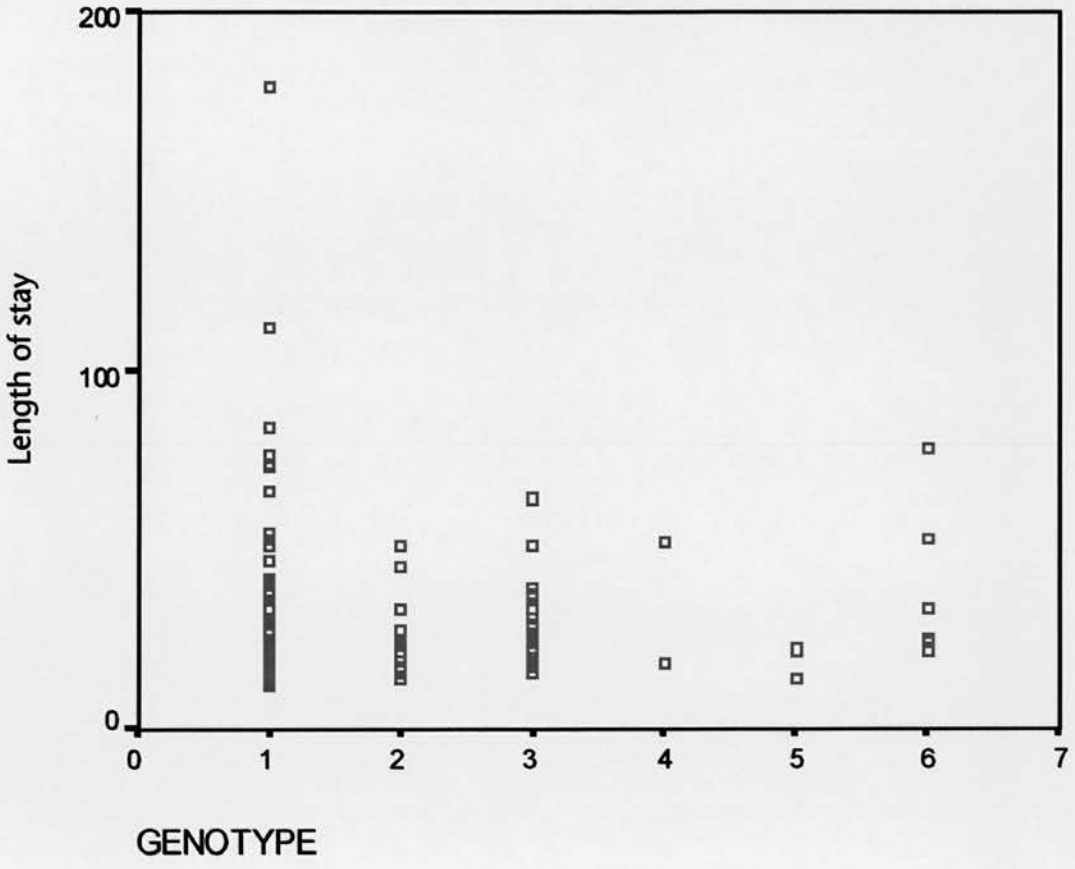
Figure 6.6 Scatter plot of the distribution of hepatic iron stain grades among patients with the various HFE genotypes



Key to HFE genotypes:

1. HC/HC
2. HY/HC
3. DC/HC
4. HY/HY
5. HD/HD
6. HY/DC

Figure 6.7 Scatter plot of the distribution of length of stay in transplant unit (days) according to HFE genotype



Key to HFE genotypes:

1. HC/HC
2. HY/HC
3. DC/HC
4. HY/HY
5. HD/HD
6. HY/DC

6.5 Discussion

The distribution among genotypes among the liver transplant recipients is similar to that expected, based on other studies in Celtic populations (Boulton, 2000; Jackson, 2001; Miedzybrodska, 1999) and on results obtained in other sections of this work.

Because of small numbers, it is impossible to analyse the numbers of patients in each genotype statistically with respect to the number expected. However, seven patients (3.9% of those for whom data were available) with the genotype HY/DC is possibly a greater number than expected.

None of the patients with genotype HY/HY had a raised post-transplantation transferrin saturation, while only one had a raised ferritin. In all three cases, the iron stain on the explanted liver showed no evidence of iron overload, suggesting that the haemochromatosis in these patients was not phenotypically expressed.

Although five of the seven patients with genotype HY/DC had an elevated serum ferritin, only two had transferrin saturations greater than 50% and in only one case (patient number 1 in table 6.3) was there any significant hepatocellular haemosiderosis.

Nine patients had markedly increased hepatic iron deposition (grade 3 or 4 siderosis) (table 6.4). Of the five whose genotypes were known, one was a compound heterozygote for C282Y and H63D (HY/DC), one was heterozygous for H63D (HC/DC), one homozygous for H63D (DC/DC) and two were wild type (HC/HC).

Unfortunately, because data collection was incomplete for this group of patients, it is impossible to identify any clear pattern of genotype or any definite correlation with serum ferritin or transferrin saturation values. However, it appears to be the case that a significant number of liver transplant recipients with severe hepatic iron overload do not have evidence of HFE-linked haemochromatosis, as suggested in earlier studies. Further weight is given to this conclusion by the fact that no correlation was found between HFE genotype and serum ferritin.

In addition, a wide distribution of ferritin and transferrin saturation values was found in the patients with grade 3 and 4 hepatic iron staining. As was touched upon in the introduction to this chapter, blood iron indices such as serum ferritin fail to reflect accurately the presence or absence of tissue iron overload in those patients with end-stage hepatic disease (Fiel 1999). Data were not collected on pre-transplant serum ferritin or transferrin saturation and the values reported here were obtained at time intervals

between a few months and seven years after transplantation. It is reported that patients with haemochromatosis who receive a liver transplant may have a post transplant fall in their serum ferritin and transferrin saturation to normal within six months of the transplant procedure (Powell, 1992). As accumulation of iron may take many years, it is possible that there will be a gradual reaccumulation of iron in these patients, but data on this are not yet available.

It is difficult to make any comment from these results on any influence of HFE genotype on outcome of transplantation. There was no correlation between genotype and length of admission to the transplant unit. However, length of stay could be affected by a large range of factors that were not controlled for in this study, and is not a validated measure of transplant outcome. There have been suggestions that outcome at a year after transplantation is worse in patients with haemochromatosis than in other transplant patients (Farrell, 1993; Kowdley, 1995; Poulos, 1996; Brandhagen, 2000). Data on one-year survival could not be collected for the present study: all patients included in the data presented here were still alive at the time of analysis. Patients who had died were excluded because of the failure of attempts at extracting DNA from paraffin sections of liver tissue. This fact renders it even more difficult to comment on outcome, since the data are by definition skewed in favour of patients who survived.

This study has some significant limitations in its methodology. Data collection was not complete, largely for logistical reasons. More demographic detail on the patients would have added to the value of the observations presented here (for example, it would have been useful to know the pre-transplant and histological diagnoses of the patients). Collecting these data was not feasible because of resource considerations. Additionally, some standardisation of the time in a patient's post-transplant course at which blood iron indices were assessed would have aided interpretation of these results. The lack of information on pre-transplant ferritin and transferrin saturation values is also unfortunate.

6.6 Conclusion

As has been reported in other studies, there were a number of patients who had received liver transplants for indications other than haemochromatosis who were subsequently found to be homozygous for the C282Y mutation of HFE. These patients may not have expressed phenotypic haemochromatosis. It was not possible to identify a correlation between HFE genotype and hepatic iron deposition as assessed by histological examination.

7. Iron status and HFE gene mutations in patients with coronary artery disease shown at coronary angiography

7.1 Introduction

The extent to which iron has a role in the pathogenesis of coronary artery disease has been the subject of intense debate (Sullivan, 1981; Koster, 1995; Sullivan, 1999; de Valk, 1999; Niederau, 2000; Ma, 2002).

Theoretical mechanisms certainly exist by which iron may have a role in the pathogenesis of atherosclerosis. The generation of hydroxyl radicals ($OH^{\bullet}$), which are very reactive and can attack a variety of cell constituents can occur via the Fenton and Haber-Weiss reactions (Britton, 2000). In the Fenton reaction, the hydroxyl radical is produced by the reaction of iron with hydrogen peroxide:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

Ferric iron is reduced to ferrous iron by the superoxide radical ($O_2^{\bullet -}$) in the Haber-Weiss reaction:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

Net reaction: $O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe} O_2 + OH^{\bullet} + OH^-$

Hydroxyl radicals may initiate the process of lipid peroxidation, which may be an important factor in the formation of atherosclerotic lesions (de Valk, 1999). It is also possible that iron depletion exerts a protective effect on the myocardium by mechanisms other than the inhibition of atherogenesis (Sullivan, 1999).

In healthy individuals, free plasma iron cannot be detected. However, in sera from iron-overloaded patients, in which serum iron-binding capacity is fully saturated, non-transferrin bound iron (NTBI) may be found (Batey, 1980; Gutteridge, 1985; Grootveld, 1989). Iron in this form may be particularly capable of causing tissue damage by the mechanisms mentioned above (Gutteridge, 1985). It has been shown that heterozygotes for hereditary haemochromatosis, whose serum iron, transferrin saturation and ferritin did not differ from those of controls, did nonetheless have significantly higher NTBI than controls (de Valk, 2000).

In 1981, Sullivan advanced the argument that the difference in the incidence of heart disease in men and women was explicable by differences in the levels of stored iron. As a corollary of this, iron depletion should protect against the development of ischaemic heart disease (Sullivan, 1981). Sullivan has subsequently made the case that the change over time in the risk of coronary artery disease parallels changes in iron stores as reflected in serum ferritin values (Sullivan, 1983; Sullivan, 1989).

There is some evidence in support of Sullivan's hypothesis. A study of 1931 men in eastern Finland indicated that an elevated serum ferritin was a strong risk factor for acute myocardial infarction and that the dietary intake of iron was strongly associated with this disease (Salonen, 1992). A further study conducted on essentially the same population after the discovery of HFE concluded that male carriers of C282Y are at a two-fold increased risk for first acute myocardial infarction compared with non-carriers (Tuomainen, 1999). A Dutch study of over 12 000 postmenopausal women found an association between heterozygosity for C282Y and cardiovascular death, especially in women with other classical risk factors (Roest, 1999). Women who were smokers, hypertensive and carriers of C282Y had an 18.85-fold increased risk of cardiovascular death compared with women without these risk factors. Women who smoked and were hypertensive but did not carry C282Y had a relative risk of 2.06 compared with non-smoking, normotensive non-carriers of C282Y. A positive association was also found between serum ferritin and ultrasonic measurement of progression of carotid atherosclerosis over a five-year follow-up period (Kiechl, 1997). Two groups have suggested that blood donors have a lower risk of coronary heart disease than non-donors, which might reflect a protective effect of a reduction in body iron stores (Meyers, 1997; Salonen, 1999).

By no means all the published studies have supported the existence of a relationship between atherosclerosis and iron stores and/or HFE gene mutations, however. Nassar *et al* studied 300 patients, half of whom manifested early onset (before the age of 50) coronary artery disease (Nassar, 1998). The other half of the study population presented for the first time with coronary artery disease over the age of 65 years. Males in the early onset group had a higher plasma ferritin than those in the later onset population. There was, however, no correlation with carriage of HFE mutations. Ferritin did not differ significantly between women in the two groups. No other data on coronary risk factors were collected in this study. 546 Italian patients with angiographically confirmed severe coronary artery disease were compared with 303 patients found to have non-diseased coronary arteries on angiography (Bozzini, 2002). The authors collected a full data set to enable assessment of coronary risk factors and found no correlation between

coronary artery disease and iron stores or HFE mutations (only C282Y was assessed). Similar negative results were produced by a Brazilian study of 160 individuals who had survived myocardial infarction and had severe coronary atherosclerotic on angiogram (Calado, 2000). Age, sex, and race-matched healthy individuals were used as a control population. No data on iron stores were presented, but there was no increase in the prevalence of C282Y or H63D in the population with coronary artery disease. A possible criticism of this study is that the control population, while free of symptoms of coronary artery disease, did not undergo coronary angiography and the true prevalence of coronary artery disease among the controls should probably not be assumed to be zero. HFE genotypes (C282Y and H63D) were determined in subjects from two case control studies: the ECTIM study designed to identify genetic polymorphisms affecting the susceptibility to myocardial infarction in patients and controls from France and the UK; and the GENIC study, examining the genetic susceptibility to brain infarction in patients from 12 French departments of neurology. Additionally, genotypes were obtained on participants in the AXA study, which was designed to investigate risk factors for early stages of atherosclerosis (Hetet, 2001). In this study, healthy individuals underwent Doppler ultrasonographic assessment of atherosclerosis in the femoral and carotid arteries. The results of the AXA study suggested a correlation between H63D and atheroma in smokers. The other two studies did not show any significant relationship between HFE mutations and risk either of coronary heart disease or brain infarction.

Other studies (Baer, 1994; Eichner, 1998) also failed to support a correlation between iron and atherosclerosis, and in a meta-analysis of published studies, Danesh and Appleby concluded that there was no evidence for a strong epidemiological association between iron status and coronary heart disease (Danesh, 1999). Two more recent large studies have also failed to show a correlation between elevated serum iron parameters and HFE gene mutations. Waalen and colleagues (Waalen, 2002) screened in excess of 30 000 men and women attending a health appraisal centre and found no consistent association between either of the common HFE gene mutations, serum ferritin or serum transferrin saturation and coronary heart disease, as assessed by review of medical records and completion of a questionnaire. Similar results were obtained in a cross-sectional evaluation of 2326 inhabitants of the Australian town of Busselton (Fox, 2002).

7.2 Aim

Against this background of conflicting evidence, the aim of the present study was to examine the hypothesis that iron stores and /or mutations in the HFE gene are risk factors for coronary artery disease. Further, to test the hypothesis that levels of potentially more reactive iron in the form of non-transferrin-bound iron (NTBI) might correlate more closely with coronary artery disease than levels of serum ferritin, NTBI was measured in a number of the study's subjects.

7.3 Method

7.3.1 *Subject recruitment*

286 patients attending for consecutive day-case coronary angiography lists over a number of months at the Royal Infirmary of Edinburgh were enrolled. Shortly after arrival at the hospital, patients were given a brief verbal description of the study and a sheet of written information (Appendix 6) by one of the cardiology clinical nurse specialists. When they had read the information sheet, written informed consent for inclusion in the study was sought. The general practitioners of patients agreeing to participate were informed of this by means of a pre-printed letter.

7.3.2 *Sample collection*

Blood was taken from consenting patients for determination of serum ferritin and erythrocyte sedimentation rate (ESR). A sample in EDTA was taken for extraction of DNA and later HFE gene mutation analysis and a serum sample was taken from 39 subjects out of the first 120 included (selected randomly) for measurement of non-transferrin-bound iron (NTBI). All samples were taken in the morning (usually before ten o'clock) with the patient fasting, as demanded by the protocol for coronary angiography. All samples were taken and conveyed to the laboratory at ambient temperature. The samples for ESR and ferritin were dealt with under standard laboratory conditions in the clinical chemistry and haematology laboratory respectively of the Royal Infirmary of Edinburgh. The samples for DNA extraction were stored at 4°C until DNA extraction was performed (within seven days). The samples for NTBI determination were centrifuged at 1500g for ten minutes as soon as possible after arrival in the laboratory, serum was removed and frozen at minus 75° C for several weeks before NTBI could be measured.

7.3.3 *Data collection and analysis*

The cardiology nursing staff assigned patients in the study consecutive numbers at the time that they gave consent. Study blood samples were identified by this number alone. The angiogram results were assessed by a cardiologist and reported as showing atherosclerotic disease in zero, one, two or three coronary arteries. The patients' age and smoking history, body mass index (BMI), and presence or absence of hyperlipidaemia, hypertension and diabetes mellitus were taken from the pre-angiography admission sheet, whenever this information was provided. The full case notes of the patients were not available for further extraction of data.

The author of this thesis was blinded to the angiographic and epidemiological data until all laboratory analyses had been completed. The results of the angiogram and epidemiological information were identified by study number only, and once the reporting of the angiograms was complete, anonymity was assured by the destruction of the master list relating study number to patient name. Patients with no coronary artery disease (no vessels affected) at angiography were taken as the control group and patients with one or more diseased vessels as the study group.

7.3.4 *Coronary Angiography*

Diagnostic coronary angiography was performed from the femoral artery using a standard protocol. Five views of the left coronary arterial system and two views of the right coronary arterial system were taken and each angiogram was reviewed by two independent cardiologists, who were unaware of the results of iron studies and HFE gene mutation analysis. For each angiogram a score of one was given to each diseased arterial system and a score of two to disease in the left main coronary artery. An arterial system was considered diseased if there was a lesion of greater than 50 percent of the vessel diameter in an epicardial vessel or of greater than two millimetres in the left anterior descending, the left circumflex or the right coronary systems.

7.3.5 *Assay of non-transferrin bound iron*

Professor J B Porter (King's College, London) kindly agreed to perform the non-transferrin bound iron assays. Frozen plasma samples were therefore posted to his laboratory for this purpose. For a representative method, see for example Singh, 1990.

7.4 Statistical Analysis

Correlation between number of affected coronary vessels and non transferrin-bound iron and between genotype and non transferrin-bound iron was examined by the Kruskal Wallis test for non-parametric variables. The association of disease in 0 vessels and disease in 1 or more vessels with genotype was examined with the χ^2 test. The presence of an association between ferritin and the presence of disease in 0 or 1 or more vessels was examined with the F test for equality of variances and the t-test for equality of means of non-paired data. The other characteristics of the two populations were compared by χ^2 analysis.

7.5 Results

62 patients had no significant coronary artery disease at angiography and 213 patients had angiographically identifiable coronary disease. Those 11 patients for whom there was no angiographic data could not be allocated to either group and were excluded from analysis. The characteristics of these two groups are displayed in table 7.1. Data collection was incomplete for a large number of patients. The numbers concerned are illustrated in table 7.1 for each category of data. Data on the gender of the patients were not collected in this study.

The HFE genotype was determined for 245 out of a total of 286 patients. 55.3% of the patients for whom a genotype was available were wild type for HFE, 10.7% were heterozygous for C282Y and 16.7% heterozygous for H63D. The genotyped study population contained no C282Y homozygotes, 1.5% homozygotes for H63D and 2.4% compound heterozygotes for C282Y and H63D. The distribution of these genotypes between the two study groups is illustrated in table 7.2.

There was no significant difference between the two groups in terms of the mean body mass index (BMI), age, or other cardiovascular risk factors (previous myocardial infarction (MI), hyperlipidaemia, established hypertension, diabetes mellitus (DM) or smoking habit (current versus non-smoker or ex-smoker combined)). Mean serum ferritin also did not differ between the groups. The ESR was measured as a global assessment of acute phase activation (because ferritin is an acute phase protein) and did not differ between the groups.

