

# **A GENETIC EPIDEMIOLOGIC STUDY OF HEMOCHROMATOSIS**

Omer Njajou Tchikamgoua

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**A genetic epidemiologic study of  
hemochromatosis**

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hemochromatose

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DEDICATED

*To my darling Jeanne Marie*

*and*

*In loving memory of my late brother Njajou Tchikamgoua Eric Papi*

## **Papers and manuscripts based on the studies described in this thesis**

### **Chapter 3.2**

Njajou OT, Houwing-Duistermaat JJ, Osborne RH, Vaessen N, Vergeer J, Heeringa J, Pols HAP, Hofman A, van Duijn CM. A population-based study of the effect of the *HFE* C282Y and H63D mutations on serum iron levels in the elderly. (Submitted)

### **Chapter 3.3**

Alizadeh BZ, Njajou OT, Houwing-Duistermaat JJ, Vaessen N, de Jong G, Vergeer J, Hofman A, Pols HAP, van Duijn CM. *HFE* gene mutations and liver function in the elderly. (Submitted)

### **Chapter 3.4**

Njajou OT, Alizadeh BZ, de Jong G, Vaessen N, Vergeer J, Houwing-Duistermaat JJ, Pols HAP, Hofman A, van Duijn CM. The role of the hemochromatosis C282Y and H63D mutations in type 2 diabetes. (Submitted)

### **Chapter 3.5**

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### **Chapter 4.2**

Njajou OT, de Jong G, Berghuis B, Vaessen N, Snijders PJLM, Goossens JP, Wilson JHP, Breuning MH, Oostra BA, Heutink P, Sandkuijl LA, van Duijn CM. A family with a hereditary form of iron overload distinct from classical hemochromatosis. (Submitted)

### **Chapter 4.3**

Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JWF, Breuning MH, Snijders PJLM, Rutten WPF, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P. A mutation in *SLC11A3* is associated with autosomal dominant hemochromatosis. Modified from *Nature Genetics* 2001;28:214-5.

### **Chapter 4.4**

Njajou OT, Vaessen N, Oostra BA, Heutink P, van Duijn CM. The hemochromatosis N144H mutation of *SLC11A3* gene in patients with type 2 diabetes. *Molecular Genetics and Metabolism* 2002;75:290-1.

### **Chapter 5.2**

Njajou OT, Alizadeh BZ, van Duijn CM. Increased mortality in C282Y homozygotes and compound heterozygotes for the *HFE* mutations. (Submitted)

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## **Chapter 1**

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Introduction to the thesis



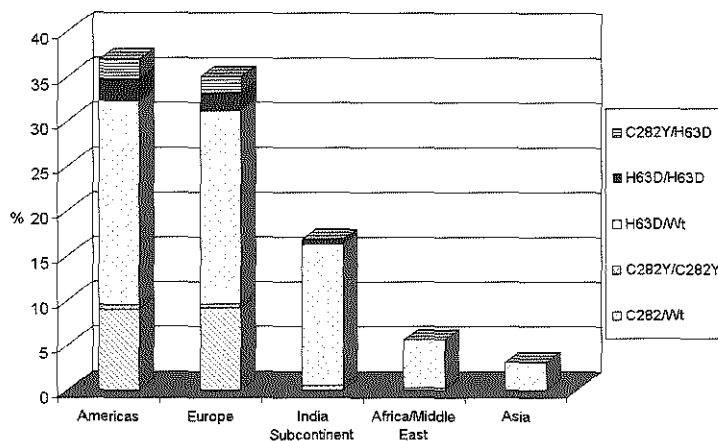
Hemochromatosis includes several disorders of iron metabolism which are characterised by pathological accumulation of iron in tissues.<sup>1</sup> Although there is debate about the definition of hemochromatosis, the disease is usually categorised into primary and secondary forms.<sup>2</sup> Primary hemochromatosis is referred to as hereditary hemochromatosis. It is an inherited disorder resulting from an inborn error of iron metabolism which leads to progressive iron loading of the parenchymal cells in the liver, pancreas and heart.<sup>1</sup> Secondary hemochromatosis is referred to as acquired hemochromatosis. These are disorders characterised by iron overload that occur as a result of chronic disorders of erythropoiesis such as thalassemia or sideroblastic anemia,<sup>2</sup> which by themselves may be hereditary. Hereditary hemochromatosis is one of the most common genetic diseases with a prevalence of 0.2 to 0.5% in populations of northern European descent.<sup>3-7</sup> Hemochromatosis can lead to multiple pathologies like cirrhosis, hepatocellular carcinoma, cardiomyopathy, diabetes mellitus, amenorrhea, impotence, cardiomyopathies, arthritis, pituitary hypogonadism and skin hyperpigmentation.<sup>8-10</sup> Early symptoms and complaints include joint pain, abdominal pain, weakness and fatigue.<sup>8</sup> Expression of the disease is modified by several factors, in particular dietary iron intake, blood donation and blood loss associated with menstruation and pregnancy. The clinical expression of the disease is 5 to 10 times more frequent in men than women and nearly 70% of carriers develop the first symptoms between 40 and 60 years of age, the age of onset being delayed in women.<sup>11</sup> The disease does not express before 20 years of age, although with family screening and periodic health examinations, asymptomatic subjects with iron overload can be identified in adulthood.

For long, the diagnosis of hemochromatosis was based on presence of excess iron in a liver biopsy in combination with levels of serum iron, serum transferrin, and total iron

binding capacity (TIBC).<sup>12,13</sup> Since the discovery of the *HFE* C282Y and H63D gene mutations,<sup>14</sup> a major gene involved in hemochromatosis, diagnostic procedures have shifted to biochemical and genetic tests. In the last decades, biochemical tests, including serum iron, ferritin and transferrin saturation level, are now widely used in combination with genetic tests.<sup>15</sup> Hemochromatosis patients can be treated successfully by iron removal, which can be done either by phlebotomy or with iron chelators.<sup>16-18</sup> This requires an early diagnosis of the disease. For Caucasians, in about 90% of cases of hereditary hemochromatosis, the cause of the disease is known but in 10% of cases, the disease is unexplained and may be due to environmental and/or genetic factors.

In caucasians, the most common form of hemochromatosis (about 80%) is due to homozygosity for the C282Y mutation or compound heterozygosity for the C282Y and H63D mutations in the *HFE* gene.<sup>4,27-29</sup> The proportion of hemochromatosis due to *HFE* mutations varies in different parts of the world. Figure 1 summarises the published frequencies of carriers of *HFE* mutations in different populations (adapted from Hanson et al, 2001).<sup>22</sup> Most C282Y and H63D carriers are found in America and Europe. About 65% of subjects from these two continents carry only the wild type allele compared to 85% in India, and about 95% in Africa, Middle East, and Asia. Some hereditary forms of hemochromatosis are due to rare or unknown genes and segregate as a recessive or dominant trait.<sup>23-26</sup>

In recent years there has been increasing interest in screening populations for hemochromatosis.<sup>4,27-29</sup> Hemochromatosis is an excellent example of a disease that meets the World Health Organisation recommendations and the US preventive services task force criteria for a screening program.<sup>30,31</sup> The disorder is common, it has a prolonged presymptomatic and early symptomatic phase, and if untreated can



**Figure 1.** Frequencies of *HFE* C282Y and H63D mutations in different populations

result in serious morbidity and premature death. Simple and effective screening tests for iron overload are available and there is a reliable confirmatory test. The treatment is safe and acceptable and in some countries the blood collected from venesection treatment is utilised by the blood transfusion services. Available data suggest that approximately one-half of males and one-fourth of females homozygous for the C282Y and H63D mutations will develop potential life-threatening complications.<sup>33</sup> However, these findings were recently disputed by others.<sup>34</sup> It is still a matter of debate whether screening for hemochromatosis should be based on the phenotype i.e. biochemical level of serum iron parameters, or based on the presence of mutations in the *HFE* gene.<sup>28,33-35</sup> At present, knowledge of the use of genetic markers for genetic screening and their relation to the full spectrum of disease is contradictory.<sup>1-10, 28,33-35</sup> The present thesis addresses these issues and aims to provide more knowledge and insights into hemochromatosis.

