# Clinical Studies in *HFE* Haemochromatosis

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

By

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## Abstract

*HFE* haemochromatosis is the most common iron overload disease. Since the discovery of the *HFE* gene is 1996, it is readily diagnosed using a genetic test rather than using liver biopsies. It is an autosomal recessive disease and the most common form of *HFE* haemochromatosis is homozygosity for p.C282Y. Homozygosity for this substitution accounts for more than 90% of haemochromatosis in Australia.

Excessive iron accumulates due to malfunction of the HFE protein that leads to excess iron absorption. As a result, excess iron builds up in various organs including liver, joints, heart, pancreas, pituitary gland and skin, that may cause end-organ damage including liver cirrhosis, cardiac failure, diabetes mellitus, hypopituitarism and skin pigmentation. Symptomatically, fatigue and arthralgia are the major complaints reported by patients with haemochromatosis.

This disease is easily treatable, as the blood contains a significant proportion of the body's iron, so excess iron can be removed via the blood through phlebotomy. Multiple studies have found that individuals with high total body iron, defined by serum ferritin of more than 1000µg/L, have the highest risk of developing complications including liver cirrhosis. In the last decade, some have suggested that patients with elevated body iron but with serum ferritin less than 1000µg/L, here defined as moderate iron overload, might not need treatment, as they might not have symptomatic manifestations of the disease. However, there have been no randomised controlled trials to examine the treatment benefits for individuals with moderately elevated iron. To answer this question, a randomised controlled trial (Mi-Iron) was conducted which was the major aim of my PhD to examine if removing excess iron would have an impact on patient-reported outcomes, particularly fatigue, as well as liver fibrosis and oxidative stress. This demonstrated that with treatment, there was an improvement in fatigue and its cognitive subcomponent, and the affect component of the arthritis score. There was also an improvement in the liver fibrosis marker, Hepascore, and oxidative stress marker plasma F2-

isoprostane, by removing excess iron in this cohort when compared to the control group who did not have iron reduction. These results, therefore, support current guidelines that all patients with haemochromatosis with elevated serum ferritin should have phlebotomy to return body iron levels to normal levels.

In the second clinical study, the relationship of serum ferritin with non-invasive markers of liver fibrosis including transient elastography, Hepascore, aspartate aminotransaminase (AST) to platelet ratio index (APRI) and Fibrosis-4 (FIB-4) to assess for liver fibrosis and cirrhosis in HFE p.C282Y homozygotes was examined. This study was conceived due to the shift from using liver biopsy to the increasing use of non-invasive techniques to assess liver fibrosis and cirrhosis. This showed that there was a linear relationship of serum ferritin with Hepascore, indicating that higher body iron is associated with more advanced liver fibrosis and cirrhosis. This relationship was also found for APRI and FIB-4 scores. These findings are important as they provide extra information in utilising these scores to assess liver fibrosis and cirrhosis in haemochromatosis.

In the third study, the hand joint arthritis in people with haemochromatosis was examined. Arthralgia is one of the major complaints of individuals with haemochromatosis and is often one of the earliest symptoms of haemochromatosis. It is often difficult to differentiate between haemochromatosis arthropathy and osteoarthritis in the hands. The second and third metacarpophalangeal joints are described to be more commonly affected in individuals with haemochromatosis. By examining the data from HealthIron, a haemochromatosis cohort extracted from a population study that assessed the burden of disease due to iron overload, I found that there was an increase in first metacarpophalangeal joint abnormalities in those with HFE p.C282Y homozygosity, comparable to the frequency of involvement of second and third metacarpophalangeal joints.

## Declaration

This is to certify that:

This thesis comprises only my original work towards the PhD and has not been submitted for any degree or diploma at this or other universities or institutions.

(ii) It compromises of my original work except where indicated in the preface and due acknowledgement has been made in the text to all other material used.

(iii) The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies, appendices and footnotes.

Signed: _____

Dr Sim Y Ong

Date: 22-10-2018

# Preface

The work presented in this thesis was carried out under the joint supervision of Prof Martin Delatycki, Bruce Lefroy Centre, Murdoch Children's Research Institute and Prof Amanda Nicoll, Department of Gastroenterology, Eastern Health.

The thesis consists of three main results chapters presented as journal publications: one has been published whilst the other two have been submitted for publication and are currently under review. All manuscripts include a list of authors that have contributed to the projects.

The Mi-Iron project was designed by the Mi-Iron team comprising of my supervisors, Prof Martin Delatycki and Prof Amanda Nicoll and collaborators including Dr Erica Wood, Prof Lawrie Powell, Prof Grant Ramm, Prof Greg Anderson, Prof John Olynyk and A/Prof Lyle Gurrin. The team also included project coordinators in various states, Lara Dolling, Michelle Wolthuizen, Jeanette Dixon, Louise Ramm, and Jennifer Kava who helped in administration, enrolment and data collection. I was also involved in recruitment, patient assessments including the performance of transient elastography and data collection in Victoria. Subsequent data management, results analysis and manuscript preparation were carried out by myself under the guidance of study statistician, A/Prof Lyle Gurrin and my supervisors. Each author listed contributed to the manuscript. The methodology of this project was published in BMJ Open in 2015 and results from this project were published in Lancet Haematology in December 2017.

The HFE Fibrosis project was performed by myself, including the design of the trial protocols, ethics committee applications and study coordination with our collaborators, Dr Richard Skoien, Prof John Olynyk, Prof Lawrie Powell and Prof Grant Ramm. Dr Thomas Worland, Dr Puraskar Pateria and Louise Ramm helped in the collection of data. I analysed the data and led the manuscript preparation under

the guidance of A/Prof Lyle Gurrin and my supervisors. The manuscript was submitted to Liver International on 22nd October 2018.

The arthritis study was based on the data collected from the HealthIron, a prospective study of long-term effects of iron overload. Data analysis and manuscript preparation were by me under the guidance of A/Prof Lyle Gurrin and my supervisors. The results of the study were submitted to Clinical gastroenterology and hepatology on 22nd October 2018.

I received a post-graduate scholarship from National Health and Medical Research Council (NHMRC) to support my candidature and Mi-Iron was funded by a NHMRC Project Grant.

## Acknowledgements

I would like to express my heartfelt gratitude to my two supervisors, Professor Martin Delatycki and Professor Amanda Nicoll. Both of them have been extremely supportive, encouraging towards my PhD and are excellent mentors. Their advice and research experience had been invaluable, navigating me through difficult periods and facilitating my growth as a researcher throughout the last few years. I am incredibly fortunate to have such patient, understanding and supportive supervisors to guide me to completion of my thesis.

I would also like to thank the Mi-Iron team, who were all tremendously helpful, friendly and co-operative, and to all the participants who had made this trial possible. Also, I would like to express my appreciation to Lara Dolling, my project coordinator in Victoria, who had provided a lot of encouragement and brought fun to my research time.

I also wish to acknowledge the assistance from Dr Thomas Worland, Dr Puraskar Pateria and Louise Ramm, who helped in the recruitment and collection of data in various states. The completion of the fibrosis study was made possible with the continuous support of Prof John Olynyk, Prof Grant Ramm, Prof Gregory Anderson, Dr Richard Skoien, Prof Lawrie Powell, and Dr Adam Testro. I would also like to thank the HealthIron investigators for their efforts and time to collect the data, allowing me to analyse the data and identifying an interesting finding in arthropathy.

Most importantly, I would like to thank my family, particularly my mum and my mother-in-law, who both have been very supportive, and taking time to travel from overseas to help us with our children, allowing me to concentrate on my PhD. Also, a special thank you to my husband, Eric, for his constant support and encouragement, motivating me to finish my PhD. Lastly, to my two beautiful children, Charlotte and Elliot, who have provided so much joy and laughter during this time.

# Publications

**Journal Publications:**

**Ong SY**, Gurrin LC, Dolling L, Dixon J, Nicoll AJ, Wolthuizen M, Wood EM, Anderson GJ, Ramm GA, Allen KJ, Olynyk JK, Crawford D, Ramm LE, Gow P, Durrant S, Powell LW, Delatycki MB. Reduction of body iron in HFE-related haemochromatosis and moderate iron overload (Mi-Iron): a multicentre, participant-blinded, randomised controlled trial. Lancet Haematol. 2017 Dec;4 (12): e607-e614.

**Ong SY**, Dolling L, Dixon JL, Nicoll AJ, Gurrin LC, Wolthuizen M, Wood EM, Anderson GJ, Ramm GA, Allen KJ, Olynyk JK, Crawford D, Kava J, Ramm LE, Gow P, Durrant S, Powell LW, Delatycki MB. Should HFE p. C282Y homozygotes with moderately elevated serum ferritin be treated? A randomised controlled trial comparing iron reduction with sham treatment (Mi-iron). BMJ Open. 2015 Aug 12;5(8):e008938.

**Ong SY**, Nicoll AJ, Delatycki M. How should hyperferritinaemia be managed? Eur J Intern Med. 2016 Sept; 33:21-7 Review.

**Abstract Publications and poster presentations:**

**Ong SY**, Worland T, Pateria P, Powell L, Gurrin L, Ramm L, Testro A, Olynk J, Ramm G, Anderson G, Skoien R, Delatycki M, Nicoll AJ. The relationship between serum ferritin and markers of liver fibrosis in a haemochromatosis cohort. Presented in Australian Gastroenterology Week 2018, Queensland.

**Sim Y Ong**, Lyle Gurrin, Nadine Bertalli, Sophie Zaloumis, Christine McLaren, Dallas English, John Hopper, Graham Giles, Greg Anderson, John Olynyk, Lawrie Powell, Katie Allen, Martin Delatycki, Amanda Nicoll. Association between arthritis phenotypes and *HFE* genotypes in the HealthIron Study. Presented in Australian Gastroenterology Week 2016, Adelaide.

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# Abbreviations

| | |
|---|---|
| AASLD | American Association of the Study of Liver Diseases |
| AIMS2 SF | Arthritis Impact Measure Scale 2 Short Form |
| ALT | alanine amino transaminase |
| APRI | aspartate aminotransaminase to platelet ratio index |
| AST | aspartate amino transaminase |
| CPPD | calcium pyrophosphate dihydrate |
| CT | Computed tomography |
| DNA | deoxyribonucleic acid |
| DMT1 | divalent metal transporter 1 |
| EASL | European Association for the Study of the Liver |
| FIB-4 | Fibrosis-4 |
| GGT | gamma glutamyl transferase |
| HADS | Hospital Anxiety and Depression Scale |
| HCC | hepatocellular carcinoma |
| HA | haemochromatosis arthropathy |
| HH | hereditary haemochromatosis |
| LDL | low-density lipoproptein |
| MCP | metacarpophalangeal |
| MRI | Magnetic resonance imaging |
| MFIS | Modified Fatigue Impact Scale |
| NAFLD | non-alcoholic fatty liver disease |
| OA | osteoarthritis |
| PI | prothrombin index |
| SF | serum ferritin |
| TE | transient elastography |
| TFR1 | transferrin receptor 1 |
| TFR2 | transferrin receptor 2 |
| TS | transferrin saturation |

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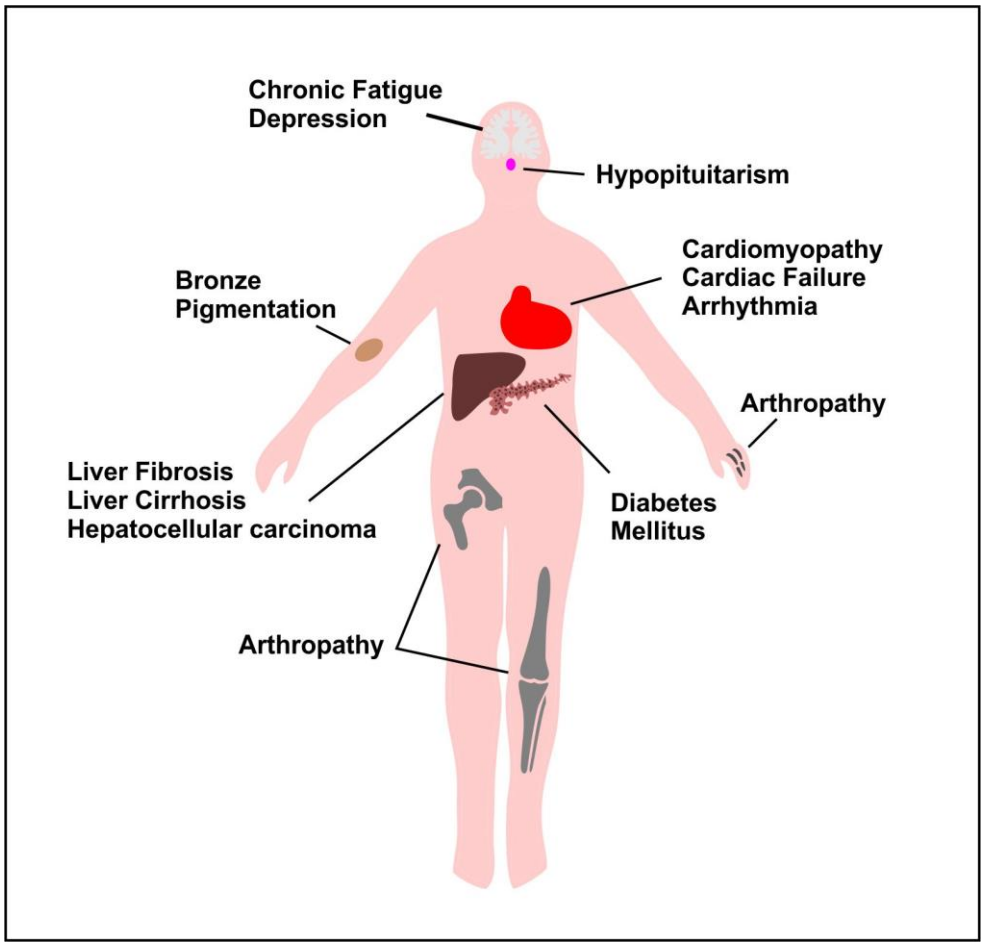
# Chapter 1: Introduction, Literature Review and Aims of the Research Presented in this Thesis

## 1.1 Introduction

Hereditary haemochromatosis (HH) is the most common iron overload disease. The most common form of HH is that due to homozygosity for the c.845G→A mutation in *HFE* which results in the p.C282Y substitution in the HFE protein (Feder, Gnirke et al. 1996). The progressive accumulation of iron can lead to organ damage, in particular the liver, joints, pituitary gland and pancreas (Figure 1.1). Early diagnosis of HH and treatment by phlebotomy to reduce toxic levels of iron can prevent some complications.

Figure 1.1: Complications from *HFE*-haemochromatosis



## 1.2 The importance of Iron and its potential toxicity

Iron is an important element in the human body. It plays a key role in haemoglobin to transport oxygen in red blood cells and in various metabolic processes including synthesis of deoxyribonucleic acid (DNA) and electron transport. There is approximately 3-4 g of iron in a human adult and the majority of iron is stored within haeme. Approximately 400-1000 mg of iron is stored in the liver. Iron is released in the circulation through recycling of senescent red blood cells by macrophages, iron absorption via enterocytes and mobilisation of the hepatic stores in response to increased iron usage. On average, a small amount of iron, approximately 1mg per day, is lost through sweat and sloughing of cells from skin and mucosal surfaces of the gastrointestinal tract. There are two sources of dietary iron, haeme and non-haeme iron. The average human adult absorbs 15- 35% of ingested haeme iron from sources including meat, poultry and fish and absorbs 2-20% of ingested non-haeme iron from sources including fruits, vegetables, legumes and cereals. Although absorption of non-haeme iron is lower than haeme iron, non-haeme iron is the larger iron source for the human body. Iron absorption through enterocytes is tightly regulated in response to the net balance of iron in the body, mainly between intracellular uptake of iron in the bone marrow and the release of iron from the recycling of the senescent red blood cells (Beaton and Adams 2012, Abbaspour, Hurrell et al. 2014).

Iron in its ferrous form ($Fe^{2+}$), is transported across the apical membrane of enterocytes by divalent metal transporter 1 (DMT1) and crosses the basolateral membrane of enterocytes through ferroportin, encoded by *SLC40A1*. Ferroportin is the only known cellular iron exporter, and is expressed highly in hepatocytes and the plasma membrane of macrophages. Iron is then oxidised to its ferric form ($Fe^{3+}$) by circulating caeruloplasmin in the plasma and hephaestin, anchored in the enterocytes. Once iron is in the plasma, it binds to transferrin to form holotransferrin, which delivers iron to different cells in the body. The cells express transferrin receptor 1 (TFR1), which is responsible for the uptake of transferrin bound iron and induces endocytosis and releases iron from transferrin to be used in

the cells for different processes, while the transferrin is recycled back to the circulation. Regulating iron homeostasis is mainly governed by hepcidin, encoded by the *HAMP* gene. Hepcidin is mainly secreted by the hepatocytes and its main role is to control the expression of ferroportin. It binds to ferroportin and causes its internalisation and degradation, thus decreasing its expression and the export of iron (Wallace 2016).

While iron is a vital element to human life, excessive iron accumulation causes the formation of free radicals and increases oxidative stress, which can subsequently lead to tissue damage. Physiologically, there is no known mechanism to remove excess iron from the body. Thus, where too much iron is absorbed as is the case in HH, end-organ damage may occur (Abbaspour, Hurrell et al. 2014).

### 1.3 Aetiology of hereditary haemochromatosis

HH can be caused by mutation(s) in a number of different genes (Table 1.1). Type 1 HH is the most common. For this review, type 1 HH will be the focus and will be referred to as HH.

The *HFE* gene on chromosome 6 was discovered in 1996. Two missense mutations, c.845G→A (which results in p.C282Y) and c.197C→G (p.H63D), were found to cause almost all *HFE*-related HH (Feder, Gnirke et al. 1996). p.C282Y homozygosity is observed in 80-90% of individuals with HH and has a prevalence between 1 in 80 and 1 in 400 in Caucasians (Merryweather-Clarke, Pointon et al. 1997, Bacon, Powell et al. 1999, Merryweather-Clarke, Pointon et al. 2000, Adams, Reboussin et al. 2005, Allen, Gurrin et al. 2008). The prevalence is highest among those of Northern European descent with the highest documented prevalence being 1 in 83 in people of Celtic descent (Ryan, O'Keane et al. 1998, Ryan and Vaughan 2000, Byrnes, Ryan et al. 2001). Whilst compound heterozygosity for p.C282Y/ p.H63D frequency is about 2% and may cause slight elevation of iron indices and hepatic stores, it remains controversial if this genotype causes HH (Lim, Rossi et al. 2004, Adams, Reboussin et al. 2005, Walsh, Dixon et al. 2006, Allen, Gurrin et al. 2008). Recent

consensus by the European Molecular Genetics Quality Network group is that this genotype is insufficient to cause HH (Porto, Brissot et al. 2016).

Table 1.1: Categories of hereditary haemochromatosis and their genetic basis

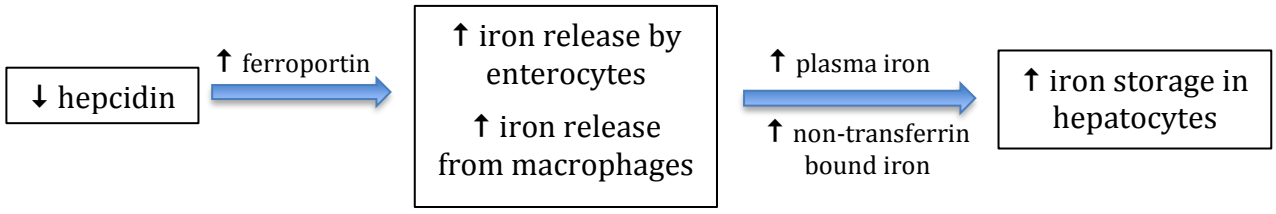
| **Category** | **Causative gene(s)** | **Inheritance Pattern** |
|---|---|---|
| Type 1 | *HFE* | Autosomal recessive |
| Type 2 (juvenile HH) | 2a- *HJV*<br>2b- *HAMP* | Autosomal recessive |
| Type 3 | *TRF2* | Autosomal recessive |
| Type 4B (Ferroportin disease) | *SLC40A1* | Autosomal dominant |

## 1.4 Pathogenesis of hereditary haemochromatosis

The HFE protein regulates the level of hepcidin. The exact mechanism is unknown but it is postulated that HFE and transferrin receptor 2 (TFR2) are sensors of iron levels through transferrin saturation (TS). Increased TS levels result in dissociation of HFE from TFR1 and increased binding of HFE to TFR2. This in turn activates hepcidin synthesis (Chua, Trinder et al. 2011, Leitman 2013). Some studies suggest that the bone morphogenetic protein-hemojuvelin complex is involved in this signalling pathway to regulate hepcidin (Andriopoulos, Corradini et al. 2009, Babitt and Lin 2011). Hepcidin interacts with ferroportin, an iron export protein found on the surface of enterocytes, hepatocytes and macrophages. Their interaction induces internalisation and degradation of ferroportin, leading to cellular iron retention and decreased the release of iron into plasma (Nemeth, Tuttle et al. 2004).

The HFE p.C282Y mutation causes disruption of the HFE protein configuration resulting in its intracellular degradation. This decreases hepcidin synthesis and increases ferroportin mediated iron export. As a result, there is increased transport of dietary iron via the enterocytes into plasma, increased release of iron from macrophages, hence increasing plasma iron and transferrin saturation and accumulation of iron in hepatocytes. As transferrin saturation increases, abnormal formation of non-transferrin bound iron occurs. This excess iron increases reactive oxygen species and leads to cellular toxicity and organ damage (Rossi 2005, Ganz and Nemeth 2011, Brissot, Pietrangelo et al. 2018) (Figure 1.2).

Figure 1.2: Role of hepcidin in *HFE*-related HH



## 1.5 Clinical features of hereditary haemochromatosis

### 1.5.1 Liver disease

The major complications from liver disease in HH are liver cirrhosis and hepatocellular carcinoma (HCC) (Beutler, Felitti et al. 2002, Powell, Dixon et al. 2006, Yen, Fancher et al. 2006, Allen, Gurrin et al. 2008, Aleman, Endalib et al. 2011). These advanced complications of haemochromatosis were commonly seen before the identification of the p.C282Y mutation in the *HFE* gene in 1990s. Since then, the genetic investigation of abnormal iron studies has increased early diagnosis and treatment of HH. From a study of 672 asymptomatic p.C282Y homozygotes including 350 with liver biopsy results, Powell et al. 2006 found that significant liver fibrosis was present in 18% of males and 5% of females with asymptomatic HH whilst liver cirrhosis was detected in 5% of males and 2% of females, and this was correlated to hepatic iron concentration and SF levels. No liver cirrhosis was detected in those with SF <1000 ug/L. Removal of iron reversed liver fibrosis in some and improved fibrosis scores in others but did not reverse liver cirrhosis (Powell, Dixon et al. 2006).

If HH is untreated, excessive iron deposition can result in liver cirrhosis, which can in turn result in HCC, the major cause of death in HH (Beaton and Adams 2006). HCC affects 1- 3% of p.C282Y homozygotes (Cauza, Peck-Radosavljevic et al. 2003, Willis, Bardsley et al. 2005) and there is 20 to 200 times increase in the incidence of HCC in HH compared to the general population (Niederau, Fischer et al. 1985, Cauza, Peck-Radosavljevic et al. 2003). It is important to recognise advanced liver fibrosis and cirrhosis in individuals with HH in order to assess the risks of morbidity and mortality by liver biopsy or by non-invasive methods, which will be further elaborated later in this chapter. HCC surveillance using ultrasound and alpha-fetoprotein every six months is recommended in all cirrhotic individuals (2010, Bacon, Adams et al. 2011). When HCC arise, liver transplantation is considered. Iron reduction before liver transplantation improves the survival from 34% to 75% at 5 years (Kowdley, Brandhagen et al. 2005, Dar, Faraj et al. 2009).

### 1.5.2 Cardiac disease

Cardiac manifestations in HFE related HH are not common and have the same prevalence as in control populations (Beutler, Felitti et al. 2002, McLaren, McLaren et al. 2008). Dilated or restrictive cardiomyopathy may occur in HH (Kremastinos, Farmakis et al. 2010). When it occurs, congestive heart failure tends to occur late in the course of disease due to a slower iron accumulation in the myocardium than in the liver (Wood 2008, Kremastinos and Farmakis 2011). Iron deposition in the Bundle of His and/or Purkinje fibres may occur, leading to conduction defects and cardiac arrhythmias. Cardiac arrhythmias have been reported in up to 25% of individuals with HH although a similar percentage was reported in the control group of that study (McDonnell, Preston et al. 1999, Beutler, Felitti et al. 2002). Interestingly, a population-based study which involved 14,485 subjects found that there is no increased risk of coronary heart disease in p.C282Y homozygotes when compared to the general population and in fact, this group had lower low-density lipoproptein (LDL) cholesterol levels when compared to wild-type controls and thus predicted 16% lower incidence of coronary heart disease than the normal population (Pankow, Boerwinkle et al. 2008). The authors postulated that the activities of liver enzymes in cholesterol metabolism and lipoprotein formation are altered due to the excess iron and thus lowering LDL with similar results found in experimental rats (Turbino-Ribeiro, Silva et al. 2003).

### 1.5.3 Endocrine disease

Diabetes mellitus may occur in HFE p.C282Y homozygotes due to the accumulation of iron in the islet cells of the pancreas, causing a loss of insulin secretory function and impaired insulin sensitivity (Creighton Mitchell and McClain 2014). The prevalence of diabetes in HH is approximately 10 - 20% and impaired glucose tolerance is seen in approximately 15 -30%. About 72% of p.C282Y homozygotes with liver cirrhosis have diabetes (Niederau, Fischer et al. 1996, McClain, Abraham et al. 2006, Hatunic, Finucane et al. 2010). A prospective study has shown that there is an improvement in glucose tolerance in individuals with HH, who have had iron

reduction therapy (Hatunic, Finucane et al. 2010). The association of diabetes risk with HH is controversial with one meta-analysis showing an association between HH and diabetes, and another failing to show this (Ellervik, Mandrup-Poulsen et al. 2001, Moczulski, Grzeszczak et al. 2001, Halsall, McFarlane et al. 2003). There is also a two- to six-fold increased risk of premature death in p.C282Y homozygotes with diabetes compared to diabetics from the general population (Ellervik, Mandrup-Poulsen et al. 2014).

Manifestations of hypogonadotropic hypogonadism such as impotence, loss of libido, amenorrhoea and osteoporosis are due to iron deposition in the pituitary gland. Hypogonadism is more likely to occur in severe iron overload. Older studies reported the prevalence of hypogonadism to be about 40% (Walsh, Wright et al. 1976, Bezwoda, Bothwell et al. 1977, Charbonnel, Chupin et al. 1981), but more recent studies have suggested the prevalence to be approximately 5% (McDermott and Walsh 2005, Uitz, Hartleb et al. 2013).