Table 7.1 Characteristics of the populations with and without coronary artery disease at angiography

		Number of affected vessels		p
		0	1-3	
	N	62	213	
	Age	62 (60.8-63.2)	60.6 (58.3-62.9)	ns
	BMI	28.4 (27.1-29.7)	28.0 (27.4-28.6)	ns
	ESR	9.9 (8.4-11.4)	12.3(8.7-15.8)	ns
	ferritin	130.9 (108.5-153.4)	123.5 (97.3-149.5)	ns
	ntbi	See text		
diabetes	yes	4	30	ns
	no	27	101	
	no data	31	82	
hypertension	yes	20	53	ns
	no	12	79	
	no data	30	81	
hyperlipidaemia	yes	17	90	ns
	no	18	40	
	no data	27	83	
smoking	current	5	21	ns
	no or ex	27	104	
	no data	30	88	
previous MI	yes	4	47	ns
	no	27	92	
	no data	30	74	

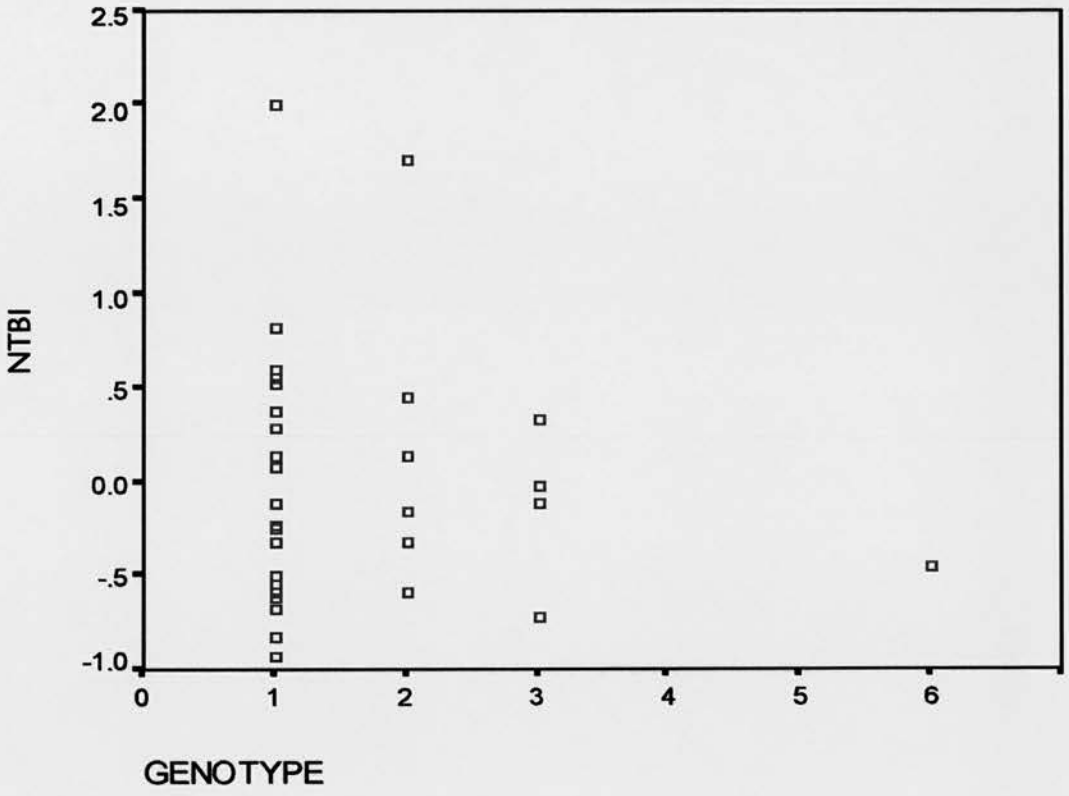
ns = not significant

Table 7.2 Distribution of HFE genotypes between the two study populations

	Number of vessels		
	0	1-3	
Genotype	n (%)		p
HC/HC	36 (66.7)	118 (64.8)	ns
HY/HC	5 (9.3)	25 (13.7)	ns
DC/HC	10 (18.5)	30 (16.4)	ns
HY/HY	0	0	ns
DC/DC	1 (1.8)	4 (2.2)	ns
HY/DC	2 (3.7)	5 (2.7)	ns

ns = not significant

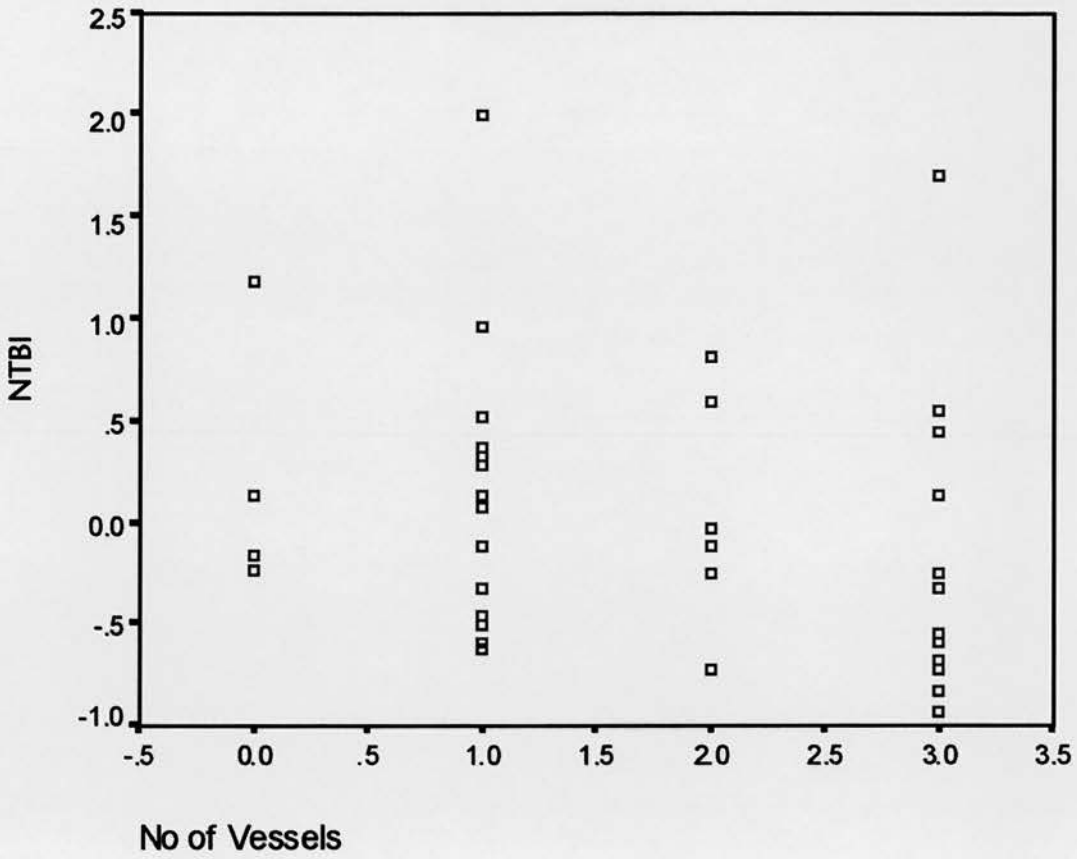
Figure 7.3 Scatter plot of the relationship between HFE genotype and non-transferrin-bound iron (NTBI)



Key to genotype

1. HC/HC
2. HY/HC
3. DC/HC
4. HY/HY
5. DC/DC
6. HY/DC

Figure 7.4 Scatter plot of the distribution of non-transferrin-bound iron (NTBI) according to angiography scores



The selection of 39 samples analysed for non-transferrin bound iron was envisaged as a pilot possibly to be followed by analysis of more samples if there was enough evidence from the results of these initial samples to justify this. Of the 39 samples in which NTBI was assayed, 33 were from patients in the group with coronary artery disease and only four from the other group (assignment of samples for NTBI measurement was random and was performed before the results of the angiography were available). Two samples were from patients excluded from analysis because of lack of angiographic data. The values ranged between –0.833 and 2.006 (normal control: –2.412; high control (thalassaemic patient): 5.368). There was no correlation between NTBI and gentoype ($p = 0.685$, Kruskal Wallis) or NTBI and number of coronary vessels diseased ($p = 0.434$, Kruskal Wallis). These data are presented as scatter plots in figures 7.3 and 7.4.

7.6 Discussion

Evidence from other studies, the details of which are given in section 7.1 above, has suggested the possibility of a relationship between body iron stores and coronary artery disease (Sullivan, 1983, Salonen, 1992, Tuomainen, 1999). Furthermore, it is postulated that iron not in the form of ferritin, but in a form more readily able to promote free radical formation and lipid peroxidation may be most implicated (McCord, 1991).

This study was designed to investigate both these postulates. In the two groups investigated, individuals with or without angiographic evidence of coronary artery disease, there was no significant difference in iron loading as expressed by the serum ferritin. In the group with evidence of coronary artery disease, 20 individuals had a ferritin <20 µg/L, indicating possible iron deficiency, and 38 had ferritin values in excess of 200 µg/L. The numbers for the group without coronary artery disease were 8 and 10 respectively. The work of Salonen and colleagues (1992) suggested a value of 200 µg/L for ferritin, above which the risk of coronary heart disease increased. However, these values are not directly comparable because Salonen's patient group was composed entirely of men whereas the present study population comprised patients of both genders.

The measurement of non-transferrin bound iron (NTBI) provides an assay of the amount of free-radical forming iron (Breuer, 2000). There is very little clinical data available to indicate whether there is a correlation between NTBI levels and coronary artery disease. It is known that individuals with iron overload, whether genetic (Gutteridge, 1985) or secondary (Hershko, 1978) have increased NTBI in their serum, whereas this is not found in normal individuals who have a largely unsaturated

transferrin (Al-Refaie, 1992). It seems reasonable to suggest, therefore that a correlation between raised serum ferritin and coronary artery disease would be mirrored in a correlation between NTBI and coronary artery disease. The present study did not find evidence to support this, although the number of individuals in whom NTBI was measured was very small (particularly in the group with no disease on coronary angiography). The small number concerned clearly limits the ability to draw general conclusions from these data. Many of the NTBI results produced negative values. This has been noted by others (Singh, 1990) and is an artefactual finding in patients with normal iron stores. It may be due to the ability of unsaturated transferrin to remove iron contamination from nitrilotriacetic acid used in the assay (Al-Rafaie, 1992).

No C282Y homozygotes were found among the patients studied. The frequency of C282Y heterozygotes was 18.5% in the group with no coronary artery disease and 16.4% in the group with abnormal angiography and that of heterozygotes for H63D was 9.3 and 13.7% respectively (table 7.2). These values are comparable to those obtained in other population studies (Boulton, 2000; Jackson, 2001; Miedzybrodska, 1999) and also in other parts of this work.

7.7 Conclusions

This study did not find evidence of an association between serum ferritin and coronary artery disease. This is an important negative result, which contributes to the on-going debate about the role of iron in the pathogenesis of coronary artery disease.

There was no correlation either between or non-transferrin bound iron (NTBI) and coronary artery disease or HFE genotype. The potential role of NTBI merits further investigation, as it is recognised that the unequal distribution of NTBI data between the control and study groups limits the advisability of drawing very definite conclusions about the role of this moiety in causing coronary artery disease.

8. Survey of General Practitioners' attitudes to and experiences of haemochromatosis

8.1 Introduction

The weight of evidence that haemochromatosis is under-diagnosed has been discussed at some length in section 2.5, above. A survey conducted in Alabama in the United States (Barton, 1997) revealed that over 85% of haemochromatosis probands were diagnosed by physicians working in primary care (66.7%), gastroenterology (10.5%) and haematology/oncology (10.5%). Many of the physicians interviewed identified difficulties in diagnosing haemochromatosis, including:

1. The misconception that haemochromatosis is rare;
2. Lack of understanding of the diagnostic criteria for haemochromatosis
3. Unfamiliarity with the multi-system disease caused by iron overload;
4. The belief that haemochromatosis could be diagnosed only in those persons with bronze skin colouration, diabetes and cirrhosis.

These difficulties were addressed in an intensive education programme (see section 2.6.2).

In 1997, a conference on Iron Overload, Public Health and Genetics was held in the United States, sponsored by the Centers for Disease Control and Prevention. As part of this conference, a working group was established to identify strategies to increase the detection of haemochromatosis. The report of their recommendations (McDonnell, 1998a) identified physician education as a priority.

As far as the researcher is aware, there is no comparable information on physicians' attitude to haemochromatosis in the United Kingdom. It is very likely that, because of the very different organisation of health care in the United Kingdom and the United States, a very significantly lower proportion of patients with haemochromatosis are diagnosed by general practitioners in the United Kingdom than by primary care physicians in the United States.

If, in the future, it is proposed to institute population screening for hereditary haemochromatosis, the participation of general practitioners is likely to be crucial to the success of the programme.

In light of this the aim of the present study was to assess the level of knowledge about haemochromatosis and some of the attitudes to the condition prevalent among general practitioners (GPs) in Edinburgh and the surrounding area by means of a postal survey.

8.2 Data collection – theoretical considerations

The group whose attitudes were to be assessed was well-defined and potentially easily accessible. It was decided to use a questionnaire to obtain the data, but a number of issues relating to the methods of data collection to be used required addressing: a questionnaire may be administered face-to-face, over the telephone, by post or by e-mail. All these methods have strengths and weaknesses. Personal interviews may be best at dealing with complex topics and at eliciting responses to questions that could be considered boring (de Vaus, 1996). In addition, the personal contact of face-to-face interviews means that these can last longer than the other two methods. Traditionally, it has been held that face-to-face interviews elicit a better response rate, particularly in comparison to postal surveys. However, Dillman (1978) contends that a well-conducted postal survey of the general public may achieve a response rate of 60-75%, which compares reasonably favourably with his achievement of 85% response in telephone surveys. When a specific, more homogeneous group (such as GPs) is surveyed, the response rate to postal surveys is about the same as that for surveys conducted by other means. This is especially so when the subject of the survey is of particular relevance to the group (de Vaus, 1996).

Issues of practicability and resource must also be considered. General Practitioners are subject to so many requests to complete questionnaires that this is viewed by many of them as an irritation (Dr Scott Murray, personal communication, 1999). It was considered, therefore, that time taken to complete the questionnaire and the degree to which those surveyed felt it an intrusion on their working day would be important factors in determining response rate. For this reason, taking into account also issues of cost and allocation of the researcher's time, it was decided to develop a postal questionnaire with a maximum length of one side of A4 paper.

The areas of knowledge about haemochromatosis to be assessed by the questionnaire were:

1. Perceptions about prevalence
2. Laboratory tests and clinical features useful and/or necessary to make the diagnosis
3. Natural history

In addition, it was desired to gather information about individual physicians' experience of patients with haemochromatosis within their own practice and their attitude to screening for haemochromatosis. The questionnaire used is reproduced in Appendix 8. In formulating questions about laboratory tests for diagnosing haemochromatosis, clinical features of the disease and its natural history, an "agree-disagree" format without any Likert-style rating scale was favoured. This was for ease of completion; the ease of analysis of the response data may have suffered as a result.

8.3 Method

Before the questionnaire was distributed to the whole study group, it was piloted it among general practitioners in two large practices, one an academic practice with six partners, the other an urban practice of eight partners. Prior discussions with one physician from each of these practices had been very helpful in the development of the format of the questionnaire and the questions themselves. Responses from the piloting exercise were favourable, and no major changes were made to the questionnaire as a result of this.

A list of mailing addresses for all general practitioners in the Lothian Health Board area was obtained from Lothian Health's Directorate of Primary Care Development in January, 1999. The list contained 540 names.

In order to raise awareness of the forthcoming study, a short paragraph about it was published in the March, 2000 issue of *GPs Only*, the newsletter for GPs published by Lothian Local Medical Committee and distributed to all GPs in the Lothian area. This gave very basic details about the study, intimated that a questionnaire about haemochromatosis would shortly be posted to all GPs, and provided contact details for the researcher.

Within a month of the distribution of the newsletter, the questionnaire was sent out with a personally signed covering letter to all the GPs in the area. The covering letter was printed on Lothian University Hospitals NHS Trust headed notepaper and

identified the researcher's position as Lecturer in Haematology. As the pilot had involved 14 GPs, a total of 526 questionnaires were posted. The Health Board van delivery service, which covers all the practices, was used to distribute the questionnaire. To facilitate the return of completed replies, every questionnaire was accompanied by a pre-addressed return envelope bearing a second class stamp. The name of the physician returning the questionnaire or of the practice in which he or she worked was not recorded.

8.4 Results

The number of completed questionnaires returned was 258. In the absence of any evidence to the contrary, non-return of questionnaires was interpreted as refusal to answer, rather than failure of the questionnaire to reach its intended recipient. The response rate was, therefore:

258 x 100/540 = 48%

Table 8.1 displays the data provided in response to question 1 about the practice sizes of the physicians returning the questionnaires.

Table 8.1 Distribution of GP practice size among respondents

List Size (number of patients)	Number (%) of respondents
<3000	20 (8)
3000-6000	65 (25)
6000-9000	83 (36)
9000-12000	47 (18)
>12000	30 (12)
No answer	3 (1)

Question 2 asked GPs how many patients with haemochromatosis they were aware of in their own practice. The majority (178 (69%)) of respondents were not aware of any patients in their practice with haemochromatosis. One respondent, whose practice size was between 6000 and 9000 was aware of seven patients with haemochromatosis in the practice. The full data are presented in table 8.3.

Table 8.2 Number of patients with haemochromatosis identified by physicians within their own practice (responses to question 2)

Number of patients	Number (percentage) of physicians identifying a given number of patients in their practice
0	178 (69)
1	48 (19)
2	18 (7)
3	7 (3)
4	2 (1)
5	0
6	0
7	1 (1)
>7	0

Question 3 asked GPs about the relative prevalence of haemochromatosis and cystic fibrosis.

37 (14%) responding physicians considered haemochromatosis commoner than cystic fibrosis; 98 (38%) thought the two conditions were "about as common" as each other; 112 (44%) considered cystic fibrosis more common; 9 (4%) failed to answer the question.

Question 4 asked about the laboratory tests that would be helpful in confirming a diagnosis of haemochromatosis. The results are displayed in table 8.3.

Table 8.3 Laboratory tests that would be helpful in confirming a diagnosis of haemochromatosis

Test	Number (%) of respondents classifying test as useful
Full blood count	130 (50)
Ferritin	209 (81)
Urea & electrolytes	239 (93)
Iron/TIBC	191 (74)
Genetic test	77 (30)

It was permitted to choose any number of the five test options in this question. A total of five respondents (2%) selected no test. Of those who selected at least one of the options provided, only five (2%) did not include either serum ferritin or iron/TIBC. One of these selected full blood count alone, two, full blood count and genetic test, and two, genetic test alone.

Question 5 asked respondents about the necessity or otherwise of given symptoms and signs to make a diagnosis of haemochromatosis. The response data are presented in table 8.4:

Table 8.4 Opinions on the necessity of various clinical signs and symptoms for the diagnosis of haemochromatosis to be made

	Number (%) of respondents identifying sign/symptom as:		
Sign/symptom	Necessary	Unnecessary	Don't know
Skin discolouration	42 (16)	175 (68)	41 (16)
Abnormal LFT	68 (27)	140 (54)	49 (19)
Diabetes	12 (4)	211 (82)	35 (14)
Arthritis	12 (4)	211 (82)	35 (14)

Question 6 asked physicians to agree or disagree with statements about the natural history of haemochromatosis. The responses are displayed in table 8.5.

Table 8.5 Opinions on the treatment and presentation of haemochromatosis

	Number (%) of respondents stating reporting following attitudes to each statement:		
Statement	Agree	Disagree	Don't know /no answer
Early treatment restores normal life expectancy	135 (52)	27 (10)	95 (37)
If untreated, it is often fatal	183 (71)	23 (9)	48 (19)
Treatment involves the use of expensive drugs	11 (4)	154 (60)	91 (35)
Younger patients are often asymptomatic	204 (79)	9 (3)	44 (17)

In response to question 7, 88 physicians (34%) said that they had ever suspected that a patient might have haemochromatosis. 178 physicians (66%) had never suspected that a patient of theirs had haemochromatosis. In question 8, those physicians who had suspected such a diagnosis were asked to state the grounds for their suspicion. 84 respondents replied to this question, often giving more than one symptom that had aroused their suspicion. The percentages that follow, therefore, add up to more than 100 and the figures to more than 84. Abnormal liver function tests (LFT) was quoted by 24 (30%) respondents with one remarking that he/she always tests for haemochromatosis as part of his screen for causes of abnormal LFT. A family history of haemochromatosis and skin discolouration were the next two most common prompts to testing for haemochromatosis, both quoted by 24 (29%) of those who responded. The changes in skin colour were variously reported as "cyanosed", "brownish", "high colour", "easily tanned" (1 respondent each) or simply "discoloured". Abnormalities in serum ferritin, iron or total iron binding capacity were the next most common, reported by 17 (20%). A diagnosis of diabetes mellitus was quoted by 11 (13%) and 8 had become suspicious on the basis of an elevated haemoglobin concentration. In 4 cases (5%), joint pains were the stimulus to further investigation, as were abdominal pain and tiredness in 2 cases (3%) each and in 1 case each (2%) recurrent transfusions, iron therapy, liver ultrasound appearance and hepatomegaly. One physician tested a patient because of previous experience with another similar patient and one reported that he/she would normally seek specialist advice before testing.

The penultimate question, question 9, asked about physicians' attitudes to screening for haemochromatosis. The response data are presented in table 8.6.

Table 8.6 Physicians' attitudes to screening for haemochromatosis

	Number (%) of respondents reporting these attitudes to statements about a screening programme for haemochromatosis		
Would you see screening as:	Agree	Disagree	No answer
A useful addition to the work of primary care	69 (27)	108 (42)	81 (31)
Something of no relevance to primary care	49 (19)	110 (43)	99 (38)
An extra burden on primary care	190 (74)	20 (8)	48 (19)

8.5 Comments from General Practitioners

At the end of the questionnaire, in question 10, respondents were given an opportunity to comment. 89 (34%) respondents commented, mostly to amplify their attitudes to screening. As they provide an interesting insight into the opinions of respondents, I have reproduced a selection below. For ease of reading, I have divided the comments into three broad groupings each under a separate sub-heading:

Expression of lack of knowledge about (aspects of) haemochromatosis

"Sorry I'm so ignorant."

"If I knew more about it I might be in a better position to judge if screening would be useful."

"Who would you be screening?"

"I don't know enough to answer this."

"Cannot comment without knowing more about frequency of condition, cost-effectiveness."

"This has brought to my attention that I know very little about the condition – I will be reading up about it in my Davidson tonight."

"Unfortunately, my knowledge of incidence etc is so poor I am unable to answer Q9 [screening] sensibly."

"Incidence has to be of an order to merit the extra workload – I have no idea what the incidence is."

Need for improved resources

"Extra resources would have to be identified to put into place primary screening."

"Too, too busy! What would be the pick up rate?"

"Depends on resources given – who is to do the work?"

"Screening would be another thing to add to the lengthening list, but don't know enough about it."

"Would have to be targeted and financed. NHS won't pay for my Multistix at present! Or in house FOB! Or in house pregnancy tests!"

"Primary care may be the place to screen, but huge burden on primary care team who already have too much to do in too little time."

"There are no resources of time or money in primary care to carry this out."

Expressions of uncertainty about justification for screening or for GPs' role in it.

" I would have to be sure a screening programme met all the criteria required."

"Primary care unable to take on any more screening – this is not our role."

"I am worn out screening! GPs should screen for common pathologies (ie 1:100 patients). Still find a lot with raised blood pressure, lipids; would find none with hereditary haemochromatosis. GPs are good at decision-making. Nurses are good at following protocols. This is a job for Mr Blair's new nurses."

"In more than 30 years I have only seen 2 patients, both in hospital practice. Suggest you work as a GP for 6 months, then you will see truly common conditions."

"Screening is only useful if treatment at an early stage improves prognosis."

"I have the feeling that this is extremely rare."

"I've only seen haemochromatosis once in 20 years of practice – I see dozens of cervical, breast and prostate cancers which would benefit more from screening."

"Never seen a case in 15 years of practice. I would think there are a lot more priorities than haemochromatosis."

"Have enough workload ++ without more screening programmes in practice. Leave the GP to screen opportunistically if he suspects the condition clinically or positive family history."

"Screening of patients with a family history OK. Regular screening tests would provide an unacceptable workload."