**Scope of this thesis**

Hemochromatosis is a heterogeneous disease both genetically and phenotypically. In chapter 2, its genetic epidemiology is reviewed in terms of heritability, genes involved and prevalence of the disease these genes explain.

The scope of the work presented in this thesis is two fold. The first aim was to study the relation of *HFE* mutations to serum iron, ferritin and transferrin saturation levels and the risk of liver diseases, diabetes and stroke. This was carried out in a population-based sample of elderly who were participants in the Rotterdam Study. This is a prospective study of risk factors for diseases and disabilities in 7983 subjects aged 55 years and over. In total, 3500 subjects were genotyped for the C282Y and H63D mutations. In chapter 3.2, we examined the effect of the major *HFE* mutations (C282Y and H63D) on serum iron levels in this population. These data are discussed in light of the possibility of genetic screening programs to detect subjects with either subclinical hemochromatosis or increased iron levels. In chapter 3.3, we examined the effect of *HFE* gene mutations on liver function in the elderly. The role of *HFE* mutations in type 2 diabetes is described in chapter 3.4. Chapter 3.5 describes the relationship between *HFE* gene mutations, atherosclerosis and stroke.

The second aim of this thesis was to identify new genes involved in hemochromatosis. This study was performed in a genetically isolated community of about 20,000 inhabitants in the south of The Netherlands. In chapter 4.2, a detailed clinical description of a large family with an atypical form of iron overload is presented. The results of a genomic screen are described in chapters 4.3 together with the identification of the gene involved. The relationship of this gene to type 2 diabetes is reported in chapter 4.4. Finally the implications of the findings of this thesis are discussed in light of the literature in chapter 5.

## References

1. Adams PC, Brissot P, Pietrangelo A, Powell LW. EASL international consensus conference on haemochromatosis. *J Hepatology* 2000;33:485-504.
2. Powell LW, Isselbacher KJ. Disorders of intermediary metabolism. In Harrison's principle of internal medicine. Harrison's online. Chapter 345.
3. Smith BN, Kantrowitz W, Grace ND, Greenberg MS, Patton TJ, Ookubo R, et al. Prevalence of hereditary hemochromatosis in a Massachusetts corporation: is Celtic origin a risk factor? *Hepatology* 1997;25:1439-46.
4. Baer DM, Simons JL, Staples RL, Rumore GJ, Morton CJ. Hemochromatosis screening in asymptomatic ambulatory men 30 years of age and older. *Am J Med* 1995;98:464-8.
5. Leggett BA, Halliday JW, Brown NN, Bryan S, Powell LW. Prevalence of hemochromatosis among asymptomatic Australians. *Br J Haematol* 1990;74:525-30.
6. Edwards CQ, Griffen LM, Goldgar D, Drummond C, Skolnick MH, Kushner JP. Prevalence of hemochromatosis among 11,065 presumably healthy blood donors. *N Engl J Med* 1988;318:1355-62.
7. Cullen LM, Gao X, Eastel S, Jazwinska EC. The hemochromatosis 845 G to A and 187 G to C mutations: Prevalence in non-Caucasian populations. *Am J Hum Genet* 1998;62:1403-7.
8. Witte DL, Crosby WH, Edwards CQ, Fairbanks VF, Mitros FA. Practice guidelines development task force of the college of American pathologists. Hereditary hemochromatosis. *Clin Chim Acta* 1996;245:139-200.
9. Edwards CQ, Cartwright GE, Skolnick MH, Amos DB. Homozygosity for hemochromatosis: clinical manifestations. *Ann Intern Med* 1980;93:519-25.
10. Powell LW, Jazwinska E, Halliday JW. Primary iron overload. In: Brock JH, Halliday JW, Pippard MJ, Powell LW, eds. Iron metabolism in health and disease. London Saunders, 1994:227-70.
11. Finch SC, Finch CA. Idiopathic hemochromatosis, an iron storage disease. A: Iron metabolism in hemochromatosis. *Medicine* 1955;34:381-430.
12. Scheuer PJ, Williams R, Muir AR. Hepatic pathology in relatives of patients with hemochromatosis. *J Pathol Bacteriol* 1962;84:53-64.
13. Basset ML, Halliday JW, Powell LW. Value of hepatic iron measurements in early hemochromatosis and determination of the critical iron level associated with fibrosis. *Hepatology* 1986;6:24-9.
14. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
15. Powell LW, George DK, McDonnell SM, Kowdley KV. Diagnosis of hemochromatosis. *Ann Intern Med* 1998;129:925-31.
16. Barton JC, McDonnell SM, Adams PC, Brissot P, Powell LW, Edwards CQ, et al. Management of hemochromatosis. *Am J Hematol* 1998;58:16-9.
17. Bomford A, Williams R. Long term results of venesection therapy in idiopathic haemochromatosis. *Quart J Med* 1976;45:611-23.

18. Crosby WH. A history of phlebotomy therapy for hemochromatosis. *Am J Med Sci* 1991;301:28-31.
19. Beutler E, Gelbart T, West C, Lee P, Adams M, Blackstone R, et al. Mutation analysis in hereditary hemochromatosis. *Blood Cells Mol Dis* 1996;22:187-94.
20. Jouanolle AM, Gandon G, Jezequel P, Blayau M, Campion ML, Yaouanq J, et al. Haemochromatosis and HLA-H. *Nature Genet* 1996;14:251-2.
21. Jazwinska EC, Cullen LM, Busfield F, Pyper WR, Webb SI, Powell LW, et al. Haemochromatosis and HLA-H. *Nature Genet* 1996;14:249-51.
22. Hanson EH, Imperatore G, Burke W. *HFE* gene and hereditary hemochromatosis: A HuGE review. *Am J Epidemiol* 2001;154:193-206.
23. Eason RJ, Adams PC, Aston CE, Searle J. Familial iron overload with possible autosomal dominant inheritance. *Aust N Z J Med* 1990;20:226-30.
24. Camaschella C, Fargion S, Sampietro M, Roetto A, Bosio S, Garozzo G, Arosio C, Piperno A. Inherited *HFE*-unrelated hemochromatosis in Italian families. *Hepatology* 1999;29:1563-4.
25. Pietrangelo A, Montosi G, Totaro A, Garuti C, Conte D, Cassanelli S, Fraquelli M, Sardini C, Vasta F, Gasparini P. Hereditary hemochromatosis without pathogenic mutations in the hemochromatosis gene. *New Engl J Med* 1999;341:725-32.
26. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, Carella M, Zelante L, Kelly AL, Cox TM, Gasparini P, Camaschella C. Juvenile hemochromatosis locus maps to chromosome 1q. *Am J Hum Genet* 64:1388-93.
27. Haddow JE, Ledue TB. Preventing manifestations of hereditary haemochromatosis through population based screening. *J Med Screen* 1994;1:16-21.
28. Edwards CQ, Griffen LM, Ajioka RS, Kushner JP. Screening for hemochromatosis: phenotype versus genotype. *Semin Hematol* 1998;35:72-6.
29. Bradley LA, Haddow JE, Palomaki GE. Population screening for hemochromatosis: a unifying analysis of published intervention trials. *J Med Screen* 1996;3:178-84.
30. Wilson JM, Junger G. The principle and practice of screening for disease. *Public Health Papers WHO* 1968;34:26-9.
31. US preventive services task force. Guide to clinical preventive services: Report of the US preventive services task force. 2nd ed. Baltimore, MD: Williams & Wilkins; 1996;231-46.
32. Edwards CQ, Kushner JP. Screening for hemochromatosis. *N Engl J Med* 1993;328:1616-20.
33. Adams PC, Valberg LS. Screening blood donors for hereditary haemochromatosis: decision analysis model comparing genotyping to phenotyping. *Am J Gastroenterol* 1999;94:1593-600.
34. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. *Lancet* 2002;359:211-8.
35. Tavill AS. Screening for haemochromatosis: phenotyping or genotyping or both? *Am J Gastroenterol* 1999;94:1430-3.