### 1.5.4 Arthropathy

Hemochromatosis arthropathy (HA) is a chronic progressive arthropathy, first described by Schumacher in 1964 (Schumacher 1964). Its frequency has been reported in the range of 24-81% in various studies, dependent on diagnostic criteria (Schumacher 1964, Carroll, Breidahl et al. 2012). The second and third metacarpophalangeal (MCP) joints are typically involved although its predilection is unknown. The hips and knees are also commonly affected, with the ankles, wrists, elbows, and shoulders being involved to a lesser extent (McDonnell, Preston et al. 1999, Powell, Dixon et al. 2006, Allen, Gurrin et al. 2008, Sahinbegovic, Dallos et al. 2010, Carroll, Breidahl et al. 2011, Wang, Gurrin et al. 2012). Articular pain is the main presenting complaint and may precede the diagnosis of HH (Niederau, Fischer et al. 1996, Sahinbegovic, Dallos et al. 2010). Such pain was reported by approximately 30% of patients with HH in a survey of 2851 people with HH (McDonnell, Preston et al. 1999) and a higher rate of 70% in a cross-sectional study of 199 patients (Sahinbegovic, Dallos et al. 2010). Although articular pain is a non-

specific symptom and is commonly reported in osteoarthritis (OA), many studies have suggested that the topography of affected joints may differentiate HA and osteoarthritis. MCP and wrist joints are more frequently affected and more severe in HA when compared to OA and the involvement of ankle and radiocarpal joints, particularly with bilateral involvement, are usually spared in OA and are suggestive of HA (Carroll, Breidahl et al. 2012, Dallos, Sahinbegovic et al. 2013, Husar-Memmer, Stadlmayr et al. 2014). Patients diagnosed with HA are often younger and more often male, when compared to patients with OA (Dallos, Sahinbegovic et al. 2013). Radiologically, both have similar features of joint space narrowing, subchondral sclerosis, erosions, osteophytes and cyst formation. However, the presence of chondrocalcinosis, a deposition of calcium pyrophosphate dihydrate crystals (CPPD crystals) in the joint cartilage, particularly in the wrists and other large joints is a late manifestation and indicates HA (Carroll, Breidahl et al. 2012, Dallos, Sahinbegovic et al. 2013).

Whilst age and male gender were shown to be associated with the development of HA (Dallos, Sahinbegovic et al. 2013), occupational and physical exertions were not linked to the development of HA (Carroll, Breidahl et al. 2011). Most studies have shown a correlation of serum ferritin level with the incidence of HA, in that there is a higher occurrence of HA with higher serum ferritin levels at diagnosis (Valenti, Fracanzani et al. 2008, Carroll, Breidahl et al. 2011). In a study of 88 HH patients, arthropathy in the second and third MCP joints was associated with a SF of more than 1000 μg/L with odds ratio 4.17 (95% CI 1.09- 13.9) (Valenti, Fracanzani et al. 2008). Similarly, another study found a strong positive association of development of HA with SF more than 1000 μg/L at diagnosis with an odds ratio of 14.0 (95% CI 1.30-150.89) (Carroll, Breidahl et al. 2011). However, other studies did not detect this association and found that HA arthropathy can develop regardless of SF levels, even when SF was only moderately elevated (Allen, Gurrin et al. 2008, Harty, Lai et al. 2011).

The impact of different *HFE* mutations on the development of HA is unclear. It is known that HA is more common in p.C282Y homozygotes and p.C282Y/p.H63D

compound heterozygotes when compared to wildtype controls (Allen, Gurrin et al. 2008, Valenti, Fracanzani et al. 2008, Wang, Gurrin et al. 2012, Dallos, Sahinbegovic et al. 2013). It has been shown that there is an increased risk of arthralgia and development of arthropathy in multiple joints including MCP joints in p.H63D homozygotes and p.H63D heterozygotes (Carroll 2006, Alizadeh, Njajou et al. 2007). This relationship was not observed in p.C282Y carriers in some studies (Beutler, Felitti et al. 2002, Willis, Scott et al. 2002, Alizadeh, Njajou et al. 2007) but not others (Ross, Kowalchuk et al. 2003, Carroll 2006).

HA is a major cause of morbidity in HH (Adams and Speechley 1996) due to lack of management options. Currently, the treatment of HA is directed at symptomatic relief with analgesia including non-steroidal anti-inflammatory medications. Anti-rheumatic medications have been reported to not have efficacy even though there have been no formal studies conducted (Carroll, Breidahl et al. 2012). Even though iron accumulation is associated with the development HA, iron depletion does not improve the course of disease or symptoms of HA unlike the other manifestations of HH, which responds to iron removal (McDonnell, Preston et al. 1999). A proportion of patients report worsening of HA symptoms after iron removal (McDonnell, Preston et al. 1999, Harty, Lai et al. 2011). Joint replacements of the hips and knees occur at a higher rate in HH when compared to controls (Sahinbegovic, Dallos et al. 2010, Wang, Gurrin et al. 2012, Elmberg, Hultcrantz et al. 2013).

### 1.5.5 Dermatologic manifestations

Skin pigmentation may occur in HH when severe iron overload is present (McLaren, McLaren et al. 2008). This phenomenon gave rise to the term “bronzed diabetes” as an early description of HH. The pigmentation is slate-grey or brown and usually occurs in a generalised pattern. Although it is mainly due to melanin deposition, iron deposition around the sweat glands can also contribute to these changes (Adams, Deugnier et al. 1997).

### 1.5.6 Fatigue

Fatigue is one of the most common symptoms reported in HH. Up to 76% of patients with HH report fatigue (Adams, Deugnier et al. 1997, McDonnell, Preston et al. 1999, Delatycki, Allen et al. 2005, Allen, Gurrin et al. 2008, McLaren, McLaren et al. 2008) (Brissot, Ball et al. 2011). It has a negative impact on quality of life (Lowry and Pakenham 2008). The relationship between fatigue and the levels of excess iron is not clear (Adams, Deugnier et al. 1997, McLaren, McLaren et al. 2008, Niewiadomski, Rode et al. 2013). A study of 410 p.C282Y homozygotes identified a positive association between fatigue and hepatic iron concentration (Adams, Deugnier et al. 1997). However, fatigue has also been reported in 34.4% of individuals with HH with normal SF, when compared to 23.6% of control participants (McLaren, McLaren et al. 2008).

Two population studies further demonstrate the uncertainty of the relationship between fatigue and iron levels. In the HealthIron study (Allen, Gurrin et al. 2008), the MFIS was significantly higher in individuals who had already received a diagnosis and had treatment for iron overload (MFIS score 28.6 ± 2.8; SF 377 ± 98 μg/L) than in individuals who were undiagnosed and unaware of the diagnosis at time of completion of the MFIS despite having significantly higher SF levels (MFIS score 17.4 ± 2.6; SF 868 ± 166 μg/L). In the HEIRS study (McLaren, McLaren et al. 2008), 51.7% of p.C282Y homozygotes previously diagnosed and treated (mean SF 186 μg/L), 39.7% of newly diagnosed with raised SF (mean SF 616 μg/L) and 34.4% of newly diagnosed HH with normal SF (mean SF 90.0 μg/L), reported fatigue.

The evidence about whether fatigue improves with iron reduction levels is also inconsistent. While reduction of iron levels improved fatigue in some individuals with HH, it did not make a difference to others (McDonnell, Preston et al. 1999, Niewiadomski, Rode et al. 2013). Among 2851 individuals with HH, 86% of those who reported symptoms including fatigue and depression, had improvement in some or all of their symptoms after phlebotomy (McDonnell, Preston et al. 1999). This supports the positive association between fatigue and reduction of iron levels. In

another study of 88 p.C282Y homozygotes, fatigue, as measured by the Modified Fatigue Impact Scale (MFIS) was present in 54%, 68% and 67% of p.C282Y homozygotes with SF of < 300 µg/L, 300 - 1000µg/L and > 1000 µg/L respectively. There was an improvement of fatigue after phlebotomy in 46%, 43% and 20% in these respective groups of SF. Paradoxically, fatigue has been reported to be worse after iron reduction in a subgroup of individuals with HH. 6 out of 11 (54%) p.C282Y homozygotes reported fatigue to be worse after normalisation of SF from > 1000 µg/L over 55 months (Niewiadomski, Rode et al. 2013).

Three possibilities arise from these data. (1) There is no relationship between HH and iron levels. (2) There is a significant psychosomatic effect of diagnosis on how individuals with HH perceive fatigue and whether a response to treatment with phlebotomy is partly a placebo effect. (3) There is a subgroup in HH who will have fatigue as a presenting complaint, making diagnosis more likely than the subgroup who are unaffected by fatigue.

### 1.5.7 Depression

Depression is a global disability that has a profound socioeconomic impact. In the general population, depression affects 1 in 6 people in Australia (Statistics. 2008) and 1 in 5 people in the United States of America and the trend is increasing in the population (Weinberger, Gbedemah et al. 2018). About 20% of patients with HH have been reported to have depression (McDonnell, Preston et al. 1999, Beutler, Felitti et al. 2002) and depression is found to be more prevalent in young male adults with higher body iron (Richardson, Heath et al. 2015).

## 1.6 Penetrance

Penetrance is the phenotypic expression of a genotype and is highly variable in HH. To date, there is no consensus as to which phenotypic characteristics should be included to define penetrance. Only some p.C282Y homozygotes develop iron overload or symptomatic disease (clinical penetrance), and some p.C282Y

homozygotes only develop biochemical expression of the genotype, known as biochemical penetrance.

### 1.6.1 Biochemical penetrance

At least 50% of HFE p.C282Y homozygotes developed biochemical penetrance with elevated SF and TS at some stage (Adams, Reboussin et al. 2005, Powell, Dixon et al. 2006, Allen, Gurrin et al. 2008). One of the population studies had shown that SF and TS were elevated in 227 p.C282Y homozygotes in 84-88% and 57-73% of male and females respectively (Adams, Reboussin et al. 2005) and in another study, 62% of 110 p.C282Y homozygotes had elevated SF at diagnosis (Powell, Dixon et al. 2006). SF and TS can decrease, remain the same or increase in HH over a period of 12 to 25 years, as shown in some studies (Andersen, Tybjaerg-Hansen et al. 2004, Olynyk, Hagan et al. 2004, Allen, Gurrin et al. 2008). For example, in the Busselton community population in Australia that identified 10 p.C282Y homozygotes without treatment, 4 subjects had increased SF, 4 subjects had unchanged SF and 2 subjects had decreased SF, in a 17-year follow-up period (Olynyk, Hagan et al. 2004). Powell et al. (Powell, Dixon et al. 2006) also found that 48.2% of 114 p.C282Y homozygotes who had initial normal SF, had a progressive rise of SF over a period up to 24 years. Gurrin et. al (Gurrin, Osborne et al. 2008) also predicted that the probability for males and females with HH, who have between SF 300µg/L and 1000µg/L, was 13%-35% and 16%-22% respectively, and would have done so by mean age 55 years.

### 1.6.2 Clinical penetrance

Clinical penetrance estimation is variable depending on the clinical symptoms and signs used to assess penetrance. Fatigue, depression and arthritis are early signs of HH but it difficult to ascertain if these symptoms are due to HH as they occur commonly in the normal population (Rossi and Jeffrey 2004). Andersen et al. (Andersen, Tybjaerg-Hansen et al. 2004) found a low clinical penetrance of 0 to 5% in HH as did Beutler et al. (Beutler, Felitti et al. 2002) who reported a penetrance of less than 1%. However, these studies did not perform liver biopsies to assess if

fibrosis or cirrhosis was present. As a result, the penetrance of the disease may have been underestimated (Allen, Gurrin et al. 2008).

By contrast, other studies found higher clinical penetrance in HH. Allen et al. (Allen, Gurrin et al. 2008) found that at least 28% males and 1% females who were p.C282Y homozygous had iron overload related disease. Among the p.C282Y homozygotes, 45% of males and 8% of females had SF > 1000μg/L, a strong predictor of liver fibrosis and cirrhosis risk in HH. The proportion of fatigue, liver disease and use of arthritis medication were all higher in male p.C282Y homozygotes with SF > 1000μg/L when compared to controls. Regardless of SF levels, more male p.C282Y homozygotes than controls were observed to have abnormal second and third MCP joints. On the other hand, female p.C282Y homozygotes with SF > 1000 μg/L were only reported to have higher use of arthritis medication and abnormal liver function tests when compared to controls (Allen, Gurrin et al. 2008). Similarly, in another study comprising 672 asymptomatic p.C282Y homozygotes, 30% of males and 11.5% females had at least one HH related condition amongst arthropathy, diabetes, hypogonadism, cardiac arrhythmia or hepatomegaly although no controls were included in the study. In addition, the presence of liver cirrhosis was confirmed by liver biopsies in 5.6% of males and 1.9% of females (Powell, Dixon et al. 2006).

The majority of p.C282Y homozygotes have moderately elevated serum ferritin levels, defined as 300-1000 μg/L (Adams, Reboussin et al. 2005, Allen, Gurrin et al. 2008), and less severe disease is seen due to the increased awareness of the disease and the accessibility of *HFE* testing. In those with moderately elevated serum ferritin levels between 300 μg/L and 1000 μg/L, one study found a similar prevalence of signs and symptoms when compared to *HFE* wild type and p.C282Y homozygotes that had normal SF levels (Allen, Bertalli et al. 2010). Powell et al. (Powell, Dixon et al. 2006) also found that hepatic fibrosis and cirrhosis correlated with hepatic iron concentration, and no hepatic cirrhosis was detected in p.C282Y homozygotes with SF less than 1000 μg/L.

## 1.7 Association with cancer

There have been reports of increased risk of cancer in HH (Osborne, Gurrin et al. 2010, Lagergren, Wahlin et al. 2016), hypothesized to be due to the role of iron in carcinogenesis. Iron induced oxidative stress and production of reactive oxygen species that cause lipid peroxidation and oxidative damage to DNA, are suggested to be responsible for the risk of development of cancer. A 14-year follow-up study found an association of colorectal and breast cancer in p.C282Y homozygotes. The hazard ratio was 2.28 for developing colorectal cancer, and in females, there was a slightly increased risk of developing breast cancer, with a hazard ratio of 1.16. (Osborne, Gurrin et al. 2010) There was insufficient data on iron studies to be able to examine if there was an association between iron levels and cancer risk.

## 1.8 Diagnosis

### 1.8.1 Transferrin saturation and serum ferritin

Transferrin saturation (TS) is the ratio of serum iron to total iron binding capacity and is widely used for the initial diagnosis of iron overload. TS is the first biochemical marker elevated in HH whilst raised serum ferritin occurs later in the course of the disease. TS is raised in approximately 70% to 80% of males and 70% of females with HH from large population studies (Adams, Reboussin et al. 2005, Allen, Gurrin et al. 2008).

There is currently no consensus on the cut off value for TS for the detection of HH. TS > 60% in males and > 50% in females has a sensitivity of 92%, specificity of 93% and a positive predictive value of 86 % to detect p.C282Y homozygotes (Tavill 2001). A cut off value of TS > 45% is often chosen as the threshold due to higher sensitivity to identify individuals with HH. However, using this cut off value means it has lower specificity and positive predictive value, as it includes minor secondary iron overload (Tavill 2001). In these cases, further clinical evaluation is needed. Fasting was

recommended to improve the specificity and sensitivity of TS measurement to detect HH but has now been shown to be unnecessary (Adams, Reboussin et al. 2007).

Ferritin is the major iron storage protein in the body, mainly in the liver, spleen and bone marrow. It is an intracellular protein, consisting of a 24-subunit protein shell, with a cavity where it binds up to 4500 iron atoms and small amounts of ferritin are secreted into the plasma (Abbaspour, Hurrell et al. 2014). The protein shell is made from two types of protein, named H and L ferritin and sequestered iron is released through ferritin degradation (Muckenthaler, Rivella et al. 2017). SF is a reflection of total body iron stores. The majority of iron is incorporated into haem to form haemoglobin. There is approximately 3 - 4 g of iron in the human body and SF is used concurrently with TS in the initial diagnosis to identify individuals with iron overload. The combination of raised TS and SF is much more sensitive as a marker of HH than an increase of one or other alone. From large population screening studies, SF is raised in 82% to 88% of male and 55% to 57% of female p.C282Y homozygotes (Adams, Reboussin et al. 2005, Allen, Gurrin et al. 2008). The upper limit of normal is generally > 300 µg/L in males and > 200 µg/L in females. Although SF is a sensitive marker for HH, it is an acute phase reactant that can be elevated in other clinical conditions. Necroinflammatory liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, viral hepatitis and other infective or inflammatory processes can result in elevated SF. In fact, these clinical conditions account for about 58% - 70% of referrals for elevated SF (Wong and Adams 2006, Dever, Mallory et al. 2010). The H-ferritin subcomponent of SF has recently been shown to enhance the expression of hepatic proinflammatory mediators such as IL-1ß by hepatic stellate cells, driving fibrogenesis in the liver (Ruddell, Hoang-Le et al. 2009). Thus, SF has now been suggested to have an additional role as a good predictor of liver fibrosis (Wood, Crawford et al. 2017).

### 1.8.2 Genetic testing

Since the identification of mutations in *HFE* as the main genetic cause of HH (Feder, Gnirke et al. 1996), testing for p.C282Y is now recommended as the first line diagnostic test for this condition. Second line genetic tests including testing for *HAMP* and *HJV* mutations should be considered if *HFE* testing is negative (Pietrangelo 2004). However, these tests may not be readily available.

### 1.8.3 Liver biopsy

Prior to the discovery of *HFE*, liver biopsy with measurement of hepatic iron was the gold standard for diagnosis of HH. It is now mainly reserved for assessing advanced liver fibrosis and cirrhosis in individuals with HH and SF>1000 μg/L. Its prognostic value to determine the presence or absence of liver cirrhosis remains important as it has clinical implications for ongoing surveillance for complications of liver cirrhosis. In some situations, liver biopsy is used for diagnosis of HH where *HFE* testing is negative. Liver biopsy is an invasive procedure and carries risks of morbidity of bleeding, infection and pain with a low mortality rate of 1: 10,000 (Barton and Adams 2010, Bacon, Adams et al. 2011, Chou and Wasson 2013). The other shortcoming of liver biopsy is the potential for sampling error due to regional variation of deposition of iron, distribution of steatosis, inflammation, and fibrosis. Different pathologists may assess the same sample differently (Regev, Berho et al. 2002). Thus, there is a shift to using non-invasive markers to assess liver fibrosis and the presence of liver cirrhosis, which will be discussed in the next section. The current recommendation for liver biopsy in individuals with *HFE*-related HH is those who have SF >1000 μg/L, abnormal liver function testing and/ or hepatomegaly (Barton and Adams 2010, Bacon, Adams et al. 2011).

### 1.8.4 Imaging techniques

Magnetic resonance imaging (MRI) can be used in the detection of iron overload and quantitative measurement of iron levels. This requires specific MRI sequences (T2*, T2, T1, R2*, R2 and R1) to assess and quantitate iron levels. *FerriScan®* is one of the methods commercially available, to detect iron overload and quantifying liver iron

concentration. Apart from being able to assess the extent of liver damage, it can aid in treatment planning with the quantification of iron levels. It has a specificity of 92% to 100% and a sensitivity of 85% to 94% in quantifying liver iron concentration (St Pierre, Clark et al. 2005). However, it is not widely available and costly, and results may be affected by movement artefact. Another MRI method to assess hepatic iron concentration is by using various gradient-recalled echo sequences and calculating the signal intensity ratio between the liver and muscle. It has a correlation coefficient of 0.87-0.92 for the liver to muscle ratio and hepatic iron concentration. This method is free and widely available, simple to implement, and is currently employed in Europe and other countries (Gandon, Olivie et al. 2004). Computed Tomography (CT) scanning may be able to detect iron overload due to increased attenuation in the liver but this test is not a sensitive method to detect iron overload. Moreover, CT is not recommended for treatment monitoring due to the risks of radiation exposure. Ultrasonography cannot detect iron overload (Hernando, Levin et al. 2014).

### 1.8.5 Non-invasive measures of liver fibrosis

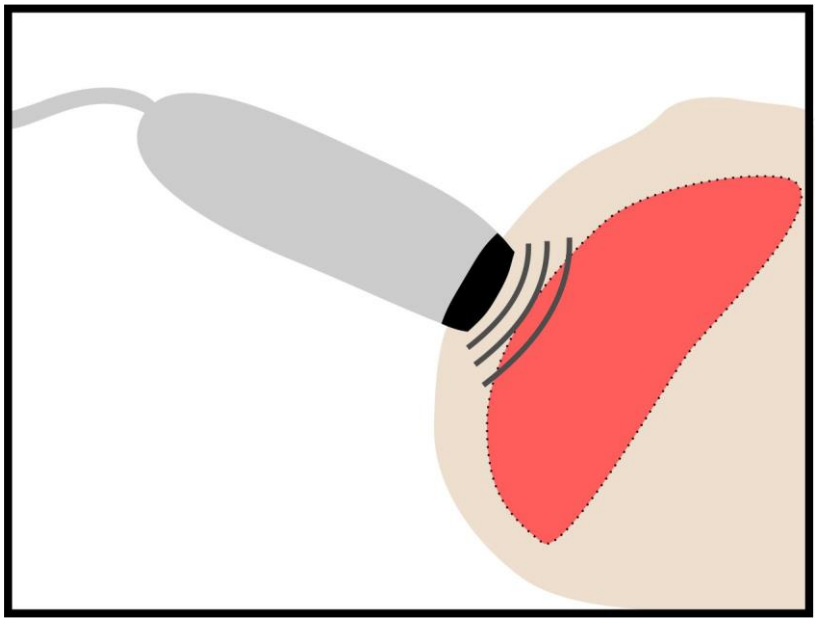
There have been major advances in non-invasive methods of assessing the presence of and degree of hepatic fibrosis. The evaluation of liver fibrosis and cirrhosis has been described using transient elastography and various blood-based tests including Hepascore and Fibrometer score, and these tests have been well validated and used for the estimation of significant hepatic fibrosis and cirrhosis in a variety of liver diseases including viral hepatitis and alcoholic liver disease (Adams, Bulsara et al. 2005, Cales, Oberti et al. 2005, Cales, Boursier et al. 2008, Manning and Afdhal 2008, Chou and Wasson 2013, Chrostek and Panasiuk 2014, Leroy, Sturm et al. 2014) (Table 1.2).

#### 1.8.5.1 Transient elastography (TE)

TE is an evaluation of the liver stiffness using a probe (most commonly Fibroscan®), which transmits mechanical waves of low frequency (50 MHz) and amplitude. As the

liver becomes progressively more fibrotic, it becomes stiffer and less elastic. The velocity of the mechanical or vibration wave correlates directly with tissue stiffness and results are converted and reported as kilopascals (kPa) (Andersen, Christensen et al. 2009) (Figure 1.3).

Figure 1.3: Transient Elastography with Fibroscan®



TE measures liver stiffness in a volume of approximately a cylinder of 1cm diameter and 5cm long, which is roughly 100 times the volume of a percutaneous liver biopsy. TE has been extensively evaluated in a number of different liver diseases initially hepatitis C, and more recently hepatitis B and NAFLD (Friedrich-Rust, Ong et al. 2008, Manning and Afdhal 2008). A meta-analysis of nine studies involving TE in mainly hepatitis C patients showed excellent results for determining the likelihood of cirrhosis, with a sensitivity of 87% and specificity of 91% (Talwalkar, Kurtz et al. 2007). The results of liver stiffness are acquired from at least ten successful valid measurements, meaning a success rate of at least 60% within the interquartile range of ≤ 30%. A cut-off value of 8.7kPa allowed correct diagnosis of those with significant fibrosis (>F2) with an area under ROC of 0.79 (Ziol, Handra-Luca et al. 2005) and a reading of more than 13kPa indicates cirrhosis of the liver is likely to be present (Di Marco, Bronte et al. 2010).

TE has been extensively studied and validated in chronic hepatitis C but not in HH. It has been shown in other iron overload diseases such as thalassaemia major to be a reliable tool for assessing liver fibrosis and cirrhosis. The liver stiffness measurements correlated positively with liver fibrosis stages and a reading of more than 13kPa predicted the presence of liver cirrhosis with an AUROC of 0.997 in predicting cirrhosis (Di Marco, Bronte et al. 2010, Fraquelli, Cassinerio et al. 2010). However, to date, whether iron overload has an impact on liver stiffness measurements has not yet been clarified. Most of the subjects in the thalassaemia population have coexistent hepatitis C, which is a confounding factor when assessing the impact of iron overload on liver stiffness measurements (Di Marco, Bronte et al. 2010, Fraquelli, Cassinerio et al. 2010, Sinakos, Perifanis et al. 2010). Among those with iron overload due to thalassaemia major, Fraquelli et al. (Fraquelli, Cassinerio et al. 2010) found that TE values were highest in the subjects who also have hepatitis C and who have SF > 1000 µg/L, suggesting that there is a relationship between iron overload and TE values.

Only two studies examined the use of TE scores in HH. Adhoute et al. (Adhoute, Foucher et al. 2008) compared TE scores to biochemical markers of liver fibrosis between HH patients and control subjects. The investigators found that there was no significant difference found in TE scores between these two groups, similar to most of the other biochemical markers, although a weak correlation between TE scores and SF levels was noted. They concluded that TE scores correlated well with these biochemical markers and were reliable tests to use in this cohort. However, no liver biopsies were available in the study group to compare the stages of fibrosis with TE scores and these biochemical markers. The study consisted only 10 patients with SF> 1000 µg/L with a mean SF of 1528.9 ± 1181.3 µg/L and the majority of the patients (n= 47) were iron depleted with a mean SF of 302.8 ± 594.2 µg/L. Although the study found that TE scores of more than 7.1kPa (suggesting significant fibrosis) were associated with diabetes and SF levels, they used a lower cut off level of SF 150µg/L in their multivariate analysis, which was within the normal range. Moreover, this association of TE scores representing significant fibrosis with SF level was not found in the univariate analysis even though a weak correlation was found between SF

levels and TE scores (Adhoute, Foucher et al. 2008). On the contrary, another study consisted of liver biopsies evaluated the use of TE in HH compared to liver biopsy, and found that TE scores were higher with severe fibrosis and they correlated well with SF and fibrosis, indicating TE can reliably assess liver fibrosis in HH (Legros, Bardou-Jacquet et al. 2015). Further studies are required to examine the relationship between SF and TE scores to establish if TE scores are affected by iron overload.

#### 1.8.5.2 Hepascore

Hepascore is a serological method of estimating liver fibrosis developed in Australia that is increasingly used in different liver diseases such as hepatitis B and C and alcoholic liver disease (Adams, Bulsara et al. 2005). It is derived from an age- and gender-specific model that inputs parameters of serum bilirubin, gamma glutamyl transferase (GGT), hyaluronic acid and alpha-2-macroglobulin. The test results in a score between 0 and 1 with a higher score being associated with a more severe liver disease. It has an area under the curve (AUC) for the receiver operating characteristic (ROC) of 0.8 for predicting significant fibrosis and 0.90 for cirrhosis. With AUROC, there is no predictive power (random guessing) of AUROC = 0.50 and perfect prediction has AUC = 1.00. A Hepascore > 0.5 has a specificity and sensitivity for significant fibrosis of 70% to 89% and 77% to 63%, respectively (Adams, Bulsara et al. 2005, Guechot, Lasnier et al. 2010). Hepascore < 0.25 makes significant fibrosis very unlikely with a negative predictive value of 0.9 (Guechot, Lasnier et al. 2010).