8.6 Discussion

The use of surveys as research tools has a number of potential pitfalls. In particular, they have been accused of being basically empiricist - of collecting a mass of facts and figures while providing nothing of theoretical value (Mills, 1959). Further, they may abstract people's opinions and actions out of the context in which they are formed, making it difficult to draw valid conclusions about the significance of the data collected. Additionally, their scope may be felt limited by the very fact that they rely on pre-written questions – a "one size fits all" approach that may not always be appropriate and may severely hamper flexibility in data collection.

Certainly, the use in this study of a questionnaire to assess GPs' opinions about hereditary haemochromatosis may be open to these criticisms and others. A longer questionnaire may have provided more detailed information and avoided the somewhat arbitrary selection of questions made for reasons of space, but would have risked a very significantly lower response rate. Face to face interviews would have allowed more in depth exploration of and expansion on physicians' views but again risked a higher rate of refusal to participate and would have been limited in practice for reasons of resource.

The issue of validity of the questionnaire as an instrument for investigating a hypothesis was raised by at least one respondent. It is difficult to know what the ideal way of validating the questionnaire would have been – a point that de Vaus (1996) makes about questionnaires in general. The questions were developed to be as open as possible, to avoid the pitfall of leading respondents to support the hypothesis under examination, that is that there may be scope for improved awareness of haemochromatosis among physicians in primary care. The aim of piloting the study before distributing it more generally was to identify questions that were unhelpful, biased or leading. The piloting process and resulting minor adjustments to the questionnaire can equally well be seen as an attempt at validation.

The aim of the questionnaire was to formulate questions which would indicate the awareness of respondents about what could be considered key areas of haemochromatosis: its prevalence; how it is diagnosed; how it may present clinically; its natural history and treatment.

In formulating a question about prevalence, I chose comparison with another genetic condition (cystic fibrosis) which has a relatively high incidence (1:2500 live births (Crompton, 1999)). The validity of this comparison could be questioned because of

the very different natural histories of the two diseases, and particularly the fact that cystic fibrosis is often diagnosed in infancy, while classical hereditary haemochromatosis never is. However, the alternative to this, supplying a choice of different incidence figures, was considered likely to be less meaningful to most of those answering the question. The hypothesis that this question was designed to test was that many GPs consider haemochromatosis a rare condition. Had a scale of incidences been used, it would have been necessary to make an arbitrary decision about the incidence at which rarity began. As it was, a large majority (82%) of the responding GPs thought that haemochromatosis was less common or about as common as cystic fibrosis, whereas, in fact, C282Y homozygosity occurs at about ten times the incidence of clinical cystic fibrosis. Notwithstanding the issue discussed in sections 2.5 and 2.6.1, above, whether the homozygous state for C282Y does or does not equate to haemochromatosis, the consensus that hereditary haemochromatosis is under-diagnosed makes the question an important one. The rarity of haemochromatosis was also a recurring topic in the free comments in response to question 10.

Questions about the diagnosis and clinical presentation of the disease present their own problems. Exactly which tests one would perform if one suspected a patient had haemochromatosis would, of course, depend on the clinical context. The hypothesis here was that most physicians would be aware of the utility of measuring body iron stores in assessing a patient with possible haemochromatosis and this was borne out by the responses. "Genetic test" was included as one of the choices for a laboratory investigation. The nature of the test was not specified, and its role in diagnosis is controversial as already discussed. It is probably not surprising and possibly appropriate, therefore, that only a minority of respondents thought it of potential use. The symptoms and signs in the question about clinical presentation were chosen to cover the "classical" textbook presentation of advanced haemochromatosis. Again, the criticism of arbitrariness can be made (and, as one respondent pointed out, no mention is made of hypogonadism). However, the aim was to assess the extent to which GPs may be relying on the presence of these symptoms and signs to guide them in their clinical decision-making. For each symptom and sign, there was a small minority of respondents who thought that it was necessary for diagnosis. 121 (47%) of respondents, however, were of the opinion that none of the four listed was required.

The statements about the natural history and treatment of haemochromatosis were chosen for their pertinence to screening. Two features of the responses to this question are remarkable: first, the large minority of respondents to every question who declared that they did not know the answer. Failure to provide an answer was also interpreted as

"do not know". Secondly, in each of the four constituent parts to the question, the large majority of those who did answer gave responses that accord with current expert opinion.

The responses to these questions taken together with the comments provided may support the contention (McDonnell, 1998a and 1998b; Barton, 1997) that there is scope for increasing the awareness of primary care physicians about haemochromatosis. Many GPs seem aware that their level of knowledge about the condition could be improved. This does not, of course, imply that they would see participation in an education programme as a priority to be included their already packed timetables. As one respondent commented:

"GPs can't be expected to know all illnesses/conditions in detail."

The small proportion of respondents who had ever suspected a diagnosis of haemochromatosis in one of their patients may reflect this knowledge deficit or the perception that it exists. The relatively low level of awareness of haemochromatosis patients by responding physicians is difficult to interpret in detail as no information was requested about the means of information gathering. For example, some practices may have the facility to call up patients with a particular diagnosis from a computerised database, while others may not have access to such information. Additionally, some GPs may have preferred to answer the question in terms of their own awareness, regardless of the availability of a database. However, it supports the contention of Ho (2001) and others (Merryweather-Clarke, 1998) that the number of known patients with haemochromatosis is far less than population prevalence studies would suggest.

That skin discolouration featured so prominently in the list of features that led respondents to suspect the diagnosis of haemochromatosis in their patients is perhaps surprising. It is clear from the data presented in table 8.4 that the majority of physicians are aware that skin discolouration is not a necessary feature in haemochromatosis. A recent series reported an incidence of skin colouration of only 5% (Bacon, 1997). Earlier reports suggested that the majority of patients displayed alteration in skin colour, and the fall in this proportion must reflect earlier diagnosis, as there is a clear correlation between hyperpigmentation and hepatic iron concentration (Adams, 1997).

Responses to questions on screening illustrated once again the substantial degree of uncertainty that exists in this population of health professionals about haemochromatosis and its relevance to their practice. Many of the comments reflected the degree to which some primary care physicians already feel swamped by among

other things, demands that they participate in screening programmes. The balance of the responses suggests that any moves to introduce population screening in primary care in the UK would require not only a substantial injection of resources but also may be likely to meet with considerable scepticism among primary care physicians about its usefulness.

8.7 Conclusion

This study is, at the time of writing, the only one to attempt a comprehensive survey of general practitioners in any part of the UK about haemochromatosis. Despite its imperfections (discussed above) it has yielded some valuable results and indicated that there appears to be significant variation in general practitioners' experience and awareness of this condition. Some are clearly aware of recent advances in diagnosis our knowledge of pathogenesis, while others are less so. It may be reasonable to assume that this variability is not confined to physicians in primary care, but may extend also into secondary and tertiary care. Respondents' comments make plain, however, that dealing with this variability may not be straightforward. It is not clear, for example, what the uptake would be of any physician education programme that was introduced.

The debate about wider population screening continues. It is clear that many physicians in primary care in the UK remain to be convinced about its utility.

9. Concluding Comments

The last decade has seen huge progress in our understanding of both the genetics of inherited iron overload and also of the molecular mechanisms responsible for the regulation of iron absorption and storage in the body in both health and disease.

The identification of the genetic basis for the most common form of hereditary haemochromatosis led initially to widespread calls for the development of a programme of population screening to enable the detection of affected individuals early in the course of the disease, before the development of any permanent organ damage caused by iron overload. In recent years, however, the early enthusiasm for population screening has waned with the realisation that the penetrance of the C282Y mutation of HFE appears to be very low. It is, nonetheless, clear that it is vital to identify those individuals who are accumulating iron early in the course of the disease if treatment is to be effective at preventing morbidity and mortality from iron overload. An increased awareness of haemochromatosis among healthcare personal may be crucial in ensuring that this can occur.

The extent to which information about progress in understanding iron overload has permeated the healthcare community is, however, uncertain. Research in the United States has demonstrated that considerable ignorance about the diagnosis, natural history and treatment of hereditary haemochromatosis persisted among healthcare workers in the late 1990s. It is difficult to be certain how well informed British health care workers are about hereditary haemochromatosis. The survey of general practitioners presented as part of this thesis suggests that there is wide variation in awareness of iron overload and in willingness to be involved in any screening programme that might be proposed. This provided a useful and interesting snapshot of opinion – and it is probably reasonable to assume that general practitioners in the southeast of Scotland would not differ dramatically in their answers from those elsewhere in the UK. However, whether such surveys are a valid tool to estimate detection rates and outcomes of haemochromatosis patients in the practices of those surveyed is by no means certain, as remarked upon by at least one of the respondents. It is this information that is crucial in the planning of any future public health strategies targeting hereditary haemochromatosis.

Speculation about the reasons for the continuing high prevalence of the C282Y mutation in the population of northern Europe has been focussed on the possibility that it may provide some measure of protection against iron deficiency and therefore afford carriers some evolutionary advantage. It is difficult in practice to demonstrate that this is the case, and the findings presented in this thesis did not show any differences in the prevalence of the various HFE genotypes in an iron deficient and a non iron deficient population. Furthermore it has become clear that it is not feasible to take advantage of the putative iron retaining tendencies of heterozygotes for C282Y to provide a population of blood donors who are able to donate more frequently without themselves becoming deficient in iron.

The results of iron studies and HFE genotyping among patients undergoing coronary angiography found in this work make a useful addition to the debate on whether iron and/or C282Y heterozygosity is a risk factor for coronary artery disease. The difficulty in controlling for other coronary risk factors goes some way to explaining the conflicting results produced by the various studies that have examined this question, but the balance may be moving against the hypothesis that either of these two variables is a significant risk factor. In principle, it is iron which is not bound to transferrin in the circulation that might be expected to be most active in the promotion of tissue damage, and it is interesting that no correlation was found in the work presented here between non-transferrin bound iron and the severity of coronary artery disease (albeit with the methodological caveats set out in chapter 7 of the thesis).

The role of iron and, by extrapolation, of the HFE mutations in other pathological states has been widely examined in the decade since the discovery of HFE. It is, of course, well known that the liver is particularly prone to damage in patients with untreated hereditary haemochromatosis. The extent to which undiagnosed hereditary haemochromatosis may be contributing to liver damage in patients coming to orthotopic liver transplantation is illustrated by the results of HFE genotyping of the patients transplanted in the Scottish Liver Transplant Unit presented as part of this thesis. This is important not only to allow the identification of patients who may be at risk of reaccumulation of iron after transplantation, but also because of considerable evidence that patients with hereditary haemochromatosis may be more prone to complications after liver transplantation than patients with other causes of hepatic failure, notwithstanding the failure of the results presented in this thesis to demonstrate such a less favourable outcome.

The role played by HFE in normal and pathological iron metabolism remains tantalisingly unclear. When eventually, the mechanisms are teased out, it may become clearer at the molecular level whether or not C282Y is likely to contribute to iron-mediated tissue damage. Many further cohort and population-based studies will doubtless also continue to add to our knowledge in this area.

References

Abboud S., Haile D.J. (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *Journal of Biological Chemistry* 275: 19906-19912

Adam D. (2001) UK changes policy on life insurance. *Nature* 414: 6

Adams P.C., Kertesz A.E., Valberg L.S. (1991) Clinical presentation of hemochromatosis: a changing scene. *American Journal of Medicine* 90: 445-449

Adams P.C. (1994) Prevalence of abnormal iron studies in heterozygotes for hereditary hemochromatosis: an analysis of 255 heterozygotes. *American Journal of Hematology* 45: 146-149

Adams P.C., Gregor J.C., Kertesz A.E., Valberg L.S. (1995) Screening blood donors for hereditary hemochromatosis: decision analysis model based on a 30-year database. *Gastroenterology* 109: 177-188

Adams P.C., Deugnier Y., Moirand R., Brissot P. (1997) The relationship between iron overload, clinical symptoms and age in 410 patients with genetic hemochromatosis. *Hepatology* 25: 162-166

Adams P.C. (1999a) Population screening for hemochromatosis *Hepatology* 29:1324-1327

Adams P.C. and Valberg L.S. (1999b) Screening blood donors for hereditary hemochromatosis: decision analysis model comparing genotyping to phenotyping. *American Journal of Gastroenterology* 94: 1593-1600

Adams P.C. (2000a) Population screening for haemochromatosis *Gut* **46**: 301-303

Adams P.C., Kertesz A.E., McLaren C.E., Barr R., Bamford A., Chakrabarti S. (2000) Population screening for hemochromatosis: a comparison of unbound iron-binding capacity, transferrin saturation and C282Y genotyping in 5,211 voluntary blood donors. *Hepatology* **31**: 1160-1164

Advisory Committee on Genetic testing. (1998) Report on genetic testing for late onset disorders. London: Health Departments of the United Kingdom

Aguilar Martinez P., Biron C., Blanc F., Masmejean C., Jeanjean P., Michel H., Schved J-F. (1997) Compound heterozygotes for hemochromatosis gene mutations: may they help to understand to understand the pathophysiology of the disease? *Blood Cells, Molecules, and Diseases* **23**: 269-276

Aguila Martinez P., Esculié-Coste C., Bismuth M., Giansily-Blaizot M., Larrey D., Schved J-F. (2001) Transferrin receptor-2 gene and non-C282Y homozygous patients with hemochromatosis. *Blood Cells, Molecules, and Diseases* **27**: 290-293

Allen K. and Williamson R. (1999) Should we genetically test everyone for haemochromatosis? *Journal of Medical Ethics* **25**: 209-214

Allen K. and Williamson R. (2000) Screening for hereditary haemochromatosis should be implemented now. *British Medical Journal* **320**: 183-184

Alper J.S., Geller L.N., Barash C.I., Billings P.R., Laden V., Natowicz M.R. (1994) Genetic discrimination and screening for hemochromatosis. *Journal of Public Health Policy* **15**: 345-358

Al-Rafaie F.N., Wickens D.G., Wonke, B., Kontoghiorghes G.J., Hoffbrand A.V. (1992) Serum non-transferrin-bound iron in beta-thalassaemia major patients treated with desferrioxamine and L1. *British Journal of Haematology* **82**: 431-436

Alvarez-Ossario L., Kirchner H., Klüter H, Schlenke P (2000) Low ferritin levels indicate the need for iron supplementation: strategy to minimize iron-depletion in regular blood donors. *Transfusion Medicine* **10**: 107-112

Andersen R.V., Tybjærg-Hansen A., Appleyard M., Birgens H., Nordestgaard B.G. (2004) Hemochromatosis mutations in the general population: iron overload progression rate. *Blood* **103**: 2914-2919

Anía B.J. and Rodríguez-Gallego C. (1999) Transfusion with blood from people with HFE gene mutations. *Lancet* **351**: 430.

Anonymous. From the NIH: use of neocytes decrease the frequency of transfusion and reduce iron overload. (1979) *Journal of the American Medical Association* **242**: 2669-1670

Arya N., Chakrabarti S., Hegele R.A., Adams P.C. (1999) HFE S65C variant is not associated with increased transferrin saturation in voluntary blood donors. *Blood Cells, Molecules and Diseases* **25**: 354-357

Asberg A., Hveem K., Thorstensen K., Ellekjter E., Kannelonning K., Fjosne U., Halvorsen T.B., Smethurst H.B., Sagen E., Bjerve K.S. (2001) Screening for hemochromatosis: high prevalence and low morbidity in an unselected population of 65,253 persons. *Scandinavian Journal of Gastroenterology* **36**: 1108-1115

Bacon B., Sadiq S.A. (1997) Hereditary hemochromatosis : presentation and diagnosis in the 1990s. *American Journal of Gastroenterology* **92**: 784-789

Bacon B.R., Olynyk J.K., Brunt E.M., Britton R.S., Wolff R.K. (1999a) *HFE* genotypes in patients with hemochromatosis and other liver diseases. *Annals of Internal Medicine* **130**: 953-962

Bacon B.R., Powell L.W., Adams P.C., Kresina T.F., Hoofnagle J.H. (1999b) Molecular medicine and hemochromatosis: at the crossroads. *Gastroenterology* **116**: 193-207

Baer D.M., Tekawa I.S., Hurley L.B. (1994) Iron stores are not associated with acute myocardial infarction. *Circulation* **89**: 2915-2918

Baer D.M., Simons J.L., Staples R.L., Rumore G.J., Morton C.J. (1995) Hemochromatosis screening in asymptomatic ambulatory men 30 years of age and older. *American Journal of Medicine* **98**: 464-468

Balan V., Baldus W., Fairbanks V., Michels V., Burritt M., Klee G. (1994) Screening for hemochromatosis: a cost-effectiveness study based on 12,258 patients. *Gastroenterology* **107**: 453-459

Barash C.I. (2000) Genetic discrimination and screening for hemochromatosis: then and now. *Genetic Testing* **4**: 213-218

Barton J.C., Barton N.H., Alford T.J. (1997) Diagnosis of hemochromatosis probands in community hospital. *American Journal of Medicine* **103**: 498-503

Barton E.H., West P.A., Rivers C.A., Barton J.C., Acton R.T. (2001) Transferrin receptor-2 (TFR2) mutation Y250X in Alabama Caucasian and African American subjects with and without primary iron overload. *Blood Cells, Molecules, and Diseases* 27: 279-284

Bassett M., Halliday J., Powell L. (1986) Value of hepatic iron measurement in early hemochromatosis and determination of the critical iron level associated with fibrosis. *Hepatology* **6**: 24-29

Bassett M.L., Halliday J.W., Bryant S., Dent O., Powell L.W. (1988) Screening for hemochromatosis. *Annals of the New York Academy of Sciences* **526**: 274-289

Bassett M.L., Leggett B.A., Halliday J.W., Webb S., Powell L.W. (1997) Analysis of population screening for haemochromatosis using biochemical and genetic makers. *Journal of Hepatology* **27**: 517-524

Batey R.G., Lai Chung Fong P., Shamir S., Sherlock S. (1980) A non-transferrin-bound serum iron in idiopathic hemochromatosis. *Digestive Diseases and Sciences* **25**: 340-346

Baty D., Terron Kwiatowski A., Mechan D., Harris A., Pippard M.J., Goudie D. (1997) Development of a multiplex ARMS test for mutations in the HFE gene associated with hereditary haemochromatosis. *Journal of Clinical Pathology* **51**: 73-74

Beckman L.E., Sjoberg K., Eriksson S., Beckman L. (2001) Haemochromatosis gene mutations in Finns, Swedes and Swedish Saamis. *Human Heredity* **52**: 110-112

Bell H., Skinningsrud A., Raknerud N., Try K. (1994) Serum ferritin and transferrin saturation in patients with chronic alcoholic and non-alcoholic liver diseases. *Journal of Internal Medicine* **236**: 315-322

Bennett M.J., Lebrón J.A., Bjorkman P.J. (2000) Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. *Nature* **403**: 46-53

Best L.G., Harris P.E., Spriggs E.L. (2001) Hemochromatosis mutations C282Y and H63D in "cis" phase. *Clinical Genetics* **60**: 68-72

Beutler E., Gelbart T., West C., Lee P., Adams M., Blackstone R., Pockros P., Kosty M., Venditti C.P., Phatak P.D., Seese N.K., Chorney K.A., Ten Elshof A.E., Gerhard G.S., Chorney M (1996) Mutation analysis in hereditary hemochromatosis. *Blood Cells, Molecules, and Diseases* **22**: 187-194

Beutler E. (1997) The significance of the 187G (H63D) mutation in hemochromatosis. *American Journal of Human Genetics* **61**: 762-764

Beutler E., Felitti V., Gelbart T., Ho N. (2000) The effect of HFE genotypes on measurements of iron overload in patients attending a health appraisal clinic. *Annals of Internal Medicine* **133**: 329-337

Beutler E., Felitti V., Ho N.J., Gelbart T. (2002a) Relationship of body iron stores to levels of serum ferritin, serum iron, unsaturated iron binding capacity and transferrin saturation in patients with iron storage disease. *Acta Haematologica* **107**: 145-149

Beutler E., Griffin M.J., Gelbart T., West C. (2002b) A previously undescribed nonsense mutation of the HFE gene. *Clinical Genetics* **61**: 40-42.