## **Chapter 2**

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*Genetic epidemiology of hemochromatosis*



In 1935, Sheldon suggested that hemochromatosis is an inborn error of iron metabolism.<sup>1</sup> Studies of familial aggregation have extended from the 1970's up to the 1990's. Hemochromatosis is indeed found more commonly in relatives of patients.<sup>2-9</sup> Studies of the transmission of the disease in families suggest that hemochromatosis segregates usually as an autosomal recessive trait.<sup>7-10</sup>

Genetic and phenotypic heterogeneity are well-recognised features in hemochromatosis and it is becoming more and more evident that several genes or environmental factors may lead to the disease. Depending on the localisation of the genetic defect and the clinical phenotype, several types of hemochromatosis are distinguished.

### ***Type 1 hemochromatosis***

Type 1 hemochromatosis (*HFE1* or simply *HFE*) is by far the most common form of hemochromatosis.<sup>11-14</sup> The culprit gene, termed *HFE*, is located on human chromosome 6p21.3 and has two major mutations, c.845G→A (C282Y) and c.187C→G (H63D).<sup>11</sup> Since its identification, over 37 allelic variants of the *HFE* gene have been described.<sup>15</sup> The localisation of the *HFE* protein in the crypt cells of the duodenum, the site of dietary iron absorption and its association with the transferrin receptor in those cells are consistent with a role in regulating iron absorption.<sup>16-18</sup> In the *HFE1* associated form of hemochromatosis, the progression of iron overload is usually slow and affected individuals do not often present with clinical signs or symptoms until the fifth or sixth decade of life.<sup>20</sup> The disease occurs with a prevalence of 0.2 to 0.5% in northern Europeans.<sup>21-23</sup> *HFE1* segregates in families as an autosomal recessive trait<sup>7-9</sup> and about 80% of clinically diagnosed probands of hemochromatosis patients are homozygous for the C282Y mutation in the *HFE* gene.<sup>11,24-25</sup> *HFE* is the most widely studied gene that is involved in

hemochromatosis. In the general white population, the carrier frequency of the C282Y mutation is estimated to be 10%, and for the H63D mutation, 22%.<sup>11-13,20-23,26,27</sup>

### ***Type 2 hemochromatosis***

Type 2 hemochromatosis (*HFE2*), also called juvenile hemochromatosis, differs from type 1 hemochromatosis.<sup>28,29</sup> This is a rare recessive form and more severe disease phenotype that affects both sexes equally in the second decade of life with rapid iron loading and early onset of cardiac symptoms, endocrine dysfunction (hypogonadotrophic hypogonadism) and premature death.<sup>29-31</sup> Kelly et al, (1998)<sup>32</sup> reported a mean onset of 22 years in patients from 3 pedigrees. The gene responsible has not yet been cloned but several genomic screens suggest linkage to human chromosome 1q in the region of D1S498 and D1S2344.<sup>33</sup> By homozygosity mapping the critical candidate region was defined in an interval of 4 cM between markers D1S442 and D1S2347. Up until now, the contribution of this gene to the occurrence of hemochromatosis is thought to be limited to a few families.<sup>32,33</sup>

### ***Type 3 hemochromatosis***

Type 3 hemochromatosis (*HFE3*) is phenotypically similar to *HFE1*. The disease has been associated to the transferrin receptor 2 (*TFR2*) gene on human chromosome 7q22.<sup>34</sup> Substitution of tyrosine by threonine at codon 250 and a 12 nucleotide deletion in exon 6, causing loss of 4 amino acid in *TFR2* (AVAQ 594-597 del) show evidence for a role in iron overload. Further evidence for a role of *TFR2* in iron metabolism is derived from the fact that the extracellular domain of *TFR2* is homologous to that of the transferrin receptor and like the latter it can bind to transferrin.<sup>35</sup> The exact role of *TFR2* in the pathogenesis of *HFE3* is still not well understood. It has been hypothesised that *TFR2* does not form a stable complex with

the *HFE* protein and binds to transferrin with very low affinity. *TFR2* mutations are rare but may occur frequently in the Italian population.<sup>35</sup>

#### ***Type 4 hemochromatosis***

Contrary to the previously described forms of hemochromatosis, type 4 hemochromatosis or *HFE4* segregates as an autosomal dominant trait.<sup>36-38</sup> The clinical phenotype of patients in this case is quite similar to that of patients with *HFE1* and *HFE2* hemochromatosis but differs in that the disease is less severe and the pattern of iron loading is distinct.<sup>36-38</sup> Type 4 hemochromatosis (*HFE4*) is associated with mutations in the gene encoding the metal transporter called *SLC11A3* alias ferroportin (*FPN1*), iron regulated transporter (*IREG1*) or metal transporter protein (*MTP1*) on human chromosome 2q.<sup>36,37</sup> These mutations are thought to be rare in the general population (chapter 4.3).

#### ***Other types of hemochromatosis***

Other forms of hereditary iron overload include neonatal hemochromatosis, hyperferritinemia cataract syndrome, aceruloplasminemia, congenital atransferrinemia and African iron overload. African iron overload is common in sub-Saharan Africa and is a distinct type of iron storage disorder.<sup>39,40</sup> It is believed to result from increased dietary iron derived from traditional home-brewed beer. The etiology of neonatal hemochromatosis and hereditary hyperferritinemia cataract syndrome is not well understood. Aceruloplasminemia, and congenital atransferrinemia are due to the absence of ceruloplasmine and transferrin respectively and are secondary forms of iron overload. The pattern of iron deposition in patients suffering from these diseases is clearly different from that of classical hemochromatosis. Each of these disorders is rare.

## Discussion

There is currently considerable uncertainty concerning the proportion of people with *HFE* mutations who will develop iron overload as well as the existence of other hemochromatosis genes or mutations. From a population-based perspective, the most important gene is *HFE* due to the high frequency of its variants. Current clinical, epidemiological, and genetic data suggest that the relationship between *HFE* and other diseases is complex.<sup>41</sup> Many questions concerning the genotype and phenotype correlation, penetrance, and expressivity remain unresolved.<sup>42</sup> For this reason genetic tests for *HFE* mutations are not recommended in population screening at present, although this approach is advocated by some.<sup>43-45</sup> Genetic testing may have a role in families in which *HFE* mutations segregate. Further, genetic tests may be helpful in confirming the diagnosis in subjects whose serum iron indices suggest hemochromatosis. In these families, genetic tests may replace for a large part the necessity of liver biopsy. Although homozygosity for the C282Y mutation may be found in up to 80% of patients with a hereditary form of the disease, for a considerable number of families the genetic origin of the disease is still unknown.

## References

1. Sheldon JH. *Haemochromatosis*. London: Oxford University Press;1935.
2. Moirand R, Jouanolle AM, Brissot P, Le Gall JY, David V, Deugnier Y. Phenotypic expression of *HFE* mutations: a French study of 1110 unrelated iron-overloaded patients and relatives. *Gastroenterology* 1999;116:372-7.
3. Brissot P, Moirand R, Jouanolle AM, Guyader D, Le Gall JY, Deugnier Y, David V. A genotypic study of 217 unrelated probands diagnosed as "genetic hemochromatosis" on "classical" phenotypic criteria. *J Hepatol* 1999;30:588-93.
4. Barton JC, Shih WW, Sawada-Hirai R, Acton RT, Harmon L, Rivers C, Rothenberg BE. Genetic and clinical description of hemochromatosis probands and heterozygotes: evidence that multiple genes linked to the major histocompatibility complex are responsible for hemochromatosis. *Blood Cells Mol Dis* 1997;23:135-45.