Hepascore has not been studied extensively in HH. In a study that assessed the atherosclerosis risk in *HFE* related HH, 44 p.C282Y homozygotes with a median SF of 741µg/L, had a median Hepascore of 0.1 (IQR 0.06 to 0.26) (Pankow, Boerwinkle et al. 2008). This suggests that the presence of significant fibrosis in this cohort of patients was extremely unlikely. In contrast, another study found that among 57 individuals with HH with a mean SF of 540µg/L ± 877µg/L, the mean Hepascore was 0.39 ± 0.37, indicating some individuals in this cohort may have had significant fibrosis (Adhoute, Foucher et al. 2008). The results from these two studies were inconsistent, demonstrating that the relationship between SF and Hepascore has not

been clearly defined. It is also unknown whether the SF may affect the Hepascore independent of the degree of fibrosis.

#### 1.8.5.3 Fibrometer$^{3G}$ V

Fibrometer was first developed by a French group to estimate the likelihood of significant liver fibrosis using a blood test comprising of prothrombin index (PI), alpha-2-macroglobulin, hyaluronic acid, AST, urea, age and platelet count. The test was then further improved to diagnose significant fibrosis in chronic hepatitis C by including gender, and was called Fibrometer$^{2G}$. Since then, the test had evolved to Fibrometer$^{3G}$ V in order to provide a more feasible, cost-effective option, and is currently formulated from platelet count, PI, alanine aminotransaminase (ALT), AST, GGT, alpha-2-macroglobulin and urea. This biomarker has an AUROC of 0.85 for predicting significant fibrosis and an AUROC of 0.9 for predicting cirrhosis. Its robustness has been evaluated in different studies and has been recommended by the French National Authority for Health for the diagnosis of liver fibrosis in hepatitis C (Cales, Boursier et al. 2010).

The combination of Fibrometer and TE was recently shown to increase the accuracy of diagnosis of significant fibrosis and cirrhosis to 92% compared with Fibrometer (84% accuracy) or TE (88% accuracy) alone. The combination has an AUROC of 0.892, improving the reliability and precision of diagnosis of significant fibrosis in chronic liver disease (Boursier, Vergniol et al. 2009, Cales, Boursier et al. 2014). It has not been tested in haemochromatosis.

Table 1.2: Summary of non-invasive liver fibrosis markers in hepatitis C.

| Marker | AUROC to detect significant fibrosis | AUROC to detect cirrhosis | References |
|---|---|---|---|
| Transient elastography | 0.79 | 0.97 | (Ziol, Handra-Luca et al. 2005) |
| Hepascore | 0.80 | 0.90 | (Adams, Bulsara et al. 2005) |
| Fibrometer$^{3G}$ V | 0.85 | 0.90 | (Cales, Boursier et al. 2010) |

## 1.9 Treatment

### 1.9.1 Phlebotomy

Phlebotomy is the mainstay of treatment for removing excess iron in individuals with HH. It is a relatively easy procedure that involves inserting a needle into a vein and withdrawing 450ml of blood from the patient can be performed in the physician's practice, blood bank or in a hospital. Typically, one unit of blood (approximately 450ml containing about 250mg of iron) is removed in each treatment. The decrease in haemoglobin triggers erythropoiesis and mobilises stored iron to make more haemoglobin for red blood cells. This results in a decrease in total body iron and is reflected by a decrease in SF. SF is used to monitor iron reduction as TS is a poor marker of iron stores (Leitman 2013).

Generally, phlebotomy is a safe procedure associated with rare serious complications such as thrombosis and infection. The more common adverse events are anaemia, bruising, and syncope. Syncope is often identified by dizziness, sweating, nausea, vomiting and pallor due to the rapid decrease of blood pressure and can potentially lead to loss of consciousness (Kim and Oh 2016).

The recommended frequency of phlebotomy is weekly to fortnightly depending on the individual's haemoglobin, haematocrit and SF. Haemoglobin and haematocrit are measured at every treatment, and SF is measured approximately every three months, or more often when the SF is approaching the normal range. Phlebotomy is postponed when anaemia is detected (Adams and Barton 2010, guidelines , Bacon, Adams et al. 2011). It can take up to one year of phlebotomy to decrease SF to normal range if SF is more than 1000 ug/L at the initiation of therapy. Hence, in surveys performed in 2851 HH patients in the United States and Canada in 1999, and a smaller survey of 210 HH patients across the United States, France, Ireland and United Kingdom in 2011, 15% of patients stated phlebotomy was inconvenient, time consuming and expressed dissatisfaction of venous access. Concerns were also raised that blood was discarded. The latter concern will perhaps dissipate over time as more countries including Australia, France, Canada and Ireland, along with some professional organisations including World Health Organisation and the United States Food and Drug Administration are accepting blood removed from individuals with HH can be used for blood transfusions. The risks of siderophilic bacterial infections causing transfusion sepsis due to *Yersinia enterocolitica*, *Listeria monocytogenes, Vibrio vulnificus* and Babsiosis pathogens remain a theoretical risk due to stringent red blood cells transport and storage (Winters, Tremblay et al. 2018). Approximately 45 to 59% of patients would consider alternative therapies to phlebotomy if available (McDonnell, Grindon et al. 1999, Brissot, Ball et al. 2011).

The clinical benefits of phlebotomy have not been fully assessed as there have been no randomised controlled trials, and such studies will be difficult to conduct with placebo treatment with the knowledge of end-organ damage with untreated iron overload greater than 1000 µg/L. There are strong indications that some symptoms such as fatigue, skin pigmentation and depression improve with phlebotomy and in some cases, the reversal of hepatic fibrosis can occur (McDonnell, Preston et al. 1999, Falize, Guillygomarc'h et al. 2006, Powell, Dixon et al. 2006). However, arthropathy and diabetes mellitus are often unchanged with phlebotomy (Adams and Barton 2010).

The current recommendations from the European Association for the Study of the Liver (EASL) and the American Association of the Study of Liver Diseases (AASLD) (guidelines , Bacon, Adams et al. 2011) are to treat all individuals with HH, who have elevated iron indices even if they are asymptomatic. There is a consensus to initiate phlebotomy in individuals with HH with SF >1000µg/L since this cohort is at a high risk of complications from HH (Adams and Barton 2010). Studies have confirmed that mortality and morbidity including arthritis, fatigue, liver fibrosis and cirrhosis are higher in p.C282Y homozygotes who have SF >1000 µg/L (Morrison, Brandhagen et al. 2003, Beaton and Adams 2006, Powell, Dixon et al. 2006, Allen, Nisselle et al. 2008). There are increasing data that individuals with HH who have moderate elevations of SF (< 1000 µg/L) may not have manifestations of HH and their SF may not further increase. Some have suggested that in these individuals, SF can be monitored rather than instituting therapy (Andersen, Tybjaerg-Hansen et al. 2004, Olynyk, Hagan et al. 2004, Adams and Barton 2010, Allen, Bertalli et al. 2010). However, no clinical trials have been conducted to assess the benefits of treatment in this cohort.

### 1.9.2 Erythrocytapheresis

An alternative to phlebotomy to remove red cells is erythrocytapheresis. This is a procedure to remove predominantly red blood cells while sparing other blood components such as platelets, plasma and coagulation factors. The whole blood of an individual is removed and passed through an automated cell separator, in which the red cells are removed and the rest of the blood components are returned to the individual. As each procedure can remove up to 800ml of red blood cells, more iron can be removed than by standard phlebotomy (Adams and Barton 2010). The volume of red cells removal is determined by gender, weight, total blood volume and haematocrit of the patient. Its efficiency was shown in a randomised trial comparing 19 patients who underwent erythrocytapheresis and 19 patients who were treated by phlebotomy, with mean SF 1103ug/L and 1676 ug/L at the initiation of therapy respectively. Those treated with erythrocytapheresis required an average of 9 treatments in 19.6 weeks to normalise SF, whilst those treated with phlebotomy

required an average of 27 treatments in 33.7 weeks (Rombout-Sestrienkova, Nieman et al. 2012). Although the erythrocytapheresis group had a lower mean SF at initiation than the phlebotomy group (p=0.04), the mean amount of iron removed per treatment was higher in the erythrocytapheresis group (mean 427mg) than the phlebotomy group (mean 205mg). At the same time, less total volume was removed in the erythrocytapheresis group (mean 4699ml) than the phlebotomy group (mean 13,016ml) (Rombout-Sestrienkova, Nieman et al. 2012). In addition, the frequency between erythrocytapheresis treatments is every two to three weeks compared to up to twice-weekly phlebotomy. Hence, there is a smaller impact on quality of life since it reduces visits for treatment, travel time and less time absent from work (Rehacek, Blaha et al. 2012, Rombout-Sestrienkova, Nieman et al. 2012). Moreover, erythrocytapheresis can maintain a euvolaemic state even with a larger volume of red cells removed as volume can be replaced by saline. Thus, it is a well tolerated and an efficacious procedure (Rehacek, Blaha et al. 2012, Rombout-Sestrienkova, Nieman et al. 2012, Evers, Kerkhoffs et al. 2014) (Figure 2.1.2).

The adverse events reported from erythrocytapheresis have been minimal and in the study of Rombout-Sestrienkova et al. 2012 (Rombout-Sestrienkova, Nieman et al. 2012), 3/19 (15.8%) patients reported 8 mild events, including 1 mild citrate reaction, presented as perioral tingling or paraesthesia, chills, twitching or tremor, due to the chelation of calcium to prevent clotting during apheresis; 1 vasovagal collapse and 6 cases of mild dizziness out of a total of 171 treatments, similar to the 5/19 (26.3%) patients in the phlebotomy who reported 1 short duration collapse and 9 cases of mild syncope out of a total of 513 treatments. A recent Cochrane review in 2017 found only three randomised controlled trials comparing the benefits and harms of erythrocytapheresis versus phlebotomy, and two are from the same author. Although there are no differences in reported adverse events between the two different treatments and short-term health-related quality of life, the evidence is weak to determine if erythrocytapheresis has more benefits or harm than phlebotomy and long-term data on mortality and health-related quality of life and other clinical outcomes are lacking (Buzzetti, Kalafateli et al. 2017).

Although the major disadvantages of erythrocytapheresis are the costs associated with apheresis equipment and the expertise required, and each treatment of erythrocytapheresis is more expensive than phlebotomy, there is no overall cost difference as fewer treatments are required for erythrocytapheresis. In fact, it is more advantageous since there is less time taken off from work (Rombout-Sestrienkova, Nieman et al. 2012).

### 1.9.3 Iron chelating agents

Iron chelation is the second-line treatment in HH when phlebotomy is not tolerated. Iron chelating agents such as desferrioxamine and deferasirox are currently off-label medications for treatment of HH.

Desferrioxamine is an iron chelator administered as a continuous subcutaneous infusion for 8-12 hours. The limitations of using desferrioxamine in HH are the compliance of patients, the need for parenteral administration and discomfort during administration (Adams and Barton 2010). Deferasirox is a newer oral iron chelator used in secondary iron overload conditions such as β-thalassaemia major. Common adverse events are diarrhoea, headache, nausea and rise in ALT and/or creatinine. These occur in less than 10% of individuals with HH treated with this agent (Phatak, Brissot et al. 2010). These side effects are dose-dependent and resolve either with dose reduction or cessation of treatment.

### 1.9.4 Proton pump inhibitors

The benefits of proton pump inhibitors, as an adjunct therapy to decrease iron absorption due to acid suppression are debatable. A randomised controlled trial conducted recently randomised 30 p.C282Y homozygotes to either 40mg of pantaprozole daily or to placebo for 12 months and initiated phlebotomy when SF was more than 100µg/L. They found that over one year, participants receiving proton pump inhibitors received 1.33 fewer phlebotomies than the control group and suggested that they can be used for long-term maintenance therapy

(Vanclooster, van Deursen et al. 2017). However, the real benefits of lifelong proton pump inhibitors for maintenance therapy is questionable due to compliance, cost and also decreasing blood available for donation (Adams 2017).

### 1.9.5 Diet

There are no significant relationships between increasing SF and dietary iron (Adams and Barton 2010). Changes to the diet cannot remove iron, and there is little effect on the rate of iron accumulation. Since iron can be efficiently removed by phlebotomy, a low iron diet plays little role in the management of HH (Gordeuk, Lovato et al. 2012).

Excess alcohol can act synergistically with iron to increase liver damage. There is a possible linear correlation between alcohol intake and increased iron absorption. In experimental models, hepcidin is down regulated with alcohol loading and HFE protein may be involved in this pathway (Flanagan, Peng et al. 2007, Ohtake, Saito et al. 2007). The likely explanation for why excess alcohol worsens liver damage in individuals with HH is that both alcohol and iron cause oxidative stress and hepatic fibrogenesis (Flanagan, Peng et al. 2007).

Whilst tea is known to inhibit iron absorption, ascorbic acid (vitamin C) enhances iron absorption by the gut and modulates iron metabolism (Kaltwasser, Werner et al. 1998, Lane and Richardson 2014). Therefore vitamin C supplementation is contraindicated in individuals with HH and iron overload (Adams and Barton 2010, guidelines).

Individuals with HH and elevated iron indices have an increased risk of primary septicaemia from *Vibrio vulnificus*. This generally results from ingestion of raw oysters or other raw shellfish or through superficial wounds in contact with seawater that contains the bacteria. *Vibrio vulnificus* is a normal marine flora that grows in warm, coastal waters. Some of these infections are fatal. Thus, it is recommended

that individuals who have HH with raised iron indices avoid raw shellfish or contact of wounds with seawater (Adams and Barton 2010).

## 1.10 Goals of treatment

There are no studies to inform what the SF end point should be. Generally, guidelines recommend a target SF of either 50 or 100µg/L (guidelines , Bacon, Adams et al. 2011). This is based on the theory that reducing iron levels to the lower end of the normal reference range will mean that there is little or no iron storage (Evers, Kerkhoffs et al. 2013). However, some have advocated that a higher end SF between 200 and 300µg/L is acceptable (Leitman 2013).

## 1.11 Prognosis

The overall survival rates in individuals with HH without complications are similar to the sex- and age-matched individuals from the general population. The cumulative survival rates are 96% at 5 years, 91% at 10 years and 88% at 15 years (Aleman, Endalib et al. 2011). Before the discovery of *HFE* gene in 1996, a longitudinal study followed 251 patients with HH diagnosed by liver biopsies up to 33 years, with a mean of 14.1 years and established that their mean survival was 21.0 years. In this cohort, 56.6% of patients had liver cirrhosis and 47.8% had diabetes and the life expectancy of patients with liver cirrhosis was reduced when compared to those without (Niederau, Fischer et al. 1996). This finding was supported by other studies and confirmed the death rate was increased thirteen-fold in individuals with HH who had liver cirrhosis compared to an age-matched population (Strohmeyer, Niederau et al. 1988, Aleman, Endalib et al. 2011, Ellervik, Mandrup-Poulsen et al. 2014). There was also a two to six-fold increased risk of premature death in p.C282Y

homozygotes with diabetes compared to diabetics from the general population (Ellervik, Mandrup-Poulsen et al. 2014).

A recent study reported lower mortality in p.C282Y homozygotes who had elevated SF but less than 1000 µg/L and had normalization of SF by phlebotomy when compared to the general population (Bardou-Jacquet, Morcet et al. 2015). p.C282Y homozygotes who had normal SF at diagnosis did not have a difference in mortality to the general population. Perhaps their lower mortality rate was due to increased medical care which resulted in lifestyle changes that were beneficial. To assess the benefits of phlebotomy in this cohort would require a randomised controlled study of treatment versus placebo (Delatycki, Gurrin et al. 2015).

## 1.12 Community Screening

The use of community screening to identify individuals with HH is the subject of debate. (Pietrangelo 2004) HH fulfills a number of criteria for screening. The prevalence of HH is high, between 1 in 80 and 1 in 400 in Caucasians (Merryweather-Clarke, Pointon et al. 1997, Bacon, Powell et al. 1999, Merryweather-Clarke, Pointon et al. 2000, Adams, Reboussin et al. 2005, Allen, Gurrin et al. 2008). Genetic testing for HH is widely available and can lead to iron reduction therapy that can prevent disease related to HH if discovered early. There is conflicting evidence in terms of acceptability of community screening. In the HEIRS study, there was an increased rate of health concerns among p.C282Y homozygotes (Wenzel, Anderson et al. 2007). However, that study included non-English speaking individuals who have been found to have more health concerns and worse psychological wellbeing, likely due to language and cultural differences (Wenzel, Anderson et al. 2007). By contrast, Delatycki et. al (Delatycki, Allen et al. 2005) found that there was no increase in anxiety or decrease in health perception among 47 p.C282Y homozygotes newly identified from 11 841 participants screened in the workplace. Individuals with HH identified by the screening program took steps to prevent disease. In addition, insurance companies in Australia have agreed that asymptomatic p.C282Y homozygotes will not be discriminated against in insurance applications if they have

normal SF or they take steps to reduce SF to the normal range (Delatycki, Allen et al. 2002). Further studies will need to be undertaken to evaluate the health economics of community screening for HH.

## 1.13 Conclusion

The above literature review serves as a background to this thesis. It has highlighted the relatively high prevalence of HH and identified that the majority of people with HH have moderately elevated iron levels, defined as SF between 300 µg/L and 1000 µg/L. Whilst there is some evidence suggesting that this cohort of patients may not exhibit signs and symptoms related to HH leading some to recommend these individuals should not have iron reduction therapy, no clinical trials have been conducted to assess the benefits of removing excess iron. More patients with HH are now diagnosed early in its course due to the availability of *HFE* gene testing. It is therefore critical to explore the need for therapy in this cohort of patients. It is important to assess the presence of liver fibrosis or cirrhosis in HH and there is an increasing use of non-invasive methods to assess liver fibrosis and cirrhosis. This warrants evaluation of the use of these methods in HH. Lastly, arthropathy in HH is debilitating and one of the early manifestations of the disease. Further study of HA is important to early recognition of the disease to prevent complications.

## 1.14 Aims of the research presented in this thesis

The aims of this research include:

1. To conduct a multi-centre, participant-blinded, randomised controlled trial of erythrocytapheresis and sham erythrocytapheresis (plasmapheresis) to assess objectively the benefits of iron reduction in HFE p.C282Y homozygotes with moderately elevated iron defined as serum ferritin between 300 µg/L and 1000 µg/L, with patient-reported outcomes and non-invasive markers of liver fibrosis and oxidative stress markers.

2. To evaluate the use of non-invasive methods including transient elastography and Hepascore, to assess liver fibrosis and cirrhosis in a haemochromatosis cohort.

3. To assess arthropathy in the hands of p.C282Y homozygotes.

# Chapter 2: Reduction of body iron in HFE-related haemochromatosis and moderate iron overload (Mi-Iron): a multicentre, participant-blinded, randomised controlled trial

## 2.1 Introduction and Summary

Evidence related to the benefits of treatment in *HFE* p.C282Y homozygotes, the most common genotype in hereditary haemochromatosis (HH), with moderately elevated SF between 300- 1000 μg/L is lacking. Some population studies had suggested there are no increase in signs and symptoms of HH in these individuals and the risk of progressing to high SF of more than 1000 μg/L, a predictor of advanced fibrosis and liver cirrhosis is low (Gurrin, Osborne et al. 2008, Allen, Bertalli et al. 2010). Thus, some clinicians have suggested a wait and observe approach for these individuals rather than treatment to normalise body iron (Adams and Barton 2010). To date, there have been no randomised control trials conducted to assess the benefits of phlebotomy versus no treatment. The Mi-Iron study, is the first randomised, participant-blinded trial conducted in p.C282Y homozygotes with moderately elevated iron with SF between 300- 1000 μg/L, using erythrocytapheresis as treatment and plasmapheresis as control, to assess patient-reported outcome measures including Modified Fatigue Impact Scale (primary outcome measure), Hospital Anxiety and Depression Scale, Medical Outcome Study Form and Arthritis Impact Measurement Scale and hepatic fibrosis with transient elastography (with Fibroscan®), Hepascore, Fibrometer and oxidative stress markers, serum and urinary F2-isoprostanes. The detailed methodology of this study had been published separately to the publication of the results and both papers have been attached in this chapter.

We found that there was a significantly greater improvement in the primary outcome, MFIS and its cognitive subcomponent in the treatment group than the control group. Similar improvement was observed in the affect component of the

AIMS2-SF. Biochemically, there was a significantly greater improvement in Hepascore and plasma F2-isoprostanes in the treatment group when compared to the control group. Collectively, our findings concluded that p.C282Y homozygotes with moderate iron overload can benefit from normalisation of iron levels and supports current clinical recommendations that all with HH and iron overload should have treatment to normalise body iron.

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# Should HFE p.C282Y homozygotes with moderately elevated serum ferritin be treated? A randomised controlled trial comparing iron reduction with sham treatment (Mi-iron)

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## ABSTRACT

**Introduction:** HFE p.C282Y homozygosity is the most common cause of hereditary haemochromatosis. There is currently insufficient evidence to assess whether non-specific symptoms or hepatic injury in homozygotes with moderately elevated iron defined as a serum ferritin (SF) of 300–1000 µg/L are related to iron overload. As such the evidence for intervention in this group is lacking. We present here methods for a study that aims to evaluate whether non-specific symptoms and hepatic fibrosis markers improve with short-term normalisation of SF in p.C282Y homozygotes with moderate elevation of SF.

**Methods and analysis:** Mi-iron is a prospective, multicentre, randomised patient-blinded trial conducted in three centres in Victoria and Queensland, Australia. Participants who are HFE p.C282Y homozygotes with SF levels between 300 and 1000 µg/L are recruited and randomised to either the treatment group or to the sham treatment group. Those in the treatment group have normalisation of SF by 3-weekly erythrocytapheresis while those in the sham treatment group have 3-weekly plasmapheresis and thus do not have normalisation of SF. Patients are blinded to all procedures. All outcome measures are administered prior to and following the course of treatment/sham treatment. Patient reported outcome measures are the Modified Fatigue Impact Scale (MFIS-primary outcome), Hospital Anxiety and Depression Scale (HADS), Medical Outcomes Study 36-item short form V.2 (SF36v2) and Arthritis Impact Measurement Scale 2 short form (AIMS2-SF). Liver injury and hepatic fibrosis are assessed with transient elastography (TE), Fibrometer and Hepascore, while oxidative stress is assessed by measurement of urine and serum F2-isoprostanes.

**Ethics and dissemination:** This study has been approved by the Human Research Ethics Committees of Austin Health, Royal Melbourne Hospital and Royal Brisbane and Women's Hospital. Study findings will be disseminated through peer-reviewed publications and conference presentations.

**Trial registration:** Trial identifier: NCT01631708; Registry: ClinicalTrials.gov

## INTRODUCTION

Hereditary haemochromatosis (HH), an iron overload disorder, is the most common genetic condition in Northern Europeans among whom approximately 1 in 200 are homozygous for the HFE p.C282Y amino acid substitution, the cause of most HH. Not all p.C282Y homozygotes develop morbidity.[1] About 20% of males and 40% of females with this genotype do not develop iron overload.[2] At least 28% of males and 1% of females develop iron overload-related disease such as liver cirrhosis, diabetes mellitus and cardiomyopathy.[2] These individuals generally have severe iron overload as indicated by a serum ferritin (SF) of greater than 1000 µg/L. Therefore, the question arises as to whether the largest group of p.C282Y homozygotes, those with raised SF but SF less than 1000 µg/L (defined here as moderate iron overload), require venesection treatment to normalise SF. Answering this question is important since if treatment is beneficial, introduction of community screening for HH should be considered whereas if there is no benefit from treatment, p.C282Y homozygotes with moderate iron overload do not need to undertake this somewhat onerous intervention.

Major management guidelines for HH recommend treatment of those with HH and SF above the upper limit of normal and recommend that SF be reduced to 50–100 µg/L.[3 4] The rationale for normalisation of SF in those with severely elevated SF (>1000 µg/L) is clear as severe morbidity and mortality can be prevented.[5 6] There is little evidence that treatment is beneficial in those with moderate iron overload however. There have been no randomised controlled trials to objectively assess whether returning iron levels to normal improves symptoms in p.C282Y homozygotes with moderately elevated SF. Reasons why such studies have not been carried out likely include the fact that treatment of HH is relatively safe and therefore commentators have adopted the stance that treatment is unlikely to result in harm while there are theoretical reasons why harm may result from not normalising SF.[7] In addition, blinding is far more complex in a trial of venesection than in a placebo-controlled pharmaceutical trial.

HFE p.C282Y homozygotes with moderately elevated SF do not have an increase in frequency of HH-related symptoms when compared to controls in cross-sectional studies.[8 9] Such cross-sectional studies are not designed to identify subtle symptoms such as fatigue, however. Anecdotal evidence suggests that fatigue benefits from treatment of HH. Fatigue is a non-specific symptom that is commonly reported in individuals with HH[2 10–13] and has a negative impact on the physical and psychological quality of life.[14] There are conflicting data about the relationship between fatigue and levels of excess iron. Adams *et al*[10] found an association between fatigue and hepatic iron index in 410 p.C282Y homozygotes. In contrast, fatigue has been reported in p.C282Y homozygotes with normal SF[13 15] and has been found to be worse in some individuals following normalisation of SF by venesection treatment.[11 15] One population study identified significantly higher Modified Fatigue Impact Scale (MFIS) scores in p.C282Y homozygotes who knew their diagnosis and had their iron levels normalised compared to those who were unaware of the diagnosis and had significantly higher SF.[2] There are three possible explanations for these observations: (1) There is no relationship between fatigue and iron levels in HH; (2) there is a significant psychosomatic effect of diagnosis on how these individuals perceive fatigue; or (3) there is a subgroup of individuals who have fatigue and are more likely to be diagnosed with HH.

A recent study suggested that treated HFE p.C282Y homozygotes with moderate iron overload have decreased cardiovascular and extrahepatic cancer-related mortality rates compared to the general population, while p.C282Y homozygotes with normal SF have the same mortality rates as the general population.[7] The relationship between the mortality findings and treatment of iron overload was questioned, however.[16]

## AIMS AND HYPOTHESIS

The aim of this multicentre randomised trial is to compare the prevalence of symptoms and objective markers of disease between those in the treatment group and those in the sham treatment group.

Our hypothesis is that HFE p.C282Y homozygotes with moderately elevated SF will have few symptoms and signs of disease and decreasing SF to normal levels will not result in a greater change in patient reported outcomes or objective markers of liver injury or hepatic fibrosis compared to those whose SF levels are not lowered to the normal range.