Beutler E., Felitti V.J., Koziol J.A., Ho N.J., Gelbart T. (2002c) Penetrance of 845G→A (C282Y) HFE hereditary hemochromatosis mutation in the USA. *Lancet* **359**: 211-218

Bhavnani M., Llod D., Bhattacharyya A., Marples J., Elton P., Worwood M. (2000) Screening for genetic haemochromatosis in blood samples with raised alanine aminotransferase. *Gut* **46**: 707-710

Bodmer J.G., Parham P., Albert E.D., Marsh S.G. (1996) Putting a hold on "HLA-H". The WHO Nomenclature Committee for factors of the HLA system. *Nature Genetics* **13**: 234-235

Bomford A., Eddlestone A.L., Kennedy L.A., Batchelor J.R., Williams R. (1977) Histocompatibility antigens as markers of abnormal iron metabolism in idiopathic haemochromatosis. *Lancet* **i**: 327-329

Bonkovsky H.L., Rubin R.B., Cable E.E., Davidoff A., Pels Rijcken T.H., Stark D.D. (1999) Hepatic iron concentration: noninvasive estimation by means of MR imaging techniques. *Radiology* **212**: 227-234

Bothwell T.H. (1982) Hemochromatosis. In *Cecil's Textbook of Medicine*, 16th edition. Philadelphia: W.B. Saunders. pp 1128-1131

Bottomley S.S. (1998) Secondary iron overload disorders. *Seminars in Hematology* **35**: 77-86

Boulton F., Collins D., Inskip H., Paes H., Garlick M. (2000) A study of the iron and HFE status of blood donors, including a group who failed the initial screen for anaemia. *British Journal of Haematology* **108**: 434-439

Bozzini C., Girelli D., Tinazzi E., Olivieri O., Stranieri C., Bassi A., Trabetti E., Faccini G., Pignatti P.F., Corrocher R. (2002) Biochemical and genetic markers of iron status and the risk of coronary artery disease: an angiography-based study. *Clinical Chemistry* **48**: 622-628

Bradley L.A., Haddow J.E., Palomaki G.E. (1996a) Population screening for haemochromatosis: expectations based on a study of relatives of symptomatic probands. *Journal of Medical Screening* **3**: 171-177

Bradley L.A., Haddow J.E., Palomaki G.E. (1996b) Population screening for haemochromatosis: a unifying analysis of published intervention trials. *Journal of Medical Screening* **3**: 178-184

Brandhagen D.J., Alvarez W., Therneau T.M., Kruckeberg K.E., Thibodeau S.N., Ludwig J., Porayko M.K. (2000) Iron overload in cirrhosis – HFE genotypes and outcome after liver transplantation. *Hepatology* **31**: 456-460

Braun J., Donner H., Plock K., Rau H., Usadel K.H., Badenhoop K. (1998) Hereditary haemochromatosis mutations (HFE) in patients with type II diabetes mellitus. *Diabetologia* 41: 983-984

Breuer W., Hershko C., Cabantchik Z.I. (2000) The importance of non-transferrin bound iron in disorders of iron metabolism. *Transfusion Science* 23: 185-192

Brissot P., Bourel M., Herry D., Verger J-P., Messner M., Beaumont C., Regnouard F., Ferrand B., Simon M. (1981) Assessment of liver iron content in 271 patients: a reevaluation of direct and indirect methods. *Gastroenterology* 80: 557-565

Brissot P., Moirand R., Jouanolle A-M., Guyader D., Le Gall J-Y., Deugnier Y., David V. (1999) A genotypic study of 217 unrelated probands diagnosed as "genetic hemochromatosis" on "classical" phenotypic criteria. *Journal of Hepatology* 30: 588-593

Brissot P (2000) Clinical spectrum of hepatic disease in hemochromatosis. In *Hemochromatosis: genetics, pathophysiology, diagnosis and treatment.* Cambridge: Cambirdge University Press, pp 250-257

British Committee for Standards in Haematology (2000) Genetic haemochromatosis Abingdon: Darwin Medical Communications Ltd

Brittenham G.M. (1988) Non-invasive methods for the early detection of hereditary hemochromatosis. *Annals of the New York Academy of Sciences* 526: 199-208

Britton R.S. (2000) Mechanisms of iron toxicity. In *Hemochromatosis: Genetics, pathophysiology, diagnosis and treatment.* Cambridge: Cambridge University Press pp 229-238.

Brunt E.M., Olynyk J.K., Britton R.S., Janney C.G., Di Bisceglie A.M., Bacon B.R. (2000) Histological evaluation of iron in liver biopsies: relationship to HFE mutations. *American Journal of Gastroenterology* 95: 1788-1793

Buffone G.J., Beck J.R. (1994) Cost-effectiveness analysis for evaluation of screening programmes: hereditary hemochromatosis. *Clinical Chemistry* 40: 1631-1636

Bulaj Z.J., Griffen L.M., Jorde L.B., Edwards C.Q., Kushner J.P. (1996) Clinical and biochemical abnormalities in people heterozygous for hemochromatosis. *New England Journal of Medicine* 335: 1799-1805

Burke W., Thomson E., Khoury M.J., McDonnell S.M., Press N., Adams P.C., Barton J.C., Beutler E., Brittenham G., Buchanan A., Clayton E.W., Cogswell M.E., Meslin E.M., Motulsky A.G., Powell L.W., Sigal E., Wilfond B.S., Collins F.S. (1998) Consensus Statement – hereditary hemochromatosis *Journal of the American Medical Association* 280: 172-178

Burt M.J., George P.M., Upton J.D., Collett J.A., Frampton C.M.A., Chapman T.M., Walmsley T.A., Chapman B.A. (1998) The significance of haemochromatosis gene mutations in the general population: implications for screening. *Gut* 43: 830-836

Butterworth J.R., Cooper B.T., Rosenberg W.M.C., Purkiss M., Jobson S., Hathaway M., Briggs D., Howell W.M., Wood G.M., Adams D.H., Iqbal T.H. (2002) The role of hemochromatosis susceptibility gene mutations in protecting against iron deficiency in celiac disease. *Gastroenterology* 123: 444-449.

Calado R.T., Franco R.F., Pazin-Filho A., Simões M.V., Marin-Neto J.A., Zago M.A. (2000) HFE mutations in coronary atherothrombotic disease. *Brazilian Journal of Medical and Biological Research* 33: 301-306

Camaschella C., Roetto A., Gasparini P., Piperno A., Fortina P, Surrey S., Rappaport E. (1996) Allelic associations of microsatellites of 6p in Italian hemochromatosis patients. *Human Genetics* 97: 476-481

Camaschella C., Roetto A., Calì A., De Gobbi M., Garozzo G., Carella M., Majorano N., Totaro A., Gasparini P. (2000) The gene encoding transferrin receptor 2 is mutated in a new type of hemochromatosis mapping to 7q22. *Nature Genetics* 25: 14-15

Cardoso E.M.P., Stål P., Hagen K., Cabeda J.M., Esin S., de Sousa M., Hulycrantz R. (1998) HFE mutations in patients with hereditary haemochromatosis in Sweden. *Journal of Internal Medicine* 243: 203-208

Cardoso C.S., Oliviera P., Porto G., Oberkanins C., Mascarenhas M., Rodrigues P., Kury F., de Sousa M. (2001) Comparative study of the two more frequent HFE mutations (C282Y and H63D): significant different allelic frequencies between the north and south of Portugal. *European Journal of Human Genetics* 9: 843-848_

Carella M., D'Ambrosio L., Totaro A., Grifa A., Valentino M.A., Piperno A., Girelli D., Roetto A., Franco B., Gasparini P., Camaschella C. (1997) Mutation analysis of the HLA-H gene in Italian hemochromatosis patients. *American Journal of Human Genetics* 60: 828-832

Cartwright G.E. (1974) Hemochromatosis. In *Harrison's Principles of Internal Medicine*, 7th edition. New York: McGraw-Hill; 618-619

Cazzola M., Barosi G., Bergamaschi G., Dezza L., Palestra P., Polino G, Ramella S., Spriano P., Ascari E. (1983) Iron loading in congenital dyserythropoietic anaemias anaemias and congenital sideroblastic anaemias. *British Journal of Haematology* 54: 649-654

Cazzola M., Beguin Y., Bergamaschi G., Guarnone R., Cerani P., Barella S., Cao A., Galanello R. (1999) Soluble transferrin receptor as a potential determinant of iron loading in congenital anaemias due to ineffective erythropoiesis. *British Journal of Haematology* **106**: 752-755

Chapman R.W., Morgan M.Y., Laulicht M., Hoffbrand A.V., Sherlock S. (1982) Hepatic iron stores and markers of iron overload in alcoholics and patients with idiopathic hemochromatosis. *Digestive Disease and Sciences* 27: 909-916

Cogswell M.E., McDonnell S.M., Koury M.J., Franks A.L., Burke W, Brittenham G. (1998) Iron overload, public health, and genetics: evaluating the evidence for hemochromatosis screening. *Annals of Internal Medicine* **129**:971-979

Cogswell M.E., Burke W., McDonnell S.M., Franks A.L. (1999) Screening for hemochromatosis: a public health perspective. *American Journal of Preventive Medicine* **16**: 134-140

Conrad M.E., Umbreit J.N., Moore E.G., Parmley R.T. (2000) Intestinal iron absorption and hemochromatosis. In *Hemochromatosis. Genetics, pathophysiology, diagnosis and treatment* Cambridge: Cambridge University Press. pp118-130

Conry-Cantilena C. and Klein H.G. (2000) Hemochromatosis patients as blood and tissue donors. In *Hemochromatosis genetics, pathophysiology, diagnosis and treatment.* Cambridge: Cambridge University Press. pp 564-573

Coppine H., Bensaid M., Fruchon S., Borot N., Blanché H., Roth M.P. (2003) Longevity and carrying the C282Y mutation for haemochromatosis on the HFE gene: case control study of 492 French centenarians. *British Medical Journal* **327**: 132-133

Crawford D.H., Jazwinska E.C., Cullen L.M., Powell L.W. (1998) Expression of HLA-linked hemochromatosis in subjects homozygous or heterozygous for the C282Y mutation. *Gastroenterology* **114**: 1003-1008

Crawford D.H.G. and Hickman P. (2000) Screening for hemochromatosis. *Hepatology* **31**: 1192-1193

Crawford D.H., Fletcher L.M., Hubscher S.G., Stuart K.A., Gane E., Angus P.W., Jeffrey G.P., McCaughan G.W., Kerlin P., Powell L.W., Elias E.E. (2004) Patient and graft survival after liver transplantation for hereditary hemochromatosis. Implications for pathogenesis. *Hepatology* **39**: 1655-1662

Crompton G.K., Haslett C., Chilvers E.R. (1999) Diseases of the respiratory system. In *Davidson's Principles and Practice of Medicine, 18th edition.* Edinburgh: Churchill Livingstone pp 303-392.

Crosby W.H.and O'Neil-Cutting M.A. (1984) A small-dose iron tolerance test as an indicator of mild iron deficiency. *Journal of the American Medical Association* **251**: 1986-1987

Cullen L.M., Gao X., Easteal S., Jazwinska E.C. (1998) The hemochromatosis 845G→A and 187C→G mutations: prevalence in non-Caucasian populations. *American Journal of Human Genetics* **62**: 1403-1407

Danesh J. and Appleby P. (1999) Coronary heart disease and iron status. Meta-analyses of prospective studies. *Circulation* **99**: 852-854

Datz C., Haas T., Rinner H., Sandhofer F., Patsch W., Paulweber B. (1998) 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? *Clinical Chemistry* **44**: 2429-2432

De Gobbi M., Roetto A., Piperno A., Mariani R., Alberti F., Papanikolaou G., Politou M., Lockitch G., Girelli D., Fargion S., Cox T.M., Gasparini P., Cazzola M., Camaschella C. (2002) Natural history of juvenile haemochromatosis. *British Journal of Haematology* **117**: 973-979

Delatycki M., Allen K., Williamson R. (2002) Insurance agreement to facilitate genetic testing. *Lancet* **359**: 1433

Deugnier Y., Loréal O., Turlin B., Guyader D., Jouanolle H., Moirand R., Jacquelinet C., Brissot P. (1992) Liver pathology in genetic hemochromatosis: a review of 135 homozygous cases and their bioclinical correlations. *Gastroenterology* **102**: 2050-2059

Deugnier Y., Turlin B., le Quilleuc D., Moirand R., Loréal O., Messner M., Meunier B., Brissot P., Launois B. (1997) A reappraisal of hepatic siderosis in patients with end-stage cirrhosis: practical implications for the diagnosis of hemochromatosis. *American Journal of Surgical Pathology* **21**: 669-675

Devalia V., Carter K., Walker A.P., Perkins S J., Worwood M., May A., Dooley J.S. (2002) Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (SLC11A3). *Blood* **100**: 695-697

De Valk B. and Marx J.J.M. (1999) Iron, atherosclerosis and ischemic heart disease. *Archives of Internal Medicine* **159**: 1542-1546

De Valk B., Addicks M.A., Gosriwatana I., Lu S., Hider R.C., Marx J.J.M. (2000) Non-transferrin-bound iron is present in the serum of hereditary haemochromatosis heterozygotes. *European Journal of Clinical Investigation* **30**: 248-251

De Vaus D.A. (1996) *Surveys in Social Research*, 4th edition. London: UCL Press Ltd

Di Bisceglie A.M., Axiotis C.A., Hoofnagle J.H., Bacon B.R. (1992) Measurements of iron status in patients with chronic hepatitis. *Gastroenterology* **102**:2108-2113

Dillman D.A. (1978) *Mail and Telephone surveys: the total design method.* New York: Wiley

Distante S., Berg J.P., Lande K., Haug E., Bell H. (2000) HFE gene mutation (C282Y) and phenotypic expression among a hospitalised population in a high prevalence area of haemochromatosis. *Gut* **47**: 575-579

Donovan A., Brownlie A., Zhou Y., Shepard J., Pratt S.J., Moynihan J., Paw B.H., Drejer A., Barut B., Zapata A., Law T.C., Brugnara C., Lux S.E., Pinkus G.S., Pinkus J.L., Kingsley P.D., Palis J., Fleming M.D., Andrews N.C., Zon L.I. (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403**: 776-781

Doran T.J., Bashir H.V., Trejaut T., Bassett M.L., Halliday J.H.W., Powell L.W. (1981) Idiopathic haemochromatosis in the Australian population: HLA linkage and recessivity. *Human Immunology* **2**:191-200

Douabin V., Deugnier Y., Jouanolle A.M., Moirand R., Macqueron G., Gireau A., Le Gall J.Y., David V. (1997) Polymorphisms in the haemochromatosis gene. *International Symposium: Iron in Biology and Medicine.* Saint-Malo, France. p267.

Dyrszka H., Eberhardt G., Eckert G. (1979) The distribution of HLA-antigens in German patients with idiopathic haemochromatosis. *Klinische Wochenschrift* **57**: 529-531

EASL International Consensus Conference on Haemochromatosis (2000). *Journal of Hepatology* **33**: 485-504

Edwards C.Q., Skolnick M.H., Dadone M.M., Kushner J.P. (1982) Iron overload in hereditary spherocytosis: association with HLA-linked hemochromatosis. *American Journal of Hematology* **13**: 101-109

Edwards C.Q., Griffin L.M., Goldgar D., Drummond C., Skolnick M.H., Kushner J.P. (1988) Prevalence of hemochromatosis among 11,065 presumably healthy blood donors. *New England Journal of Medicine* **318**: 1355-1362

Edwards C.Q., Griffen L.M., Kaplan J., Kushner J.P. (1989) Twenty-four hour variation of transferrin saturation in treated and untreated haemochromatosis homozygotes. *Journal of Internal Medicine* **226**: 373-379

Edwards C.Q. and Kushner J.P. (1993) Screening for hemochromatosis. *New England Journal of Medicine* **328**: 1616-1620

Edwards C.Q., Griffen L.M., Ajioka R.S., Kushner J.P. (1998) Screening for hemochromatosis: phenotype versus genotype. *Seminars in Hematology* **35**: 72-76

Edwards C.Q., Ajioka R.S., Kushner J.P. (2000) Hemochromatosis: a genetic definition. In *Hemochromatosis: Genetics, pathophysiology, diagnosis and treatment* Cambridge: Cambridge University Press

Eichner J.E., Qi H., Moore W.E., Schechter E. (1998) Iron measures in coronary angiography patients. *Atherosclerosis* **136**: 241-245

Ellervik C., Mandrup-Poulsen T., Nordestgaard B.G., Larsen L.E., Appleyard M., Frandsen M., Petersen P., Schlichting P., Særmark T., Tybjærg-Hansen A., Birgens H. (2001) Prevalence of hereditary haemochromatosis in late-onset type 1 diabetes mellitus: a retrospective study. *Lancet* **358**: 1405-1409

El-Sareg H.B., Inadomi J.M., Kowdley K.V. (2000) Screening for hereditary hemochromatosis in siblings and children of affected patients: a cost-effectiveness analysis. *Annals of Internal Medicine* **132**: 261-269

European Haemochromatosis Consortium (1999) Polymorphism in intron 4 of HFE does not compromise haemochromatosis mutation results. *Nature Genetics* **23**: 271

Farrell F.J., Nguyen M., Woodley S., Imperial J.C., Garcia-Kennedy R., Man K., Esquival C.O., Keeffe E.B. (1994) Outcome of liver transplantation in patients with hemochromatosis. *Hepatology* **20**: 404-410

Feder J.N., Gnirke A., Thomas W., Tsuchihashi Z., Ruddy D.A., Basava A., Dormishian F., Domingo R., Jr., Ellis M.C., Fullan A., Hinton L.M., Jones N.L., Kimmel B.E., Kronmal G.S., Lauer P., Lee V.K., Loeb D.B., Mapa F.A., McClelland E., Meyer N.C., Mintier G.A., Moeller N., Moore T, Morikang E, Prass C.E., Quintana L., Starnes S.M., Schatzman R.C., Brunke K.J., Drayna D.T., Risch N.J., Bacon B.R., Wolff R.K. (1996) A novel MHC class I – like gene is mutated in patients with hereditary haemochromatosis. *Nature Genetics* **13**: 399-408

Feder J.N., Tsuchihashi Z., Irrinki A., Lee V.K., Mapa F.A., Morikang E., Prass C.E., Starnes S.M., Wolff R.K., Parkkila S., Sly W.S., Schatzman R.C. (1997) The hemochromatosis founder mutation in HLA-H disrupts beta 2-microglobulin interaction and cell surface expression. *Journal of Biological Chemistry* **272**: 14025-14028

Feder J.N., Penny D.M., Irrinki A., Lee V.K., Lebrón J.A., Watson N., Tsuchihashi Z., Sigal E., Bjorkman P.J., Schatzman R.C. (1998) The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proceedings of the National Academy of Sciences of the USA* **95**: 2492-2497

Fiel M.I., Schiano T.D., Bodenheimer H.C. Jr., Thung S. N., King T.W., Varma C.R., Miller C.M., Brunt E.M., Starnes S., Prass C., Wolff R.K., Bacon B.R. (1999) Hereditary hemochromatosis in liver transplantation. *Liver Transplantation and Surgery* **5**: 50-56

Finch C.A., Cook J.D., Labbe R.F., Culala M (1977) Effects of blood donation on iron stores as evaluated by serum ferritin. *Blood* **50**: 441-447

Finch S.C. and Finch C.A. (1955) Idiopathic hemochromatosis, an iron storage disease. *Medicine (Baltimore)* **34**: 381-430

Finch C.A. (1994) Regulators of iron balance in humans. *Blood* **84**: 1697-1702

Fleming M.D., Trenor C.C. 3rd, Su M.A., Foernzler D., Beier D.R., Dietrich W.F., Andrews N.C. (1997) Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genetics* **16**: 383-386

Fleming R.E., Migas M.C., Holden C.C., Waheed A., Britton R.S., Tomatsu S., Bacon B.R., Sly W.S. (2000) Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary haemochromatosis. *Proceedings of the National Academy of Sciences of the USA* **97**: 2214-2219

Fleming R.E., Sly W.S. (2001) Ferroportin mutation in autosomal dominant hemochromatosis: loss of function, gain in understanding. *Journal of Clinical Investigation* **108**: 521-522

Fox, C.J., Cullen D.J., Knuiman M.W., Cumpston G.N., Divitini, M.L., Rossi, E., Gochee P.A., Powell, L.W., Olynyk J.K. (2002) Effects of body iron stores and haemochromatosis gentoypes on coronary heart disease outcomes in the Busselton health study. *Journal of Cardiovascular Risk* **9**: 287-293

Franks A.L. and Burke W. (1999) Will the real hemochromatosis please stand up? *Annals of Internal Medicine* **130**: 1018-1019

Frayling T., Ellard S., Grove J., Waker M., Hattersley A.T. (1998) C282Y mutation in HFE (haemochromatosis) gene and type 2 diabetes. *Lancet* **351**: 1933-1934

Fleming R.E., Migas M.C., Zhou X., Jiang J., Britton R.S., Brunt E.M., Tomatsu S., Waheed A., Bacon B.R., Sly W.S. (1999) Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1. *Proceedings of the National Academy of Sciences of the USA* **96**: 3143-3148

Fleming R.F. and Sly W.S. (2002) Mechanisms of iron accumulation in hereditary hemochromatosis. *Annual Review of Physiology* **64**: 663-680

Gambino R., Desvarieux E., Orth M., Matan H., Ackattupathil T., Lijoi E., Wimmer C., Bower J., Gunter E. (1997) The relation between chemically measured total iron-binding capacity concentrations and immunologically measured transferrin concentrations in human serum. *Clinical Chemistry* **43**: 2408-2412

Garry P.J., Van der Jagt D.J., Wayne S.J., Koehler K.H., Rhyne R.L., Simon T.L. (1991) A prospective study of blood donations in healthy elderly persons. *Transfusion* **31**: 686-692

Garry P.J., Koehler K.M., Simon T.L. (1995) Iron stores and iron absorption: effects of repeated blood donations. *American Journal of Clinical Nutrition* **62**: 611-620

Garry P.J., Montoya G.D., Baumgartner R.N., Liang H.C., Williams T.M., Brodie S.G. (1997) Impact of HLA-H mutations on iron stores in healthy elderly men and women. *Blood Cells, Molecules, and Diseases* **23**: 277-287

Gayle C.B. (1992) Hemochromatosis and dietary iron supplementation: implications from US mortality, morbidity and health survey data. *Journal of the American Dietetic Association* **92**: 208-212

George D.K., Evans R.M., Crofton R.W., Gunn I.R. (1995) Testing for haemochromatosis in the diabetic clinic. *Annals of Clinical Biochemistry* **32**: 521-526

George D.K., Ramm G.A., Walker N.I., Powell L.W., Crawford D.H.G. (1999) Elevated serum type IV collagen: a sensitive indicator of the presence of cirrhosis in haemochromatosis. *Journal of Hepatology* **31**: 47-52

Girelli D., Olivieri O., De Franceschi L., Corrocher R., Bergamaschi G., Cazzola M. (1995) A linkage between hereditary hyperferritinaemia not related to iron overload and autosomal dominant congenital cataract. *British Journal of Haematology* **90**: 931-934

Girelli D., Bozzini C., Roetto A., Alberti F., Daraio F., Colombari R., Olivieri O., Corrocher R., Camaschella C. (2002) Clinical and pathologic findings in hemochromatosis type 3 due to a novel mutation in transferrin receptor 2 gene. *Gastroenterology* **122**: 1295-1302

Gochee P.A., Powell L.W., Cullen D.J., Du Sart D., Rossi E., Olynyk J.K. (2002) A population-based study of the biochemical and clinical expression of the H63D hemochromatosis mutation. *Gastroenterology* **122**: 646-651

Griffiths W.J., Kelly A.L., Smith S.J., Cox T.M. (2000) Localization of iron transport and regulatory proteins in human cells. *Quarterly Journal of Medicine* **93**: 575-587

Griffiths W.J.H., Sly W.S., Cox T.M. (2001) Intestinal iron uptake determined by divalent metal transporter is enhanced in HFE-deficient mice with hemochromatosis. *Gastroenterology* **120**: 1420-1429

Grootveld M., Bell J.D., Halliwell B., Aruoma O.I., Bomford A., Sadler P.J. (1989) Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *Journal of Biological Chemistry* **264**: 4417-4422.