5. Simon M, Alexandre JL, Fauchet R, Genetet B, Bourel M. The genetics of hemochromatosis. *Prog Med Genet* 1980;4:135-68.
6. Simon M, Alexandre JL, Bourel M, Le Marec B, Scordia C. Heredity of haemochromatosis: a study of 106 families. *Clin Genet* 1977;11:327-41.
7. Saddi R, Feingold J. Idiopathic hemochromatosis: an autosomal recessive disease. *Clin Genet* 1974;5:234-41.
8. Simon M, Bourel M, Genetet B, Fauchet R. Idiopathic hemochromatosis: demonstration of recessive transmission and early detection by family HLA typing. *New Engl J Med* 1977;297:1017-21.
9. Saddi R, Feingold J. Hemochromatose idiopathique. Maladie recessive autosomique. *Rev Francaise Etude Clinique Biol* 1969;14:238-51.
10. Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL, Martin NG. Effects of *HFE* C282Y and H63D polymorphism and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000;66:1246-58.
11. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
12. Beutler E, Gelbart T, West C, Lee P, Adams M, Blackstone R, et al. Mutation analysis in hereditary hemochromatosis. *Blood Cells Mol Dis* 1996;22:187-94.
13. Borot JC, Roth M, Malfroy L, Demangel C, Vinel JP, Pascal JP, et al. Mutations in the MHC class I-like candidate gene for hemochromatosis in French patients. *Immunogenetics* 1997;45:320-4.
14. Jazwinska EC, Cullen LM, Busfield F, Pyper WR, Webb SI, Powell LW, et al. Haemochromatosis and HLA-H. *Nature Genet* 1996;14:249-51.
15. Pointon JJ, Wallace D, Merryweather-Clarke AT, Robson KJ. Uncommon mutations and polymorphisms in the hemochromatosis gene. *Genet Test* 2000;4:151-61.
16. Parkilla S, Wahed A, Britton RS, Feder JN, Tsuchihashi Z, Schatzman RC, Bacon BR, Sly WS. Immunohistochemistry of HLA-H, the protein defective in patients with hereditary hemochromatosis, reveals unique pattern of expression in gastrointestinal tract. *Proc Natl Acad Sci USA* 1997;94:2534-9.
17. Lebron JA, Bennet MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA, Feder JN, Bjorkman PJ. Crystal structure of the hemochromatosis protein *HFE* and characterisation of its interaction with transferrin receptor. *Cell* 1998;93:111-23.
18. Parkilla S, Wahed A, Britton RS, Bacon BR, Zhou XY, Tomatsu S, Fleming RE, Sly WS. Association of the transferrin receptor in human placenta with *HFE*, the protein defective in hereditary hemochromatosis. *Proc Natl Acad Sci USA* 1997;94:3198-202.
19. Finch SC, Finch CA. Idiopathic hemochromatosis, an iron storage disease. A: Iron metabolism in hemochromatosis. *Medicine* 1955;34:381-430.
20. Merryweather-Clarke AT, Pointon JJ, Shearman JD, Robson KJH. Global prevalence of putative haemochromatosis mutations. *J Med Gen* 1997;34:275-8.

21. Baer DM, Simons JL, Staples RL, et al. Hemochromatosis screening in asymptomatic ambulatory men 30 years of age and older. *Am J Med* 1995;98:464-8.
22. Smith BN, Kantrowitz W, Grace ND, Greenberg MS, Patton TJ, Ookubo R, et al. Prevalence of hereditary hemochromatosis in a Massachusetts corporation: is Celtic origin a risk factor? *Hepatology* 1997;25:1439-46.
23. Edwards CQ, Griffen LM, Goldgar D, Drummond C, Skolnick MH, Kushner JP. Prevalence of hemochromatosis among 11,065 presumably healthy blood donors. *New Engl J Med* 1988;318:1355-62.
24. Jouanolle AM, Gandon G, Jezequel P, Blayau M, Campion ML, Yaouanq J, et al. Haemochromatosis and HLA-H. *Nature Genet* 1996;14:251-2.
25. Adams PC, Brissot P, Pietrangelo A, Powell LW. EASL international consensus conference on haemochromatosis. *J of Hepatology* 2000;33:485-504.
26. Burke W, Thomson E, Khoury MJ, McDonnell SM, Press N, Adams PC, et al. Hereditary hemochromatosis: gene discovery and its implication for population-based screening. *JAMA* 1998;280:172-8.
27. Beutler E, Gelbart T. HLA-H mutations in Ashkenazi Jewish population. *Blood Cells Mol Dis* 1997;23:95-8.
28. Camaschella C, Roetto A, Cicilano M, Pasquero P, Bosio S, Gubetta L, et al. Juvenile and adult hemochromatosis are distinct genetic disorders. *Eur J Hum Genet* 1997;5:371-5.
29. Cazzola M, Cerani P, Rovati A, Iannone A, Claudiani G, Bergamaschi G. Juvenile genetic hemochromatosis is clinically and genetically distinct from the classical HLA-related disorder. *Blood* 1998;92:2979-81.
30. Charlton RW, Abrahams C, Bothwell TH. Idiopathic hemochromatosis in young subjects. *Arch Path* 1967;83:132-40.
31. Cazzola M, Ascari E, Barosi G, Claudiani G, Dacco M, Kaltwasser JP, Panaiotopoulos N, Schalk KP, Werner EE. Juvenile idiopathic haemochromatosis: a life threatening disorder presenting as hypogonadotropic hypogonadism. *Hum Genet* 1983;65:149-54.
32. Kelly AL, Rhodes DA, Roland JM, Schofield P, Cox TM. Hereditary juvenile haemochromatosis: a genetically heterogeneous life-threatening iron-storage disease. *Quart J Med* 1998;91:607-18.
33. Roetto A, Totaro A, Cazzola M, Ciciliano M, Bosio S, D'Ascola G, et al. Juvenile hemochromatosis locus maps to chromosome 1q. *Am J Hum Genet* 1999;64:1388-93.
34. Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo M, Majorano N, Totaro A, et al. The gene TFR2 is mutated in a new type of hemochromatosis mapping to 7q22. *Nature Genet* 2000;25:14-5.
35. Griffiths W, Cox T. Haemochromatosis: novel gene discovery and the molecular pathophysiology of iron metabolism. *Hum Molec Genet* 2000;9:2377-82.
36. Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JWF, Breuning MH, Snijders PJLM, Rutten WPF, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P. A mutation in *SLC11A3* is associated with autosomal dominant hemochromatosis. *Nature Genet* 2001;28:214-5.



37. Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A. Autosomal dominant hemochromatosis is associated with a mutation in the ferroportin (*SLC11A3*) gene. *The J Clin Invest* 2001;108:619-23.
38. Pietrangelo A, Montosi G, Totaro A, Garuti C, Conte D, Cassanelli S, Fraquelli M, Sardini C, Vasta F, Gasparini P. Hereditary hemochromatosis without pathogenic mutations in the hemochromatosis gene. *New Engl J Med* 1999;341:725-32.
39. Baer JC, Edwards CQ, Bertoli LF, Shroyer TW, Hudson SL. Iron overload in African Americans. *Am J Med* 1995;99:616-23.
40. Gangaidzo IT, Moyo VM, Saugweme T, Khumalo H, Charakupa RM, Gomo ZAR, et al. Iron overload in urban Africans in the 1990s. *Gut* 1999;45:278-83.
41. Powell LW, Jazwinska E, Halliday JW. Primary iron overload. In: Brock JH, Halliday JW, Pippard MJ, and Powell LW. Iron metabolism in health and disease. WB Saunders, London, pp. 227-270.
42. Haddow JE, Bradley LA. Hereditary hemochromatosis: to screen or not. Conditions for screening are not yet fulfilled. *BMJ* 1999;319:23.
43. Brintenham GM, Franks AL, Rickles FR. Research priorities in hereditary hemochromatosis. *Ann Intern Med* 1998;129:993-6.
44. McDonnell SM, Phatak PD, Felitti V, Hover A, McLaren GD. Screening for hemochromatosis in primary care settings. *Ann Intern Med* 1998;129:962-70.
45. Cogswell M, Burk W, McDonnell S, Franks AL. Screening for hemochromatosis: a public health perspective. *Am J Prev Med* 1999;16:134-4.