## METHODS AND ANALYSIS

The Mi-iron (Moderately increased iron levels) study is a multicentre, randomised single-blinded trial being conducted in Victoria (Austin Hospital and the Royal Melbourne Hospital) and Queensland (Royal Brisbane and Women's Hospital) that started in August 2012 and is due to conclude in December 2015. Figure 1 summarises the methodology of the Mi-iron Study.

### Participants

*Inclusion criteria*

1. Age 18–70 years inclusive
2. HFE p.C282Y homozygous
3. SF between 300 and 1000 µg/L
4. Previously or currently raised TS

*Exclusion criteria*

1. HH due to other genotypes
2. Venesection in the past 2 years for treatment of HH
3. Other risk factor(s) for liver injury including hepatitis B (HBV) or C (HCV), excess alcohol consumption (>60 g/day in males, 40 g/day in females), body mass index (BMI) of ≥35 kg/m$^2$
4. Pregnant females

### Study intervention

Apheresis is being used as the study intervention. All procedures are conducted using the Haemonetics MCS Plus apheresis system.

### Randomisation and stratification

Participants are randomised to either the treatment group to have erythrocytapheresis or the sham treatment group to have plasmapheresis. Randomisation is by computer generated random number sequence. Randomisation is stratified by gender, initial SF (300–599 µg/L or 600–1000 µg/L as a binary variable) and site.

### Maintenance of blinding

The participant is blinded as to which arm of the study he/she has been randomised by being connected to the apheresis machine with the machine and the tubing not visible to the individual. This is achieved by the participant's arm being passed through an opaque black curtain (figure 2). Thus participants are unaware of whether they are having red blood cells (RBCs) or plasma removed and are unaware of whether or not they

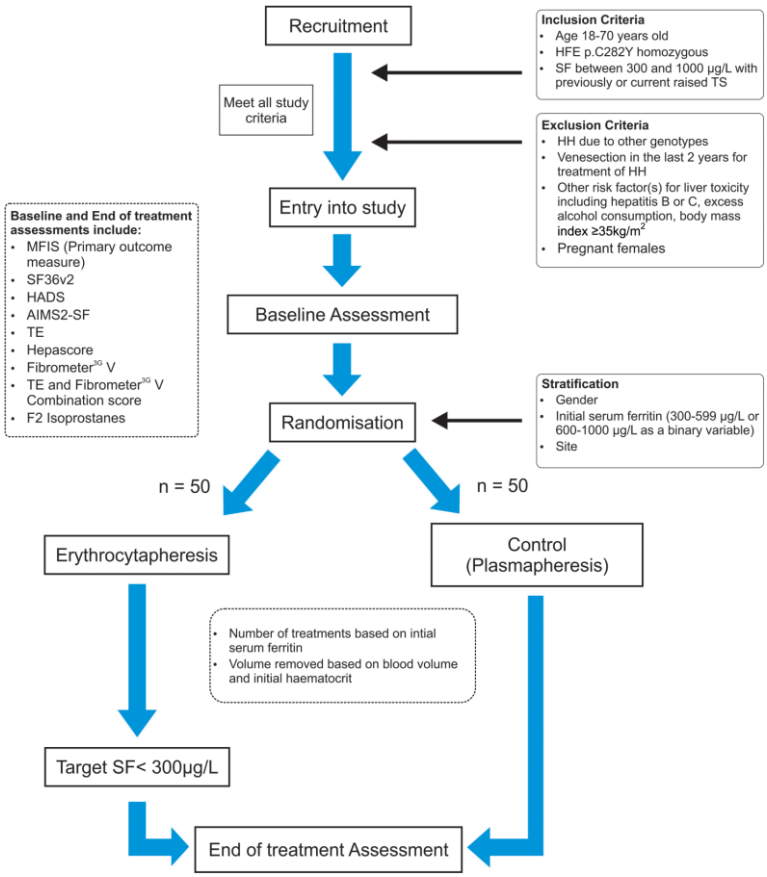


**Figure 1** Flow chart of methodology for the Mi-iron Study. TS, transferrin saturation; SF, serum ferritin; MFIS, Modified Fatigue Impact Scale; SF36v2, Medical Outcomes Study 36-item short form V.2; HADS, Hospital Anxiety and Depression scale; AIMS2-SF, Arthritis Impact Measurement Scales 2 short form; TE, transient elastography.

are having their iron levels reduced. Staff performing the apheresis are trained to not inadvertently reveal the treatment arm of the participant through strict adherence to study protocol and careful use of language in describing what is being performed. A member of the research team is present during the intervention to ensure blinding is maintained by monitoring the procedure and conversation between apheresis staff with the participant.

Those undergoing plasmapheresis have the same volume of plasma removed as the volume of RBCs removed had they been randomised to the erythrocytapheresis arm. Therefore, the risk of symptoms due to reduction in circulating blood volume is the same for individuals in both arms of the study.

## Intervention

Treatments are administered every 3 weeks. The volume of RBCs/plasma removed is individualised based on the individual's blood volume and haematocrit. Haematocrit is measured at the start of each erythrocytapheresis treatment while in the sham treatment group, mock blood tests are taken to ensure the participant's experience is identical irrespective of the treatment group to which they have been randomised. The volume removed is calculated based on the height, weight, pretreatment haematocrit and the target haematocrit (30–35%; figure 3). Treatments are ceased in the treatment group when SF is below 300 µg/L. For the sham group, the calculated number of treatments is equivalent to as if their SF was normalised in the RBC group.

Participants in the sham arm are offered erythrocytapheresis or phlebotomy to normalise SF at their choice of venue on completion of the study.

## Safety blood test monitoring

All participants have SF measured 2–3 weeks after the expected last treatment to ensure the SF level has decreased to the normal range (<300 µg/L) for those in the treatment group before proceeding to the end of trial assessment. Participants in both arms of the study have the same blood test to ensure blinding. Serum B12,

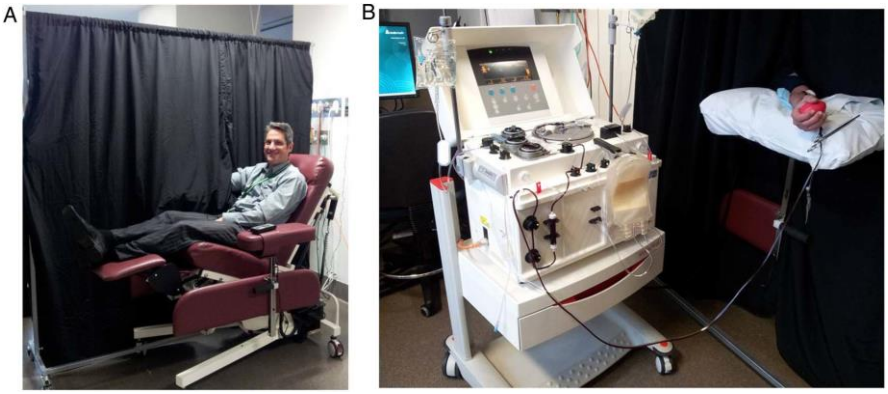


**Figure 2** A black opaque curtain prevents the participant from seeing the apheresis machine and therefore the individual cannot see if red blood cells or plasma is removed. (A) View from the patient's perspective, (B) View from the apheresis machine side of the curtain.

folate, iron studies and full-blood count are checked approximately mid-treatment in both groups for safety monitoring.

### Target SF

There have been no definitive studies conducted to demonstrate what the target SF should be at the end of treatment in an individual with HH. Guidelines recommend a target SF of less than 50–100 µg/L. In this study, we have chosen to reduce SF to anywhere in the normal range, that is, a SF of 20–300 µg/L, based on the expectation that an individual with HH whose SF is in the normal range should have similar symptoms to those without HH and who have a normal SF.

### Outcome measures

The outcome measures being assessed are patient-reported outcome scales to assess symptoms, as well as markers of liver injury, hepatic fibrosis and oxidative stress. These are administered at baseline and the end of erythrocytapheresis/ sham apheresis treatment.

#### Patient-reported outcome scales

1. *Modified Fatigue Impact Scale (MFIS)* is the primary outcome measure for this study. The MFIS is a 21-item scale that measures the impact of fatigue on three independent subscales of physical, cognitive and psychosocial functioning.[18] Participants rate their fatigue in the past month on a five-point Likert-type scale. The total score ranges from 0 to 84 with higher scores reflecting greater fatigue. Subscale scores, physical (0–36); cognitive (0–40); and psychosocial (0–8), can also be derived.
2. *Medical Outcomes Study 36-item short form V.2 (SF36v2)* is a 36-item generic health survey to measure health and well-being[19] that has been previously used in various HH studies to measure quality of life.[2 12 20 21] It assesses eight different health components (physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, mental health) and provides a summary score for both physical and mental components. It is a norm-based scoring system and thus can be used to compare participant scores to the general population.
3. *Hospital Anxiety and Depression Scale (HADS)* is composed of a 14-item total scale (HADS-T) consisting of two seven-item independent subscales, the Anxiety (HADS-A) and Depression (HADS-D) subscales.[22] Participants rate how they have felt in the past week on a four-point Likert-type scale. Scores on each scale can be interpreted in ranges: normal (0–7); mild (8–10); moderate (11–14); and severe (15–21). Higher scores on each subscale or the entire scale indicate

$$\text{Hct post} = \frac{(\text{TBV} \times \text{Donor Hct}/100) - (\text{Target RBC volume} \times \text{Bowl Hct}/100)}{\text{TBV} - \text{Target RBC volume} + \text{Compensation Volume} + (30\text{ml} \times \text{cycles})}$$

**Nadler's Formula**

Female TBV (ml) = 183 + 0.000356 x height $(\text{cm})^3$ + 33 x weight (kg)

Male TBV (ml) = 604 + 0.0003668 x height $(\text{cm})^3$ + 32.2 x weight (kg)

**Figure 3** Equation to estimate postcollection haematocrit (Hct post) based on total blood volume (TBV) using Nadler's fomula.[17] Adopted from the Haemonetics MCS Plus apheresis system manual. TBV, total blood volume; RBC, red blood cell; Hct, haematocrit.

greater anxiety, depression or both. This scale has been found to be valid and reliable in various populations.[23 24]

4. *Arthritis Impact Measurement Scales 2 short form (AIMS2-SF)* is a 26-item validated scale that assesses the impact of arthritis on five core domains of the participants.[25 26] It measures physical functioning, symptoms, affect, role and social interactions of the individuals. A five-point Likert-type scale is used to rate how participants have felt in the past month. The higher the raw score, the greater the impact of arthritis on the participant. Use of arthritis medication at baseline and end of trial will also be compared.
5. To assess the fidelity of the blinding process, the participants are asked which treatment group they believe they were allocated to at the completion of the study, before unblinding.

### Liver injury and hepatic fibrosis markers

Transient elastography (TE) and blood tests for components of Hepascore and Fibrometer$^{3G}$ V are collected from individuals at baseline and end of the trial.

### Transient elastography

Fibroscan is a method of TE that evaluates liver stiffness using an ultrasound probe to measure the velocity of a mechanical wave that is pulsed through the liver. As the liver becomes progressively more fibrotic, it becomes harder and less elastic. The velocity of the wave correlates directly with tissue stiffness and results are reported in kilopascals (kPa).[27] TE has been evaluated in a number of different liver diseases, including HBV and HCV, alcoholic liver disease, non-alcoholic fatty liver disease and HH.[28 29] A recent meta-analysis of nine studies involving TE showed excellent results for diagnosing cirrhosis, with a sensitivity of 87% and specificity of 91%.[30] Adhoute *et al*[31] have shown that TE measurements correlate with Hepascore measurements in individuals with HH. The results of liver stiffness are acquired from at least 10 successful valid measurements with a success rate of at least 60% within the IQR of ≤30%. A cut-off value of 8.7 kPa was sensitive for the diagnosis of those with significant fibrosis (≥F2), with an area under the curve (AUC) for the receiver operating characteristic (ROC) of 0.79[32] and a reading of more than 13 kPa was highly predictive that cirrhosis of the liver was present in a cohort with iron overload due to β-thalassaemia.[33]

### Hepascore

Hepascore is derived from an age-specific and gender-specific model that inputs parameters of serum bilirubin, γ glutamyl transferase (GGT), hyaluronic acid and α2-macroglobulin. The test results in a score between 0 and 1 with a higher score being associated with more severe liver disease. In a HCV cohort, Hepascore demonstrated an AUC for the ROC of 0.8 for predicting significant fibrosis (≥F2) and 0.90 for predicting cirrhosis.[34] A score >0.5 was found to have a specificity of 70% and sensitivity of 77% to detect significant fibrosis (≥F2) in a large HCV cohort.[35] A Hepascore <0.25 can exclude significant fibrosis with a negative predictive value of 0.9.[35] In a study that included the Hepascore in *HFE* related HH, 44 p.C282Y homozygotes had a median score of 0.1.[36]

### Fibrometer$^{3G}$ V

Fibrometer$^{3G}$ V is formulated from the platelet count (PLT), prothrombin index (PI), and the alanine amino transaminase (ALT), aspartate amino transaminase (AST), GGT, α2-macroglobulin and urea levels. This biomarker had an AUROC of 0.85 for predicting significant fibrosis and an AUROC of 0.9 for predicting cirrhosis in a HCV cohort.[37] Its robustness has been evaluated in different studies and has been recommended by the French National Authority for Health for the estimation of liver fibrosis in HCV.

The combination of Fibrometer$^{3G}$ V and TE has recently been shown to increase the accuracy of diagnosing significant fibrosis and cirrhosis to 92% compared with Fibrometer$^{3G}$ V (84% accuracy) or TE (88% accuracy) alone. The combination has an AUROC of 0.89, improving the reliability and precision of diagnosis of significant fibrosis in chronic liver disease.[38 39] Fibrometer has not been tested in HH.

### *Oxidative stress marker*

Iron is a strong pro-oxidant and there is evidence that markers of oxidative stress are elevated in individuals with elevated iron indices due to HH.[40–43] To assess oxidative stress, F2-isoprostanes, a validated marker of cellular lipid oxidative damage,[44] are being measured in urine and blood. While elevated makers of oxidative stress are not necessarily related to symptoms of disease, we will be able to assess the relationship between this early marker of tissue injury and the other markers being measured, including iron indices, Hepascore, Fibrometer, TE score and the scores for the various clinical scales being administered. We will also assess whether F2-isoprostanes are positively impacted by normalisation of iron indices in the erythrocytapheresis group.

## Sample size calculation

Data from the Melbourne HealthIron study[2] were used to calculate SDs of the MFIS score of 14.1 and 17.8 for male and female C282Y homozygotes, respectively. Using a conservative value for the SD of 18, a sample size of 50 in each treatment group ensures an 80% chance (statistical power) that a treatment effect of a mean difference of 10 MFIS units (well above a clinically relevant difference on this scale which runs from 0 to 84) is reflected in a p value less than 0.05. Summary statistics from figure 2 of Adams *et al*[34] show the mean Hepascore changing from 0.20 in patients with METAVIR fibrosis grade 0 or 1 (F0 or F1), through 0.45 in those with F2, to close to 1.0 in

those with F3 or F4. The within-fibrosis grade SD of Hepascore is approximately 0.20 in the F2 group and it is much lower in the remaining groups. Using this SD, a sample size of 50 in each treatment group of the trial delivers statistical power of 85% to detect a treatment effect of 0.12 on the Hepascore scale. A change of this magnitude is similar to the observed mean difference in Hepascore between adjacent fibrosis groups presented in Adams *et al*.[34] Accommodating the stratified design, the regression-based statistical analysis will result in minimal loss of power provided that only the average initial measures and not the treatment effects are different between strata.

### Statistical analysis

The primary analysis addressing the research hypothesis will be a comparison of the change in scores for all outcome scales, biochemical tests and TE scores, from baseline to end of treatment, between those who have had their iron levels returned to normal and those who were in the sham treatment group, with assignment to the comparison groups based on intention to treat. This analysis will be implemented using a linear regression model of the final measure on each scale including as covariates the value of the initial measure, gender, initial SF (300–599 µg/L or 600–1000 µg/L as a binary variable) and site (Melbourne or Brisbane as a two category variable). In a separate analysis, this model with the Hepascore as the outcome measure will be extended to include the quantity of iron removed (calculated as 1 g iron per litre of RBCs removed by erythrocytapheresis) to determine whether there is an association between the change in Hepascore and the reduction in iron level. Similar analyses will be performed for F2-isoprostanes, TE and patient-reported outcome scales.

## CONCLUSION

This is the first randomised controlled trial of treatment for HH. It will demonstrate whether there is any benefit in the short term from normalisation of SF in HFE p.C282Y homozygotes with moderately elevated SF. This has implications for management of this group of individuals and may assist in determining whether introduction of community screening for HH should be considered.

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**Contributors** SYO, LD, LCG and MBD wrote the first draft of the manuscript. SYO is involved in participant recruitment and assessment. LD and MW coordinate the study in Melbourne and are responsible for data management. JLD and LER coordinate the study in Brisbane. AJN and LWP participated in design of the trial and are involved in participant recruitment and assessment. LCG contributed to trial design and is the study statistician involved in randomisation and data analysis. EMW, GJA, GAR, KJA, JKO, DC, JK, PG and SD contributed to design of the trial. MBD is the chief investigator of the study, conceived the study, was involved in design of the study and is involved in recruitment and assessment of participants.

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**Competing interests** None declared.

**Ethics approval** This study has been approved by the Human Research Ethics Committees of Austin Health, Royal Melbourne Hospital and Royal Brisbane and Women's Hospital.

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# Reduction of body iron in *HFE*-related haemochromatosis and moderate iron overload (Mi-Iron): a multicentre, participant-blinded, randomised controlled trial

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## Summary

**Background** The iron overload disorder hereditary haemochromatosis is most commonly caused by HFE p.Cys282Tyr homozygosity. In the absence of results from any randomised trials, current evidence is insufficient to determine whether individuals with hereditary haemochromatosis and moderately elevated serum ferritin, should undergo iron reduction treatment. This trial aimed to establish whether serum ferritin normalisation in this population improved symptoms and surrogate biomarkers.

**Methods** This study was a multicentre, participant-blinded, randomised controlled trial done at three centres in Australia. We enrolled people who were homozygous for HFE p.Cys282Tyr, aged between 18 and 70 years, with moderately elevated serum ferritin, defined as 300–1000 µg/L, and raised transferrin saturation. Participants were randomly assigned, via a computer-generated random number, to undergo either iron reduction by erythrocytapheresis (treatment group) or sham treatment by plasmapheresis (control group). Randomisation was stratified by baseline serum ferritin (<600 µg/L or ≥600 µg/L), sex, and study site. Erythrocytapheresis and plasmapheresis were done every 3 weeks, the number of procedures and volume of red cells or plasma removed determined on the basis of each patient's haemoglobin, haematocrit, and serum ferritin concentration, as well their height and weight. In the erythrocytapheresis group, the target was to reduce serum ferritin to less than 300 µg/L. The number of procedures for the control group was based on the initial serum ferritin and prediction of decrease in serum ferritin of approximately 120 µg/L per treatment. The primary outcome was patient-reported Modified Fatigue Impact Scale (MFIS) score, measured at baseline and before unblinding. Analyses were by intention to treat, including the safety analysis. The trial is registered with ClinicalTrials.gov, number NCT01631708, and has been completed.

**Findings** Between Aug 15, 2012, and June 9, 2016, 104 participants were randomly assigned to the treatment (n=54) and control (n=50) groups, of whom 94 completed the study (50 in the treatment group and 44 in the control group). Improvement in MFIS score was greater in the treatment group than in the control group (mean difference –6·3, 95% CI –11·1 to –1·4, p=0·013). There was a significant difference in the cognitive subcomponent (–3·6, –5·9 to –1·3, p=0·0030), but not in the physical (–1·90 –4·5 to 0·63, p=0·14) and psychosocial (–0·54, –1·2 to 0·11, p=0·10) subcomponents. No serious adverse events occurred in either group. One participant in the control group had a vasovagal event and 17 participants (14 in the treatment group and three in the control group) had transient symptoms assessed as related to hypovolaemia. Mild citrate reactions were more common in the treatment group (32 events [25%] in 129 procedures) compared with the control group (one event [1%] in 93 procedures).

**Interpretation** To our knowledge, this study is the first to objectively assess the consequences of iron removal in individuals with hereditary haemochromatosis and moderately elevated serum ferritin. Our results suggest that serum ferritin normalisation by iron depletion could be of benefit for all individuals with hereditary haemochromatosis and elevated serum ferritin levels.

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## Introduction

*HFE*-associated hereditary haemochromatosis is the most common autosomal recessive disease among white European populations, with roughly one in 200 having homozygosity for the p.Cys282Tyr mutation, which places them at increased risk of iron overload.[1] Hereditary haemochromatosis results from elevated dietary iron absorption and subsequent iron deposition in various organs, resulting in potentially severe tissue damage because of the capacity of iron to induce oxidative stress.[2] The most serious, life-threatening clinical manifestations of hereditary haemochromatosis are liver cirrhosis and increased incidence of hepatocellular carcinoma. Iron deposition in the joints, pancreas, pituitary gland, and heart can also lead to arthralgia and arthritis, diabetes mellitus, sexual dysfunction, and cardiac failure.[3] Iron overload has also been reported to cause psychosocial effects, such as fatigue and depression, affecting quality of life.[4,5] Men are particularly at risk of morbidity from hereditary haemochromatosis, with at least 28% of men

## Research in context

### Evidence before this study

We searched PubMed up to Oct 20, 2017, without language restrictions using the search terms "haemochromatosis", "treatment", "phlebotomy", "venesection", "erythrocytapheresis", and "randomised". Although there are ample data to support the benefit of normalisation of body iron in people with hereditary haemochromatosis due to HFE p.Cys282Tyr homozygosity with serum ferritin of more than 1000 µg/L, few data exist to inform the question of whether treatment is needed for those with serum ferritin above the normal range but less than 1000 µg/L.

### Added value of this study

Our study, to our knowledge, is the first randomised, blinded study of iron depletion therapy by red cell removal compared with sham therapy in people with HFE p.Cys282Tyr homozygosity and moderate iron overload. Our results identified clinical and biochemical benefits of body iron normalisation for these people.

### Implications of all the available evidence

This study provides evidence that all individuals with hereditary haemochromatosis who have iron overload, as indicated by serum ferritin above the normal range, could benefit from normalisation of body iron.

See Online for appendix

homozygous for HFE p.Cys282Tyr in a large, unselected sample of the general population of Australia satisfying criteria for documented iron overload-related disease.[5]

Because of the potential complications of hereditary haemochromatosis, all management guidelines recommend removing excess iron from all people with hereditary haemochromatosis who have an elevated serum ferritin concentration, irrespective of whether they are symptomatic or not.[6,7] Treatment is generally by regular venesection, although erythrocytapheresis or iron chelation are occasionally used.[1]

Evidence for the benefit of iron depletion in people with HFE p.Cys282Tyr homozygosity and serum ferritin greater than or equal to 1000 µg/L is strong, as the risk of developing iron overload-related disease, including liver cirrhosis, is high in this group.[5,8] However, the evidence on the need for treatment in those with serum ferritin above the normal range (approximately 300 µg/L) but less than 1000 µg/L (moderate iron overload), the most common group among people with HFE p.Cys282Tyr homozygosity, is less clear. Some experts have suggested that individuals in this category might reasonably be observed rather than treated: the so-called watch and wait approach.[9,10] Although previous cohort studies have not shown evidence of increased risk of morbidity in those with moderate iron overload,[11,12] these studies were not specifically designed to answer the question of whether treatment is clinically beneficial to such individuals. It is unknown whether such individuals benefit from prophylactic treatment. There is, therefore, a need for objective evidence to inform the management of people with hereditary haemochromatosis who have moderate iron overload. We aimed to answer whether reduction of total body iron was beneficial for these individuals, as assessed with patient-reported outcomes, and non-invasive markers of liver fibrosis and oxidative stress.

## Methods

### Study design and participants

The methods of the Moderately Increased Iron (Mi-Iron) study have been published previously.[13] Mi-Iron was a multicentre, participant-blinded, randomised controlled trial done at three centres in Australia (appendix).

Patient inclusion criteria were HFE p.Cys282Tyr homozygosity, age 18–70 years, serum ferritin between 300 µg/L and 1000 µg/L, and raised transferrin saturation, with no venesection treatment for hereditary haemochromatosis in the 2 years before study entry. Exclusion criteria were other HFE genotypes, pregnancy, or risk factors of concomitant liver disease (hepatitis, alcohol intake ≥40 g per day for women and ≥60 g per day for men, or body-mass index [BMI] >35 kg/m²). Participants were recruited over 4 years through referral from pathology laboratories that did *HFE* testing, the Australian Red Cross Blood Service, medical professionals, and Haemochromatosis Australia, a patient support group. All participants provided written informed consent. The study was approved by the Human Research Ethics Committees of Austin Health, Melbourne, Melbourne Health, and Royal Brisbane and Women's Hospital.

### Randomisation and masking

Participants were stratified according to serum ferritin (<600 µg/L or ≥600 µg/L), sex, and study site, and subsequently randomly assigned to either the treatment group to receive erythrocytapheresis (and thus reduction of body iron, while remaining euvolaemic at the end of the procedure) or the control (sham) group to receive plasmapharesis (procedure performed without reduction of body iron). Randomisation was done with a computer-generated random number sequence in permuted blocks of length 6 generated by the main study statistician, LCG. Participants were enrolled by the study coordinators and site investigators who assigned them to trial groups after randomisation. Participants were blinded to the procedure they were undergoing by use of a full-length black curtain to conceal the apheresis machine behind it. Apheresis staff received training to ensure blinding.

### Procedures

Erythrocytapheresis and plasmapharesis were done with at least a 3 week interval between each procedure. The

number of treatments and volume of red cells or plasma removed were based on each patient's haemoglobin, haematocrit, and serum ferritin concentrations, as well as their height and weight. Blood sampling was done before each procedure to assess these parameters. In the erythrocytapheresis group, the target serum ferritin concentration was less than 300 µg/L. The number of procedures for the control group was based on the initial serum ferritin and a predicted decrease in serum ferritin of approximately 120 µg/L per treatment, as suggested in a study by Rombout-Sestrienkova and colleagues.[14] All participants were blinded to the results of investigations and blood samples, which were explained as being used for safety testing. A blood test was obtained 1 week before the end of treatment assessment for both groups to ensure that serum ferritin concentrations were less than 300 µg/L for the treatment group.

Participants completed questionnaires at baseline and the end of treatment (prior to unblinding), including the Modified Fatigue Impact Scale (MFIS),[15] Medical Outcomes Study Health Survey Version 2 (SF36v2),[16] Hospital Anxiety and Depression Scale (HADS),[17] and Arthritis Impact Measurement 2 Short-form Scale (AIMS2-SF).[18] At the end of treatment, before unblinding, participants were asked whether they thought they were in the treatment or control group.

We used surrogate biomarkers to assess liver fibrosis, including transient elastography with Fibroscan (Echosens, Paris, France),[19] Hepascore,[20] and Fibrometer 2G, 3G, and VCTE (Echosens).[21,22] We assessed oxidative stress by measuring urinary and plasma $F_2$-isoprostane concentrations with mass spectrometry as previously described.[2,23–27] Surrogate biomarkers were measured at baseline and at the end of treatment, before unblinding.