Gruenheid S., Canonne-Hergaux F., Gauthier S., Hackam D.J., Grinstein S., Gros P. (1999) The iron transporter protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *Journal of Experimental Medicine* **189**: 831-841

Guidelines for the Blood Transfusion Services in the UK, 6th edition (2002). London: the Stationery Office.

Gunshin H., Mackenzie B., Berger U.V., Gunshin Y., Romero M.F., Boron W.F., Nussberger S., Gollan J.L., Hediger M.A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **388**: 482-487

Gutteridge J.M., Rowley D.A., Griffiths E., Halliwell B. (1985) Low-molecular- weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. *Clinical Science* **68**: 463-467

Guyader D., Jacquelinet C., Moirand R., Turlin B., Mendler M.H., Chaperon J., David V., Brissot P., Adams P., Deugnier Y. (1998) Noninvasive prediction of fibrosis in C282Y homozygous hemochromatosis. *Gastroenterology* **115**: 929-936

Haddow J.E. and Bradley L.A. (1999) Hereditary haemochromatosis: to screen or not. *British Medical Journal* **319**:531-532

Halliday J.W., Russo A., Cowlishaw J.L., Powell L.W. (1977) Serum ferritin in the diagnosis of haemochromatosis. A study of 43 families. *Lancet* **ii**: 621-624

Hamill R.L., Woods J.C., Cook B.A. (1991) Congenital atransferrinemia: a case report and review of the literature. *American Journal of Clinical Pathology* **96**: 215-218

Halpern Z., Rahmani R., Levo Y. (1985) Severe hemochromatosis: the predominant clinical manifestation of congenital dyserythropoietic anaemia type 2. *Acta Haematologica (Basel)* **78**: 33-36

Hamill R.L., Woods J.C., Cook B.A. (1991) Congenital atransferrinemia: a case report and review of the literature. *American Journal of Clinical Pathology* **96**: 215-218

Hamilton L.D., Gubler C.J., Cartwright G.E., Wintrobe M.M. (1950) Diurnal variation in the plasma iron level of man. *Proceedings of the Society for Experimental Biology and Medicine* 75:65-68

Heilmeyer L., Keller W, Viwell O., et al (1961) Kongenitale Atransferrinämie bei einem sieben Jahre alten Kind. *Deutsche medizinische Wochenschrift* **86**: 1745-1751

Hershko C., Graham G., Bates G.W., Rachmilewitz E.A. (1978) Non-specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *British Journal of Haematology* **40**: 255-263.

Hetet G., Elbaz A., Gariepy J., Nicaud V., Arveiler D., Morrison C., Kee F., Evans A., Simon A., Amarenco P., Cambien F., Grandchamp B. (2001) Association between haemochromatosis gene mutations and the risk of cardiovascular diseases. *European Journal of Clinical Investigation* **31**: 382-388

Ho G.T. and Stanley A.J. (2001) Haemochromatosis: where are all the patients? *Scottish Medical Journal* **46**: 99-100

Holström P., Marmur J., Eggertsen G., Gåfvels M., Stål P. (2002) Mild iron overload in patients carrying the HFE S65C gene mutation: a retrospective study in patients with suspected iron overload and healthy controls. *Gut* **51**: 723-730

International Committee for Standardization in Haematology (1978) The measurement of total and unsaturated iron binding capacity in serum. *British Journal of Haematology* **38**: 281-290

Jackson H.A., Carter K., Darke C., Guttridge M.G., Ravine D., Hutton R.D., Napier J.A., Worwood M. (2001) HFE mutations, iron deficiency and overload in 10 500 blood donors. *British Journal of Haematology* **114**: 474-484

Jacobs A., Miller F., Worwood M., Beamish M.R., Wardrop C.A. (1972) Ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. *British Medical Journal* **4**: 206-208

Jacolot S., Le Gac G., Scotet V., Quere I., Mura C., Ferec C. (2004) HAMP as a modifier gene that increases the phenotypic expression of the HFE pC282Y homozygous genotype. *Blood* **103**: 2835-2840

Jaime J.C., Cazarez R., Mares M.A., Marfil L.J., Harrison C.R. (1988) Iron stores in remunerated blood donors as evaluated by plasma ferritin levels. *Transfusion* **28**: 62-65

Jazwinska E.C., Lee S.C., Webb S.I., Halliday J.W., Powell L.W. (1993) Localization of the hemochromatosis gene close to D6S105. *American Journal of Human Genetics* **56**: 428-433

Jazwinska E.C., Cullen L.M., Busfield F., Pyper W.R., Webb S.I., Powell L.W., Morris C.P., Walsh T.P. (1996) Haemochromatosis and HLA-H. *Nature Genetics* **14**: 399-408

Jeffrey G. and Adams P.C. (1999a) Blood from patients with hereditary hemochromatosis – a wasted resource? *Transfusion* **39**: 549-550

Jeffrey G.P., Chakrabarti S., Hegele R.A., Adams P.C. (1999b) Polymorphism in intron 4 of HFE may cause overestimation of C282Y homozygote prevalence in haemochromatosis. *Nature Genetics* **22**: 325-326

Kawabata H., Yang R., Hirama T., Vuong P.T., Nakami T., Said J.W., Koeffler H.P. (1999) Molecular cloning of transferrin receptor 2: a new member of the transferrin receptor-like family. *Journal of Biological Chemistry* **274**: 20826-20832

Kawabata H., Nakamaki T., Ikonomi P., Smith R.D., Germain R.S., Koeffler H.P. (2001). Expression of trasnferrin receptor 2 in normal and neoplastic hematopoietic cells. *Blood* **98**: 2714-2719

Kiechl S., Willeit J., Egger G., Poewe W., Oberhollenzer F. (1997) Body iron stores and the risk of carotid atherosclerosis: prospective results from the Brunek study. *Circulation* **96**: 3300-3307

Kirschbaum N.E. and Foster P.A. (1995) The polymerase chain reaction with sequence specific primers for the detection of the factor V mutation associated with activated protein C resistance. *Thrombosis and Haemostasis* 74:874-878

Koster J.F. and Sluiter W. (1995) Is increased tissue ferritin a risk factor for atherosclerosis and ischaemic heart disease? *British Heart Journal* 73: 208

Kowdley K.V., Hassanein T., Kaur S., Farrell F.J., van Thiel D.H., Keeffe E.B., Sorrell M.F., Bacon B.R., Weber F.L. Jr., Tavill A.S. (1995) Primary liver cancer and survival in patients undergoing liver transplantation for hemochromatosis. *Liver Transplantation and Surgery* **1**: 237-241

Kowdley K.V., Trainer T.D., Saltzman J.R., Pedrosa M., Krawitt E.L., Know T.A., Susskind K., Pratt D., Bonkovsky H.L., Grace N.D., Kaplan M.M. (1997) Utility of hepatic iron index in American patients with hereditary hemochromatosis: a multicenter study. *Gastroenterology* **113**: 1270-1277

Krawczak M., Cooper D.N., Schmidtke J. (2001) Estimating the efficacy and efficiency of cascade genetic screening. *American Journal of Human Genetics* **69**: 361-370

Kühn L.C. (1998) Iron and gene expression: molecular mechanisms regulating cellular iron homeostasis. *Nutrition Reviews* **56** (2): S11-S19

Lebrón J.A., West A.P. Jr., Bjorkman P.J. (1999) The hemochromatosis protein HFE competes with transferrin for binding to the transferrin receptor. *Journal of Molecular Biology* **294**: 239-245

Lee M.H. and Means R.T. (1995) Extremely elevated serum ferritin levels in a university hospital: associated disease and clinical significance. *American Journal of Medicine* **98**: 566-571

Lee P.L., Gelbart T., West C., Halloran C., Beutler E. (1999) The human Nramp2 gene: characterization of the the gene structure, alternative splicing, promoter region and polymorphisms. *Blood Cells, Molecules and Diseases* **24**: 199-215

Lee P.L., Halloran C., West C., Beutler E. (2001a) Mutation analysis of the transferrin receptor-2 gene in patients with iron overload. *Blood Cells, Molecules, and Diseases* **27**: 285-289

Lee P.L., Gelbart T., West C., Halloran C., Felitti V., Beutler E. (2001b) A study of the genes that may modulate the expression of hereditary hemochromatosis: transferrin receptor-1, ferroportin, ceruloplasmin, ferritin light and heavy chains, iron regulatory proteins-1 and –2, and hepcidin. *Blood Cells, Molecules, and Diseases* **27**: 783-802

Leggett B.A., Halliday J.W., Brown N.N., Bryant S., Powell L.W. (1990) Prevalence of haemochromatosis amongst asymptomatic Australians. *British Journal of Haematology* **74**: 525-530

Levstik M. and Adams P.C. (1998) Eligibility and exclusion of hemochromatosis patients as voluntary blood donors. *Canadian Journal of Gastroenterology* **12**: 61-63

Levy J.E., Montross L.K., Andrews N.C. (2000) Genes that modify the hemochromatosis phenotype in mice. *Journal of Clinical Investigation* **105**: 1209-1216.

Lindmark B. and Eriksson S. (1985) Regional differences in the idiopathic hemochromatosis gene frequency in Sweden. *Acta Medica Scandinavica* **218**: 299-304

Lipschitz D.A., Cook J.D., Finch C.A. (1974) A clinical evaluation of serum ferritin as an index of iron stores. *New England Journal of Medicine* **290**: 1213-1216

Lipinski M., Hors J., Saleun J.P., Saddi R., Passa P., Lafaurie S., Feingold N., Dausset J. (1978) Idiopathic hemochromatosis: linkage with HLA. *Tissue Antigens* **11**: 471-474

Lloyd D.A., Adams P., Sinclair N.R., Stiller C.R., Valberg L.S. (1978) Histocompatibility antigens as markers of abnormal iron metabolism in patients with idiopathic hemochromatosis and their relatives. *Canadian Medical Association Journal* **119**: 1051-1056

Looker A.C. and Johnson C.L. (1998) Prevalence of elevated serum transferrin saturation in adults in the United States. *Annals of Internal Medicine* **129**: 940-945

Lucotte G. (1998) Celtic origin of the C282Y mutation of hemochromatosis. *Blood Cells, Molecules, and Diseases* **24**: 433-438

Lucotte G., Champenois T., Sémonin O. (2001) A rare case of a patient heterozygous for the hemochromatosis mutation C282Y and homozygous for H63D. *Blood Cells, Molecules, and Diseases* **27**: 892-893

Ludwig J., Hashimoto E., Porayko M.K., Moyer T.M., Baldus W.P. (1997) Hemosiderosis in cirrhosis: a study of 447 native livers. *Gastroenterology* **112**: 882-888

Lundvall O., Weinfeld A., Lundin P. (1971) Iron storage in porphyria cutanea tarda. *Acta Medica Scandinavica* **188**: 37-53

Ma J. and Stamfer M.J. (2002) Body iron stores and coronary heart disease. *Clinical Chemistry* **48**: 601-603

McCullen M.A., Crawford D.H., Hickman P.E. (2002) Screening for hemochromatosis. *Clinica Chimica Acta* **315**: 169-186

McCune C.A., Al-Jader L.N., May A., Hayes S.L., Jackson H.A., Worwood M. (2002) Hereditary haemochromatosis: only 1% of adult HFE C282Y homozygotes in South Wales have a clinical diagnosis of iron overload. *Human Genetics* **111**: 538-543

McCord JM (1991) Is iron sufficiency a risk factor in ischemic heart disease? *Circulation* **83**: 1112-1114

MacDonald R. (1964) Hemochromatosis and Hemosiderosis. Springfield, Illinois: Charles C. Thomas

McDonnell S.M., Witte D.L., Cogswell M.E., McIntyre R. (1998a) Strategies to increase detection of hemochromatosis. *Annals of Internal Medicine* **129**: 987-992

McDonnell S.M., Phatak P.D., Felitti V., Hover A., McLaren G.D. (1998b) Screening for hemochromatosis in primary care settings. *Annals of Internal Medicine* **129**: 962-970

McDonnell S.M., Hover A., Gloe D., Ou C-Y., Cogswell M.E., Grummer-Strawn L. (1999a) Population-based screening for hemochromatosis using phenotypic and DNA testing among employees of health maintenance organisations in Springfield, Missouri *American Journal of Medicine* **107**: 30-37.

McDonnell S.M., Grindon A.J., Preston B.L., Barton J.C., Edwards C.Q., Adams P.C. (1999b) A survey of phlebotomy among persons with hemochromatosis. *Transfusion* **39**: 651-656

McKie A.T., Marciani P., Rolfs A., Brennan K., Wehr K., Barrow D., Miret S., Bomford A., Peters T.J., Farzaneh F., Hediger M.A., Hentze M.W., Simpson R.J. (2000) A novel iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron into the circulation. *Molecular Cell* **5**: 299-309

McKie A.T., Barrow D., Latunde-Dada G.O., Rolfs A., Sager G., Mudaly E., Mudaly M., Richardson C., Barlow D., Bomford A., Peters T.J., Raja K.B., Shirali S., Hediger M.A., Farzaneh F, Simpson R.J. (2001) An iron-regulated ferric-reductase associated with the absorption of dietary iron. *Science* **291**: 1755-1759

McLaren C.E., McLachlan G.J., Halliday J.W., Webb S.I., Leggett B.A., Jazwinska E.C., Crawford D.H.G., Gordeuk V.R., McLaren G.D., Powell L.W. (1998) Distribution of transferrin saturation in an Australian population: relevance to the early diagnosis of hemochromatosis. *Gastroenterology* **114**: 543-549

McLaren G.D. (1989) Reticuloendothelial iron stores and hereditary hemochromatosis: a paradox. *Journal of Clinical and Laboratory Medicine* **113**: 137-138

MacSween R.N.M. and Jackson J.M. (1966) Haemochromatosis: a clinico-pathological review of 37 cases. *Scottish Medical Journal* **11**: 395-400

MacSween R.N.M. and Scott R.A. (1973) Hepatic cirrhosis: a clinico-pathological review of 520 cases. *Journal of Clinical Pathology* **26**: 936-942

Maniatis T., Fritsch E.F., Sambrook J. (1982) *Molecular cloning, a laboratory manual.* Cold Spring Harbour, NY.

Mann S., Wade V.J., Dickson D.P., Reid N.M., Ward R.J., O'Connell M., Peters T.J. (1988) Structural specificity of haemosiderin iron cores in iron-overload diseases. *FEBS Letters* **234**: 69-72

Mattman A., Huntsman D., Lockitch G., Langlois S., Buskard N., Ralston D., Butterfield Y., Rodrigues P., Jones S., Porto G., Marra M., De Sousa M., Vatcher G. (2002) Transferrin receptor 2 (TfR2) and HFE mutational analysis in non-C282Y iron overload: identification of a novel TfR2 mutation. *Blood* **100**: 1075-1077

Merryweather-Clarke A., Pointon J., Shearman J., Robson K. (1997) Global prevalence of putative haemochromatosis mutations. *Journal of Medical Genetics* **34**: 275-278

Merryweather-Clarke A.T., Worwood M., Parkinson L., Mattock C., Pointon J.J., Shearman J.D., Robson K.J.H. (1998) The effect of HFE mutations on serum ferritin and transferrin saturation in the Jersey population. *British Journal of Haematology* **101**: 369-373

Merryweather-Clarke A.T., Cadet E., Bomford A., Capron D., Viprakasit V., Miller A., McHugh P.J., Chapman R.W., Pointon J.J., Wimhurst V.L., Livesey K.J., Tanphaichitr V., Rochette J., Robson K.J. (2003) Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. *Human Molecular Genetics* **12**: 2241-2247

Meyers D.G., Strickland D., Maloley P.A. Seburg J.K., Wilson J.E., McManus J.F. (1997) Possible association of a reduction in cardiovascular events with blood donation. *Heart* **78**: 188-193

Miedzybrodska Z., Loughlin S., Baty D., Terron A., Kelly K., Dean J., Greaves M. (1999) Haemochromatosis mutations in north-east Scotland. *British Journal of Haematology* **106**: 385-387

Milman N. (2000) Inheritance of hemochromatosis: family studies. In *Hemochromatosis: Genetics, pathophysiology, diagnosis and treatment.* Cambridge, Cambridge University Press.

Mills C.W. (1959) *The Sociological Imagination.* New York: Harper & Row

Moirand R., Adams P.C., Bicheler V., Brissot P., Deugnier Y. (1997a) Clinical features of genetic haemochromatosis in women compared with men. *Annals of Internal Medicine* **127**: 105-110

Moirand R, Mortaji A.M., Loréal O., Paillard F., Brissot P., Deugnier Y. (1997b) A new syndrome of liver iron overload with normal transferrin saturation. *Lancet* **349**: 95-97

Moirand R., Jouanolle A-M., Brissot P., Le Gall J-Y., David V., Deugnier Y. (1999) Phenotypic expression of HFE mutations: a French study of 1110 unrelated iron-overloaded patients and relatives. *Gastroenterology* **116**: 372-377

Montosi G., Donovan A., Totaro A., Garuti C., Pignatti E., Cassanelli S., Trenor C.C., Gasparini P., Andrews N.C., Pietrangelo A. (2001) Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SCL11A3) gene. *Journal of Clinical Investigation* **108**: 619-623

Morita H., Ikeda S., Yamamoto K., Morita S., Yoshida K., Nomoto S., Kato M. (1995) Hereditary ceruloplasmin deficiency with hemosiderosis: a clinicopathological study of a Japanese family. *Annals of Neurology* **37**: 646-656

Morrison E.D., Brandhagen D.J., Phatak P.D., Krawitt E.L., El-Serag H.B., Gordon S.C., Galan M.V., Tung B.Y., Ioannou G.N., Kowdley K.V. (2003) Serum ferritin level predicts advanced hepatic fibrosis among US patients with phenotypc hemochromatosis. *Annals of Internal Medicine* **138**: 627-633

Moodie S.J., Ang L., Stenner J.M., Finlayson C., Khotari A., Levin G.E., Maxwell J.D. (2002) Testing for haemochromatosis in a liver clinic population: relationship between ethnic origin, HFE gene mutations, liver histology and serum iron markers. *European Journal of Gastroenterology and Hepatology* **14**: 223-229

Moyo V.M., Mandishona E., Hasstedt S.J., Gangaidzo I.T., Gomo Z.A.R., Khumalo H., Saungweme T., Kiire C.F., Paterson A.C., Bloom P., MacPhail P., Rouault T., Gordeuk V.R. (1998) Evidence of genetic transmission in African iron overload. *Blood* **91**: 1076-1082

Mura C., Raguenes O., Férec C. (1999) HFE mutations in 711 hemochromatosis probands: evidence for S65C implication in mild form of hemochromatosis. *Blood* **93**: 2502-2505

Nassar B.A., Zayed E.M., Title L.M., O'Neill B.J., Bata I.R., Kirkland S.A., Dunn J., Dempsey G.I., Tan M.H., Johnstone D.E. (1998) Relation of HFE gene mutations, high iron stores and early onset coronary artery disease. *Canadian Journal of Cardiology* **14**: 215-220

National Committee for Clinical Laboratory Standards (1990) Determination of serum iron and total iron-binding capacity: proposed standard H17-P. Villanova, Pennsylvania, NCCLS.

Newton C.R. and Graham A. (1997) *PCR* 2nd edition. BIOS scientific publishers Ltd.