## **Chapter 3**

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Population-based studies



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## Chapter 3.1

### Introduction

Hereditary hemochromatosis is a disorder of iron metabolism in which there is increased absorption and progressive storage of iron in body organs leading to multi-organ damage and multiple pathologies. About 80% of Caucasian patients with hemochromatosis are homozygous for the C282Y mutation in the *HFE* gene. The role of another common polymorphism found in the *HFE* gene, the H63D, is less clear. Various questions are unanswered with regard to genotype and phenotype correlation and the potential for screening based on *HFE* mutations. One of the questions (still open) that we address in this chapter is how the C282Y and H63D mutations relate to serum iron levels. Important parameters such as sensitivity, specificity and positive predictive values are necessary in the evaluation of screening strategies and need to be estimated in a population-based sample. We aimed to estimate these parameters in a population-based sample, the Rotterdam Study (chapter 3.2). Further, we assessed the relation of the *HFE* gene to pathology. In this thesis, we focussed on the major diseases associated with *HFE*, i.e. liver diseases (chapter 3.3), diabetes (chapter 3.4) and stroke (chapter 3.5).



## **A population-based study of the effect of the *HFE* C282Y and H63D mutations on serum iron levels in the elderly**

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### **Summary**

The C282Y and H63D mutations in the *HFE* gene are important causes of hemochromatosis. In the elderly, these mutations might be associated with increased morbidity because of life-long accumulation of iron. In a population-based sample of elderly, we determined the value of genotyping for *HFE* mutations to screen for subclinical hemochromatosis. *HFE* genotype frequencies were determined in a random group of 2095 subjects (aged 55 years and over). In this group, we selected within the six genotype categories a total of 347 individuals and measured their serum transferrin saturation, iron and ferritin levels. We also estimated the heritability and parameters needed to evaluate screening, including the sensitivity, specificity, positive and negative predictive values (PPV, NPV) of *HFE* genotypes. Iron parameters were significantly increased in subjects homozygous, heterozygous or compound heterozygous. The effect of the mutations was more pronounced in men than in women. For the H63D mutation, an allele dose effect was observed. The *HFE* gene explained about 5% of the variability in serum iron indices. The PPV for hemochromatosis for the C282Y homozygotes was 100% in men and 67% in women. The NPV of the wild type allele was 97% for both men and women. The sensitivity of both mutations was 70% for men and 52% for women and the specificity was 62% for men and 64% for women. Our study shows that the *HFE* C282Y and H63D mutations are determinants of iron parameters in the elderly and will be effective in detecting individuals at high risk of hemochromatosis. However, when screening based on these two mutations some individuals with subclinical hemochromatosis will be missed.

## Introduction

Hereditary hemochromatosis is a disease characterised by iron accumulation in the tissue of several organs including the liver, heart, pancreas, pituitary and joints.<sup>1</sup> Iron deposition may result in a wide range of common conditions such as arthritis, diabetes, myocardial infarction, stroke and cancer.<sup>2,3</sup> Several mutations involved in the pathogenesis of hemochromatosis have been described.<sup>4</sup> The two most common are the 845 G→A (C282Y) transition and the 187 C→G (H63D) transversion in the *HFE* gene on chromosome 6.<sup>5</sup> Most of our knowledge about the genotype-phenotype relationship is derived from studies of patients diagnosed with hereditary hemochromatosis. Over 80% of Caucasian patients are homozygous for the C282Y mutation, suggesting this is by far the most common cause of hereditary hemochromatosis.<sup>5,6</sup> Further, compound heterozygotes, i.e. carriers of both the *HFE* C282Y and H63D mutations, have been found to be at increased risk of hereditary hemochromatosis.<sup>7</sup> However, these findings are based on series of patients derived from families with hereditary hemochromatosis. In the general population, subclinical hemochromatosis can exist undetected for many years. Since iron accumulation can be prevented by phlebotomy, there is increasing interest in genetic screening for genetically determined high-risk groups based on *HFE*. However, Beutler et al, (2002),<sup>8</sup> reported that in subjects older than 26 years, only 30% had subclinical hemochromatosis based on serum transferrin saturation. Although there is a debate about the relevance of screening for hemochromatosis, important parameters such as the sensitivity, specificity, positive and negative predictive values (PPV, NPV) of *HFE* genotyping have not been investigated in a population-based study. Increased levels of serum iron, ferritin and transferrin saturation have been found in subjects homozygous or heterozygous for the C282Y and H63D mutations as well as compound heterozygotes.<sup>8-13</sup> However, it is not clear to what extent the relationship between the



C282Y or H63D heterozygosity and iron status can be explained by subjects who are compound heterozygotes. Further, a significant additive effect on iron status of the H63D mutation was found,<sup>10</sup> suggesting that individuals heterozygous or homozygous for the common H63D mutation may be at increased risk for hemochromatosis and related disorders. Up until now, most population-based studies have targeted relatively young populations.<sup>8,10,14</sup> The mean (SD) age of the largest study<sup>8</sup> conducted to date in caucasians was 58.3 (13.8) years. Due to their subtle effect on iron status, the effect of heterozygosity may be pronounced in older people. Iron accumulation is likely to be more pronounced in elderly men. We know of only one study in the elderly that investigated the effect of heterozygosity for *HFE* C282Y or H63D on iron levels.<sup>15</sup> However, this study included only women who are prior to menopause relatively protected from iron accumulation due to menstruation. The aim of this investigation was to study the contribution of the *HFE* C282Y and H63D mutations to serum iron indices in an elderly population (55 years and over) and to evaluate the usefulness of genetic screening to identify subjects with subclinical hemochromatosis.

## **Materials and Methods**

### **Participants**

The present investigation was conducted within the Rotterdam Study, a population-based study of subjects aged 55 years and over living in a suburb of Rotterdam, The Netherlands. Baseline examination took place in 1990. Details on this cohort study are described elsewhere.<sup>16</sup> Briefly, information on current health status and medical history was obtained by means of a structured interview using a standardised questionnaire. Participants attended the research centre for several clinical and laboratory tests. Fasting blood samples were collected by venepuncture and serum

was kept frozen until analysis. In total 7983 people participated in the Rotterdam Study (response rate 78%). From each participant, written informed consent was obtained. The study was approved by the medical ethics committee of the Erasmus Medical Centre. From the total cohort, we randomly selected a group of 2095 subjects and estimated the frequency of the *HFE* C282Y and H63D mutations. Among the subjects genotyped, we measured the serum transferrin saturation, iron and ferritin levels in a total of 347 individuals selected equally within the six genotype groups based on *HFE* C282Y and H63D, i.e., those without any mutation (the wild type homozygotes Wt/Wt), the H63D heterozygotes (H63D/Wt), the C282Y heterozygotes (C282Y/Wt), the H63D homozygotes (H63D/H63D), the compound heterozygotes (C282Y/H63D) and the C282Y homozygotes (C282Y/C282Y). Five samples with evidence of hemolysis were excluded from the study. A transferrin saturation level above 45% is proposed as a cut off value for the diagnosis of hemochromatosis in population-based screening.<sup>17</sup> In the present study, individuals with a transferrin saturation above 45% were considered as cases of subclinical hemochromatosis.

### **Laboratory analysis**

Genomic DNA was extracted from frozen buffy coat using the salting out protocol as described elsewhere.<sup>18</sup> Fragments of DNA were amplified by the Polymerase Chain Reaction (PCR) using oligonucleotides primers described previously.<sup>5</sup> The 25µl PCR reaction tube contained 100ng of each primer, 2.5µl of 10x PCR buffer, 2.5mM dNTP, 50 mM MgCl<sub>2</sub>, 5 U/µl Ampli Taq Gold and 2 µl (~ 50 ng) DNA. We carried out 15 minutes of initial denaturation at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1.5 minutes at 72°C. Restriction digestion was performed directly on the PCR products by addition of 0.1 U/µl Sna BI (Perkin-Elmer) for codon 282 or 0.1 U/µl Dpn II (Perkin-Elmer) for codon 63 and incubating overnight at 37°C.