### Outcomes

The primary outcome was change in MFIS score between baseline and the end of treatment. Secondary outcomes were patient-reported outcomes, as measured by SF36v2, HADS, and AIMS2-SF; assessment of liver fibrosis according to transient elastography, Hepascore, Fibrometer, and oxidative stress; and the fidelity of blinding. Safety and adverse events were recorded during procedures by the apheresis nursing staff who administered the erythrocytapheresis and plasmapheresis procedures. Other adverse events were recorded by the research team members at study visits.

### Statistical analysis

The sample size calculation has been described in detail previously.[13] Briefly, using an SD of 18, a sample size of 50 in each treatment group would deliver a statistical power of 80% to detect a treatment effect of a mean difference of 10 MFIS units with an α of 0·05. With an SD of 0·20, a sample size of 50 will ensure statistical power of 85% to detect a treatment effect of 0·12 on the Hepascore scale with an α of 0·05.

We compared differences in scores at baseline and the end of treatment between the control and treatment groups. The analyses were based on the intention-to-treat principle in participants who had completed the study, with estimates of the treatment effect generated by fitting a linear regression model to data from all participants adjusting for the stratification factors, serum ferritin concentration (300–599 µg/L and 600–999 µg/L), sex, and study site. Fibrometer 2G, 3G, and VCTE results were log-transformed for analysis. Unless otherwise stated, within-group summary statistics are presented as the sample mean and SE. We did the statistical analyses with Stata software (version 13.1). This study is registered with ClinicalTrials.gov, number NCT01631708.

### Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## Results

Between Aug 15, 2012, and Jun 9, 2016, 128 individuals were screened and 104 participants were randomised (figure). Of the 24 individuals screened but not included in randomisation, 22 were ineligible for the study and two were eligible but withdrew before randomisation. Ten participants withdrew after randomisation, of whom two in the treatment group and five in the control group withdrew for personal reasons (eg, not having time to be in the trial) without starting treatment, and two in the treatment group and one in the control group withdrew after starting the study but without completing the end-of-trial assessment.

Of the 94 participants who completed the study, 50 were randomly assigned to the treatment group and 44 to the control group. 93 of the 94 participants were newly diagnosed with hereditary haemochromatosis and thus had not had treatment for the condition before the study. One participant in the control group had undergone venesection treatment more than 2 years before study entry. 75 participants were diagnosed through routine blood tests or family history (38 in the treatment group and 37 in the control group) and 18 participants presented with symptoms including lethargy, fatigue, or generally feeling unwell (11 in the treatment group and seven in the control group). Data on mode of diagnosis were missing from one individual in the treatment group (appendix).

Table 1 shows the baseline demographics of all participants. The mean number of treatments for participants was similar between the two groups (2·6 [SD 1·9], 95% CI 2·1 to 3·1 in the treatment group *vs* 2·1 [1·1], 1·8 to 2·5 in the control group, p=0·18). The difference in mean serum ferritin between baseline and the end of

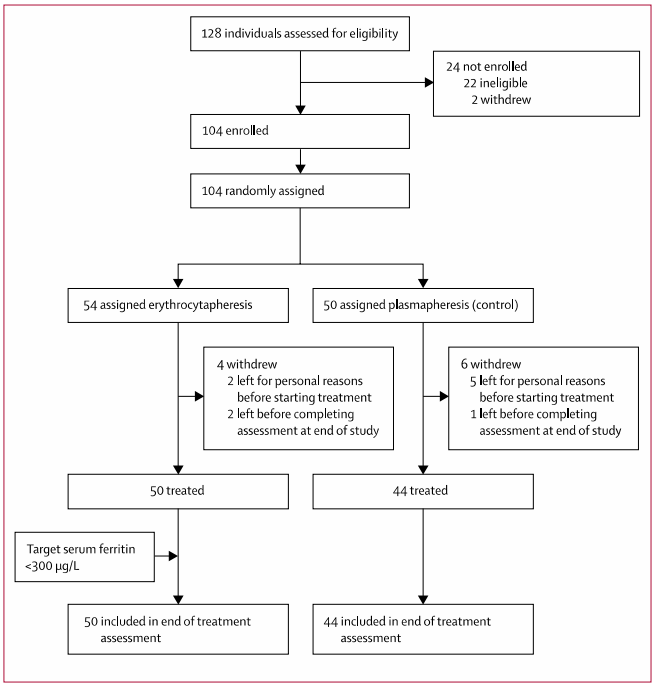


*Figure:* **Trial profile**

treatment was significant for the treatment group (decrease of 314·9 µg/L [185·5], –367·7 to –262·2, p<0·0001) but not for the control group (decrease of 30·8 µg/L [138·6], –73·0 to 11·3, p=0·15). Similarly, there was a significant reduction in mean transferrin saturation in the treatment group (baseline transferrin saturation 63·5% [17·0], 58·8 to 68·1 *vs* end of treatment transferrin saturation 45·4% [15·9], 40·9 to 50·0, p<0·0001) but not for the control group (baseline transferrin saturation 64·2% [18·6], 59·0 to 69·5 *vs* end of treatment transferrin saturation 61·7% [18·1], 95% CI 56·2 to 67·2, p=0·64). The mean baseline alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were in the normal range and similar in both groups (table 1). ALT and AST concentrations remained in the normal range for both groups at the end of treatment (treatment group ALT 22·6 U/L [8·9], 20·1 to 25·1 *vs* control group ALT 28·4 U/L [15·7], 23·7 to 33·2; treatment group AST 20·9 U/L [6·5], 19·1 to 22·8 *vs* control group AST 23·3 U/L [8·1], 20·8 to 25·7). The mean weight of erythrocytes removed per treatment in the treatment group (mean 436·5 g [112·3], 404·6 to 468·5) was similar to the weight of plasma removed per procedure in the control group (434·7 g [121·6], 397·7 to 471·7; p=0·77). The mean total weight of iron removed was about 1·2 g with erythrocytapheresis, whereas the control group had close to no iron removed (mean total 0·0008 g).

The mean decrease in MFIS score, the primary outcome, was greater in the treatment group (–6·8 [SE 1·6], 95% CI –10·0 to –3·6) than for the control group (–1·4 [1·7], –4·8 to 2·1, p=0·013; table 2). Of the MFIS subscales, the only significant difference between the groups was in the cognitive component (treatment –3·9 [0·78], –5·5 to –2·4 *vs* control –0·80 [0·83], –2·5 to 0·86, p=0·0030; table 2).

There was no significant difference between change in scores for the mental (p=0·44) and physical (p=0·31) components of SF36v2 between the groups (table 2). Similarly, there were no significant difference in change in score between the groups for the total HADS score, which was generally less than 10 at baseline (p=0·26; table 2). Among the five components of the AIMS2-SF, the mean affect component was the only one that was significantly different between the treatment group and the control group. The AIMS2-SF affect component improved by about half a unit on a baseline mean of about two units in the treatment group, whereas there was no change in the mean component for controls (p=0·034; table 2).

As could be expected in an unselected group of people with HFE p.Cys282Tyr homozygosity and serum ferritin less than 1000 µg/L, there was little evidence of liver fibrosis as measured by transient elastography in either group at baseline (table 1). The mean change in transient elastography scores between treatment and control groups was also similar between groups (table 2). There was no evidence of liver fibrosis as measured by Hepascore in either group at baseline (table 1). However, after treatment, Hepascore decreased in the treatment group and increased in the control group (p=0·049; table 2). There was little evidence of advanced liver fibrosis as measured by Fibrometer 2G, 3G, or VCTE in either group at baseline (table 1) and little evidence of change in either group after treatment, so there was no discernible difference in change between groups (table 2). The mean change in plasma $F_2$-isoprostanes was larger for the treatment group than the control group (p=0·038), although this effect was not seen for urinary $F_2$-isoprostanes (table 2).

There were no differences between groups when participants were asked about which group of the study they believed they had been assigned to, indicating successful blinding of participants to the randomised treatment allocation (appendix).

Apheresis treatments were well tolerated, with no serious adverse events reported in either group. One participant in the control group had a vasovagal event and 17 participants (14 in the treatment group and three in the control group) had transient symptoms assessed as related to hypovolaemia. Mild citrate reactions were more common in the treatment group (32 events [25%] in 129 procedures)

| | Erythrocytapheresis (n=54) | Plasmapheresis (control; n=50) |
|---|---|---|
| **Sex** | | |
| Male | 34 (63%) | 32 (64%) |
| Female | 20 (37%) | 18 (36%) |
| **Mean age (years)** | | |
| Male | 37·3 (14·2; 32·3–42·3) | 43·5 (13·1; 38·8–48·2) |
| Female | 49·1 (13·3; 42·8–55·3) | 45·3 (15·3; 37·7–52·9) |
| **Mean body-mass index (kg/m²)** | | |
| Male | 25·4 (3·8; 24·1–26·8) | 26·8 (3·8; 25·4–28·2) |
| Female | 26·3 (4·7; 24·2–28·5) | 26·6 (3·6; 24·8–28·4) |
| **Serum ferritin 600–1000 µg/L (n=30)** | | |
| Male | 14 (26%) | 13 (26%) |
| Serum ferritin (µg/L) | 723·5 (93·3; 669·6–777·4) | 705·5 (69·2; 663·7–747·3) |
| Female | 4 (7%) | 1 (2%) |
| Serum ferritin (µg/L) | 715·5 (173·6; 439·2–991·8) | 861 |
| **Serum ferritin 300–599 µg/L (n=64)** | | |
| Male | 20 (37%) | 19 (38%) |
| Serum ferritin (µg/L) | 403·5 (77·7; 367·1–439·0) | 436·2 (87·0; 394·5–478·4) |
| Female | 16 (30%) | 17 (34%) |
| Serum ferritin (µg/L) | 414·3 (72·5; 375·6–452·9) | 402·3 (82·6; 359·8–444·8) |
| **Serum markers** | | |
| Transferrin saturation (%) | 63·5 (17·0; 58·8–68·1) | 64·2 (18·6; 59·0–69·5) |
| Alanine aminotransferase (U/L) | 29·3 (13·4; 25·4–33·1) | 32·6 (23·9; 25·4–39·9) |
| Aspartate aminotransferase (U/L) | 23·9 (9·0; 21·4–26·5) | 26·0 (13·2; 22·0–30·0) |
| **Patient-reported outcome measures** | | |
| MFIS | | |
| Total | 26·5 (17·1; 21·8–31·2) | 24·9 (17·1; 20·1–29·8) |
| Cognitive | 13·2 (8·7; 10·8–15·6) | 11·7 (7·3; 9·7–13·8) |
| Physical | 11·3 (8·2; 9·1–13·6) | 10·9 (8·9; 8·4–13·5) |
| Psychosocial | 2·0 (1·9; 1·5–2·5) | 2·2 (2·2; 1·6–2·9) |
| SF36v2 | | |
| Mental component summary | 48·3 (12·3; 44·9–51·7) | 47·4 (14·6; 43·2–51·6) |
| Physical component summary | 49·6 (7·4; 47·6–51·7) | 51·0 (7·7; 48·8–53·2) |
| (Table 1 continues in next column) | | |

| | Erythrocytapheresis (n=54) | Plasmapheresis (control; n=50) |
|---|---|---|
| (Continued from previous column) | | |
| **HADS** | | |
| Total | 9·0 (6·8; 7·1–10·9) | 9·2 (7·6; 7·0–11·4) |
| Anxiety | 5·7 (3·9; 4·6–6·8) | 5·5 (4·3; 4·3–6·8) |
| Depression | 3·3 (3·3; 2·4–4·2) | 3·7 (4·1; 2·5–4·9) |
| **AIMS2** | | |
| Physical | 0·59 (0·65; 0·41–0·77) | 0·66 (0·75; 0·45–0·88) |
| Affect | 2·0 (1·9; 1·5–2·5) | 2·1 (2·2; 1·4–2·7) |
| Symptom | 0·85 (1·8; 0·37–1·3) | 0·90 (1·5; 0·47–1·3) |
| Social | 4·9 (1·5; 4·5–5·3) | 4·8 (2·2; 4·2–5·4) |
| Work | 1·1 (2·0; 0·47–1·7) | 1·9 (3·2; 0·93–2·9) |
| **Hepatic fibrosis markers** | | |
| Transient elastography score (kPa) | 4·9 (1·6; 4·5–5·4) | 4·9 (1·3; 4·5–5·2) |
| Hepascore* | 0·21 (0·12; 0·18–0·25) | 0·20 (0·12; 0·16–0·24) |
| Fibrometer2G (log)* | 0·16 (0·091; 0·13–0·19) | 0·18 (0·13; 0·14–0·22) |
| Fibrometer3G (log)* | 0·18 (0·11; 0·15–0·22) | 0·21 (0·14; 0·16–0·25) |
| Fibrometer VCTE (log)* | 0·12 (0·067; 0·10–0·14) | 0·14 (0·14; 0·10–0·19) |
| **Oxidative stress markers** | | |
| $F_2$-isoprostanes plasma (pmol/L)* | 929·2 (201·1; 871·5–987·0) | 884·4 (255·8; 804·7–964·1) |
| $F_2$-isoprostanes urine (pmol/L)* | 396·5 (163·5; 350·1–443·0) | 382·9 (219·8; 315·2–450·5) |

Data are mean (SD; 95% CI) or n (%). MFIS=Modified Fatigue Impact Scale. SF36v2=Medical Outcomes Study 36-item short form version 2. HADS=Hospital Anxiety and Depression Scale. AIMS2-SF=Arthritis Impact Measurement Scales 2 short form. *Data available for participants who completed the study.

***Table 1:*** **Baseline demographics and clinical characteristics**

compared with the control group (one event [1%] in 93 procedures).

In a post-hoc analysis, we also compared participants in the treatment group who were diagnosed as having symptoms at baseline with those who were asymptomatic. The change in MFIS in response to ferritin normalisation was not significantly different (–2·7 [SE 3·7], 95% CI –10·1 to 4·6, p=0·46) between treatment group participants with symptoms (–8·8 [3·2], –15·3 to –2·3) and those who were asymptomatic and were diagnosed because of family history or by screening blood tests (–6·1 [1·7], –9·6 to –2·6).

## Discussion

Our results showed that iron depletion in individuals with hereditary haemochromatosis with moderately elevated serum ferritin improved mental wellbeing. Iron depletion was also associated with improvement in Hepascore and plasma $F_2$-isoprostanes.

Hereditary haemochromatosis is common in white European populations, but who should be treated with iron depletion therapy and how aggressively they should

| | Patients assessed | Change in erythrocytapheresis group | Change in plasmapheresis (control) group | Adjusted mean difference | p value |
|---|---|---|---|---|---|
| **Patient reported outcome measures** | | | | | |
| MFIS | | | | | |
| Total | 93 | −6·8 (1·6; −10·0 to −3·6) | −1·4 (1·7; −4·8 to 2·1) | −6·3 (2·5 ; −11·1 to −1·4) | 0·013 |
| Cognitive | 94 | −3·9 (0·78; −5·5 to −2·4) | −0·80 (0·83; −2·5 to 0·86) | −3·6 (1·2 ; −5·9 to −1·3) | 0·0030 |
| Physical | 93 | −2·3 (0·83; −4·0 to −0·70) | −0·60 (0·89; −2·4 to 1·2) | −1·9 (1·3; −4·5 to 0·63) | 0·14 |
| Psychosocial | 94 | −0·58 (0·22; −1·0 to −0·15) | −0·068 (0·23; −0·52 to 0·39) | −0·54 (0·33; −1·2 to 0·11) | 0·10 |
| SF36v2 | | | | | |
| Mental component summary | 88 | 2·1 (1·3; −0·41 to 4·6) | 1·2 (1·4; −1·5 to 3·9) | 1·5 (1·9; −2·3 to 5·4) | 0·44 |
| Physical component summary | 88 | 1·4 (0·87; −0·29 to 3·2) | 0·30 (0·94; −1·6 to 2·2) | 1·4 (1·4; −1·3 to 4·1) | 0·31 |
| HADS | | | | | |
| Total | 91 | −2·0 (0·64; −3·3 to −0·72) | −1·1 (0·69; −2·5 to 0·28) | −1·1 (0·95; −3·0 to 0·80) | 0·26 |
| Anxiety | 92 | −1·5 (0·42; −2·3 to −0·64) | −0·49 (0·45; −1·4 to 0·40) | −0·98 (0·61; −2·2 to 0·24) | 0·12 |
| Depression | 93 | −0·62 (0·30; −1·2 to 0·022) | −0·51 (0·33; −1·2 to 0·13) | −0·28 (0·46; −1·2 to 0·63) | 0·54 |
| AIMS2-SF | | | | | |
| Physical | 93 | −0·071 (0·11; −0·28 to 0·14) | 0·044 (0·11; −0·18 to 0·27) | −0·13 (0·16; −0·46 to 0·19) | 0·42 |
| Affect | 89 | −0·48 (0·15; −0·78 to −0·17) | 0·00 (0·17; −0·33 to 0·33) | −0·51 (0·24; −0·98 to −0·038) | 0·034 |
| Symptom | 94 | 0·00 (0·21; −0·41 to 0·41) | 0·11 (0·22; −0·32 to 0·55) | −0·19 (0·31; −0·81 to 0·43) | 0·54 |
| Social | 93 | −0·13 (0·20; −0·51 to 0·26) | 0·00 (0·21; −0·42 to 0·42) | −0·21 (0·30; −0·80 to 0·40) | 0·48 |
| Work* | 67 | 0·48 (0·53; −0·58 to 1·5) | −0·68 (0·54; −1·8 to 0·39) | 1·2 (0·82; −0·47 to 2·8) | 0·16 |
| **Hepatic fibrosis markers** | | | | | |
| Transient elastography score (kPa) | 94 | 0·052 (0·21; −0·37 to 0·48) | −0·12 (0·23; −0·57 to 0·34) | 0·16 (0·33; −0·49 to 0·82) | 0·63 |
| Hepascore | 94 | −0·012 (0·017; −0·091 to 0·0073) | 0·030 (0·018; −0·0062 to 0·065) | −0·051 (0·026; −0·10 to −0·00018) | 0·049 |
| Fibrometer 2G (log) | 88 | 0·075 (0·050; −0·026 to 0·17) | 0·034 (0·057; −0·078 to 0·15) | 0·068 (0·077; −0·086 to 0·22) | 0·38 |
| Fibrometer 3G (log) | 88 | 0·055 (0·050; −0·045 to 0·15) | −0·026 (0·060; −0·14 to 0·085) | 0·11 (0·075; −0·034 to 0·26) | 0·13 |
| Fibrometer VCTE (log) | 89 | 0·15 (0·062; 0·023 to 0·27) | −0·050 (0·069; −0·19 to 0·088) | 0·19 (0·097; −0·0015 to 0·39) | 0·052 |
| **Oxidative stress markers** | | | | | |
| $F_2$-isoprostanes plasma (pmol/L) | 91 | −62·9 (34·7; −131·8 to 6·1) | 37·6 (37·5; −36·9 to 112·1) | −113·7 (53·9; −220·9 to −6·5) | 0·038 |
| $F_2$-isoprostanes urine (pmol/L) | 92 | −1·4 (30·8; −62·5 to 59·8) | 4·5 (33·6; −62·2 to 71·3) | −26·7 (46·7; −119·6 to 66·1) | 0·57 |

Data are mean (SE; 95% CI). Changes in the erythrocytapheresis and plasmapheresis control groups were calculated as the mean difference in scores between baseline and follow-up. Mean difference was the comparison of the change between control and treatment groups, adjusted for serum ferritin groups, sex, and site. MFIS=Modified Fatigue Impact Scale. SF36v2=Medical Outcomes Study 36-item short form version 2. HADS=Hospital Anxiety and Depression Scale. AIMS2-SF=Arthritis Impact Measurement Scales 2 short form. *The work component of AIMS2-SF was only assessed in the subset of participants who were still in paid employment (not retired, or doing unpaid, volunteer , or carer work).

***Table 2:*** **Outcome measures**

be treated remain important clinical questions. There are surprisingly few objective data on this topic and no evidence from randomised controlled trials. Our study, to our knowledge, the first randomised, blinded study of iron depletion therapy by red cell removal compared with sham therapy, provides good evidence that normalising body iron stores has a clinical benefit for people with HFE p.Cys282Tyr homozygosity and moderate iron overload.

Change in total MFIS was greater in the treatment group than in the control group, which was mainly due to improvement in the cognitive component. It is unknown what change in MFIS score is clinically meaningful. Although the baseline mean MFIS score in this study was lower than the minimum MFIS score for a diagnosis of fatigue (38),[28] this does not necessarily mean that treated individuals did not obtain benefit from the treatment. A difference of six units in the MFIS score between the treatment and control groups could represent psychosocial benefit in the treated cohort. It is possible that the higher baseline MFIS in the treatment group than in the control group, despite not being a significant difference, contributed to the greater change detected in the treatment group. However our finding of an improvement in the affect component of AIMS2-SF in the treatment group supports the notion that normalisation of iron can positively affect mental wellbeing. These patient-reported findings were accompanied by improvement in the non-invasive hepatic fibrosis marker Hepascore and plasma $F_2$-isoprostanes, a marker of oxidative stress, in the treatment group.

There was no change in any outcome measure that was significantly greater in the control group than in the treatment group. This finding suggests that the normalisation of body iron in people with HFE p.Cys282Tyr homozygosity is unlikely to be harmful. Indeed, we have shown in this study that, in many instances, treatment is beneficial for those with moderate iron overload. Overall, we considered 21 comparisons between treatment and control interventions in eight domains (four each that were patient-reported and surrogate markers). Of these comparisons, six of eight domains and 16 of 21 comparisons favoured the treatment over control including 13 of 14 patient-reported outcomes, although not all differences were statistically significant. Notwithstanding the positive correlation between comparisons in some domains, such summary statistics are unlikely if the treatment offered no benefit in any domain.

The mechanism by which venesection results in reduced fatigue is uncertain. Reduction in body iron leading to reduced oxidative stress is thought to be the most likely explanation and is supported by our observation of a reduction in plasma $F_2$-isoprostanes in the treatment group. Also supporting this mechanism is the finding of increased $F_2$-isoprostanes in individuals with chronic fatigue compared with controls.[29] It is also possible that the mechanism is unrelated to iron levels and could be a non-specific effect of red blood cell removal.

Apheresis staff and other study staff were trained to use neutral language to ensure that unblinding did not occur through accidental reference to whether a participant was undergoing erythrocytapheresis or plasmapheresis. The effectiveness of the blinding of participants to treatment was supported by similar proportions of participants in each group who believed they were treated, were in the sham group, or were unsure to which group they had been assigned (appendix). Double blinding was not possible because apheresis staff needed to implement different procedures for the treatment and control groups.

About 70% of the participants in the study were asymptomatic and diagnosed through family history or routine iron studies as part of a health check. It is therefore unlikely that our results are due to offering treatment only to individuals with the most severe and advanced forms of clinical disease. Moreover, in an exploratory analysis, we did not find a difference in the primary outcome, the MFIS, in response to normalisation of serum ferritin in participants who were diagnosed as a result of having symptoms compared with those who were asymptomatic. This finding suggests that serum ferritin normalisation should be recommended for all individuals with hereditary haemochromatosis and raised serum ferritin, irrespective of how the diagnosis is made. This recommendation is supported by results from a cohort study that showed decreased cardiovascular and extrahepatic cancer-related mortality in individuals with HFE-related hereditary haemochromatosis with moderately elevated serum ferritin that was normalised by venesection therapy.[30]

There is uncertainty as to the appropriate target serum ferritin concentration after iron depletion therapy for hereditary haemochromatosis. Some experts recommend a lower limit of 50 µg/L[9] whereas others recommend 100 µg/L.[31] We chose an endpoint of serum ferritin less than 300 µg/L for this study on the assumption that if a clinical benefit of iron normalisation exists, then it should be seen by reducing serum ferritin into the normal range and should not require reduction to the lower serum ferritin levels recommended in various guidelines. We were also concerned that aiming for a serum ferritin endpoint of 50–100 µg/L could result in iron deficiency anaemia and fatigue that might have confounded the outcome of the study. Our study was not designed to answer the question of how low the concentration of serum ferritin should be to signal the end of treatment for iron overload due to hereditary haemochromatosis. It would be interesting to do a similar study with sufficient power to allow for some treated individuals to be assigned to have their serum ferritin lowered to just below 300 µg/L and others to around 50 µg/L to assess any differences in outcomes between these groups.

Debate continues as to whether screening for hereditary haemochromatosis should be instituted.[32,33] There are roughly 1 million people each in the USA and Europe, and almost 100 000 in Australia who have or will get moderately elevated serum ferritin due to *HFE* mutations.[5] Our data suggest that people with HFE p.Cys282Tyr homozygosity with moderate iron overload, who are apparently asymptomatic, can benefit from normalisation of body iron. These findings add weight to the case for introducing screening for hereditary haemochromatosis in the community.

The limitations of this study include the fact that the trial was, unavoidably, single blinded rather than double blinded. It is also possible that a larger sample size would have allowed clearer differences between the groups to be identified. Additionally, reduction of final serum ferritin to 50–100 µg/L in the treatment group might also have resulted in clearer differences between the groups.

In conclusion, our results show both patient-reported and surrogate marker evidence of benefit from normalisation of iron levels in people with HFE p.Cys282Tyr homozygosity with moderate iron overload and support recommendations for the treatment of all individuals in this category, irrespective of the means of diagnosis.

**Contributors**

LCG, AJN, EMW, GJA, GAR, KJA, JKO, DC, PG, LWP, and MBD conceived and designed the study. SYO and LCG analysed the data. SYO, LD, JD, MW, LER, SD, LWP, and MBD acquired the data. All authors interpreted the data and contributed to the manuscript.

**Declaration of interests**

We declare no competing interests.

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## Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Ong S Y, Gurrin L C, Dolling L, et al, A. Reduction of body iron in *HFE*-related haemochromatosis and moderate iron overload (Mi-Iron): a multicentre, participant-blinded, randomised controlled trial. *Lancet Haematol* 2017; **4:** e607–14.