Nicolas G., Bennoun M., Devaux I., Beaumont C., Grandchamp B., Kahn A., Vaulont S. (2001) Lack of hepcidin gene expression and sever tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proceedings of the National Academy of Sciences of the USA* **98**: 8780-8785

Niederau C., Fischer R., Pürschel A., Stremmel W., Häussinger D., Strohmeyer G. (1996) Long-term survival in patients with hereditary hemochromatosis. *Gastroenterology* **110**:1107-1119

Niederau C., Niederau C.M., Lange S., Littauer A., Abdel-Jalil N., Maurer M., Häussinger D., Strohmeyer G. (1998) Screening for hemochromatosis and iron deficiency in employees and primary care patients in western Germany. *Annals of Internal Medicine* **128**: 337-345

Niederau C. (2000) Iron overload and atherosclerosis. *Hepatology* **32**: 672-674
Niederau C., Strohmeyer G. (2002) Strategies for early diagnosis of haemochromatosis. *European Journal of Gastroenterology and Hepatology* **14**: 223-229

Njajou O., Vaessen N., Joosse M., Berhuis B., van Dongen J.W., Breuning M.H., Snijders P.J., Rutten W.P., Sandkuijl L.A., Oostra B.A., van Duijn C.M., Heutink P. (2001) A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. *Nature Genetics* **28**: 213-214

O'Brien T., Barrett B., Murray D.M., O'Sullivan D.J. (1990) Usefulness of biochemical screening of diabetic patients for hemochromatosis. *Diabetes Care* **13**: 532-534

Olsson K.S., Ritter R., Rosén U., Heedman P.A., Staugård F. (1983) Prevalence of iron overload in central Sweden. *Acta Medica Scandinavica* **213**: 145-150

Olynyk J., Hall P., Ahern M., Kwiatek R., Mackinnon M. (1994) Screening for genetic haemochromatosis in a rheumatology clinic. *Australia and New Zealand Journal of Medicine* **24**: 22-25

Olynyk J.K., Cullen D.J., Aquilia S., Rossi E., Summerville L., Powell L.W. (1999) A population-based study of the clinical expression of the hemochromatosis gene. *New England Journal of Medicine* **341**: 718-724

O'Mahony S., O'Brien P.A., Whelton M.J. (1987) Genetic haemochromatosis and congenital spherocytosis. *Lancet* **i**: 282

Papanikolaou G., Politau M., Terpos E., Fourlemadis S., Sakellaropoulos N., Loukopoulis D. (2000) Hereditary hemochromatosis: HFE mutation analysis in Greeks reveals genetic heterogeneity. *Blood Cells, Molecules, and Diseases* **26**: 163-168

Papanikolaou G., Samuels M.E., Ludwig E.H., MacDonald M.L., Franchini P.L., Dube M.P., Andres L., MacFarlane J., Sakellaropoulos N., Politou M., Nemeth E., Thompson J., Risler J.K., Zaborowska C., Babakaiff R., Radomski C.C., Pape T.D., Davidas O., Christakis J., Brissot P, Lockitch G., Ganz T., Hayden M.R., Goldberg Y.P. (2004) Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. *Nature Genetics* **36**: 77-82

Park C.H., Valore E.V., Waring A.J., Ganz T. (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Journal of Biological Chemistry* **276**: 7806-7810

Parkkila S., Waheed A., Britton R.S., Feder J.N., Tsuchihashi Z., Schatzman R.C., Bacon B.R., Sly W.S. (1997) Immunohistochemistry of HLA-H, the protein defective in patients with hereditary hemochromatosis, reveals unique pattern of expression in gastrointestinal tract. *Proceedings of the National Academy of Sciences of the USA* **94**: 2534-2539

Pederson N.S. and Morling N. (1978) Iron stores in blood donors evaluated by serum ferritin. *Scandinavian Journal of Haematology* **20**: 70-76

Phatak P.D., Guzman G., Woll J.E., Robeson A., Phelps C.E. (1994) Cost-effectiveness of screening for hereditary hemochromatosis. *Archives of Internal Medicine* **154**: 769-776

Phatak P.D., Sham R.L., Raubertas R.F., Dunnigan K., 0'Leary M.T., Braggins C., Cappuccio J.D. (1998) Prevalence of hereditary hemochromatosis in 16031 primary care patients. *Annals of Internal Medicine* **129**: 954-961

Phillips R.A., van Slyke D.D., Hamilton P., Dole V.P., Emerson K., Archibald R.A. (1950) Measurement of specific gravities of whole blood and plasma by standard copper sulfate solution. *Journal of Biological Chemistry* **183**: 305-307

Philpott C.C. (2002) Molecular aspects of iron absorption: insights into the role of HFE in hemochromatosis. *Hepatology* **35**: 993-1001.

Pietrangelo A., Montosi G., Totaro A., Garuti C., Conte D., Cassanelli S., Fraquelli M., Sardini C., Vasta F., Gasparini P. (1999) Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. *New England Journal of Medicine* **341**: 725-731

Pietrangelo A. (2002) Physiology of iron transport and the hemochromatosis gene. *American Journal of Physiology. Gastrointestinal and Liver Physiology.* **282**: G403-G414

Pietrangelo A. (2004) Non-HFE hemochromatosis. *Hepatology* **39**: 21-29

Pigeon C., Ilyin G., Courselaud B., Leroyer P., Turlin B., Brissot P., Loreal O. (2001) A new mouse liver-specific gene, encoding a protein homologous to the human antimicrobial peptide hepcidin, is expressed during iron overload. *Journal of Biological Chemistry* **276**: 7811-7819

Piperno A., Arosio C., Fargion S., Roetto A., Nicoli C., Girelli D., Sbaiz L., Gasparini P., Boari G., Sampietro M., Camaschella C. (1996) The ancestral hemochromatosis haplotype is associated with severe phenotype expression in Italian patients. *Hepatology* **24**: 43-46

Piperno A., Sampietro M., Pietrangelo A., Arosio C., Lupica L., Montosi, G., Vergani A., Fraquelli M., Girelli D., Pasquero P., Roetto A., Gasparini P., Fargion S., Conte D., Camaschella C. (1998) Heterogeneity of hemochromatosis in Italy. *Gastroenterology* **114**: 996-1002

Piperno A., Arosio C., Fossati L., Viganò M., Trombini P., Vergani A., Mancia G. (2000) Two novel nonsense mutations of HFE gene in five unrelated Italian patients with hemochromatosis. *Gastroenterology* **119**: 441-444.

Pippard M.J. and Hoffbrand A.V. (1999) Iron. In *Postgraduate Haematology*, fourth edition. Oxford: Butterworth-Heinemann. pp 23-46

Porto G., Alves H., Rodrigues P., Cabeda J.M., Portal J.M., Ruivo A., Justiça B., Wolff R., De Sousa M. (1998) Major histocompatibility complex class I associations in iron overload: evidence for a new link between the HFE H63D HFE mutation, HLA-A29, and non-classical forms of hemochromatosis. *Immunogenetics* **47**: 404-410

Poulos J.E. and bacon B.R. (1996) Liver transplantation for hereditary hemochromatosis. *Digestive Diseases* **14**: 316-322

Powell L.W. (1992) Does transplantation of the liver cure genetic hemochromatosis? *Journal of Hepatology* **16**: 259-261

Powell L.W. (1996) Hemochromatosis: the impact of early diagnosis and therapy. *Gastroenterology* **110**: 1304-1307

Powell L.W. (2000) Broadsheet number 54: hereditary hemochromatosis. *Pathology* **32**: 24-36

Power T.E. and Adams P.C. (2001) Psychosocial impact of C282Y mutation testing for hemochromatosis. *Genetic Testing* **5**: 107-110

Pratiwi R., Fletcher L.M., Pyper W.R., Do K-A, Crawford D.H.G., Powell L.W., Jazwinska E.C. (1999) Linkage disequilibrium analysis in Australian haemochromatosis patients indicates bipartite association with clinical expression. *Journal of Hepatology* **31**: 39-46

Press R.D., Flora K., Gross C., Rabkin J.M., Corless C.L. (1998) Hepatic iron overload. Direct HFE (HLA-H) mutation analysis vs quantitative iron assays for the diagnosis of hereditary hemochromatosis. *American Journal of Clinical Pathology* **109**: 577-584

Qiagen HotStarTaq *PCR Handbook*, (1999)

Raha-Chowdhury R., Bowen D.J., Burnett A.K., Worwood M. (1995) Allelic associations and homozygosity at loci from HLA-B to D6S299 in genetic haemochromatosis. *Journal of Medical Genetics* **32**: 446-452

Read E.J., Herron R.M., Hughes D.M. (1993) Effect of non-monetary incentives on safety of blood donations. *Transfusion* **33**: 45S

Reyes M., Blanck H.M., Khoury M.J. Screening for iron overload due to hereditary hemochromatosis. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available at: www.cdc.gov/nccdphp/dnpa/hemochromatosis/screening.html updated 23.05.02.

Ritter B., Safwenberg J., Olsonn K.S. (1984) HLA as a marker of the haemochromatosis gene in Sweden. *Human Genetics* **68**: 62-66

Rivard S.R., Mura C., Simard H., Simard R., Grimard D., Le Gac G., Raguenes O., Ferec C., DE Braekeleer M. (2000) Mutation analysis in the HFE gene in patients with hereditary haemochromatosis in Saguenay-Lac-Saint-Jean (Quebec, Canada). *British Journal of Haematology* **108**: 854-858

Rochette J., Pointon J.J., Fisher C.A., Perera G., Arambepola M., Arichcha D.S., De Silva S., Vandwalle J.L., Monti J.P., Old J.M., Merryweather-Clarke A.T., Weatherall D.J., Robson K.J. (1999) Multicentric origin of hemochromatosis gene (HFE) mutations. *American Journal of Human Genetics* **64**: 1056-1062

Roest M., van der Schouw Y.T., de Valk B., Marx J.J.M., Tempelman M.J., de Groot P.G., Sixma J.J., Banga J.D. (1999) Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation* **100**: 1268-1273

Roetto A., Totano A., Cazzola M., Cicalano M., Bosio S., D'Ascola G., Carella M., Zelante L., Kelly A.L., Cox T.M., Gaparini P., Camaschella C. (1999) The juvenile hemochromatosis locus maps to chromosome 1q. *American Journal of Human Genetics* **64**: 1388-1393

Roetto A., Alberti F., Daraio F., Calì A., Cazzola M., Totaro A., Gasparini P., Camaschella C. (2000) Exclusion of ZIRTL as candidate gene of juvenile hemochromatosis and refinement of the critical interval on 1q21. *Blood Cells, Molecules and Diseases* **26**: 205-210

Roetto A., Totaro A., Piperno A., Piga A., Longo F., Garozzo G., Calì A., De Gobbi M., Gasparini P., Camaschella C. (2001) New mutations inactivating transferrin receptor 2 in hemochromatosis type 3. *Blood* **97**: 2555-2560

Roetto A., Merryweather-Clarke A.T., Daraio F., Livesey K., Pointon J.J., Barbabietole G., Piga A., Mackie P.H., Robson K.J.H., Camaschella C. (2002) A valine deletion of ferroportin 1: a common mutation in hemochromatosis type 4? *Blood* **100**: 733-734

Rosenberg W., Howell M., Roderick P., Eccles D., Day I. (1999) Hereditary haemochromatosis should be more widely known about. *British Medical Journal* **318**: 1486-1487

Rossi E., Olynyk J., Cullen D.J., Papadopoulos G., Bulsara M., Summerville L., Powell L.W. (2000) Compound heterozygous hemochromatosis genotype predicts increased iron and erythrocyte indices in women. *Clinical Chemistry* **46**: 162-166.

Roy C.N., Enns C.A. (2000) Iron homeostasis: new tales from the crypt. *Blood* **96**: 4020-4027

Royal Infirmary of Edinburgh (2000) Extraction of DNA from peripheral blood. *Department of Haematology Standard Operating Procedure 97.02d*

Ryan E., O'Keane C., Crowe J. (1997) Hemochromatosis in Ireland and HFE. *Blood Cells, Molecules, and Diseases* **24**: 428-432

Ryan E., Byrnes V., Coughton B., Flanagan A-M., Barrett S., O'Keane J.C., Crowe J. (2002) Underdiagnosis of hereditary haemochromatosis: lack of presentation or penetration? *Gut* **51**: 108-112.

Saddie R. and Feingold J. (1969) Idiopathic haemochromatosis: an autosomal recessive disease. *Clinical Genetics* **5**: 234-241

Sallie R.W., Reed W.D., Shilkin K.B. (1991) Confirmation of the efficacy of hepatic tissue iron index in differentiating genetic haemochromatosis from alcoholic liver disease. *Gut* **32**: 207-210

Salonen J.T., Nyyssönen K., Korpela H., Tuomilehto J., Seppänen R., Salonen R. (1992) High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. *Circulation* **86**: 803-811

Sanchez A.M., Schreiber G.B., Bethel J., McCurdy P.R., Glynn S.A., Williams A.E., Gilcher R. (2001) Prevalence, donation practices and risk assessment of blood donors with hemochromatosis. *Journal of the American Medical Association* **286**: 1475-1481

Scheuer P.J., Williams R., Muir A.R. (1962) Hepatic pathology in relatives of patients with haemochromatosis. *Journal of Pathology and Bacteriology* **84**: 53-64

Schwarz-Eywill M., Heilig B., Bauer H., Breibart A., Pezzutto A. (1992) Evaluation of serum ferritin as a marker for adult Still's disease activity. *Annals of the Rheumatic Diseases* **51**: 683-685

Scoog D.P. (1993) Laboratory evaluation of iron deficiency. In *Hematology. Clinical and Laboratory Practice.* St Lois: Mosby – Year Book, Inc pp 273-279

Searle J., Kerr J.F.R., Halliday J.W., Powell L.W. (1994) Iron storage disease. In *Pathology of the liver* 3rd edition. McSween R.N.M., Anthony P.P., Scheuer P.J., Burr A.D., Portmann B.C., eds. London: Churchill Livingstone, pp 219-241

Sham R.L., Ou C-Y., Cappuccio J., Braggins C., Dunnigan K., Phatak P.D. (1997) Correlation between genotype and phenotype in hereditary hemochromatosis: analysis of 61 cases. *Blood Cells, Molecules, and Diseases* **23**: 314-320

Shaheen N.J., Bacon B.R., Grimm I.S. (1998) Clinical characteristics of hereditary hemochromatosis patients who lack the C282Y mutation. *Hepatology* **28**: 526-529

Sheldon J.H. (1935) Haemochromatosis. London: Oxford University Press

Simon M., Pawlotsky Y., Bourel M., Fauchet R., Genetet R. (1975) Hémochromatose idiopathique: maladie associée à l'antigène tissulaire HL-A3? *Nouvelle Presse Médicale* **4**: 1432

Simon M., Alexandre J.L., Fauchet R., Genetet B., Bourel M. (1980) The genetics of haemochromatosis. *Progress in Medical Genetics* **4**: 135-168

Simon M., Le Mignon L., Fauchet R., Yaouanq J., David V., Edan G., Bourel M. (1987) A study of 609 haplotypes marking for the hemochromatosis gene: 1 mapping of the gene near the HLA-A locus and characters required to define a heterozygous population and 2 hypothesis concerning the underlying cause of hemochromatosis-HLA association. *American Journal of Human Genetics* **41**: 89-105

Singh B.M., Grunewald R.A., Press M., Muller B.R., Wise P.H. (1992) Prevalence of haemochromatosis amongst patients with diabetes mellitus. *Diabetes Medicine* **9**: 730-731

Singh S., Hider R.C., Porter J.B. (1990) A direct method for quantification of non-transferrin-bound iron. *Analytical Biochemistry* **186**: 320-323

Smith B.N., Kantrowitz W., Grace N.D., Greenberg M.S., Patton T.J., Ookubo R., Sorger K., Semeraro J.G., Doyle J.R., Cooper A.G., Kamat B.R., Maregni L.M., Rand W.M. (1997) Prevalence of hereditary hemochromatosis in Massachusetts corporation: is Celtic origin a risk factor? *Hepatology* **25**: 1439-1446

Starr R.T. (1980) Use of an alumina column in estimating total iron binding. *Clinical Chemistry* **26**: 156-158

Statland B.E., Winkel P., Bokelund H. (1976) Variation of serum iron concentration in young healthy men: within-day and day-to-day changes. *Clinical Biochemistry* **9**: 26-29

Strohmeyer G., Niederau C. (2000) Diabetes mellitus and hemochromatosis. In *Hemochromatosis: Genetics, pathophysiology, diagnosis and treatment*. Cambridge, Cambridge University Press.

Sullivan J.L. (1981) Iron and the sex difference in heart disease risk. *Lancet* **i**: 1293-1294

Sullivan J.L. (1983) The sex difference in ischemic heart disease. *Perspectives in Biology and Medicine* **26**: 657-671

Sullivan J.L. (1989) The iron paradigm of ischemic heart disease. *American Heart Journal* **117**: 1177-1188

Sullivan J.L. (1999) Iron and the genetics of cardiovascular disease. *Circulation* **100**: 1260-1263

Tan L., Khan M.K., Hawk J.C. III (1999) Use of blood therapeutically drawn from hemochromatosis patients. *Transfusion* **39**: 1018-1026

Task Force on Genetic Testing of the NIH-DOE Working Group on Ethical, Legal and Social Implications of Human Genome Research. (1997) Promoting safe and effective genetic testing in the United States. Bethesda: National Institute of Health

Tavill A.S. (1999) Screening for hemochromatosis: phenotyping or genotyping or both. *American Journal of Gastroenterology* **94**: 1430-1432

Thorstensen K., Asberg A., Kvitland M., Svaasand E., Hveem K., Bjerve K.S. (2000a) Detection of an unusual combination of mutations in the HFE gene for hemochromatosis. *Genetic Testing* **4**: 371-376

Trinder D., Oates P.S., Thomas C., Sadleir J., Morgan E.H. (2000) 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* **46**: 270-276

Trousseau A. (1865) Glycosurie; diabète sucré. *Clinique médical de l'Hôtel de Dieu de Paris* 2nd edition, **2**: 663-698. Paris: Baillière

Troisier M. (1871) Diabète sucré. *Bulletin de la Société de l'Anatomie (Paris)* **16**: 231-235

Totaro A., Grifa A., Carella M., D'Ambrosio L., Valentino M., Roth M.P., Borot N., Coppin H., Roetto A., Camaschella C., Gasparini P. (1997) Hereditary hemochromatosis: A *Hpa*I polymorphism within the HLA-H gene. *Molecular and Cellular Probes* **11**: 229-230.

Tsuchihashi Z., Hansen S., Quintana L., Kronmal G.S., Mapa F.A., Feder J.N., Wolff R.K. (1998) Transferrin receptor mutation analysis in hereditary hemochromatosis patients. *Blood Cells, Molecules, and Diseases* **24**: 317-321

Tuomainen T.P., Salonen R., Nyyssönen K., Salonen T.J. (1997) Cohort study of relation between donating blood and risk of myocardial infarction in 2682 men in eastern Finland. *British Medical Journal* **314**: 793-794

TuomainenT.P., Kontula K., Nyyssönen K., Lakka T.A., Heliö T., Salonen J.T. (1999) Increased risk of acute myocardial infarction in carriers of the hemochromatosis gene Cys282Tyr mutation. *Circulation* **100**: 1274-1279

Umbreit J.N., Conrad M.E., Moore E.G., Latour L.F. (1998) Iron absorption and cellular transport: the mobilferrin/paraferritin paradigm. *Seminars in Hematology* **35**: 13-26

University of Edinburgh (1995) DNA from paraffin sections. *Department of Pathology Standard Operating Procedure.*

U.S. Preventive Services Task Force (1996) *Guide to clinical preventive services*, 2nd edition. Baltimore, MD: Williams and Wilkins.

von Recklinghausen F.D. (1889) Über Hämochromatose. *Tageblatt der Versammlung Deutscher Naturforscher und Ärtzte in Heidelberg* **62**: 324-325

Vulpe C.D., Kuo Y-M., Murphy T.L., Cowley L., Askwith C., Libina N., Gitschier J., Anderson G.J. (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature Genetics* **21**: 195-199

Waalen, J., Felitti V., Gelbart T., Ho N.J., Beutler E. (2002) Prevalence of coronary heart disease associated with HFE mutations in adults attending a health appraisal center. *Americal Journal of Medicine* **113**: 472-479

Waheed A., Parkkila S., Zhou X.Y., Tomatsu S., Tsuchihashi Z., Feder J.N., Schatzman R.C., Britton R.S., Bacon B.R., Sly W.S. (1997) Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta 2-microglobulin, intracellular processing, and cell surface expression of HFE in COS-7 cells. *Proceedings of the National Academy of Sciences of the USA* **94**: 12384-12389

Waheed A., Parkkila S., Saarnio J., Fleming R.E., Zhou X.Y., Tomatsu S., Britton R.S., Bacon B.R., Sly W.S. (1999) Association of HFE protein with transferrin receptor in crypt enterocytes in human duodenum. *Proceedings of the National Academy of Sciences of the USA.* **96**: 1579-1584

Wallace D.F., Dooley J.S., Walker A.P. (1999) A novel mutation of HFE explains the classical phenotype of genetic hemochromatosis in a C282Y heterozygote. *Gastroenterology* **116**: 1409-1412

Wallace D.F., Pedersen P., Dixon J.L., Stephenson P., Searle J.W., Powell L.W., Subramaniam V.N. (2002) Novel mutation in ferroportin 1 is associated with autosomal dominant hemochromatosis. *Blood* **100**: 692-694

Ward R.J., O'Connell M.J., Dickson D.P., Reid N.M., Wade V.J., Mann S., Bomford A., Peters T.J. (1989) Biochemical studies of the iron cores and polypeptide shells of haemosiderin isolated from patients with primary or secondary haemochromatosis. *Biochimica et Biophysica Acta* **993**: 131-133

West A.P. Jr., Bennett M.J., Sellers V.M., Andrews N.C., Enns C.A., Bjorkman P.J. (2000) Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE. *Journal of Biological Chemistry* **275**: 38135-38138

Wetterhall S.F., Cogswell M.E., Kowdley, K.V. (1998) Public health surveillance for hereditary hemochromatosis. *Annals of Internal Medicine* **129**: 980-986

Willis G., Jennings B., Goodman E., Fellows I.W., Wimperis J.Z. (1997) A high prevalence of HLA-H 845A mutations in hemochromatosis patients and the normal population in eastern England. *Blood Cells, Molecules, and Diseases* **23**: 288-291

Willis G., Wimperis Z., Smith K.C., Fellows I. W., Jennings B.A. (1999) Haemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet*: **354**: 221-222

Willis G., Fellows I.W., Wimperis J.Z. (2000) Deaths attributed to haemochromatosis are rare in Britain. *British Medical Journal* **320**: 1146

Willis G., Scott D.G., Jennings B.A., Smith K., Bukhari M., Wimperis J.Z. (2002) HFE mutations in an inflammatory arthritis population. *Rheumatology (Oxford)* **41**: 176-179

Wilson J.M.G. and Junger G. (1968) *The principles and practice of screening for disease.* Public Health Paper 34, Geneva, Switzerland: World Health Organisation.