The products were electrophoresed on a 3 % agarose gel containing ethidium bromide in presence of a 1Kb DNA standard. Control samples of known status (normozygous, heterozygous and homozygous) for each mutation were included at the PCR stage. Amplification with the primer for codon 282 produces a PCR product of 390 bp, which will not digest the wild type and will digest the mutant to produce 276 bp and 113 bp pieces. Amplification with the primer for codon 63 produces a PCR product of 208 bp, that digests the wild types to produce 138 bp and 70 bp pieces but will not digest the mutant.

Serum iron indices were assessed in stored serum samples (-80°C). Serum iron was measured by immuno(chemiluminescence) assay using Roche/Hitachi 717 kit (Roche). Serum ferritin (µg/l) was measured by colorimetric assay using the Elecsys (Roche). Serum transferrin was measured by immunoturbidimetric assay using Tinaquant kit (Roche) and the transferrin saturation derived as follows: Serum iron (µmol/l) / [2\*transferrin (µmol/L)]/100.

### **Statistical analysis**

Genotype frequencies were estimated by gene counting and allele frequencies calculated from genotype frequencies. Population means of iron indices were estimated by taking the sum of the means of serum iron indices for the genotype weighted with the genotype frequency. To study the relation between iron parameters and *HFE* genotypes, linear regression models were fitted for males and females separately. In these models, the effect of the compound heterozygotes was included as an interaction between the C282Y and H63D mutations. More parsimonious models were considered by assuming additive effects for the two mutations. The models assume that the effect in subjects who are homozygous is twice that of those heterozygous. Additivity was tested for each mutation by comparing the model with

the genotypes as additive effect (coding 0 for WtWt, 1 for the heterozygotes and 2 for the homozygotes) to the model including the genotypes as a categorical variable (codominant model). The models were compared by using the F-statistic. Heritability (percentage variance) of iron parameters due to *HFE* mutations was computed based on the final model and using the population frequencies of the various genotypes. Using the population-based frequencies of the mutations by genotype and the prevalence of subclinical hemochromatosis, the positive predictive value (PPV: the proportion of people with a positive test who have the disease), the negative predictive value (NPV: the proportion of people with a negative test who do not have the disease), the sensitivity (the probability that the test correctly classifies people with preclinical disease as positive) and specificity (the probability that the test classifies as negative those who will not have the disease) were estimated as described elsewhere.<sup>19,20</sup>

## **Results**

Table 1 shows the findings of the genotyping of the *HFE* gene for the C282Y and H63D mutation in the random population-based series of 2095 subjects. The genotypes are ordered by frequency; the Wt/Wt was the most common *HFE* genotype (63.1% in women) and the C282Y/C282Y genotype was the most rare genotype (0.2% in men). Genotype frequencies were in Hardy-Weinberg equilibrium proportions for both men and women. There was only a small non significant decrease in the frequency of the C282Y and H63D mutation by age. In women, the allele frequency of the C282Y mutation varied from 6.4 % in those aged 55-64 years to 6.2% in those 75 years and older, while in men the C282Y allele frequency varied from 6.0 % to 5.0%.

**Table 1.** Frequency of *HFE* genotypes in a population-based random sample of 2095 subjects

Genotype	Men	Women
Wt/Wt*	604 (60.6)	693 (63.1)
H63D/Wt	250 (25.1)	241 (21.9)
C282Y/Wt	99 (9.9)	107 (9.7)
H63D/H63D	29 (2.9)	30 (2.7)
C282Y/H63D	13 (1.3)	23 (2.1)
C282Y/C282Y	2 (0.2)	4 (0.4)

\* Wt = Wild type (absence of mutation).

Values are numbers (%) of individuals.

For the H63D mutation, the frequency of the genotypes also did not vary significantly by age group. The frequency for those 55 to 64 was 14% compared with 19% for those 75 years and over.

Table 2 shows the population prevalence of subclinical hemochromatosis and the weighted means of serum transferrin saturation, iron and ferritin for men and women.

**Table 2.** Population prevalence of subclinical hemochromatosis and weighted means (SD) of serum iron parameters

	Men n=169	Women n=173
Prevalence of subclinical hemochromatosis (%)	0.06	0.03
Transferrin Saturation in %	29.32 (12.36)	26.28 (10.12)
Iron in ug/ml	17.47 (6.28)	16.03 (5.38)
Ferritin in ng/ml	205.46 (152.84)	155.11 (113.25)

The population prevalence of hemochromatosis was higher in men compared to women. We observed that men had higher levels of serum transferrin saturation, iron and ferritin compared to women. The mean (SD) age was 67.12 (6.59) years for men and 67.08 (6.32) years for women. When testing the effect of each mutation on the iron parameters, additivity for C282Y was rejected whereas additivity for H63D could not be rejected for all outcomes (data not shown). When testing for interaction between the two *HFE* mutations, a significant interaction between the C282Y and H63D alleles was found for serum transferrin saturation ( $P=0.004$ ), for serum iron ( $P=0.003$ ) and for serum ferritin ( $P=0.006$ ) (data not shown). This suggests that the effect of the two mutations in compound heterozygotes is higher than that predicted from the effect in heterozygotes. Table 3 shows the effect of *HFE* mutations on iron parameters. Serum transferrin saturation, iron and ferritin levels were significantly higher ( $P<0.001$ ) for those homozygous for the C282Y mutation in men as well as women compared to those homozygous for the wild type. There was a significant interaction between sex and C282Y homozygosity for all iron parameters, suggesting a more pronounced effect in men. Compound heterozygosity was associated with a significant effect on all serum iron indices except for ferritin in women. Also for the compound heterozygotes, there was a significant difference in effect between men and women. The H63D mutation was associated with significantly increased levels of serum transferrin and iron in men and women. Heterozygosity for the C282Y mutation was not significantly associated with serum iron, ferritin and transferrin saturation when analysing men and women separately. The effects were similar in men and women. When combining men and women, we observed a significant association of the C282Y heterozygous state with higher levels of serum transferrin saturation ( $P<0.03$ , data not shown).

**Table 3.** Differences in means serum iron indices between carriers of *HFE* mutations and the wild type

Genotype	Men N=169		Women n=173	
		P value <sup>#</sup>		P value <sup>#</sup>
SERUM TRANSFERRIN SATURATION in % (range)				
H63D/Wt* <sup>†</sup>	3.75 (1.26 to 6.25)	0.003	4.12 (1.80 to 6.43)	0.001
H63D/H63D <sup>†</sup>	7.52 (2.52 to 12.5)		8.24 (3.60 to 12.86)	
C282Y/Wt	3.77 (-0.9 to 8.44)	0.11	3.45 (-0.72 to 7.62)	0.10
C282Y/H63D	16.54 (11.19 to 21.88)	<0.001	8.67 (4.22 to 13.12)	<0.001
C282Y/C282Y	46.46 (31.48 to 61.44)	<0.001	18.88 (10.85 to 26.91)	<0.001
SERUM IRON in ug/ml (range)				
H63D/Wt* <sup>†</sup>	1.80 (0.46 to 3.14)	0.009	1.88 (0.59 to 3.16)	0.004
H63D/H63D <sup>†</sup>	3.61 (0.92 to 7.28)		3.76 (1.18 to 6.32)	
C282Y/Wt	1.03 (-1.48 to 3.54)	0.42	1.30 (-1.02 to 3.61)	0.27
C282Y/H63D	7.77 (4.90 to 10.64)	<0.001	3.43 (0.95 to 5.90)	0.007
C282Y/C282Y	16.84 (8.8 to 24.88)	<0.001	6.62 (2.16 to 11.08)	0.004
SERUM FERRITIN in ng/ml (range)				
H63D/Wt* <sup>†</sup>	40.09 (-6.53 to 86.71)	0.09	9.00 (-25.42 to 43.41)	0.61
H63D/H63D <sup>†</sup>	80.18 (-13.06 to 173.42)		18.00 (-50.84 to 86.42)	
C282Y/Wt	2.11 (-85.29 to 89.51)	0.96	44.60 (-17.44 to 106.63)	0.16
C282Y/H63D	200.84 (100.89 to 300.79)	<0.001	44.16 (-22.08 to 110.40)	0.19
C282Y/C282Y	477.95 (198.00 to 757.89)	0.001	211.65 (92.13 to 331.17)	0.001

\*Wt = Wild type.