## Appendix

**Supplementary Table 1. Mode of diagnosis of study participants with HFE-related hereditary haemochromatosis**

| Diagnosis | Treatment (n=50) | Control (n=44) | Total (n=94) |
|---|---|---|---|
| **Routine blood tests/ family history** | 38/50 (76%) | 37/44 (84%) | 75/94 (79.8%) |
| **Symptoms including lethargy/ fatigue/ generally unwell** | 11/50 (22%) | 7/44 (16%) | 18/94 (19.1%) |
| **Missing** | 1/50 (2%) | 0 (0%) | 1/94 (1.1%) |

**Supplementary Table 2. Response to the question of whether subjects believed they had been in the treatment (Yes) or control (No) arm of the study asked immediately prior to unblinding**

| | Treatment (n=50) | Control (n=44) | p-value |
|---|---|---|---|
| **Yes** | 10 /50 (20%) | 10/44 (22.7%) | 0.60 |
| **No** | 9/50 (18%) | 6/44 (13.6%) | |
| **Not sure** | 29/50 (58%) | 28/44 (63.6%) | |
| **Missing** | 2/50 (4%) | 0 (0%) | |

**Sites involved:**

1. Queensland: Royal Brisbane and Women's hospital
   Principal investigator: Professor Lawrie Powell
   Number of participants recruited: 54

2. Victoria: Austin Health
   Principal investigator: Professor Martin Delatycki
   Number of participants recruited: 36

3. Victoria: Royal Melbourne Hospital
   Principal investigator: A/Prof Amanda Nicoll
   Number of participants recruited: 14

# Chapter 3: The relationship between serum ferritin and non-invasive markers of liver fibrosis in HFE-haemochromatosis

## 3.1 Introduction and Summary

There is an increased use of non-invasive liver fibrosis markers in clinical practice to help determine the presence and stage of liver fibrosis. These markers are often used instead of liver biopsies due to the invasive nature and associated risks of biopsy, and variabilities of sampling and assessment by pathologists (Regev, Berho et al. 2002, Chou and Wasson 2013). These markers are readily available and can be used easily to monitor liver fibrosis progression. Transient elastography, Hepascore, APRI and FIB-4 have been widely used and validated in hepatitis C but not in haemochromatosis. The relationship between serum ferritin, a surrogate marker for iron load, and these markers remain undetermined (Adhoute, Foucher et al. 2008, Legros, Bardou-Jacquet et al. 2015). It is unclear what the impact of iron load on these markers is and hence, this study aimed to address this area of deficit.

The study included 150 p.C282Y homozygotes, with 26 participants having SF more than 1000 µg/L. Results of four different non-invasive markers, TE, Hepascore, APRI and FIB-4 were obtained from the cohort. Overall, the majority of the cohort with mean SF 753.87 ± 71.24 µg/L (95% CI: 613.09, 894.64), did not have evidence of liver fibrosis and the mean SF was higher in those who had evidence of significant liver fibrosis or cirrhosis from the non-invasive markers. There was a positive relationship between SF and Hepascore, APRI and FIB-4. The relationship between SF and TE did not reach statistical significance although there was a positive trend. These non-invasive liver fibrosis markers may be useful tools to detect and monitor liver fibrosis in haemochromatosis.

## 3.2 PDF of Submitted Manuscript

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# The relationship between serum ferritin and non-invasive markers of liver fibrosis in HFE-haemochromatosis

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| Key Words: | HAEMOCHROMATOSIS, HEPATIC FIBROSIS, TRANSIENT ELASTOGRAPHY, HEPASCORE, LIVER FIBROSIS MARKERS |
| | |

**Title: The relationship between serum ferritin and non-invasive markers of liver fibrosis in *HFE*-haemochromatosis**

**Short title: Serum ferritin and liver fibrosis markers in haemochromatosis**

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**Abbreviations**

Hereditary haemochromatosis (Rahman, *et al*)

Transient elastography (TE)

Area under the receiver-operating characteristic curve (AUROC)

Serum ferritin (SF)

Gamma-glutamyl transferase (GGT)

Aspartate aminotransferase (AST)

AST to platelet ratio index (APRI)

Fibrosis-4 (FIB-4)

Alanine aminotransferase (ALT)

Body mass index (BMI)

Transferrin saturation (TS)

International normalised ratio (INR)



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**Summary**

Non-invasive markers of liver fibrosis such as transient elastography (TE) and serum biomarkers are increasingly used in assessing hepatic fibrosis in chronic liver diseases. The relationship between these markers in *HFE*-associated hereditary haemochromatosis and serum markers of iron overload has not been determined. This study aimed to investigate the relationship between serum ferritin (SF) levels and non-invasive markers of liver fibrosis in HH. From Aug 2012 to Jan 2018, *HFE* p.C282Y homozygotes with a SF>300µg/L were recruited prospectively. TE and serum biomarker panels of hepatic fibrosis, Hepascore, APRI and FIB-4 were measured. SF was log transformed and linear regression analysis was used to examine the relationship between SF and the liver fibrosis markers, adjusted for site. One hundred and fifty participants were recruited with a mean SF of 753±71.2µg/L. Of these, 26 had SF>1000µg/L with a mean SF 1855.7±331.3µg/L. The mean SF was significantly higher in individuals with higher Hepascore, APRI and FIB-4, (estimated regression coefficient= 0.13,p<0.01; 0.08,p<0.01; 0.22,p<0.01 respectively) and a positive trend with TE (0.54, p=0.053). In conclusion, there was a positive correlation between SF and non-invasive liver fibrosis markers Hepascore, APRI, FIB-4, and likely TE and these should be considered for monitoring for fibrosis in HH.

**Keywords:**

Haemochromatosis; Hepatic fibrosis; Transient elastography; Hepascore; Liver fibrosis markers

## Introduction

Hereditary haemochromatosis (Rahman, *et al*) is an inherited iron overload disorder and is most commonly caused by homozygosity for the p.C282Y substitution in the HFE protein (Feder, *et al* 1996). The prevalence of homozygosity for this mutation is between 1 in 80 and 1 in 400 in Caucasians and is highest among those with Northern European decent (Adams, *et al* 2005b, Allen, *et al* 2008, Merryweather-Clarke, *et al* 2000, Ryan, *et al* 1998). Iron accumulation can result in end organ damage in the liver, heart, and pancreas. Early diagnosis and treatment by phlebotomy to remove excess iron can prevent these complications (Powell, *et al* 2006).

Due to the availability of genetic testing for *HFE* mutations to diagnose HH and advances in non-invasive methods to assess the degree of hepatic fibrosis, liver biopsy is performed less frequently than was the case prior to the discovery of the causative gene mutation. Liver biopsy is currently recommended for individuals with *HFE*-related HH with SF >1000μg/L if the individual is over the age of 40 years, has abnormal liver function tests and/or hepatomegaly (Bacon, *et al* 2011, Barton and Adams 2010). Liver biopsy is an invasive procedure and can result in significant morbidities such as bleeding, infection, and pain (Chou and Wasson 2013). Moreover, there is potential sampling error due to regional variation of deposition of iron and distribution of steatosis, inflammation, and fibrosis. Different pathologists may assess the same sample differently due to regional variation in the biopsy, resulting in large inter-observer variability (Regev, *et al* 2002). As a result, a number of non-invasive methods that can assess a larger volume of the liver

to determine the presence and severity of liver fibrosis are being developed and utilised.

Transient elastography (TE), a rapid and non-invasive test of liver stiffness for the estimation of liver fibrosis, is widely used in liver diseases such as hepatitis B and C, alcoholic liver disease and non-fatty alcoholic liver disease (Andersen, *et al* 2009, Friedrich-Rust, *et al* 2008, Manning and Afdhal 2008). It uses a mechanical wave pulsed through the liver, with the rebounding velocity measured by ultrasound and converted to a stiffness estimate. The results of liver stiffness are acquired from at least ten valid measurements with a success rate of at least 60% within the interquartile range of ≤30%, to be considered acceptable. In patients with hepatitis C, compared to liver histology, a TE cut-off value of 8.7kPa allowed correct diagnosis of those with significant fibrosis (>F2) with an area under the receiver-operating characteristic curve (AUROC) of 0.79, and a cut-off value of 14.5kPa with an AUROC of 0.97, identified liver cirrhosis (Ziol, *et al* 2005). One study found TE to be reliable for estimating liver fibrosis in thalassemia major, a secondary iron overload disease (Di Marco, *et al* 2010, Fraquelli, *et al* 2010, Ziol, *et al* 2005). This study found that TE values were highest in individuals with thalassemia major who had serum ferritin (SF) >1000µg/L, suggesting that there is a relationship between iron overload and TE values (Fraquelli, *et al* 2010). Similarly, a strong relationship was found between TE and both SF and liver iron concentration in individuals with thalassemia major without hepatitis C, which was a confounding factor in liver stiffness measurements (Perifanis,

*et al* 2008, Sinakos*, et al* 2010). In contrast, no correlation was found between SF and TE scores in another thalassemia study (Di Marco*, et al* 2010).

There are a number of non-invasive tests of liver fibrosis based on serum analytes such as Hepascore, aspartate aminotransferase (AST) to platelet ratio index (APRI) and Fibrosis-4 (FIB-4). Hepascore is derived from an algorithm based on the age, sex, serum bilirubin, gamma-glutamyl transferase (GGT), hyaluronic acid and α-2-macroglobulin (Adams*, et al* 2005a). Hepascore provides values between 0 and 1, with a higher score being associated with a more severe liver disease. In hepatitis C, Hepascore demonstrated an AUROC of 0.8 for predicting significant fibrosis (cut-off 0.5) and an AUROC of 0.90 for cirrhosis (cut-off 0.75). Hepascore >0.5 has a specificity of 70% - 89% and sensitivity of 63% - 77% for significant fibrosis (Adams*, et al* 2005a, Guechot*, et al* 2010). Hepascore <0.25 can exclude significant fibrosis with a negative predictive value of 0.9 (Guechot*, et al* 2010).

The APRI index is a simple, non-expensive test to predict significant fibrosis and cirrhosis in hepatitis C (Wai*, et al* 2003). A threshold of 0.7 predicts significant fibrosis with 77% sensitivity and 72% specificity, while a threshold of 1.0 predicts severe fibrosis with 61% sensitivity and 64% specificity, and also predicts cirrhosis, with 76% sensitivity and 72% specificity (Lin*, et al* 2011). Similarly, the FIB-4 test is an inexpensive non-invasive test that combines platelet count, alanine aminotransferase (ALT), AST and age, and has a 94.7% negative predictive value with a sensitivity of 74.3%, to exclude

significant fibrosis if the index is <1.45. It also has a good positive predictive value of 82.1% with a specificity of 98.2% if the index is >3.25 to diagnose significant fibrosis, and an AUROC of 0.91 to identify cirrhosis. Overall, it is postulated that the FIB-4 index may reduce the need of liver biopsy by 70% (Vallet-Pichard*, et al* 2007).

Although these non-invasive tests of liver fibrosis had been thoroughly studied in liver diseases such as Hepatitis C, there are a lack of studies employing these tests in HH. In HH, Adhoute *et al*.(Adhoute*, et al* 2008) found a weak correlation between SF levels and TE scores, although univariate analysis did not confirm this association. The study did not include liver histology and most of the individuals with HH were treated and had normal SF at the time of administration of TE (Adhoute*, et al* 2008). Another study, which included liver histology, found TE scores were higher in those with more severe liver fibrosis and there was a positive correlation between SF and TE scores, indicating TE is reliable in detecting liver fibrosis in HH (Legros*, et al* 2015). Thus, whether iron overload has an impact on liver stiffness measurements remains unclear and further studies are required to assess the impact of iron overload on TE scores. Only one study has examined Hepascore in HH and it found no differences between the HH cohort and controls. However, most of the HH subjects were iron depleted with SF in the normal range (Adhoute*, et al* 2008).

To date, it is unclear if iron overload has an impact on the markers of liver fibrosis in HH. The aim of our study was to examine the relationship between

SF, used as a surrogate marker of iron load, and the non-invasive markers of liver fibrosis, TE, Hepascore, APRI score and FIB-4 index in HH.

**Methods**

From August 2012 to January 2018, individuals homozygous for *HFE* p.C282Y, 18 years of age or older, with a SF more than 300µg/L were recruited prospectively from Austin Health and Eastern Health in Victoria, the Royal Brisbane and Women's Hospital in Queensland and the Fiona-Stanley Hospital in Western Australia, through referrals from doctors, pathology companies that perform *HFE* genetic testing, and the Australian Red Cross Blood Service. All participants provided written informed consent. Individuals were excluded if they had significant alcohol intake (>60 g/day for males and >40 g/day for females), body mass index (BMI) of more than 35kg/m$^2$, were pregnant, or had known liver disease from another cause including hepatitis B or C or autoimmune liver disease. Clinical information including age, sex, BMI, the presence of diabetes mellitus and history of phlebotomy were recorded.

Blood was collected for measurement of SF, transferrin saturation (TS) and for parameters for measuring non-invasive markers of fibrosis including platelets, ALT, AST, GGT, bilirubin, prothrombin time, international normalised ratio (INR), alpha-2-macroglobulin and hyaluronic acid, and TE was performed on all participants. Non-invasive biomarker panel scores were calculated according to the following formulae:

- Hepascore: y/(1+y), where y = exp [(-4.185818 – 0.0249 x age + 0.7464 x sex (male = 1, female = 0) + 1.0039 x $\alpha$-2-macroglobulin (g/L)

+ 0.0302 x hyaluronic acid (μg/L) + 0.0691 x bilirubin (μmol/L) – 0.0012 x GGT (U/L)] (Adams, *et al* 2005a).

- APRI: [(AST (U/L) / upper limit of normal) x 100/ platelet count ($10^9$/L)] (Wai, *et al* 2003).
- FIB-4: [age (years) x AST (U/L)] / [platelet count ($10^9$/L) x ALT (U/L)$^{½}$] (Vallet-Pichard, *et al* 2007).

Retrospective data from 2013-2018 was obtained from the gastroenterology databases of Fiona-Stanley Hospital, Fremantle Hospital, Austin Health, and Eastern Health based on the same inclusion criteria. Only data from individuals who had at least one of TE or Hepascore and had elevated SF more than 300μg/L were included.

Human Research Ethics Committee approvals were obtained from Austin Health (HREC/15/Austin/56 and HREC/12/Austin/19), Eastern Health (LR81/2015), Metro North Hospital and Health Service (HREC/15/QRBW/502) and South Metropolitan Health Service (15-147).

Statistical Analysis

Simple linear regression was performed to assess the relationship between SF and each fibrosis marker, adjusted for site. SF was log-transformed for analysis, using Stata software (version 13.1).

**Results**

Between August 2012 and January 2018, 131 participants were recruited prospectively and data on 19 individuals were collected retrospectively. There

were 102 (68%) males and 48 (32%) females and the average age for females (49.29 ± 2.04) was slightly older than for males (42.84 ± 1.42, p=0.01). The average BMI for both sexes was similar (26.69 ± 0.38 kg/m$^2$ for males, and 26.09 ± 0.62 kg/m$^2$ for females, p=0.42). Twenty-five participants (16.7%) had a history of phlebotomy, with the number of treatments known for 20 participants (11.3 ± 2.44, 95% CI: 6.20, 16.40). Only one participant had a liver biopsy and none of the participants had diabetes mellitus (Table 1).

The mean SF of the cohort was 753.87 ± 71.24 µg/L (95% CI: 613.09, 894.64) and the mean TS was 65.32 ± 1.56 % (95% CI: 62.24, 68.40). Twenty-six participants had SF >1000 µg/L, with a mean SF of 1855.73 ± 331.30 µg/L (95% CI: 1173.41, 2538.05) in that group. The means of the biochemical parameters measured for the entire cohort, including platelets, ALT, AST, GGT and INR were within normal range (Table 1). Overall, the cohort had values of non-invasive fibrosis markers (TE, Hepascore, APRI and FIB-4) within the normal range.

Based on the results of the non-invasive markers, the majority of the participants did not have liver fibrosis. Only a small number of participants were suspected to have significant fibrosis or liver cirrhosis (Table 2). The mean SF was higher in those whose non-invasive tests suggested the presence of significant fibrosis or liver cirrhosis (Table 2). When adjusted for site, the mean SF correlated with Hepascore (estimated regression coefficient= 0.13, p <0.01), APRI (0.08, p <0.01) and FIB-4 (0.22, p<0.01) (Figure 1). Although not reaching statistical significance (estimated regression

coefficient= 0.54, p=0.053), there was a trend for SF to correlate with TE scores (Figure 1).

## Discussion

It is desirable to assess the stage of liver fibrosis in haemochromatosis for prognosis and in monitoring disease progression using non-invasive methods, as liver biopsies are not always the most suitable modality for monitoring due to their invasive nature and associated risks. As liver biopsies are becoming infrequently performed in HH since genetic testing allows confident diagnosis for most, understanding the relationship between SF and non-invasive modalities for assessment of hepatic fibrosis and liver damage for both progression and prognosis is desirable for use in clinical practice. SF not only reflects body iron stores but has also been shown to be a proinflammatory and profibrogenic factor in the liver, representing a stronger predictor of liver fibrosis when compared to hepatic iron concentration (Wood*, et al* 2017). Our results showed that the majority of individuals with haemochromatosis from our cohort did not have evidence of significant liver fibrosis based on the results of non-invasive markers and most had SF <1000µg/L. Our results support multiple studies that found that those who have a SF <1000µg/L are unlikely to have severe liver fibrosis, and thus liver biopsy is not indicated (Legros*, et al* 2015).

Our study found weak, but significant, positive correlations between SF and the hepatic fibrosis biomarker panels Hepascore, APRI and FIB-4. This supports the utility of SF as a predictor of hepatic fibrosis in HH as a previous

study has demonstrated (Wood, *et al* 2017). Biomarker panel scores were significantly higher in individuals with elevated SF, demonstrating a higher likelihood of significant liver fibrosis and cirrhosis with increased SF levels, which also reflects body iron stores in HH. There was a trend to statistical significance when TE was correlated with SF. TE has been shown to be a reliable tool for assessing risk of liver fibrosis in many studies, including in iron overload diseases such as thalassemia (Fraquelli, *et al* 2010, Sinakos, *et al* 2010). One of the participants had an SF of 9588 µg/L with a TE of 2.8kPa. Since it is unlikely for *HFE*-induced iron overload to cause such a high ferritin level, it is possible that this participant had other causes for the very high SF other than iron overload such as non-alcoholic steatohepatitis due to a high BMI of 30kg/m$^2$ and concurrent alcohol consumption, and thus had normal TE testing. Sub-analysis excluding this outlier found a significant association of TE with SF >1000µg/L (estimated regression coefficient 0.80, p= 0.007), consistent with the results reported in Legros et al. (Legros, *et al* 2015).

Liver histology, although regarded as the "gold standard" for assessment of liver fibrosis, has its limitations due to the potential for sampling error and non-uniform deposition of iron. Biochemical markers such as APRI, FIB-4, Hepascore and TE may have advantages for assessing liver fibrosis in haemochromatosis, as they are not subjected to the limitations of liver biopsies and provide a more global assessment. Moreover, they have been validated in liver diseases such as hepatitis C as dependable markers to assess liver fibrosis.

Whilst the duration of exposure to increased iron levels had been shown to be an important factor in the development of hepatic fibrosis (Olynyk*, et al* 2005), Powell, Dixon, Ramm et al. (Powell*, et al* 2006) demonstrated that hepatic iron concentration and SF were significantly associated with the presence of liver fibrosis and cirrhosis. SF was also shown to be a better predictor of fibrosis, independent of hepatic iron, age, steatosis and alcohol (Wood*, et al* 2017). Powell, Dixon, Ramm et al. (Powell*, et al* 2006) also reported improvement in liver fibrosis after normalization of body iron, through repeated liver biopsies. However, in current practice, repeat liver biopsies are rarely justified given their risks and the availability of various non-invasive methods to monitor liver injury. Our findings are consistent with non-invasive modalities including Hepascore, APRI, FIB-4 and TE providing useful information in the assessment of liver fibrosis both at initial diagnosis and for monitoring, especially in those who have initial elevated SF and indications of advanced liver fibrosis or cirrhosis. Future studies to include assessment of liver fibrosis using non-invasive methods before and after treatment of those with SF >1000µg /L would be of value.

In conclusion, our results indicate that there is a positive relationship between non-invasive markers and SF, a predictor of liver fibrosis. Although TE only showed a trend to significance with SF, it is currently widely used in clinical practice due to its availability and strong data in other liver diseases. A combination of TE with other non-invasive markers such as Hepascore, APRI and FIB-4 may be useful to assess and monitor liver fibrosis or cirrhosis in individuals with HH.

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SYO wrote the manuscript and SYO, AJN and MBD designed the study. The study was analysed by SYO and LCG. SYO, TW, PP, LER, JKO, LWP, MBD recruited and collected data for the study. All authors critically reviewed the manuscript and approved the submitted version. The authors thank Dr Danny Con for his advice on the results. This study was supported by the Victorian Government's Operational Infrastructure Support Program.

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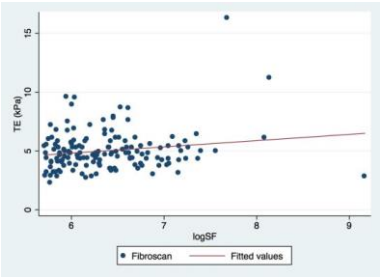
**Table 1:** Characteristics and non-invasive biomarker panel scores of participants. Data are expressed as mean ± standard error (95% confidence interval).

| Characteristic | n | Parameter |
|---|---|---|
| Sex | | |
| Male : Female | 102: 48 | 68% : 32% |
| Mean Age (years) | | |
| Male : Female | 102: 48 | 42.84 ± 1.42 : 49.29 ± 2.04 |
| BMI (kg/m$^2$) | | |
| Male : Female | 99 : 46 | 26.69 ± 0.38 : 26.09 ± 0.62 (40.02, 45.67) : (24.84, 27.34) |
| Received phlebotomy | 25 | 16.7% |
| No. of phlebotomies | 20 | 11.3 ± 2.44 (6.20, 16.40) |
| **Biochemical Parameter** | | |
| Serum ferritin (µg/L) | 150 | 753.87 ± 71.24 (613.09, 894.64) |
| Serum ferritin > 1000 µg/L | 26 (17.3%) | 1855.73 ± 331.30 (1173.41, 2538.05) |
| Transferrin saturation (%) | 150 | 65.32 ± 1.56 (62.24, 68.40) |
| Platelets (x10$^9$/L) | 144 | 232.76 ± 4.22 (224.42, 241.10) |
| ALT (U/L) | 150 | 35.33 ± 2.03 (31.31, 39.34) |
| AST (U/L) | 136 | 26.52 ± 1.08 (24.38, 28.66) |
| GGT (U/L) | 150 | 31.27 ± 3.90 (23.55, 38.98) |
| INR | 133 | 1.0± 0.01 (0.98, 1.01) |
| **Non-invasive fibrosis markers** | | |
| Transient elastography (kPa) | 148 | 5.03 ± 0.14 (4.74, 5.31) |
| Hepascore | 133 | 0.23 ± 0.01 (0.20, 0.26) |
| APRI | 134 | 0.33 ± 0.01 (0.30, 0.35) |
| FIB-4 | 134 | 0.92 ± 0.04 (0.84, 1.00) |

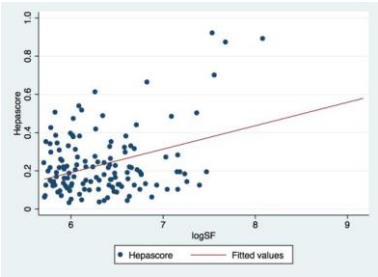
**Table 2:** Mean scores of non-invasive liver fibrosis markers, mean SF and relationship between SF and liver fibrosis markers using linear regression, adjusted for site. Data are presented with standard errors and 95% confidence intervals.

| | n | Mean score | Mean SF (µg/L) | Estimated regression coefficient (SE), p-value* |
|---|---|---|---|---|
| **Transient Elastography (kPa)** | | | | |
| < 8.7<br>No fibrosis | 142 | 4.79 ± 0.10<br>(4.58, 4.99) | 717.89 ± 71.19<br>(577.15, 858.62) | 0.54 (0.28),<br>p = 0.053 |
| ≥8.7 and < 14.5<br>Advanced fibrosis | 5 | 9.58 ± 0.44<br>(8.36, 10.80) | 1064 ± 591.64<br>(-578.65, 2706.65) | |
| ≥14.5<br>Cirrhosis | 1 | 16.3 | 2170 | |
| **Hepascore** | | | | |
| < 0.5<br>Minimal/ absent fibrosis | 123 | 0.20 ± 0.01<br>(0.18, 0.22) | 602.02 ± 26.89<br>(548.80, 655.25) | 0.13 (0.03),<br>p < 0.01 |
| 0.5- 0.84<br>Significant fibrosis | 7 | 0.57 ± 0.03<br>(0.50, 0.65) | 886.43 ± 238.18<br>(303.63, 1469.23) | |
| ≥ 0.85<br>Cirrhosis | 3 | 0.89 ± 0.01<br>(0.83, 0.96) | 2436.67 ± 420.09<br>(629.15, 4244.18) | |
| **APRI** | | | | |
| ≤ 0.7<br>No fibrosis | 130 | 0.31 ± 0.01<br>(0.29, 0.33) | 690.8 ± 44.22<br>(603.31, 778.30) | 0.08 (0.03),<br>p < 0.01 |
| 0.7- 1.0<br>Significant fibrosis | 2 | 0.76 ± 0.05<br>(0.10, 1.43) | 605.0 ± 52.0<br>(-55.72, 1265.72) | |
| >1.0<br>Severe fibrosis/ cirrhosis | 2 | 1.14 ± 0.10<br>(-0.08, 2.36) | 798.0 ± 63.0<br>(-2.49, 1598.50) | |
| **FIB-4** | | | | |
| <1.45<br>Absence of significant fibrosis | 120 | 0.81 ± 0.03<br>(0.75, 0.86) | 638.04 ± 37.28<br>(564.22, 711.86) | 0.22 (0.08),<br>p < 0.01 |
| 1.45- 3.25<br>Inconclusive | 13 | 1.72 ± 0.07<br>(1.57, 1.86) | 1159.31 ± 249.79<br>(615.06, 1703.56) | |
| >3.25<br>Advanced fibrosis/ cirrhosis | 1 | 3.69 | 974 | |

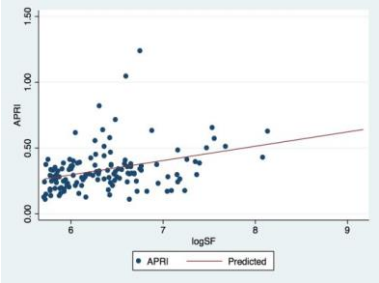
**Figure 1:** Correlation of non-invasive liver fibrosis markers with Serum Ferritin (SF). (a) Transient Elastography (TE); (b) Hepascore; (c) Aspartate aminotransferase to platelet ratio (APRI); (d) Fibrosis-4 (FIB-4).



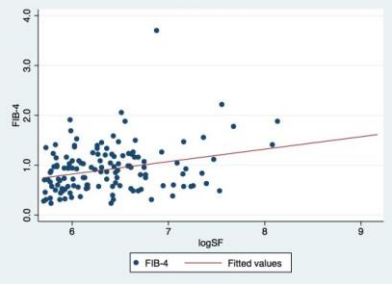

**A) TE**




**B) HEPASCORE**




**C) APRI**




**D) FIB-4**

# Chapter 4: The incidence of first metacarpophalangeal joint arthropathy is increased in HFE p.C282Y homozygotes

## 4.1 Introduction and Summary

Haemochromatosis arthropathy (HA) is a progressive disabling disease, present in up to 80% individuals with haemochromatosis, and often precedes the diagnosis of HH. The exact mechanism of the development of HA is unknown and the association with SF and TS is uncertain (Allen, Gurrin et al. 2008, Valenti, Fracanzani et al. 2008, Carroll, Breidahl et al. 2011). In addition, the impact of different genotypes on HA is unclear. Currently, HA is managed symptomatically with analgesia, as removing excess iron is not effective (Carroll, Breidahl et al. 2012). Early diagnosis will lead to institution of therapy to prevent the progression of HH. This study aimed to examine the incidence of MCP abnormalities with different genotypes, defined by the presence of bony spurs, effusion or joint tenderness.