Witte D.L., Crosby W.H., Edwards C.Q., Fairbanks V.F., Mitros F.A. (1996) Hereditary hemochromatosis. *Clinica Chimica Acta* **245**: 139-200

Witte D.L. (1997) Mild liver enzyme abnormalities: eliminating hemochromatosis as cause. *Clinical Chemistry* **43**: 1535-1538

Wolf G and Wessling-Resnick M. (1994) An integrin-mobilferrin iron transport pathway in intestine and hemopoietic cells. *Nutrition Reviews* **52**: 387-389

Worwood M., Darke C., Trenchard P (1991) Hereditary haemochromatosis and blood donations. *British Medical Journal* **302**: 593

Worwood M. (2000) Early detection of genetic hemochromatosis: should all young adults be offered the genetic test? *Genetic Testing* **4**: 219-228

Yang Q., McDonnell S.M., Khoury M.J., Cono J., Parrish R.G. (1998) Hemochromatosis-associated mortality in the United States from 1979 to 1992: an analysis of multiple-cause mortality data. *Annals of Internal Medicine* **129**: 946-953

Zhou X.Y., Tomatsu S., Fleming R.E., Parkkila S., Waheed A., Jiang J., Fei Y., Brunt E.M., Ruddy D.A., Prass C.E., Schatzman R.C., O'Neill R., Britton R.S., Bacon B.R., Sly W.S. (1997) HFE gene knockout produces mouse model of hereditary hemochromatosis. *Proceedings of the National Academy of Sciences of the USA* **95**: 2492-2497

Zilva J.F., Pannall P.R., Mayne P.D. (1988) Iron metabolism. In *Clinical Chemistry in Diagnosis and Treatment* 5th edition. London: Edward Arnold; 388-400

Zimmern R.L. (1999) Genetic testing: a conceptual exploration. *Journal of Medical Ethics* **25**: 151-156

Zoller H., Koch R.O., Theurl I., Obrist P., Pietrangelo A., Montosi G., Haile D.J., Vogel W., Weiss G. (2001) Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. *Gastroenterology* **120**: 1412-1419

Presentations of this work to learned societies

Heterozygosity for the C282Y mutation of the HFE gene does not protect male blood donors from the development of iron deficiency. Oral presentation to International Society of Blood Transfusion meeting, Paris, July 2001.

Appendix 1:
Information sheet for donors

As a blood donor, you provide a vital support to the Health Service. Without donated blood, many operations could not go ahead, and the treatment of some illnesses, such as leukaemia, would be impossible. The amount of blood needed is continuing to increase, and we are looking for ways to increase the amount which is donated.

Your contribution as a regular donor is greatly appreciated and your comfort and safety during the process of donating are of the highest importance to us. As you know, donors are currently allowed to donate red cells only once every three months. The reason for this is, that with every donation of red cells you make, you lose a certain amount of iron from your body. Your body responds to this by increasing the amount of iron which is absorbed from food and drink, to make up for what has been lost. If you continue to lose iron, the absorption from food and drink may not be able to keep up, and your body may become deficient in iron. This may result in anaemia.

It is possible that some people have a greater ability than others to make up for this iron loss of blood donation. This may be related to genetic make-up. We would like to know more about the relationship between the genetic make-up, blood iron levels and blood donation, and hope that this information may be useful in determining how often any given donor can donate. We are asking you to consider helping us in this by participating in a study which will gather the required information over the course of your next few donations.

The "iron" gene

A gene has been found which is involved in determining how much iron your body takes up from food. About 10% of the population have a change in this gene, which seems to have no harmful effects and, indeed, might afford protection against becoming deficient in iron. However, some people (fewer than 1%) have a double dose of the changed gene, and they are at risk of developing illness due to iron overload in the long term. If, as a result of taking part in this study, you were shown to have the double gene, we would refer you to a specialist to see whether any further investigation might be necessary. (It could be argued that this is a benefit of screening, but there is still some doubt about the long-term significance of the double gene when it is picked up by chance in this way.)

What we are asking you to do

We would like you to agree to let us have an extra blood sample prior to each of your next few donations, so that we can monitor your exact haemoglobin and iron levels and we will carry out the tests for the "iron" gene and compare results. This will tell us whether it will be possible to use these tests to predict who can donate most frequently, and who is at risk of anaemia from donating.

If you take part, you will be given a specific appointment date and time for your next donation (to fit in with what is convenient for you – it should also save some time). You will not have to queue when you arrive. Instead of checking your haemoglobin from a finger-prick as at present, we will take a small (15 mls or 3 teaspoonsful) blood sample from a vein in your arm before you give blood. We will use this sample to check your haemoglobin before you donate and also to check your iron levels and analyse the gene referred to above.

In all other respects, your visit will be the same as usual. If you have the changed gene, this will be discussed with you at the end of the study.

If you decide to take part in the study, we will tell you General Practitioner if we find any evidence that you may be at risk of iron overload, or if you become anaemic. You must not feel under any pressure to take part. If you decide not to, your donations will be treated in exactly the same way as usual, and you will continue to receive the best care we can give while you are here.

If you would like to speak to a doctor who is not involved in the study about any aspect of it, please contact:

Dr P L Yap, Consultant, Edinburgh Blood Transfusion Service. Tel: 0131 536 5302

Appendix 2:

Questionnaire for Participants in Study of Blood Donors' Iron Levels

1. What is your weight (in stones and pounds or kilogrammes)?

2. Approximately how many times in a typical week do you eat red meat (ie beef, pork, lamb, sausages, ham, bacon, etc)? Count each meal that contains red meat as "one".

3. Are you taking iron tablets or vitamin pills at the moment? If so, please give the name of the tablets and the number you take per day.

4. Have you, as far as you remember, taken iron or vitamin tablets during the last two years?

   If you answered "YES" to Q. 4:

5. How long did you take them for?

6. What were they called? (if you can remember)

THANK YOU FOR YOUR HELP

Appendix 3:
Letter to C282Y heterozygotes

Dear Donor

Iron Study

Thank you very much for participating in this study. Whether you gave only one or up to five donations in the study, you provided valuable information. We will be in a position to let everyone know what we found in the next few weeks.

I would like to let you know that, when we tested you, we found that you have inherited one changed copy of the gene we were looking at. This gene controls how much iron the body takes from the diet. You have two copies of this (and all other genes). If both copies are changed, there is a risk of the accumulation of potentially harmful amounts of iron in the body. However, in your case, where one copy only is changed, there is no evidence that you are at risk from a build up of iron. In fact, having one changed copy of the gene seems to have no consequences at all for your health. It is quite common in the general population (about 12% of the Scottish population have it).

You can pass on this changed copy of the gene to your children. For them, as for you, there will be no ill-effects if they inherit only one changed copy. However, as the changed genes are quite common, there is a possibility that your partner may also have a changed gene. In this case, your children *could* inherit a changed gene from both of you, and therefore be at increased risk of iron accumulation.

It may be that you will wish to find out whether this might be the case with your children. One step you could consider would be to ask your partner if she would be prepared to be tested for the changed gene. This can be arranged through your GP. I enclose a letter for you to take to your GP if you would like to proceed with this. If your partner does not have the changed gene, your children should not be prone to excess iron accumulation.

Please note that the test results take 2-3 months to come back. Any health effects from excess iron take 20 or more years to occur.

If you have any questions about this, please address them to Dr J Gillon, Consultant Physician, South East Scotland Blood Transfusion Service, Lauriston Building, Lauriston Place, Edinburgh.

Yours faithfully

Andrew Stewart
Specialist Registrar in Haematology

Appendix 4:

Letter to General Practitioner of C282Y heterozygotes

Dear Doctor

Your patient has participated in a study we were running to look at iron stores and haemochromatosis (HFE) gene mutations in blood donors. You received notification of this in autumn 1999.

Your patient has been found to be heterozygous for the C282Y mutation in the HFE gene. Homozygosity for this mutation predisposes to the development of hereditary haemochromatosis. Heterozygosity, however, has not been shown to have any harmful effects at all.

About 12% of the local population are heterozygous. There is therefore a significant chance that your patient's partner (if he has one) is also heterozygous. In this situation, there would be a 50% chance of any children being homozygous, and so potentially affected by haemochromatosis.

If you patient's partner would like to be tested, this can be done by sending a red topped (full blood count) tube to the Department of Haematology at the RIE. We forward the sample to Dundee for analysis, and results usually take 2-3 months to return.

If you patient wishes his children tested, this can be arranged in the same way. We would not recommend genetic testing on any children less than 18 years old (iron overload virtually never causes any clinical problems before the mid 20s).

If anyone requires counselling about results of these tests, this can be arranged by referral to Dr PRE Johnson, Consultant Haematologist, Western General Hospital, Edinburgh.

If you require further information, please contact Dr J Gillon.

Yours faithfully

Andrew Stewart
Specialist Registrar in Haematology

Jack Gillon
Consultant Physician

Appendix 5:
Letter to Participants wild-type for C282Y

Dear Donor

Iron Study

Thank you very much for participating in this study. Whether you gave only one or up to five donations in the study, you provided valuable information. We will be in a position to let everyone know what we found in the next few weeks.

I would like to let you know that when we tested you, we found no evidence that you should be prone to accumulate excess iron in your body.

If you have any further questions about the study, please address them to Dr J Gillon, Consultant Physician, South East Scotland Blood Transfusion Service, Lauriston Building, Lauriston Place, Edinburgh.

Yours faithfully

Andrew Stewart
Specialist Registrar in Haematology

Appendix 6:

Information sheet for patients participating in coronary angiography study

There are a large number of factors which can contribute to the development of disease or narrowing of the arteries which supply the heart. Some of these, such as smoking, are very well known, but many others have been suggested. We would like to invite you to take part in a study to look at whether there is a connection between the amount of iron stored in the body and the development of heart disease.

Iron is taken into your body from food and drink. The amount taken in each day is normally closely controlled, so that only what is needed is absorbed. A recently discovered gene seems to be involved in this control mechanism. Changes in this gene are quite common, and may affect the amount of iron which is stored in the body. The effect on the amount of iron stored is nearly always very small, and we do not think that it has any effect on health at all. A very small number of people do have a larger change in the gene, which causes the build-up of large amounts of iron in the body.

If you take part in the study, all that will happen is that some extra blood (about 3 teaspoonsful) will be taken along with the blood tests you will be having anyway to prepare you for your angiogram. We will use this blood to test for the amount of iron stored in your body and to look for any changes in the iron control gene. The blood samples we use for the study will be identified by a code number, which means we will not know whose blood we are testing. You will, therefore, not receive any results from this study, and no results from it will be entered into your medical records.

You are completely free not to agree to have this extra sample taken. Whether you take part in the study or not will not affect in any way the care you receive, the way the angiogram is performed or the treatment of the results of the angiogram.

If you would like to speak to a doctor who is not involved in this study about any aspect of it, please contact Prof. C.A. Ludlam, consultant haematologist at the Royal Infirmary.

Appendix 7:
The Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium states that:

In the absence of mutation non-random mating, selection and genetic drift, the genetic constitution of the population remains the same from one generation to the next.

The Hardy-Weinberg equation relates the frequencies of a normal gene (p) and that of its abnormal counterpart (q) in a population:

$p + q = 1$
$p^2 + 2pq + q^2 = 1$

Here, p^2 is the frequency of the normal homozygote, q^2 the frequency of the abnormal homozygote and 2pq the frequency of the heterozygote.

Appendix 8:
Questionnaire for GP survey

1. What is the list size of your practice?

   ❑ <3000 ❑ 3000-6000 ❑ 6000-9000 ❑ 9000-12000 ❑ >12000

2. How many patients with haemochromatosis, if any, are there in your practice?

3. Compared with cystic fibrosis, do you think haemochromatosis is (please tick one):

   ❑ Commoner ❑ about as common ❑ rarer

4. Which of the following would be of use in confirming the diagnosis of haemochromatosis: (please tick as many as you wish)

   ❑ Full blood count ❑ ferritin ❑ U&E

   ❑ serum iron/TIBC ❑ genetic test

5. Please indicate whether or not the following symptoms and signs are necessary for a diagnosis of haemochromatosis:

Skin discolouration	❑ necessary	❑ not necessary	❑ don't know
Abnormal LFTs	❑ necessary	❑ not necessary	❑ don't know
Diabetes	❑ necessary	❑ not necessary	❑ don't know
Arthritis	❑ necessary	❑ not necessary	❑ don't know

6. Please indicate whether you agree or disagree with the following statements about haemochromatosis:

   Early treatment restores normal life expectancy

   ❑ agree ❑ disagree ❑ don't know

   If untreated, it is often fatal

   ❑ agree ❑ disagree ❑ don't know

   Treatment involves use of expensive drugs

   ❑ agree ❑ disagree ❑ don't know

   Younger patients are often asymptomatic

   ❑ agree ❑ disagree ❑ don't know

7. Have you ever suspected that a patient may have haemochroatosis?

❑ YES ❑ NO

8. If you answered "yes" to Q.7, what led you to suspect haemochromatosis?

9. Would you see a screening programme for haemochromatosis as (you may indicate more than one)

- A useful addition to the work of Primary care ❑ agree ❑ disagree
- Something of no relevance to Primary care ❑ agree ❑ disagree
- An extra burden on Primary Care ❑ agree ❑ disagree

10. Any other comments?

Appendix 9:

Raw data from studies reported in Chapter 5

A. Normal control subjects

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
1	41	F	141	7.0	293	97	5
2	32	F	133	8.9	330	94	
3	38	F	123	6.8	308	91	1
4	39	F	128	5.8	257	98	1
5	37	M	131	5.2	208	96	1
6	69	F	135	5.3	322	91	1
7	32	F	118	4.4	251	94	1
8	64	M	144	7.6	254	88	1
9	62	F	147	7.5	291	95	1
10	70	M	142	5.9	227	91	3
11	55	F	131	7.4	271	85	1
12	50	F	126	5.1	309	91	1
13	31	F	119	5.7	233	82	1
14	63	M	144	7.8	333	85	3
15	44	F	130	5.4	164	96	3
16	50	F	137	5.5	195	89	2
17	44	M	154	5.7	201	93	5
18	25	F	135	5.9	194	91	6
19	87	F	132	9.4	251	92	5
20	17	F	120	9.5	282	91	2
21	29	F	137	7.2	214	92	5
22	47	M	146	8.5	183	89	1
23	59	F	129	8.8	293	92	
24	51	F	139	7.9	263	93	
25	64	F	137	5.1	223	94	1
26	34	F	135	5.3	285	88	1
27	77	F	138	5.4	233	89	1
28	65	M	150	6.8	228	92	1
29	89	F	129	8.8	308	89	2
30	61	M	134	6.8	174	85	1
31	54	M	143	9.4	281	91	

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
32	62	M	146	8.9	207	92	1
33	49	F	137	6.5	257	94	5
34	82	M	134	8.2	233	95	1
35	31	F	128	5.5	356	89	1
36	60	M	175	9.6	253	86	3
37	43	F	144	6.4	313	82	3
38	30	M	146	5.3	262	85	2
39	57	F	136	5.5	276	87	1
40	42	F	133	8.0	375	88	1
41	19	F	131	5.8	265	87	1
42	49	F	127	4.4	282	90	3
43	28	F	120	5.2	238	85	1
44	51	F	123	8.0	383	93	1
45	70	F	127	7.4	234	84	3
46	51	F	123	6.7	278	92	2
47	63	F	138	6.0	271	85	1
48	31	F	139	8.8	285	88	
49	41	F	125	9.2	321	92	1
50	73	M	132	6.0	188	92	3
51	51	F	126	8.7	258	88	3
52	39	M	139	6.4	212	83	1
53	69	F	120	8.1	283	99	
54	79	M	148	7.0	381	93	3
55	30	F	138	7.7	222	91	3
56	28	F	147	8.4	195	84	1
57	85	F	120	7.8	309	90	1
58	56	M	140	6.2	237	91	1
59	44	F	123	7.0	252	95	1
60	39	M	154	5.3	230	91	1
61	53	M	148	5.2	246	92	2
62	50	M	136	5.0	258	92	3
63	34	F	136	5.7	273	96	1
64	67	F	126	6.0	356	90	3
65	17	F	138	6.0	257	86	1
66	43	F	129	6.8	301	90	1
67	64	M	152	4.5	189	99	1
68	30	F	123	9.9	326	92	3

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
69	62	M	137	5.3	325	89	1
70	68	M	131	7.5	267	85	3
71	88	F	137	6.2	307	91	1
72	71	M	137	4.9	185	91	1
73	58	M	142	5.8	290	91	1
74	64	F	128	4.8	247	88	1
75	35	F	125	7.2	234	93	1
76	79	F	139	7.1	232	93	1
77	46	M	137	4.3	285	93	1
78	39	F	126	5.3	227	91	3
79	48	F	127	5.2	252	94	1
80	74	F	129	6.7	326	94	1
81	24	F	142	6.0	272	93	1
82	48	M	142	8.1	238	95	1
83	33	M	136	7.3	312	91	1
84	32	F	116	7.0	167	86	1
85	40	M	147	6.8	341	89	2
86	47	M	154	7.5	178	99	1
87	54	M	142	7.7	214	92	1
88	75	F	126	7.0	230	95	3
89	39	M	154	5.7	211	93	3
90	14	M	130	7.6	353	83	3
91	22	F	135	5.9	264	88	1
92	82	F	115	7.0	364	87	1
93	41	M	158	4.5	218	94	3
94	71	F	136	7.9	263	93	
95	54	M	136	6.6	259	90	1
96	30	F	136	7.4	385	92	1
97	67	F	116	5.2	218	94	3
98	22	F	143	7.6	273	92	3
99	51	F	129	6.1	243	91	
100	29	M	146	5.4	329	94	3
101	74	F	145	6.6	306	95	3
102	45	M	150	5.5	231	88	1
103	48	F	117	6.3	378	92	1
104	32	F	125	5.7	241	88	1
105	46	M	132	8.2	394	97	1

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
106	16	F	116	7.4	318	82	1
107	55	F	132	7.8	272	90	2
108	42	F	130	8.6	253	98	1
109	82	M	134	6.6	272	92	2
110	61	F	135	4.9	267	92	1
111	31	F	139	5.4	264	91	1
112	65	F	141	6.5	186	84	3
113	31	F	135	6.5	374	91	1
114	44	F	145	6.9	276	96	1
115	45	M	155	5.2	185	88	1
116	84	F	131	5.0	192	87	3
117	55	F	151	8.0	173	91	1
118	67	M	146	8.0	269	92	1
119	72	F	132	5.9	343	95	3
120	71	F	125	5.6	254	93	1
121	78	M	135	8.6	244	90	1
122	51	F	135	9.6	327	92	1
123	45	M	155	7.9	209	86	1
124	70	F	128	9.1	324	91	3
125	41	F	138	7.7	331	91	1
126	50	F	123	7.5	232	92	1
127	91	F	126	8.8	232	81	1
128	83	F	123	5.7	302	95	1
129	62	F	123	8.4	257	94	1
130	28	F	126	4.7	242	86	1
131	61	F	144	5.8	162	90	1
132	49	F	132	6.0	311	91	1
133	47	M	143	5.5	252	91	3
134	30	M	167	6.2	237	88	1
135	30	M	134	9.8	270	89	1
136	22	F	120	7.5	327	98	1
137	22	F	123	6.1	249	91	3
138	55	F	123	6.5	244	87	3
139	71	F	134	7.3	223	88	1
140	75	M	159	7.3	196	91	
141	25	M	152	9.0	241	79	1
142	72	F	143	6.1	282	91	1

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
143	72	F	143	6.1	282	91	1
144	36	F	119	6.0	203	88	1
145	53	F	124	6.2	203	88	1
146	51	F	146	5.3	247	89	1
147	48	M	142	11.0	230	89	2
148	59	M	136	7.4	242	90	
149	60	M	159	9.6	214	87	3
150	28	F	127	6.0	150	96	1
151	74	M	152	9.9	277	86	3
152	80	F	122	6.8	281	100	1
153	30	F	137	89	285	89	1
154	26	F	115	7.7	259	86	1
155	41	M	134	10.0	280	89	1
156	54	F	136	8.9	212	91	3
157	18	M	157	8.4	243	83	1
158	44	F	131	7.4	305	94	1
159	55	M	157	7.6	354	94	3
160	19	F	117	7.6	281	89	1
161	55	F	139	7.0	288	94	3
162	73	M	141	10.5	293	90	1
163	59	F	115	6.6	198	89	
164	45	M	135	4.6	306	92	1
165	84	F	119	8.0	294	99	3
166	39	M	143	4.7	246	89	3
167	88	F	117	7.7	318	95	1
168	36	F	129	6.0	318	89	1
169	55	M	143	73	332	84	1
170	60	F	144	8.4	256	89	3
171	59	F	121	6.2	294	93	1
172	81	F	139	4.6	214	88	1
173	60	F	127	7.5	230	93	1
174	65	F	115	7.1	277	92	1
175	60	F	128	5.4	268	86	1
176	41	M	137	9.3	189	84	3
177	59	F	136	7.7	281	89	1
178	48	M	146	6.1	248	91	3
179	78	F	124	5.7	305	93	3

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
180	58	F	128	4.9	252	94	3
181	49	F	131	6.0	294	93	3
182	62	M	150	5.3	175	88	1
183	54	F	125	7.8	310	83	1
184	27	F	124	6.5	217	94	1
185	33	F	140	8.9	215	94	1
186	43	M	139	5.9	167	85	2
187	38	M	153	5.4	229	92	3
188	56	F	127	7.9	252	91	3
189	18	F	116	6.5	247	92	1
190	46	M	148	7.1	212	99	1
191	30	F	119	6.8	279	90	1
192	85	F	138	5.1	190	89	3
193	38	F	127	6.7	255	89	2
194	40	M	144	6.3	260	91	1
195	18	F	118	83	343	93	1
196	69	F	116	5.7	270	87	1
197	31	F	124	8.2	165	96	1
198	24	F	131	7.4	358	91	1
199	53	F	134	7.9	316	97	5
200	74	F	132	6.9	36	83	1
201	36	M	150	9.2	264	94	3
202	67	M	139	6.1	180	91	3
203	35	F	131	7.8	215	89	1
204	37	F	121	5.5	188	92	1
205	62	M	135	8.0	241	95	2
206	62	M	152	7.4	219	95	1
207	39	F	137	8.6	256	89	1
208	85	F	121	6.8	344	97	1
209	52	F	138	7.5	210	92	2
210	77	F	118	5.9	238	82	1
211	39	F	132	10.2	305	96	1
212	18	F	125	7.3	254	84	1
213	35	F	134	5.9	198	94	1
214	20	F	133	5.3	276	95	1
215	68	F	135	8.5	383	91	1
216	22	F	132	6.6	392	90	1