<sup>†</sup> One degree of freedom.<sup>#</sup> P value of difference compared to wild type value.

Together the *HFE* genotypes explained about 6% of the variability of serum transferrin saturation in men and 5% in women whereas the proportion of variability of serum iron and ferritin was less than 5% in men and women.

Twenty-one men (13%) and 14 women (8%) had serum transferrin saturation over 45%. When using the cut-off point of 45% to define subjects with subclinical hemochromatosis, all men (100%) and 67% of the women homozygous for C282Y showed levels of serum transferrin saturation above the cut off. None of the subjects were known by their treating physician with a diagnosis of hemochromatosis. Table 4 shows the PPV, NPV, sensitivity and specificity of *HFE* genotypes. The PPV for subclinical hemochromatosis varied from 100% in men homozygous for C282Y to 2.8% in women heterozygous for H63D while the sensitivity varied from 3.6% for men homozygous for C282Y to 37% for men heterozygous for H63D.

**Table 4.** Positive and negative predictive values, sensitivity and specificity of *HFE* genotypes

Genotype	Men n=169		Women n=173	
	NPV* (%)	Specificity (%)	NPV (%)	Specificity (%)
Wt/Wt	97.2	62	97.4	64
	PPV† (%)	Sensitivity (%)	PPV (%)	Sensitivity (%)
H63D/Wt	8.1	36.6	2.8	18.2
C282Y/Wt	8.3	14.8	2.9	8.3
H63D/H63D	12.1	6.3	7.4	5.9
C282Y/H63D	34.8	8.1	17.9	11.1
C282Y/C282Y	100	3.6	66.7	7.9
All <i>HFE</i> genotypes	9.8	69.5	4.7	51.5

\*NPV: Negative predictive value.

†PPV: Positive predictive value.



For all *HFE* genotypes, the PPV was 10% for men and 5% for women, the sensitivity was 70% for men and 52% for women and the specificity was 62% for men and 64% for women. The NPV for men as well as women was over 97%.

## **Discussion**

In this population-based sample of elderly, 0.3% were homozygous and 10% heterozygous for the C282Y while 3% were homozygous and 23% heterozygous for the H63D mutation. These frequencies are very similar to that observed in younger populations<sup>21,22</sup> and in a sample of elderly men.<sup>23</sup> In line with the findings of Beutler et al, (2002),<sup>8</sup> we did not find evidence for a significant decrease in the frequency of the *HFE* C282Y mutation or H63D mutation with age. This finding suggests that these mutations may remain putative risk factors for chronic diseases throughout late age.

Serum transferrin saturation and serum iron levels were significantly increased in those homozygous or compound heterozygous for C282Y as well as H63D in both men and women. Subjects heterozygous for the H63D or C282Y mutation showed a modest but statistically significant increase in serum transferrin when combining the findings of men and women (adjusted for age and sex). In those homozygous and in compound heterozygous, the effect of *HFE* was significantly stronger in men. Although the H63D mutation has been regarded as non-functional, our results show that the H63D mutation is associated with increased serum iron and transferrin saturation independent of the presence of the C282Y allele. This finding is in line with previous reports of large population-based studies.<sup>10,14</sup> Serum ferritin showed the weakest association with the two *HFE* mutations studied. A significantly higher serum ferritin was observed only for the C282Y homozygotes (men and women) and

compound heterozygous men. This is for a large part explained by the fact that serum ferritin has by far the largest standard deviation. Therefore the power to assess a relation between the *HFE* mutations and serum ferritin was very low.

In this study we considered patients with serum transferrin levels above 45% as having subclinical hemochromatosis. Earlier studies have shown that using this cut-off point, 98% of patients with hereditary hemochromatosis may be detected.<sup>17</sup> None of the patients with a serum transferrin saturation level over 45% in our study was known by themselves or their treating physician with a clinical diagnosis of hemochromatosis. Using serum transferrin saturation above 45%, the PPV is high (100%) for men homozygous for C282Y as well as women homozygous for the C282Y mutation (67%). Thus, screening for C282Y homozygosity is helpful to identify high risk groups. These carriers do have a high risk of iron overload. These findings appear to be at odds with those of Beutler et al, (2002)<sup>8</sup> who reported that less than 1% of C282Y homozygous subjects develop frank clinical hemochromatosis and 30% have subclinical hemochromatosis. This contradictory finding may be related to the case definition of hemochromatosis. Beutler et al, (2002)<sup>8</sup> used a combination of serum iron parameters and signs and symptoms of hemochromatosis to define the disease. Moreover, signs and symptoms of hemochromatosis are very unspecific and subjects 'non-affected' at the time of the study may develop the disease later. We therefore used the "golden standard" method of population-based screening for hemochromatosis using transferrin saturation above 45%.<sup>17</sup> Serum transferrin saturation over 45% is a risk factor for several diseases including liver pathology as seen in the study of Beutler et al, (2002)<sup>8</sup>.

Although this study suggests that screening for the C282Y mutation may be effective to detect subjects with increased transferrin saturation, whether the *HFE* C282Y and

H63D mutations can be used to detect persons with subclinical hemochromatosis in the population depends not only on the PPV but also on the sensitivity and the specificity. We found that screening for hemochromatosis based on *HFE* genotypes has a sensitivity of 70% for men and 53% for women and a specificity of 62% for men and 64% for women. These values suggest that many subclinical cases will be undetected when screening based on the *HFE* genotypes. At first this observation is at odds with the findings that 80% of patients with hemochromatosis are explained by homozygosity for the C282Y mutation. However, these findings are based on patients with hereditary forms of iron disease<sup>6,25</sup> and the number of patients with a genetic (*HFE*) origin may therefore be high. The situation is very different in the general population. Although our study suggests that the *HFE* C282Y and H63D mutations are determinants of physiological iron levels, the *HFE* genotypes explained no more than 5% of the variability in serum iron indices. This observation is in agreement with estimates derived from a sample of twins<sup>10</sup> and points towards the existence of other factors than *HFE* genotypes that account for the serum levels of iron. These may concern genetic as well as environmental factors. Our findings in the general elderly population suggest that the value for screening for high iron based on *HFE* genotypes is limited.

In conclusion, our study shows that *HFE* C282Y and H63D are common mutations in the elderly. At late age, these mutations may still be determinants of serum transferrin, iron and ferritin. Heterozygosity for the C282Y and H63D mutations is associated with small effects on serum iron parameters. Although the PPV is high in C282Y homozygotes, screening for hemochromatosis by *HFE* genotyping is of limited value because of the low sensitivity leaving a large number of subjects with increased serum transferrin saturation levels undetected.

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## References

1. Bhavnani M, Lloyd D, Bhattacharyya A, Marples J, Elton P, Worwood M. Screening for genetic hemochromatosis in blood samples with raised alanine aminotransferase. *Gut* 2000;46:707-10.
2. Powell LW, Jazwinska E, Halliday JW. Primary iron overload. In: Brock JH, Halliday JW, Pippard MJ, and Powell LW. Iron metabolism in health and disease. WB Saunders, London, pp. 227-70.
3. Edwards CQ, Griffen LM, Goldgar D. Prevalence of hemochromatosis among 11,605 presumably healthy blood donors. *New Engl J Med* 1988;318:1355-62.
4. de Villiers JN, Hillerman R, Loubser L, Kotze JM. Spectrum of mutations in the *HFE* gene implicated in hemochromatosis and porphyria. *Hum Mol Genet* 1999;8:1517-22.
5. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
6. Jouanolle, AM, Gandon G, Jezequel P, Blayau M, Campion ML, Yaouanq J, et al. Haemochromatosis and HLA-H. *Nature Genet* 1996;14:251-2.
7. de Valk B, Witlox RS, van der Schouw YT, Marx JJ. Biochemical expression of heterozygous hereditary hemochromatosis. *Eur J Intern Med* 2000;11:317-21.
8. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. *Lancet* 2002;359:211-8.
9. Rossi E, Bulsara MK, Olynyk JK, Cullen DJ, Summerville L, Powell LW. Effect of hemochromatosis genotype and lifestyle factors on iron and red cell indices in a community population. *Clinical Chemistry* 2001;47:202-8.
10. Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL and Martin NG. Effects of *HFE* C282Y and H63D polymorphism and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000;66:1246-58.