Results from this study revealed that there was an increased incidence of HA involving the first MCP joint in p.C282Y homozygotes compared to other genotypes. HA involving the first MCP had a similar incidence to HA affecting the commonly described second and third MCP joints. This finding will increase the awareness of clinicians to identify HA, and possibly consider the diagnosis of HH earlier. In addition, having the p.H63D substitution may confer a risk to developing arthropathy. The study did not identify a relationship between the development of MCP abnormalities with TS >50%, after a follow-up period of 12 years, reinforcing the conclusion that development of HA is independent of iron indices.

## 4.2 PDF of Submitted Manuscript




**Incidence of first metacarpophalangeal joint arthropathy is increased in HFE p.C282Y homozygotes**

| | |
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| Keywords: | arthropathy, hemochromatosis, Metacarpophalangeal joints, Arthritis |
| | |

**Title**: Incidence of first metacarpophalangeal joint arthropathy is increased in *HFE* p.C282Y homozygotes

**Short title**: Arthropathy and *HFE* genotypes

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**Abbreviations:**

HA: Hemochromatosis arthropathy

*HFE*: Homeostatic iron regulator

HH: Hereditary hemochromatosis

MCP: Metacarpophalangeal

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SYO drafted the manuscript and performed the statistical analysis under the guidance of LCG. Revision of the manuscript was performed by AJN, LCG, JLH, GJA, JKO and MBD. MBD, KJA and AJN designed the study and CEM, DRE, JLH, GGG, GJA, JKO and LP were involved in developing the study. MBD and KJA secured the funding from NHMRC for HealthIron. AJN, KJA and MBD performed the clinical examinations. NAB and LCG performed data entry and organization.

Word count: 2057

**Abstract**

**Objectives**

Arthropathy affects up to 80% of people with *HFE*-related hereditary hemochromatosis. Arthropathy affecting the second and third metacarpophalangeal (MCP) joints, are hallmark features of HH. The relationship between arthropathy and common *HFE* genotypes is unclear. This study aimed to investigate the association between different *HFE* genotypes and hand arthropathy and to identify the most commonly affected hand joints in HH.

**Methods**

From the Melbourne Collaborative Cohort Study, 1438 participants were invited to the HealthIron study of the disease burden of HH, consisting of all *HFE* p.C282Y homozygotes and a stratified random sample of other genotypes. 818/1438 participants underwent a physical examination, completed questionnaires and provided a blood sample. Clinicians blinded to the genotype examined the MCP joints of both hands for bony spurs, effusions and tenderness. Statistical analysis using logistic regression models, adjusted for gender and menopause status, examined the association between different *HFE* genotypes and the presence of any features of arthropathy in either hand.

**Results**

The presence of any abnormalities in either hand was increased for the first MCP joint of p.C282Y homozygotes when compared with wild-type and other *HFE* genotypes (OR 1.31 (95%CI: 0.74, 2.30)), as was also the case for second and third MCP joints

(OR 1.41, 95%CI: 0.83, 2.40; OR 1.34, 95%CI: 0.79, 2.28). p.H63D heterozygotes also exhibited increased rates of second and third MCP joint changes when compared with wild-type genotypes.

**Conclusions**

*HFE* p.C282Y homozygotes have increased arthropathy of the first MCP joints. p.H63D heterozygotes might also have a predisposition to the development of arthropathy.

**Keywords**:

Arthropathy; Hemochromatosis; Metacarpophalangeal joints.

**Word count: 2041**

**Introduction**

*HFE*-associated hereditary hemochromatosis is an autosomal recessive disorder that affects approximately 1 in 200 people of Northern European descent (1-3), characterized by iron accumulation in different organs including the liver, pancreas, heart, skin and joints, causing a significant impact on physical health and quality of life (4, 5).

Hemochromatosis arthropathy (HA), a chronic progressive arthropathy, has a variable frequency of 24-81%, mainly in *HFE* p.C282Y homozygotes and p.C282Y/p.H63D compound heterozygotes (6, 7). The most common feature is joint pain (5, 8). Symptoms of HA may *precede* the diagnosis of HH (8, 9). The second and third metacarpophalangeal (MCP) joints of the hand and other larger joints such as the hip, elbow and knee joints are often involved (10, 11).

Although the radiological features of HA (subchondral sclerosis, joint space narrowing, erosions and periarticular bone cyst formation) are similar to OA, the symmetrical involvement of particular joints; the presence of hook-like osteophytes on the radial side of the metacarpal heads; and chondrocalcinosis in the wrist and knee joints favour the diagnosis of OA (8). However, chondrocalcinosis is a late manifestation of the disease and is unlikely to help in early diagnosis of HA (6, 8, 9, 12).

The pathophysiology of HA remains unknown. It is considered to be a non-inflammatory condition; however, erosive and inflammatory joint changes have been reported by low field magnetic resonance imaging (13). Pathologic findings from

surgical specimens suggest that there is iron deposition in the synovium of the joints, and iron retention in infiltrating macrophages and neutrophils, resulting in progressive chondral resorption and joint destruction (11, 14). Iron depletion with venesection therapy does not reduce iron deposition in the synovium or joint damage, and does not improve symptoms, resulting in high rates of joint replacement surgeries (7, 9, 15-17).

The development of HA maybe associated with different factors. HA is more common in *HFE* p.C282Y homozygotes, p.C282Y/p.H63D compound heterozygotes, males, and increasing age (5, 8, 10). Unlike osteoarthritis, occupational and physical exertions are not known to be associated with the development of HA (7). The relationship between the development of HA and serum ferritin level is unclear, with conflicting results. Some authors have shown an association with high serum ferritin levels (>1000 µg/L), while others have found that HA development is independent of serum ferritin levels (5, 7, 18, 19). A recent study also concluded that persistently elevated transferrin saturation for more than six years is associated with worse joint symptoms, regardless of serum ferritin levels (20). Other than p.C282Y homozygotes and p.C282Y/p.H63D compound heterozygotes, it is possible that other genotypes may play a role in the development of HA. It has been shown that the risk of arthralgia, and the development of HA with the involvement of multiple joints are increased in p.H63D homozygotes and p.H63D heterozygotes, but this relationship is not observed in p.C282Y heterozygotes (21-24). The aim of this study was to identify the association of hand arthropathy in individuals with different *HFE* genotypes when compared to individuals with the wild type genotype.

**Methods**

Participants were enrolled through the Melbourne Collaborative Cohort Study, a prospective study examining the influence of diet and lifestyle factors on the development of common chronic diseases (25). In this study, 41,528 participants (age range of 40 – 69 years of age) were recruited between 1990 and 1994 through the electoral roll, advertisements and community announcements in local media. A blood sample at baseline was collected and aliquoted as blood spots on Guthrie cards and stored at room temperature, and 1 mL samples of buffy coats and plasma were stored in liquid nitrogen.

Samples from participants who reported being born in Ireland, United Kingdom, New Zealand and Australia were processed. Southern European participants enrolled in the cohort were excluded from this study due to a lower prevalence of *HFE* mutations in this population. The DNA samples were extracted from Guthrie cards or from frozen buffy coats and genotyped for the *HFE* p.C282Y and p.H63D substitutions. Subsequently, between 2004 and 2006, letters of invitation were sent to a sample of 1438 participants, which included all p.C282Y homozygotes (n=203) and a stratified random sample from each of the other five *HFE* genotype groups (p.C282Y/p.H63D compound heterozygote, p.C282Y heterozygotes, p.H63D homozygotes, p.H63D heterozygotes and wild type for p.C282Y and p.H63D). Consenting participants provided a cheekbrush DNA sample to confirm their genotype, and a blood sample for iron indices and liver enzyme levels, and completed a computer-assisted personal interview. The study physicians (AJN, KJA and MBD), who were blinded to the genotype of participants, examined the MCP joints for joint effusions, bony spurs and

tenderness for each digit. The presence of these features was recorded as binary data (present/absent) for each digit.

*Statistical Analysis*

The presence of any one feature of bony spur, effusion or tenderness for each of the five MCP joints on either hand was combined for analysis. Using logistic regression with gender and menopausal status as covariates, odds ratios were used to examine the association between *HFE* genotypes and clinical features of the MCP joints, with the cohort with the *HFE* wild-type genotype as the control. Logistic regression was also performed to identify the association between log-transformed serum ferritin, transferrin saturation and MCP abnormalities. Analysis was performed by Stata 13.1 (Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).



**Results**

Of the 1438 participants, 619 did not have a physical examination and 148 participants did not have genotype information available. Complete data were available for 818 participants. This included 109 p.C282Y homozygotes; 144 p.C282Y/p.H63D compound heterozygotes; 12 p.H63D homozygotes; 197 p.C282Y heterozygotes; 96 p.H63D heterozygotes and 260 wild-types (Table 1). The low number of p.H63D homozygotes is attributable to the study design that did not stratify on *HFE* genotype for those without p.C282Y substitutions. This group is therefore not considered further in this paper. Eighty-seven of the 109 p.C282Y homozygotes had baseline and follow-up iron indices recorded. The mean baseline serum ferritin and transferrin saturation was 770.2µg/L ± 125.5 (520.6, 1019.8) and 62.3% ± 2.8 (56.8, 67.8), respectively. The follow-up serum ferritin was 378.7µg/L ± 40.9 (297.3, 460.1)

and transferrin saturation was 56.3% ± 2.3 (51.8, 60.9). Of the 87 p.C282Y homozygotes, 37 had abnormalities in the MCP joints when examined at follow-up, and 26/37 had transferrin saturation of more than 50%. There was no significant relationship between MCP abnormalities and baseline serum ferritin and transferrin saturation (p=0.09; p=0.44) and no relationship was found in those who had transferrin saturation more than 50% at follow-up (p=0.09).

The presence of any one feature of bony spur, effusion or tenderness in either hand of the first MCP joint had a higher odds ratio [1.31 ± 0.38 (0.74, 2.30)] in p.C282Y homozygotes when compared to controls and other *HFE* genotypes (Table 2). Twenty-two percent of p.C282Y homozygotes had features of HA arthropathy in the first MCP joint compared to 17% of wild types.

In the second and third MCP joints, features of HA arthropathy were seen at similar frequencies (26-27%) in p.C282Y homozygotes, p.C282Y/p.H63D compound heterozygotes and p.H63D heterozygotes (Table 2). The odds ratios of the presence of abnormal features in the second and third MCP joints in these three genotype groups were consistently increased when compared to the wild-type controls and p.C282Y heterozygotes (Table 2).

Only a small number of participants (n=1 to 7) had abnormalities in either the fourth or fifth MCP joints in one or both hands (Table 2).

**Discussion**

This study is the first to analyze blinded examinations of hand MCP joints in individuals with different *HFE* genotypes and to compare them to wild type controls. We clearly demonstrate the novel finding of increased incidence of arthropathy of the first MCP in p.C282Y homozygotes, and confirm an earlier observation of arthropathy in individuals with one or two copies of the p.H63D substitution.

The strengths of the study include the prospective design and recruitment of participants from the community, and the blinding of the examiners to the participants' genotype and serum ferritin. The study had a good representation of the general Caucasian population in Victoria and included different genotypes with a control (wild type for *HFE*) group for comparison.

It is widely accepted that clinical and radiological abnormalities are observed in the second and third MCP joints in *HFE* p.C282Y homozygotes ([6], [9], [18]). Our study confirms these earlier observations, and extends this knowledge by demonstrating an increased frequency of abnormalities in the first MCP joint in p.C282Y homozygotes. The frequency of first MCP joint arthropathy (22.02%) was similar to the more commonly reported second and third MCP joint involvement (26.61%). It is possible that the presence of bony spurs, joint effusions or tenderness in the first MCP joint may be an indicator of HH arthropathy, especially in p.C282Y homozygotes. The fourth and fifth MCP joints were the least affected in all genotype groups.

There was also an increased rate of arthropathy in the second and third MCP joints in p.H63D heterozygotes, similar to the rate in p.C282Y homozygotes and

p.C282Y/p.H63D compound heterozygotes. We were unable to explore the corresponding risk in p.H63D homozygotes due to the small sample size in our study. However, a previous study found that arthralgia was significantly increased in both p.H63D homozygotes and heterozygotes at an early age, with an increase in osteophytes in multiple joints (23). An increased incidence of osteoarthritis of the ankle joints has also been associated with the p.H63D substitution, although the p.H63D sample size in that study was small (24). The mechanism by which the presence of the p.H63D mutation increases the risk of developing hand arthropathy compared to the general population remains unclear as this mutation confers only a very low risk of developing iron overload-related disease.

Arthropathy has previously been reported to be unrelated to serum ferritin from this cohort (5) and sub-analysis did not detect a relationship between MCP abnormalities and those who had transferrin saturation of more than 50% at more than 12 years of follow-up. This is contrary to the findings of Bardou-Jacquet et al. (20), who found an association of worse self-reported joint symptoms if the p.C282Y homozygotes have transferrin saturation more than 50%, for more than 6 years. This difference may be explained by the different methods used to categorize abnormalities, whereby the joints were examined objectively in our study, compared to self-reported questionnaires of joint symptoms which may include arthralgia from different causes.

One of the limitations of our study was the lack of radiographic information to assess bony changes, and examination was limited to MCP joints. Radiological examination and clinical examination of joints other than the hand were beyond the scope of this study due to the large number of participants and limited resources.

Normalization of body iron does not appear to improve the symptoms of arthropathy, so awareness of this complication of HH may not have a direct clinical benefit until newer approaches based on an increased understanding of the joint disease emerge. However, identifying hand arthropathy may lead to early diagnosis of HH for some patients. Further studies with radiographic information on the joint changes are needed to confirm our findings.

In conclusion, this is the first study to demonstrate an increased risk of arthropathy in the first MCP joint in *HFE* p.C282Y homozygotes, in addition to the disease in the second and third MCP joints. It also revealed increased rates of arthropathy in the second and third MCP joints in p.H63D heterozygotes, p.C282Y homozygotes, and p.C282Y/p.H63D compound heterozygotes, suggesting the p.H63D substitution may also predispose to the development of arthropathy.



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Table 1. Number of participants and genotypes

| Genotype | Number |
|---|---|
| Wild type (CC) | 260 |
| p.C282Y heterozygote (CY) | 197 |
| p.H63D heterozygote (HD) | 96 |
| Compound heterozygote (p.C282Y/p.H63D) (YD) | 144 |
| p.H63D homozygote (DD) | 12 |
| p.C282Y homozygotes (YY) | 109 |

Table 2. Estimated odds ratio (95% confidence interval) of the presence of clinical features of arthropathy (bony spurs and/or effusion and/or tenderness) in the metacarpophalangeal (MCP) joints of either hand within different *HFE* genotypes when compared to wild type controls, adjusted for gender and menopause status.

| MCP Joint | Wild type n=260 | p.C282Y heterozygote n=197 | p.H63D heterozygote n=96 | p.C282Y/p.H63D compound heterozygote n=144 | p.C282Y homozygote n=109 |
|---|---|---|---|---|---|
| 1st MCP | - | 0.75 (0.44, 1.29) | 0.72 (0.37, 1.42) | 0.55 (0.29, 1.04) | 1.31 (0.74, 2.30) |
| n= | 45 (17.31%) | 25 (12.69%) | 13 (13.54%) | 15 (10.42%) | 24 (22.02%) |
| 2nd MCP | - | 0.84 (0.51, 1.38) | 1.39 (0.79, 2.43) | 1.53 (0.94, 2.49) | 1.41 (0.83, 2.40) |
| n= | 52 (20.0%) | 32 (16.24%) | 25 (26.04%) | 39 (27.08%) | 29 (26.61%) |
| 3rd MCP | - | 0.86 (0.53, 1.40) | 1.32 (0.76, 2.31) | 1.46 (0.90, 2.37) | 1.34 (0.79, 2.28) |
| n= | 54 (20.77%) | 34 (17.26%) | 25 (26.04%) | 39 (27.08%) | 29 (26.61%) |
| 4th MCP | - | 0.12 (0.02, 0.94) | 0.63 (0.17, 2.30) | 1.07 (0.41, 2.82) | 2.67 (1.15, 6.19) |
| n= | 12 (4.62%) | 1 (0.51%) | 3 (3.13%) | 7 (4.86%) | 3 (2.75%) |
| 5th MCP | - | 0.52 (0.13, 1.98) | 1.34 (0.39, 4.57) | 0.44 (0.09, 2.13) | 0.88 (0.23, 3.39) |
| n= | 8 (3.08%) | 3 (1.52%) | 4 (4.17%) | 2 (1.39%) | 3 (2.75%) |

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| n= | 8 (3.08%) | 3 (1.52%) | 4 (4.17%) | 2 (1.39%) | 3 (2.75%) |

# Chapter 5: Conclusions and Future directions

This PhD project aimed to investigate different areas in HH where the evidence had been deficient or inconclusive. This included addressing if treatment should be instituted for patients with moderately elevated serum ferritin (300 – 1000 µg/L), understanding the relationship between serum ferritin and non-invasive methods to assess liver fibrosis for use in clinical practice, as well as assessing the MCPs for haemochromatosis arthropathy.

## 5.1 Reduction of body iron in HFE-related haemochromatosis and moderate iron overload (Mi-Iron): a multicentre, participant-blinded, randomised controlled trial

The current guidelines suggest all patients with haemochromatosis and elevated iron should have treatment, although data on treatment for moderately elevated iron in haemochromatosis patients was lacking and no clinical trials had been conducted to answer this question.

Mi-Iron is the first randomised, patient-blinded controlled trial to assess the benefits of treatment in a cohort of HFE p.C282Y homozygotes with moderately elevated iron. The findings from this study identified an improvement in the primary outcome, fatigue, particularly in the cognitive component after removal of iron when compared to the control group. This finding is further supported by an improvement in the affect component of the arthritis score, suggesting there is an overall improvement of the mental wellbeing of these patients after removal of iron. These improvements are unlikely a placebo effect as the participants were blinded to their groups and blinding was found to be successful in the trial.

Fatigue and arthritis are common problems in the community. However, there is no data on MFIS and AIMS2-SF in the normal population to serve as a baseline. Therefore, it may be of interest to assess the baseline MFIS and AIMS2-SF of the

general population and compare the effects of phlebotomy to the haemochromatosis cohort, although such a trial may be difficult to conduct.

Apart from improvements in the patient-reported outcomes, the study also found biochemical evidence of improvement in the liver fibrosis marker, Hepascore and oxidative stress marker, plasma F2-isoprostanes, in the treatment group. Although some of the outcomes in the treatment group did not reach statistical significance when compared to the control group, there was still an overall improvement. A study with higher power may be able to elicit a more obvious difference. Seventy percent of the participants were apparently asymptomatic, diagnosed either through family history or routine blood testing. The results demonstrated that treatment is beneficial, even in those who are asymptomatic.

The study was a short-term study. It would be of interest to engage in a long-term follow-up study to further assess these outcomes and to assess if there are any changes in liver fibrosis status.

The target end point of treatment remains controversial. The current guidelines recommend a target SF of 50 μg/L. The study reduced SF in the treatment group to the normal range of less than 300 μg/L and was able to show improvements in MFIS, AIMS2-SF, Hepascore and plasma F2-isoprostanes. Whether there are further benefits to decrease SF to a lower limit remains questionable as lower SF may have the risk of anaemia and fatigue. Further studies with different target end points are recommended to determine the target SF.

In summary, the study strongly suggests that there are benefits in returning the total body iron to normal levels in haemochromatosis patients, including those with moderate iron overload and provide evidence for the current guidelines that recommend iron normalization in all individuals with iron overload.

## 5.2 The relationship between serum ferritin and non-invasive markers of liver fibrosis in HFE-haemochromatosis

There has been an increased use of non-invasive markers of liver fibrosis in other liver diseases such as Hepatitis C, and there is a need to assess these markers in haemochromatosis, for prognosis and disease monitoring, especially with liver biopsies being less commonly performed due to its invasive nature and associated risks.

This study examined the relationship between SF with TE, Hepascore, APRI and FIB-4. The majority of the cohort did not exhibit evidence of significant fibrosis as most of the participants had SF <1000 μg/L, a level known to have a low risk of iron-overload related complications. The availability of genetic testing means that early diagnosis and treatment is more common than in the past and thus progression to severe disease is less often seen. Our results support the recommendation that liver biopsies are not indicated in individuals with haemochromatosis and SF <1000 μg/L (Legros, Bardou-Jacquet et al. 2015).

SF is a marker of iron stores and had been found to be a good predictor of hepatic fibrosis progression in HH (Wood, Crawford et al. 2017). Our study found a positive correlation between SF and Hepascore, APRI and FIB-4, with a higher likelihood of significant liver fibrosis or cirrhosis with higher SF levels. Although the relationship between SF and TE did not reach statistical significance, there was a positive trend and TE has been shown to be reliable in HH with SF >1000 μg/L and other iron overload diseases such as β-thalassemia (Fraquelli, Cassinerio et al. 2010, Legros, Bardou-Jacquet et al. 2015). The limitations of this study include the lack of liver histology as the "gold standard" to assess liver fibrosis stage to compare to non-invasive markers. However, regional and assessment variability continues to be a concern for liver histology. Moreover, SF has been found to be a better predictor of liver fibrosis than hepatic iron concentration obtained from liver biopsies (Wood, Crawford et al. 2017). Legros et al. demonstrated that an algorithm that

incorporated TE and SF can reliably determine fibrosis in 61% in individuals with HH who have SF >1000 µg/L (Legros, Bardou-Jacquet et al. 2015). It would be of interest if future studies can confirm these findings and include other biochemical panels such as enhanced liver fibrosis biomarker and Forns index for assessment since TE may not be available in some facilities.

In summary, understanding the relationship between SF and different non-invasive liver fibrosis markers enables clinicians to utilise these tests more confidently in clinical practice for determining prognosis and disease monitoring in HH.

## 5.3 The incidence of first metacarpophalangeal joint arthropathy is increased in HFE p.C282Y homozygotes

This study found that there was an increased incidence of bony spurs, tenderness or effusion features in the first MCP joint of p.C282Y homozygotes, similar to the incidence seen in the second and third MCP joints, which are commonly described in HH. The study physicians who examined these features, were blinded to the genotypes and SF levels of subjects, providing an objective assessment. However, the lack of radiographic information may underestimate the number of asymptomatic individuals who had joint changes, as demonstrated by a study which showed 15% of asymptomatic patients had small erosions, joint space narrowing or osteophytes on MRI (Frenzen, Schafer et al. 2013). As the scope of the study was limited to MCP joints, abnormalities in other joints such as wrists, ankles, hips and knees were not examined, potentially affecting the assessment of HA. A study to include various joints to assess its association with different genotypes, including imaging may be difficult to conduct due to availability, costs and radiation exposure, depending on the type of imaging utilised.

The study also found that p.H63D may confer a risk to the development of HA as the study demonstrated that there was an increased rate of abnormalities in the second and third MCP joints in p.H63D heterozygotes. Similarly, this mutation had been

associated with joint abnormalities in other studies (Carroll 2006, Alizadeh, Njajou et al. 2007). However, there is a lack of understanding of how the p.H63D mutation may increase the risk of HA compared to the general population as this mutation has a very low risk of causing iron overload related disease. The current evidence remains controversial and requires a larger study of p.C282Y homozygotes and p.H63D homozygotes to assess HA.

A recent study reported an association of worse joint symptoms with the presence of TS >50% for more than 6 years (Bardou-Jacquet, Laine et al. 2017). Our study did not demonstrate the association between abnormal MCP joints and TS >50% in our 12 year follow-up period. The discrepancy may be due to more self-reported joint symptoms in the other study, including the possibility of symptoms due to non-haemochromatosis arthritis.

In summary, the new finding of increased abnormalities in the first MCP joint in HH provides a potential avenue for clinicians to detect HA allowing earlier diagnosis of HH enabling earlier treatment and prevention of complications such as liver cirrhosis.

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# Appendix

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Review Article

## How should hyperferritinaemia be investigated and managed?

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ABSTRACT

Hyperferritinaemia is commonly found in clinical practice. In assessing the cause of hyperferritinaemia, it is important to identify if there is true iron overload or not as hyperferritinaemia may be seen in other conditions such as excess alcohol intake, inflammation and non-alcoholic fatty liver disease. Assessment of whether the serum ferritin level is elevated or not should take into account body mass index, gender and age. This review article provides an overview of the different causes of hyperferritinaemia, differentiating those due to iron overload from those not due to iron overload, and provides an algorithm for clinicians to use in clinical practice to carry out appropriate investigations and management.



### 1. Introduction

Hyperferritinaemia is a common finding during a medical assessment and has a number of causes. It can be difficult to determine if the finding is due to iron overload or whether it is part of the acute phase response. It is important to perform appropriate investigations to assist in diagnosis and management. This review provides an approach to investigation and management of hyperferritinaemia and highlights the common clinical conditions causing this.

A comprehensive search for relevant articles was performed through PubMed until September 2015 with keywords related to hyperferritinaemia, haemochromatosis, non-alcoholic fatty liver disease, alcohol liver disease, phlebotomy and erythrocytapheresis.

*Abbreviations:* SF, serum ferritin; HH, hereditary haemochromatosis; NAFLD, non-alcoholic fatty liver disease; HCC, hepatocellular carcinoma; TS, transferrin saturation; HJV, hemojuvelin; NASH, non-alcoholic hepatic steatohepatitis; HHCS, hereditary hyperferritinaemia-cataract syndrome; PCT, porphyria cutanea tarda; UROD, uroporphyrinogen decarboxylase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ANA, anti-nuclear antibody; SMA, smooth muscle antibody; MRI, magnetic resonance imaging; TE, therapeutic erythrocytapheresis.

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### 2. Serum ferritin and hyperferritinaemia

Ferritin is an intracellular protein shell that contains about 4000 iron atoms. There is about 3–4 g of iron in a human adult and the majority is incorporated into haem that forms haemoglobin, which transports oxygen in red blood cells. Ferritin measured in the serum (SF) is a reflection of body iron stores such that the more iron present in the body, the higher SF tends to be.

A reference range is defined as a set of interval values which 95% of the target population falls into [1]. Conventionally, the reference range for SF is considered to be 30–300 μg/L for men and postmenopausal women and 15–200 μg/L for premenopausal women, although different laboratories have different reference ranges [2]. Age, gender, menopausal status, weight and lifestyle factors, such as alcohol intake and smoking can influence SF [2–5].