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
217	70	F	147	6.9	253	90	1
218	30	M	139	9.9	297	93	3
219	22	M	158	5.1	269	88	1
220	70	F	137	5.3	257	91	2
221	78	F	141	8.8	319	89	1
222	62	F	151	8.4	352	89	5
223	36	F	131	7.8	372	94	3
224	43	F	137	7.4	185	90	1
225	37	M	138	6.6	245	86	3
226	41	F	124	8.7	291	86	1
227	37	F	137	7.6	244	93	3
228	49	M	151	6.6	269	92	1
229	46	M	133	5.1	266	92	1
230	45	M	149	8.0	254	91	1
231	21	F	140	5.2	237	93	3
232	71	M	139	5.4	271	96	2
233	70	F	149	9.2	355	90	3
234	20	M	141	7.6	224	95	1
235	67	M	145	6.5	177	90	1
236	77	F	131	7.2	301	92	1
237	78	F	119	6.9	331	93	3
238	82	F	142	5.9	206	95	6
239	77	F	115	5.8	278	94	1
240	60	F	147	6.5	237	96	1
241	57	M	154	6.9	253	87	1
242	54	F	130	6.6	240	95	1
243	85	F	130	7.9	255	99	1
244	20	F	125	5.7	305	90	1
245	72	F	126	7.2	269	90	1
246	32	F	125	7.7	394	91	3
247	32	F	124	8.0	242	83	1
248	69	F	128	7.6	292	89	3
249	34	F	144	6.4	258	92	1
250	44	F	138	6.9	318	98	2
251	55	M	157	8.2	215	86	1
252	32	F	136	10.4	251	83	1
253	20	F	117	6.6	299	97	2

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
254	67	F	119	7.2	227	95	3
255	56	F	139	6.7	295	89	3
256	33	M	155	6.0	259	88	3
257	73	F	133	4.8	247	93	6
258	37	F	117	7.7	333	86	1
259	68	F	121	9.3	292	90	1
260	66	F	129	7.9	264	95	1
261	59	F	129	5.0	289	94	3
262	65	F	131	5.5	269	86	1
263	70	F	117	6.2	329	85	1
264	19	F	126	5.7	234	89	3
265	22	M	143	10.6	295	89	1
266	37	M	152	7.8	198	87	1
267	33	M	134	10.2	253	96	3
268	28	F	125	7.4	362	92	1
269	19	M	139	8.7	179	85	3
270	63	F	118	6.4	325	84	1
271	90	M	146	8.4	281	91	1
272	28	F	116	6.8	277	88	1
273	38	F	139	8.3	235	92	1
274	38	F	123	8.0	245	84	1
275	29	F	148	6.1	276	82	2
276	66	F	162	5.8	247	97	1
277	30	F	119	8.4	316	87	1
278	49	M	137	7.7	259	95	1
279	57	F	128	6.4	389	98	1
280	52	F	116	7.3	315	99	2
281	77	M	134	7.6	241	97	1
282	73	F	136	6.5	264	92	1
283	61	M	159	5.6	245	93	1
284	22	F	124	7.4	258	92	2
285	54	F	155	7.3	207	95	
286	66	F	115	9.6	273	86	1
287	46	F	144	7.5	305	92	3
288	31	F	130	4.6	302	95	3
289	26	M	142	7.3	230	91	2
290	49	M	148	10.8	233	83	2

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
291	81	F	130	7.0	355	87	1
292	78	F	157	7.9	324	91	1
293	34	F	116	7.8	200	89	1
294	34	F	116	10.2	166	98	1
295	50	M	145	7.3	219	88	1
296	37	M	133	8.9	263	90	1
297	38	F	140	12.0	343	90	
298	41	M	161	4.6	220	92	1
299	42	F	137	5.3	229	94	1
300	30	F	139	7.6	222	86	2
301	63	F	136	10.0	302	96	3
302	50	F	135	5.9	249	95	2
303	69	F	131	4.8	264	93	1
304	66	F	131	9.1	263	93	1
305	67	F	124	9.3	234	91	2
306	53	M	157	7.7	213	92	6
307	44	F	137	6.2	351	96	1
308	26	F	140	7.2	300	93	
309	17	F	142	8.9	248	86	6
310	30	F	141	7.2	344	91	1
311	57	F	126	8.6	334	91	1
312	31	F	119	7.4	388	94	2
313	32	F	132	7.7	264	90	2
314	40	M	148	6.5	329	91	5
315	42	F	128	8.7	254	90	1
316	31	F	127	8.1	389	92	2
317	67	M	143	7.8	183	92	1
318	56	F	131	6.8	185	89	2
319	17	M	147	5.8	307	92	1
320	26	F	131	8.0	394	84	1
321	62	F	133	6.5	228	100	1
322	58	F	146	8.5	273	80	1
323	89	M	135	7.1	315	97	3
324	32	F	133	7.1	236	90	3
325	33	M	145	6.8	226	91	
326	36	F	128	7.9	331	93	1
327	31	F	130	8.5	314	89	3

Study number	Age (years)	Sex	Hb (g/L)	WCC (10^9/L)	Platelets (10^9/L)	MCV (fL)	Genotype
328	58	F	132	4.9	291	94	1
329	69	M	134	6.6	209	86	1
330	54	F	143	8.7	331	99	1
331	69	M	143	9.1	313	94	1
332	60	F	130	5.9	190	88	3
333	78	F	136	5.9	193	96	1
334	69	F	116	8.2	252	88	1
335	33	F	125	9.9	382	92	5
336	56	F	140	7.2	265	85	1
337	54	F	150	9.6	344	93	1
338	19	F	128	7.3	243	87	1
339	70	F	128	5.2	273	90	1
340	42	M	153	4.9	223	85	4
341	32	F	139	5.8	293	92	
342	33	F	133	6.3	229	96	5
343	62	F	124	7.5	395	98	1
344	41	F	125	5.8	277	89	3
345	73	F	127	5.8	281	89	1
346	58	F	134	8.1	317	90	1
347	41	F	133	6.8	233	97	1
348	62	F	122	6.8	298	100	2
349	79	M	158	8.9	264	97	2
350	81	M	141	5.5	186	83	3
351	85	F	125	5.4	263	89	3
352	34	F	130	6.8	157	95	2

B. Iron deficient subjects

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
1	52	F	84	3	1
2	11	F	98	2	1
3	78	F	105	9	1
4	51	F	109	5	1
5	47	F	88	3	
6	29	F	73	2	1
7	43	F	93	6	
8	65	F	104	4	
9	90	M	67	7	3
10	43	F	92	4	3
11	35	F	103	3	3
12	77	F	93	5	3
13	39	F	90	7	1
14	38	F	100	4	1
15	24	F	83	5	2
16	35	F	96	3	1
17	72	F	93	5	1
18	30	M	104	3	1
19	62	F	69	10	3
20	29	M	91	3	
21	50	M	124	7	
22	83	F	86	6	3
23	67	M	119	7	
24	36	M	99	3	1
25	45	F	110	8	
26	78	F	79	7	2
27	43	F	103	5	1
28	43	F	123	5	2
29	32	F	113	6	
30	91	F	82	11	
31	80	F	99	7	1
32	64	F	143	13	
33	76	M	138	9	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
34	44	F	90	3	3
35	44	F	112	3	
36	50	F	98	3	3
37	36	F	83	5	3
38	82	M	86	13	3
39	36	F	88	4	1
40	60	M	69	5	3
41	17	F	123	7	1
42	37	F	77	3	
43	37	F	121	13	
44	18	F	98	4	1
45	79	F	44	7	1
46	51	F	104	4	1
47	38	M	101	3	3
48	23	F	70	5	3
49	68	M	146	13	1
50	30	F	109	5	1
51	70	M	77	12	1
52	F	50	99	10	
53	36	F	102	5	1
54	41	M	56	6	
55	39	F	96	3	1
56	77	M	87	10	1
57	41	F	89	4	
58	28	F	97	7	1
59	70	F	87	7	5
60	68	F	51	2	1
61	59	F	94	6	3
62	34	F	104	4	1
63	48	F	107	7	1
64	27	M	116	5	1
65	86	F	98	8	1
66	61	F	99	4	1
67	32	F	118	8	2
68	35	F	88	3	1
69	73	F	93	6	
70	44	F	95	4	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
71	51	F	104	6	1
72	73	F	103	4	1
73	70	M	103	10	1
74	81	F	109	8	1
75	41	F	88	3	
76	66	F	78	8	1
77	45	F	114	3	3
78	39	F	77	3	1
79	53	F	105	9	1
80	72	F	104	4	2
81	34	F	107	4	2
82	31	M	115	10	1
83	79	F	52	8	1
84	83	F	75	11	
85	65	F	111	11	1
86	85	F	68	11	3
87	51	F	99	11	1
88	65	F	123	13	3
89	58	F	91	13	1
90	39	F	115	13	1
91	25	F	101	11	1
92	53	F	108	10	
93	22	F	111	8	2
94	29	F	105	5	3
95	65	M	65	2	1
96	50	F	55	12	1
97	49	F	101	4	1
98	49	F	108	4	3
99	51	F	104	12	1
100	31	F	93	4	3
101	35	F	97	5	1
102	48	F	111	12	1
103	50	F	111	8	5
104	57	M	92	4	1
105	79	F	98	13	1
106	74	F	114	8	
107	76	M	104	7	

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
108	68	F	109	8	1
109	87	F	96	13	
110	79	M	97	7	3
111	77	M	101	7	3
112	51	F	103	7	1
113	38	F	72	3	1
114	10	F	112	3	3
115	51	F	109	6	1
116	87	F	73	11	
117	28	F	98	5	1
118	49	M	103	6	
119	49	M	116	12	3
120	88	F	77	6	1
121	51	F	67	6	1
122	30	F	109	5	1
123	88	F	84	8	1
124	35	F	113	10	1
125	24	F	127	9	1
126	76	F	87	7	1
127	78	F	51	5	2
128	73	F	125	12	2
129	83	F	98	9	1
130	26	F	106	6	1
131	19	F	72	3	3
132	45	F	94	3	1
133	23	F	111	4	1
134	52	F	105	5	1
135	69	F	88	7	3
136	41	F	129	9	1
137	36	F	79	7	1
138	87	F	88	11	1
139	74	M	114	5	1
140	87	M	103	11	1
141	77	F	94	9	1
142	37	F	109	7	3
143	77	M	89	5	1
144	40	F	84	3	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
145	90	F	70	5	1
146	71	M	103	10	1
147	52	F	94	6	3
148	15	F	112	4	1
149	40	F	111	2	3
150	70	F	79	5	3
151	82	M	73	7	3
152	71	M	93	6	1
153	78	F	76	9	1
154	41	F	97	6	3
155	76	M	113	14	3
156	69	F	94	5	2
157	43	F	88	4	1
158	40	F	101	3	1
159	56	M	80	7	2
160	88	M	99	8	
161	83	F	75	13	3
162	80	F	95	12	1
163	15	F	93	5	3
164	47	F	105	6	3
165	40	F	12	5	1
166	86	M	99	11	2
167	30	F	93	3	1
168	29	F	79	3	1
169	70	M	82	8	1
170	82	F	98	10	3
171	47	F	97	6	3
172	76	F	72	6	1
173	40	F	116	6	1
174	30	F	104	10	1
175	50	F	63	6	6
176	43	M	99	7	1
177	52	F	79	3	1
178	23	F	109	7	5
179	86	F	60	10	1
180	63	F	84	9	2
181	65	F	112	7	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
182	57	F	145	14	2
183	19	F	118	13	1
184	61	F	63	5	3
185	37	F	102	14	3
186	46	M	130	8	3
187	40	F	90	5	3
188	54	F	111	6	1
189	43	F	137	10	1
190	75	F	88	12	1
191	79	F	83	3	1
192	28	F	124	12	1
193	93	F	108	11	3
194	48	M	101	6	6
195	31	F	107	5	3
196	18	M	91	7	3
197	44	F	104	5	
198	49	M	89	4	1
199	57	F	96	5	3
200	92	F	88	9	1
201	86	F	61	11	1
202	37	F	111	8	1
203	62	F	115	12	1
204	90	F	82	6	1
205	62	F	125	10	3
206	45	F	106	7	1
207	76	M	96	6	2
208	70	F	106	11	1
209	51	F	103	5	2
210	29	F	124	10	1
211	42	F	106	4	1
212	39	F	118	6	1
213	37	F	101	3	1
214	31	M	109	7	3
215	62	M	93	10	3
216	78	F	105	10	1
217	82	F	88	8	3
218	91	M	99	10	2

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
219	77	F	91	7	3
220	89	M	92	12	1
221	59	F	96	4	3
222	50	F	109	5	3
223	40	F	102	5	3
224	91	F	78	9	1
225	41	M	153	10	2
226	35	F	99	3	2
227	46	F	81	3	2
228	72	M	70	4	1
229	46	F	99	4	1
230	40	F	92	4	1
231	36	F	95	7	1
232	45	F	96	5	1
233	37	F	133	9	1
234	40	F	111	2	3
235	29	F	89	2	
236	68	M	77	6	1
237	81	F	107	14	1
238	22	F	124	5	1
239	91	F	90	13	1
240	52	F	78	5	1
241	48	F	76	4	3
242	48	F	66	7	1
243	66	F	99	8	6
244	47	F	108	4	1
245	47	F	100	3	1
246	88	M	105	12	
247	48	F	114	4	1
248	49	F	92	6	5
249	60	M	93	8	1
250	41	M	79	6	1
251	21	F	105	5	1
252	28	F	105	4	1
253	35	F	104	3	1
254	83	F	109	9	1
255	66	F	96	7	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
256	35	F	97	11	3
257	86	F	94	13	5
258	77	M	76	5	1
259	44	F	87	4	1
260	37	M	76	14	
261	81	F	76	14	1
262	88	F	71	7	1
263	32	F	81	8	1
264	78	M	114	8	1
265	38	F	101	4	1
266	43	F	99	4	1
267	36	F	108	4	1
268	79	F	83	6	1
269	51	F	98	6	1
270	78	M	87	5	1
271	48	F	91	6	3
272	72	M	118	5	1
273	90	F	70	12	1
274	47	F	105	4	1
275	46	F	85	3	1
276	52	F	115	8	1
277	21	M	118	5	1
278	47	F	86	3	3
279	51	F	94	7	3
280	40	F	129	7	3
281	41	F	112	4	3
282	36	F	124	5	3
283	65	F	99	4	1
284	25	M	110	5	1
285	45	F	96	4	1
286	81	F	54	4	1
287	88	F	106	8	1
288	87	F	101	8	1
289	43	F	110	4	1
290	72	M	113	14	2
291	87	F	55	11	1
292	47	F	106	4	2

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
293	43	F	109	7	2
294	80	F	105	7	1
295	55	M	88	4	3
296	73	F	86	4	1
297	85	F	101	10	3
298	68	F	97	12	3
299	47	F	131	5	1
300	73	M	129	11	1
301	78	F	117	13	1
302	48	F	99	3	3
303	31	F	111	4	3
304	29	F	72	2	3
305	65	F	126	10	2
306	79	F	107	6	1
307	29	F	123	4	1
308	39	F	103	3	1
309	41	F	104	4	1
310	18	F	100	3	?
311	86	F	98	10	1
312	36	F	121	9	1
313	41	F	96	2	3
314	77	F	103	9	1
315	74	M	98	14	2
316	60	M	87	4	1
317	45	F	91	9	1
318	74	F	105	8	1
319	35	F	107	4	1
320	77	F	114	9	?
321	52	F	106	5	3
322	79	F	86	10	1
323	47	F	105	7	1
324	16	M	135	8	1
325	31	F	92	6	1
326	89	F	68	5	1
327	79	F	102	10	1
328	69	M	72	5	1
329	44	M	76	8	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
330	42	F	78	3	3
331	88	F	99	9	2
332	86	F	95	12	1
333	68	F	109	9	1
334	72	F	82	4	3
335	75	M	118	10	1
336	30	F	110	3	1
337	45	M	89	13	5
338	48	F	96	5	1
339	41	F	88	3	1
340	50	F	106	5	?
341	86	F	109	9	2
342	43	F	124	4	?
343	66	F	106	9	1
344	48	F	122	10	?
345	46	F	96	3	5
346	45	F	122	12	1
347	45	F	107	4	3
348	30	F	95	4	1
349	52	F	99	5	1
350	35	F	101	5	6
351	42	F	149	6	?
352	35	F	124	8	5
353	78	M	86	6	1
354	86	F	115	8	2
355	50	F	108	3	3
356	82	F	92	11	3
357	42	F	102	4	1
358	78	M	125	7	3
359	87	F	81	7	1
360	90	F	74	11	1
361	3	M	113	6	3
362	47	F	94	6	1
363	77	M	108	6	1
364	76	F	106	6	?
365	72	M	89	9	3
366	90	F	94	11	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
367	29	M	135	9	1
368	68	F	106	6	1
369	49	F	113	5	1
370	70	F	94	7	1
371	91	M	76	10	2
372	20	F	86	5	3
373	39	M	106	3	1
374	85	F	125	12	6
375	82	F	108	11	6
376	44	F	116	10	?
377	68	F	11	6	1
378	42	M	129	12	1
379	40	F	113	11	1
380	82	F	99	8	1
381	44	F	92	3	1
382	78	F	96	5	1
383	54	F	126	8	1
384	24	F	94	7	1
385	63	F	111	6	1
386	78	F	70	7	1
387	85	F	71	4	?
388	35	F	120	6	1
389	39	F	124	12	2
390	41	F	107	6	1
391	78	M	90	5	6
392	80	M	117	13	1
393	84	M	100	4	12
394	40	F	101	4	2
395	71	M	107	9	3
396	63	M	111	4	2
397	56	M	96	2	1
398	48	M	112	6	1
399	40	M	117	2	3
400	30	F	116	5	-
401	79	F	69	4	3
402	66	M	113	5	1
403	85	F	87	10	6

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
404	68	M	107	5	3
405	74	F	98	7	-
406	30	F	76	1	1
407	31	F	102	3	1
408	44	F	102	8	1
409	82	F	100	7	1
410	71	F	140	9	1
411	32	F	121	11	1
412	80	F	89	5	1
413	45	F	94	3	1
414	59	M	117	6	1
415	86	M	49	3	3
416	35	F	98	4	1
417	64	M	92	6	1
418	48	F	82	7	1
419	59	F	108	9	2
420	83	F	62	13	
421	88	F	101	9	
422	85	F	63	13	1
423	70	M	116	7	1
424	32	F	119	7	3
425	60	F	104	3	6
426	88	M	94	10	1
427	83	M	85	14	1
428	74	F	109	11	1
429	44	F	73	2	3
430	26	F	124	7	1
431	19	F	130	14	3
432	39	F	99	3	3
433	18	F	98	14	1
434	18	F	127	13	1
435	27	F	86	3	1
436	42	F	101	6	3
437	65	M	76	4	2
438	67	F	112	7	1
439	89	F	109	7	1
440	72	F	95	8	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
441	47	F	103	5	1
442	42	F	66	2	3
443	74	M	69	5	3
444	89	F	86	6	1
445	38	F	90	5	1
446	72	F	94	8	1
447	79	F	95	7	1
448	71	M	52	4	1
449	23	F	93	6	1
450	41	F	76	8	1
451	65	F	112	6	1
452	50	F	91	9	3
453	66	F	94	4	2
454	69	M	141	9	1
455	38	F	94	4	1
456	72	F	86	11	1
457	66	F	95	7	3
458	39	F	111	6	3
459	79	F	86	7	3
460	21	F	122	6	1
461	19	M	102	7	3
462	19	F	105	5	3
463	79	F	112	10	1
464	60	M	90	8	1
465	37	F	81	7	1
466	81	F	95	10	6
467	24	M	84	5	1
468	84	M	108	11	1
469	39	F	106	8	-
470	78	F	106	8	1
471	80	F	62	7	1
472	44	F	106	4	3
473	96	F	58	6	3
474	71	F	116	4	3
475	79	M	61	4	2
476	42	F	113	9	1
477	76	F	83	4	1

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
478	82	M	98	7	1
479	47	M	55	3	2
480	64	F	112	6	3
481	70	M	55	3	1
482	35	F	109	4	1
483	29	F	119	5	3
484	45	F	107	1	3
485	47	F	101	5	1
486	76	F	105	7	3
487	47	F	110	3	2
488	38	F	63	2	1
489	77	M	126	6	1
490	39	F	89	4	-
491	43	F	77	2	1
492	46	M	119	6	6
493	83	F	98	8	1
494	77	M	104	8	-
495	47	F	111	4	3
496	36	F	114	11	1
497	71	F	98	10	2
498	67	F	95	6`	2
499	43	F	123	7	2
500	96	F	92	`13	1
501	80	M	85	8	1
502	88	F	113	14	1
503	81	M	110	6	1
504	83	M	69	6	1
505	32	F	80	3	1
506	44	F	117	6	1
507	57	F	90	8	1
508	58	F	89	4	3
509	20	F	114	4	1
510	48	F	104	3	2
511	74	M	87	6	2
512	46	F	108	4	1
513	40	F	82	6	1
514	77	M	96	11	-

Study number	Age (years)	Sex	Hb (g/L)	Ferritin (µg/L)	Genotype
515	52	F	101	12	3
516	41	F	93	7	3
517	77	F	117	9	1
518	58	F	109	7	1
519	36	M	106	5	-
520	85	M	98	7	1