An Australian population study compared the levels of SF between age-matched cohorts in 1995 and 2005 and demonstrated SF increased by 21% in men and 10% in women over time with a significant increase in the number of individuals with elevated SF above the conventional 300 μg/L [2]. This trend was similarly detected in an American and Canadian population study where 26% of men and 13% of women without *HFE* mutations had an SF above the conventional cut off of 300 μg/L and 200 μg/L respectively [6]. Thus, considering different age, gender and lifestyle factors is important when interpreting the normal reference range of SF, and to establish if there is true iron overload.

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## 3. What causes hyperferritinaemia?

The main causes of hyperferritinaemia are shown in Table 1. Iron overload, the most common cause of which is hereditary haemochromatosis (HH), is often assumed to be the cause of hyperferritinaemia. However, between 58%–70% of those referred for hyperferritinaemia do not have iron overload [7]. This is because SF is an acute phase reactant and can be elevated in conditions due to infective or inflammatory processes. The most common causes of non- iron overload hyperferritinaemia include non-alcoholic fatty liver disease (NAFLD) and alcohol ingestion [7,8]. In patients with inflammatory disorders such as sepsis, malignancy or autoimmunity, the SF may also be raised. Other rare causes of hyperferritinaemia include hereditary hyperferritinaemia-cataract syndrome, porphyria cutanea tarda and acaeruloplasminaemia.

## 4. Hyperferritinaemia due to iron overload

### 4.1. Primary iron overload

#### 4.1.1. Hereditary haemochromatosis

HH refers to a group of inherited conditions of iron dysregulation characterised by excessive iron absorption. As the human body has no means of excreting iron, in HH, total body accumulation occurs, with subsequent damage to a wide range of organs. There are four types of HH (Table 1) with type 1 being by far the most common.

*4.1.1.1. Type 1 HH.* Type 1 HH is due to mutations in *HFE* on chromosome 6 and is the most common cause of HH. There are two *HFE* mutations that account for almost all type 1 HH, c.845G → A and c.187C → G that result in HFE p.C282Y and p.H63D respectively. p.C282Y homozygosity represents the most common genotype in individuals with HH and is found in 60–95% of affected individuals [6,9–11]. The disease is most common in people of northern European ancestry with the incidence of p.C282Y homozygosity ranging from 1 in 80 to 1 in 200 [6,12,13]. Compound heterozygosity for p.C282Y and p.H63D accounts for ~2% of HH and studies have demonstrated that p.C282Y homozygotes have significantly higher SF and transferrin saturation (TS) compared to other *HFE* genotypes (p.C282Y/p.H63D, p.H63D/p.H63D) [6,14]. Homozygosity for c.187C → G (p.H63D) does not cause clinically significant iron overload and is not a cause of haemochromatosis.

Untreated, type 1 HH can result in serious complications including hepatic cirrhosis, hepatocellular carcinoma (HCC), cardiomyopathy, diabetes mellitus and hypogonadotropic hypogonadism. In addition, symptoms including arthritis and fatigue can have a significant impact on the quality of life of affected individuals. A longitudinal population study found that a minimum of 28.4% of male and 1.2% of female p.C282Y homozygotes had iron overload related diseases [14]. A far lower proportion of those compound heterozygous (p.C282Y/p.H63D) have evidence of iron overload disease compared to those who are p.C282Y homozygous [6,14–16].

Severe disease including hepatic cirrhosis and HCC is almost exclusively seen in those with a SF > 1000 μg/L [14,17–19], and those who develop SF > 1000 μg/L will generally have done so by 55 years of age [20]. However, the majority of p.C282Y homozygotes have SF < 1000 μg/L [6,14] and not everyone with type 1 HH with SF < 1000 μg/L develops symptoms of iron overload [14,20–25]. To date, there are three published longitudinal studies with a range of follow up period from 12 to 25 years comprising a total of 105 p.C282Y homozygotes [14,22,23]. Andersen and colleagues found that the mean SF and TS only increased slightly in their cohort of p.C282Y homozygotes over a follow up period of up to 25 years [22]. Olynyk and colleagues found that among ten p.C282Y homozygotes, only four had increased SF over 17 years whilst the other six had stable or decreased SF [23]. Subsequently, Gurrin and colleagues predicted that there is a 25% chance of developing SF > 1000 μg/L in males and 18% chance in females over 12 years in those with baseline SF < 1000 μg/L and those with normal SF at baseline has less than 15% chance of developing SF > 1000 μg/L over 10 to 15 years [20].

While there is strong evidence that individuals with HFE-related HH and SF ≥ 1000 μg/L should be treated to prevent complications [14,17–19], there is a lack of evidence about the optimal management for p.C282Y homozygotes with SF < 1000 μg/L. Thus, further research is required to provide evidence for the management of this group.

*4.1.1.2. Type 2 HH.* Type 2 HH or juvenile haemochromatosis is a rare autosomal recessive disease that has an onset of symptoms between the first to third decades of life, and affects males and females equally. The predominant features are hypogonadotropic hypogonadism and cardiomyopathy, with cardiac involvement being the most common cause of death [26]. Other manifestations include liver fibrosis or cirrhosis, diabetes mellitus and arthropathy [27,28]. In Type 2 HH, the SF concentration is usually > 1000 μg/L and TS close to 100%. Mutations in two genes are known to be responsible for type 2 HH. Hemojuvelin (*HJV*) mutations account for more than 90% of type 2 HH, while mutations in *HAMP*, the gene that encodes hepcidin, accounts for the remainder [29].

*4.1.1.3. Type 3 HH.* Type 3 HH is a rare autosomal recessive condition caused by mutations in *TFR2* that maps to chromosome 7q22. It was first described in 1999 by Camaschella and colleagues in two Sicilian families [30]. The clinical presentations are similar to type 1 HH and onset occurs in adulthood in most individuals although earlier onset has been described [31,32].

*4.1.1.4. Type 4 HH.* Type 4 HH, also known as the ferroportin disease, is an autosomal dominant condition, and is due to mutations in *SLC40A1* on chromosome 2. *SLC40A1* encodes ferroportin, which is a transmembrane iron transporter that mediates iron export out of enterocytes [33]. Similar to HFE, ferroportin is regulated by hepcidin [34]. Most mutations lead to loss of protein function, therefore reducing iron export from cells particularly from reticuloendothelial macrophages. Consequently, iron accumulation occurs resulting in high SF and decreased availability of iron for transferrin, reflected in low or normal TS. This form of Type 4 HH is described as "classical ferroportin disease" where SF can be extremely high (> 10,000 μg/L). Mild anaemia is often present and hence there may be decreased tolerance to therapeutic phlebotomy [35]. The less common "non-classical ferroportin disease" is associated

**Table 1**
Major causes of hyperferritinaemia.

| Iron overload |
|---|
| Primary iron overload |
| • Type 1 haemochromatosis: autosomal recessive. Gene: *HFE* |
| • Type 2 haemochromatosis: autosomal recessive. Genes: *HJV*; *HAMP* |
| • Type 3 haemochromatosis: autosomal recessive. Gene: *TFR2* |
| • Type 4 haemochromatosis: autosomal dominant. Gene: *SLC40A1* (Ferroportin disease) |
| • Acaeruloplasminaemia. Gene: *CP* (caeruloplasmin) |
| Secondary iron overload |
| • Multiple blood transfusions |
| • Excess parenteral iron administration |
| **Inflammatory and immunological causes** |
| NAFLD and/or obesity |
| Alcohol |
| Infections |
| Systemic inflammation including autoimmune and rheumatological conditions |
| Malignancy |
| Hepatic failure |
| **Others** |
| Hereditary hyperferritinaemia-cataract syndrome |
| Porphyria cutanea tarda |

with hepcidin resistant ferroportin and affected individuals present with features similar to type 1 HH with raised SF and TS [36].

#### 4.1.2. Acaeruloplasminaemia

Acaeruloplasminaemia is a rare autosomal recessive disease that typically presents in adulthood with various neurological symptoms including involuntary movements and ataxia, diabetes mellitus and retinal degeneration. Due to the homozygous/compound heterozygous mutations in *CP*, the gene that encodes caeruloplasmin, there is an absence of caeruloplasmin ferroxidase activity, affecting iron transport and processing, and subsequently results in iron accumulation in various organs, including the brain, liver and pancreas. SF is usually very high and is accompanied by absent or low serum caeruloplasmin and copper levels [37,38].

### 4.2. Secondary iron overload

Several haematological disorders cause ineffective erythropoiesis requiring multiple blood transfusions. The most common of these is β-thalassaemia major and intermedia [39]. These autosomal recessive conditions cause abnormal haemoglobin formation, subsequent destruction of red blood cells and anaemia. Regular blood transfusions for anaemia result in secondary iron accumulation and an elevated SF.

## 5. Non-iron overload causes of hyperferritinaemia

### 5.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a major global health problem in populations with high rates of obesity and type 2 diabetes, and is now one of the most common causes of liver disease. It is estimated to affect 20–30% of the adult population in developed countries [40–42]. NAFLD is the hepatic manifestation of the metabolic syndrome, and consists of a wide spectrum of liver damage severity ranging from simple steatosis to non-alcoholic hepatic steatohepatitis (NASH), and may progress to liver cirrhosis. HCC can develop in individuals with NAFLD with or without cirrhosis [43].

Increased SF is found in 30% of individuals with NAFLD [44] and an Australian population study found a positive correlation between SF and body mass index (BMI) [2]. A BMI of ≥25 kg/m$^2$ was associated with higher levels of SF in men aged more than 35 years of age and post-menopausal women, when compared to those with a BMI < 25 kg/m$^2$ of similar age. The range of SF was 413 to 696 μg/L in men greater than 35 years of age with BMI ≥ 25 kg/m$^2$ and 249 μg/L to 422 μg/L in post-menopausal women with BMI of ≥25 kg/m$^2$ [2]. Comparatively, the range of SF was 350 to 511 μg/L in men more than 35 years of age with BMI < 25 kg/m$^2$ and 222 μg/L to 323 μg/L in post-menopausal women with BMI of <25 kg/m$^2$ [2].

There has been increasing evidence of an association between increased SF and increased glucose levels, hyperlipidaemia and insulin resistance in NAFLD in various populations [4,45–47]. Oxidative stress caused by iron has been proposed as the mechanism of the development of steatosis and fibrosis in NAFLD [48–52]. Studies have also examined SF as a biomarker to predict the development of NAFLD and NASH, and to predict advanced fibrosis [53–56]. In addition, high SF levels have also been shown to be associated with the presence of type 2 diabetes mellitus in the general population [57].

### 5.2. Alcohol

Alcohol consumption increases oxidative stress and iron stores secondary to down-regulation of hepcidin [58]. Excess alcohol consumption accounts for about 10–20% of hyperferritinaemia [7,8,58]. Liver injury from alcohol consumption ranges from simple steatosis, steatohepatitis, through to liver cirrhosis. Alcoholic liver disease is usually observed when consumption exceeds 60 g/day of alcohol but may occasionally be seen in individuals who consume less than this. Steatosis is usually reversible following 4–6 weeks of abstinence although progression to fibrosis with or without cirrhosis occurs in up to 15% despite abstinence [59,60].

Regular alcohol consumption can elevate SF up to 50% [61]. A cross sectional study showed alcohol consumption of one standard drink per day (10 g alcohol) is associated with 21% higher SF in women aged ≤49 years compared to women who abstain from alcohol consumption [2–4]. Thus, it is critical to ascertain the amount, frequency and pattern of alcohol intake in hyperferritinaemia. It is advisable to repeat measurement of SF following four weeks of abstinence if regular alcohol intake is occurring.

### 5.3. Systemic inflammation, infections and malignancy

SF is an acute phase reactant and is often elevated in systemic inflammation caused by autoimmune diseases, rheumatological diseases, infections and malignancy [62]. Proinflammatory cytokines, especially interleukin 6, stimulate the production of hepcidin resulting in internalisation and degradation of ferroportin and decrease in iron export and sequestration of iron in cells [63]. As a result, there is decreased availability of iron for erythropoiesis even though iron stores are paradoxically increased in cells, causing elevated SF and anaemia of inflammation [64]. This is also known as the anaemia of chronic disease.

### 5.4. Hepatic failure

Hepatic iron accumulation is commonly seen in the presence of hepatic cirrhosis. This tends to be most marked in those with cirrhosis due to excess alcohol consumption [65]. High SF in those with hepatic cirrhosis is a predictor of poor prognosis and early mortality in hepatic failure [66].

### 5.5. Hereditary hyperferritinaemia-cataract syndrome

Hereditary hyperferritinaemia-cataract syndrome (HHCS) is an autosomal dominant condition caused by mutations in the *FTL* gene that encodes the light chain of ferritin. These individuals have hyperferritinaemia with normal or low TS, and early onset of cataracts due to precipitation of ferritin in the lens [67]. The cataracts may be asymptomatic but can be diagnosed in children [68,69]. Some L-ferritin mutations can cause benign hyperferritinaemia without cataracts but with a very high glycosylation of SF, compared to low glycosylation of SF in HHCS [70,71]. There is no iron overload in HHCS.

### 5.6. Porphyria cutanea tarda

Porphyria cutanea tarda (PCT) is the most common form of porphyria. It affects the haem biosynthesis pathway. Eighty percent of PCT is sporadic, and is a result of inhibition of uroporphyrinogen decarboxylase (UROD) activity in the liver (type I PCT). In a minority, PCT is due to heterozygous mutation of *UROD* that encodes uroporphyrinogen decarboxylase (type II PCT) [72]. Bullous skin lesions and increased sensitivity to light are the classical features of PCT [73]. Hepatitis C infection is considered one of the most common associations with PCT and other triggers include excessive alcohol consumption, oestrogen use, or HIV infection [73,74]. Increased iron deposition in the liver due to reduced production of hepcidin plays a role in the development of PCT and thus, there is an increased risk of PCT in individuals with HH [75].

## 6. Approach to investigation of hyperferritinaemia

A systematic approach is required to determine the cause of hyperferritinaemia such as shown in Fig. 1. A critical question in evaluation of hyperferritinaemia is whether or not it is due to iron overload.

### 6.1. Clinical history/physical examination

Pertinent clinical details include existing or past medical problems, family history to delineate hereditary causes, use of iron supplements, alcohol consumption and blood transfusions. Physical examination should include measuring weight and height to calculate BMI and to check for the presence of bullous skin lesions.

### 6.2. Blood tests

TS is the ratio of serum iron to total iron binding capacity, and is useful to differentiate between iron overload and other causes of hyperferritinaemia. A TS > 45% for women and TS > 50% for men may suggest the presence of iron overload in the setting of raised SF [76]. However, a population study of 101,168 participants found that approximately 30% of p.C282Y homozygotes had normal TS even though 40% of them had raised SF levels [77]. Fasting TS is not necessarily more accurate than a non-fasting test [77]. It is important to note that a normal TS does not exclude iron overload. Individuals with ferroportin disease generally have a normal or low TS in the setting of iron overload and elevated SF [35,78,79]. Thus, TS is a useful clinical tool, but it is neither 100% specific or sensitive in differentiating between iron overload and non-iron overload causes of hyperferritinaemia.

Fasting glucose and lipid profile, the inflammatory markers C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) and *HFE* testing for p.C282Y and p.H63D should be requested in hyperferritinaemia even with normal liver function. These tests will indicate if there is an inflammatory, infective, malignant or hereditary cause.

If there is elevation of SF with abnormal liver function, the following investigations should also be considered: (1) serum caeruloplasmin and copper to assess for Wilson disease or acaeruloplasminaemia; (2) IgG levels, anti-nuclear antibody (ANA), smooth muscle antibody (SMA) and liver kidney microsomal antibodies for autoimmune liver diseases (3) hepatitis A IgM, hepatitis B surface antigen, hepatitis C antibody, and cytomegalovirus and Epstein–Barr virus IgM for infective causes and (4) protease inhibitor typing for alpha-1-antitypsin deficiency.

### 6.3. Liver imaging

Imaging can be helpful in investigating the cause and hepatic impact of hyperferritinaemia. Liver ultrasound can reveal hepatomegaly, fatty infiltration of liver, biliary disease, liver cirrhosis and the presence of portal hypertension reflected by recanalisation of the umbilical vein, portal vein flow reversal, and splenomegaly [80].

Magnetic resonance imaging (MRI) techniques provide a non-invasive method for the detection of iron overload, quantification of liver iron concentration and the degree of fibrosis. FerriScan® has been validated against liver biopsy and is widely used to measure liver iron concentration, however, the software for this is not available in all centres. It can aid in treatment planning with the quantification of iron levels. It has a specificity of 92% to 100%, and a sensitivity of 85% to 94% in quantifying liver iron concentration [81]. Alternatively, the T2* MRI sequence used to quantitate cardiac iron concentration in patients with haemoglobinopathy disorders, is now increasingly being used to quantitate liver iron levels [82]. MR elastography and MRI diffusion-weighted imaging are both accurate ways to estimate liver fibrosis and hence degree of liver damage [83].

### 6.4. What to do if an individual with hyperferritinaemia is HFE homozygous/compound heterozygous?

If the individual is HFE p.C282Y homozygote or p.C282Y/p.H63D compound heterozygote, and has SF >1000 μg/L particularly if liver

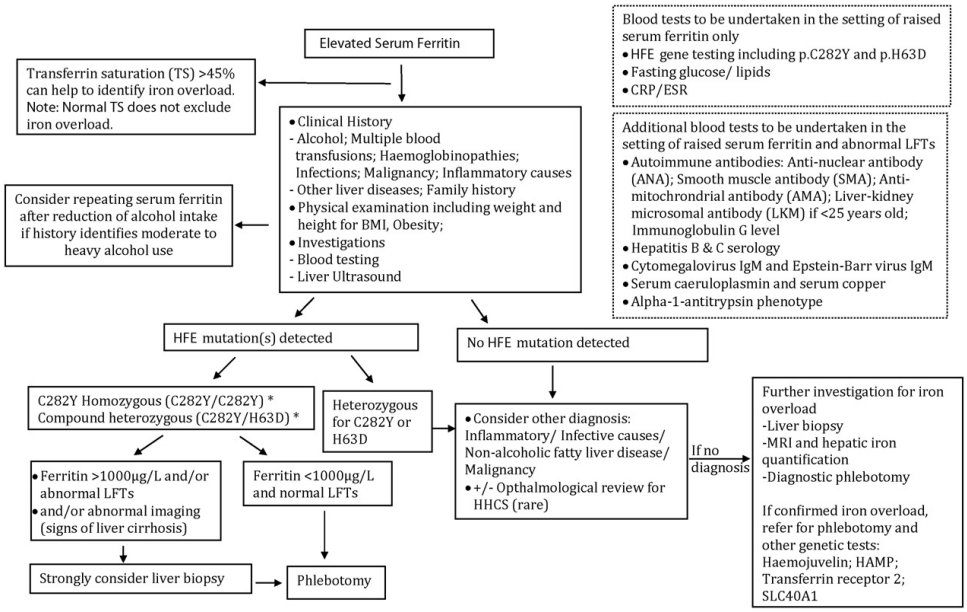


**Fig. 1.** Algorithm of approach to hyperferritinaemia. *Recommend family screening.

function is abnormal or liver imaging shows suggestive signs of liver cirrhosis, a liver biopsy should be performed to assess if liver fibrosis and/or cirrhosis is present. Such individuals should be referred for specialist care. Liver biopsy is not required in HFE homozygous/compound heterozygous individuals with SF < 1000 μg/L unless there is hepatomegaly and/or abnormal liver function, as a number of studies have shown that liver cirrhosis is very unlikely to be present in this setting [14,20–25].

Liver biopsy is considered the gold standard for diagnosis, staging and grading of liver damage. Although a generally safe procedure, it is invasive and carries a small risk of adverse events including bleeding and pain and has a mortality risk of 0.1% to 0.3% [84,85]. There is also sample and observer variability that can lead to potential misdiagnosis [86]. Thus, routine liver biopsy is now no longer used for diagnostic purposes unless the aetiology is unclear or it is required for assessing hepatic injury (fibrosis and cirrhosis). In addition to assessing liver injury, liver biopsy can also be utilised to measure the hepatic iron index (HII = micromoles per gramme divided by patient age) to confirm iron overload. A HII of more than 1.9 indicates iron overload [87].

### *6.5. What to do if the individual with hyperferritinaemia does not have HFE mutations?*

If the individual has no *HFE* mutations detected or is only a carrier of a single p.C282Y or p.H63D mutation, hyperferritinaemia is very unlikely to be due to HH. Other common causes of hyperferritinaemia (without iron overload) such as NAFLD, alcoholic liver disease, inflammatory and infective causes (Table 1) need to be considered as outlined in the algorithm (Fig. 1). HHCS is a rare disorder but an ophthalmological review for the presence of cataracts can help to exclude the condition if no other causes are found. Other forms of HH need to also be considered.

Further investigations to assess whether iron overload is present may be appropriate if no cause is apparent from the initial investigations. This can be by liver biopsy with hepatic iron quantitation, liver iron quantitation by MRI imaging of the liver or quantitative phlebotomy. Quantitative phlebotomy can diagnose iron overload by removing blood and assessing the impact on SF and haemoglobin. One unit of red blood cell (450mls of whole blood) is equivalent to removing approximately 250 mg of iron [88]. Since there is 3–4 g of iron in humans, removal of 3–4 g of iron (12–16 phlebotomies) without causing anaemia from iron deficiency, is diagnostic of iron overload [89]. If iron overload is confirmed, testing of *HJV*, *HAMP*, *TR2* and *SLC40A1* for types 2–4 HH should be considered.

## 7. Treatment of iron overload

### *7.1. Phlebotomy*

Phlebotomy is the mainstay of treatment for removing excess iron in individuals with hyperferritinaemia due to iron overload. Typically, one unit of whole blood (approximately 250 mg of iron) is removed and the decrease in haemoglobin triggers erythropoiesis and mobilises stored iron to make more red blood cells. Therefore a decrease in total body iron is reflected by a decrease in SF. SF is used to monitor iron reduction as TS is a poor marker of iron stores [88]. The frequency of phlebotomy depends on the individual's haemoglobin, haematocrit and SF and can be up to twice weekly when SF is more than 1000 μg/L. Haemoglobin and haematocrit are measured at every treatment and SF is measured approximately every three months, or more often when the SF is approaching the normal range. Treatments are postponed when the individual is anaemic or has low haematocrit to decrease the risk of adverse events [90–92]. Problems that can be associated with such therapy include anaemia, severe cardiac failure, hypoproteinaemia, vasovagal reactions, difficulty with venous access, bruising and nerve damage [93–95]. From a survey of 2851 HH patients, 12% had issues with phlebotomies mainly because of venous access and the time required to have the procedure [96].

The clinical benefits of phlebotomy have not been fully assessed, as there have been no randomised controlled trials in HH. There are suggestions that some symptoms such as fatigue, skin pigmentation, depression and hepatic fibrosis can improve with phlebotomy and reduction in iron overload [96–98]. The current recommendations from the European Association for the Study of the Liver (EASL) and the American Association of the Study of Liver Diseases (AASLD) are to treat all individuals with HH, who have elevated SF even if they are asymptomatic [90,92]. There is a consensus to initiate phlebotomy in individuals with HH with SF > 1000 μg/L since this cohort is at a high risk of complications from HH including hepatic cirrhosis [91]. There is some evidence that individuals with HH who have moderate elevations of SF (<1000 μg/L) may not have manifestations of HH and their SF may not further increase over many years [24]. As a result, some have suggested that in these individuals, SF can be monitored rather than instituting therapy [22–24]. A French study concluded that phlebotomy decreases the mortality in individuals with HFE-related HH with moderately raised SF when compared to the general population [99]. If this was the case, individuals with HH with normal SF at diagnosis should have decreased mortality but this was not found in the study. The study also had a short follow up duration and information on one third concerning the amount of iron removed was missing, thus likely affecting the interpretation of data on mortality [100].

The literature does not provide any guidance on the ideal target SF at the end of phlebotomy treatment, although, most guidelines recommend a target of either 50 or 100 μg/L [90,92]. This is based on the theory that reducing iron levels to the lower end of the normal reference range will mean that there is little or no iron overload in tissues [101]. It is possible that a higher final SF of between 200 and 300 μg/L is acceptable [88].

### *7.2. Erythrocytapheresis*

Therapeutic erythrocytapheresis (TE) is a procedure employed mainly in haematological conditions such as sickle cell disease and polycythaemia to remove predominantly red blood cells through an automated cell separator, while returning other blood components such as platelets, plasma and coagulation factors to the individual. However, TE is increasingly used as a treatment in HH especially in individuals who are intolerant of phlebotomy. As each procedure can remove up to 800 ml of red blood cells, more iron can be removed than by standard phlebotomy [93]. Its efficiency was shown in a randomised trial comparing TE and phlebotomy in individuals with HFE-related HH where an average of nine TE treatments were required to normalise SF compared to 27 phlebotomies [102]. The frequency of TE treatment is every two to three weeks and thus is less frequent compared to twice-weekly phlebotomies. The other advantages of TE are an overall shorter duration of therapy to normalise SF [102,103], its feasibility in patients with hypoproteinaemia and cardiac failure and a lower rate of hypovolaemia related reactions [93]. TE can maintain a euvolaemic state even with a larger volume of red cells removed as volume can be replaced by saline. Thus, it is a generally well-tolerated and efficacious procedure [101–103]. The major drawbacks are the need for apheresis equipment and expertise in administering the treatment, and that each treatment is more expensive than phlebotomy [104]. However, one study found the overall cost of treatment was similar to phlebotomy due to the reduced number of treatments [102].

### *7.3. Iron chelation therapy*

Iron chelation is the main treatment for secondary iron overload, such as β-thalassaemia, and is the second-line treatment in HH when phlebotomy is not tolerated. Desferrioxamine is an iron chelator administered as a continuous subcutaneous infusion for 8–24 h. The limitations of using desferrioxamine are the compliance of patients, the need for parenteral administration, discomfort during administration

[91] and side effects including neurotoxicity, and opportunistic infections [105]. A more widely used iron chelator is deferasirox, which can be administered orally as a daily dose. It is effective in reducing excess iron from secondary iron overload [106,107]. Deferasirox may be considered in HH where affected individuals cannot tolerate phlebotomy and clinical trials have shown that it is well tolerated and effective in reducing iron overload in HH [108,109]. Adverse events from deferasirox are dose dependent and include diarrhoea, headache, nausea and rise in ALT and/or creatinine, although these side effects occur in less than 10% of individuals with HH and they generally resolved either with dose reduction or cessation [108].

## 8. Conclusions

Hyperferritinaemia is a common clinical presentation and it is important to have a systemic approach to identify the cause, particularly to confirm whether or not it is due to iron overload. Although HH is a common cause of hyperferritinaemia, it is important to exclude other common aetiologies including NAFLD, alcohol consumption and inflammatory conditions. When the aetiology of hyperferritinaemia is unclear, referral to a specialist such as a hepatologist or haematologist is advised for further investigations and treatment. Phlebotomy is the first line treatment for decreasing iron overload but other modalities such as TE and iron chelation should be considered in individuals who do not tolerate phlebotomy.

## Conflict of interests

The authors declare no conflict of interests.

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