11. Bulaj ZJ, Griffen LM, Jorde LB, Edwards CQ, Kushner JP. Clinical and biochemical abnormalities in people heterozygous for hemochromatosis. *N Engl J Med* 1996; 335:1799-805.
12. Bacon BR, Powell LW, Adams PC, Kresina TF, Hoofnagle JH. Molecular medicine and hemochromatosis: At the crossroads. *Gastroenterology* 1999;116:193-201.
13. Adams PC. Prevalence of abnormal iron stores in heterozygotes for hereditary hemochromatosis: an analysis of 255 heterozygotes. *Am J Hematol* 1994;45:146-9.
14. Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population based study of the clinical expression of the hemochromatosis gene. *N Engl J Med* 1999;341:718-24.
15. Rossi E, Olynyk JK, Cullen DJ, Papadopoulos G, Bulsara M, Summerville L, Powell LW. Compound heterozygous hemochromatosis genotype predicts iron and erythrocyte indices in women. *Clinical Chemistry* 2000;46:162-6.
16. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of diseases and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol* 1991;4:403-22.
17. McLaren CE, McLachlan GJ, Halliday JW, Webb SI, Leggett BA, Jazwinska EC, et al. Distribution of transferrin saturation in an Australian population: relevance to the early diagnosis of hemochromatosis. *Gastroenterology* 1998;114:543-9.
18. Miller SA, Dykes DD, Polesky, HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 1988;16:1215.
19. Holtzman NA, Marteau TM. Will genetics revolutionize medicine? *N Engl J Med* 2000;343:141-4.
20. Wald NJ, Hackshaw AK, Frost CD. When can a risk factor be used as a worthwhile screening test? *BMJ* 1999;319:1562-5.
21. Baer DM, Simons JL, Staples RL. Hemochromatosis screening in asymptomatic ambulatory men 30 years of age and older. *Am J Med* 1995;98:464-8.
22. Cullen LM, Summerville L, Glassick TV, Crawford DHG, Powell LW, Jazwinska EC. Neonatal screening for hemochromatosis defect. *Blood* 1997;90:4236-7.
23. Willis G, Wimperis JZ, Smith KC, Fellows IW, Jennings BA. Haemochromatosis gene C282Y homozygotes in an elderly male population. *Lancet* 1999;354:221-2.
24. Burt MJ, George PM, Upton JD, Collett JA, Frampton CMA, Chapman TM, Walmsley TA, Chapman BA. The significance of haemochromatosis gene mutations in the general population: implications for screening. *Gut* 1998;43:830-6.
25. Jazwinska EC, Cullen LM, Busfield F, Pyper WR, Webb SI, Powell LW, et al. Haemochromatosis and HLA-H. *Nature Genet* 1996;14:249-51.



## ***HFE* gene mutations and liver function in the elderly**

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### **Summary**

Accumulation of iron in carriers of the mutations in the hemochromatosis gene (*HFE*) may result in liver damages at old age. We studied the effects of *HFE* mutations and body iron indexes on liver function and mortality in the elderly. We genotyped 2095 subjects for the C282Y and H63D mutations who were randomly derived from the Rotterdam Study, a population based study of elderly people. For 1576 of the subjects genotyped, serum liver function tests (LFT) were available. Serum iron, ferritin and transferrin saturation were measured in a total of 342 subjects selected within each genotype category. There was no difference in the mean of alanin aminotransferase, alkaline phosphatase and total protein by *HFE* genotypes. Serum alanin aminotransferase increased significantly with increasing serum ferritin. Increased total bilirubin was associated significantly ( $P=0.001$ ) with high serum iron and transferrin saturation levels and in women also with higher serum ferritin levels. Mean bilirubin in men heterozygous (9.9, 95%CI 9.4-10.5) and homozygous (11.1, 95%CI 8.8-13.9) and women homozygous (8.8, 95%CI 7.4-10.5) for the H63D mutation was significantly higher compared to men (9.1, 95%CI 8.8-9.4) and women (7.7, 95%CI 7.5-7.9) homozygous for the wild type allele. Serum bilirubin was significantly ( $P<0.001$ ) associated with lower mortality rate in men. There was no evidence that *HFE* mutations affect liver function. However, levels of serum iron indices are associated with liver functioning, suggesting that in addition to *HFE* other determinants of iron metabolism may be important. The association of the H63D mutation with higher bilirubin, which in turn is associated with a significant reduction in mortality, is of interest.

## Introduction

Hereditary hemochromatosis is an autosomal recessive disorder that leads to increased intestinal absorption of iron with progressive deposition in tissues, especially the liver.<sup>1,2</sup> The disease affects Caucasian populations of predominantly northern European descent, with a carrier rate of 1 in 10 and a prevalence rate of homozygotes up to 1 in 200-400.<sup>3,4</sup> In over 80% of patients, the disease is explained by mutations in the *HFE* gene.<sup>1,5</sup> The *HFE* gene has been mapped to the short arm of chromosome 6.<sup>5</sup> The predominant mutation is a single base transition, G845A, leading to substitution of a cysteine residue by tyrosine at position 282 (C282Y) of the *HFE* protein.<sup>1,5</sup> The other more common mutation is the C187G transversion leading to a substitution of histidine by aspartic acid at position 63 (H63D) of the *HFE* protein.<sup>1,5</sup> Although the functional effects of H63D are disputed, several reports show mild increase in body iron indexes in carriers.<sup>6,7</sup> At old age, H63D and C282Y mutations may lead to a mild form of iron overload in subjects heterozygous for the mutation.<sup>8</sup> Even in modest tissue concentrations, iron may potentiate liver cell injury.<sup>9</sup> *HFE* C282Y and H63D mutations are reported to be a co-factor for chronic liver diseases.<sup>10,11</sup> A relationship between *HFE* mutations and liver disease was recently reported.<sup>12</sup> We studied the effects of the *HFE* mutations and serum iron indices on liver function assessed by liver function tests (LFT) in an elderly population. Further, the liver function tests found to be related to *HFE* mutations were evaluated for their clinical relevance by associating them to overall mortality.



## **Materials and Methods**

### **Design of the Study**

The present study was conducted in the framework of the Rotterdam Study, a population-based study of chronic and disabling disorders in the elderly. The Rotterdam Study consists of 7983 (response rate 78%) inhabitants aged 55 years and over living in the district of Ommoord in Rotterdam. Full subjects' recruitment, data acquisition and baseline examinations, on which this study is based, took place between 1990 and 1993 by means of a structured interview using a standardised questionnaire. Blood samples were collected on the day of baseline examination by venepuncture. The study was approved by the medical ethics committee of the Erasmus Medical Centre and informed consent was obtained from all subjects. Participants were followed up to 11.3 years. Information on the vital status of all participants was obtained at regular intervals from municipal health authorities in Rotterdam. At the time of this study, follow up on the vital status was completed for 7549 persons and 444 subjects (5.6%) had incomplete follow up. As persons with yet undetermined liver function tests were excluded ( $n=3223$ ), 4316 individuals were included in the survival analysis.

We studied the relation between *HFE* mutations and liver function measured by serum liver function tests. From the total cohort, 2095 subjects were randomly selected and were genotyped for the *HFE* C282Y and H63D mutations. Of these subjects, 1576 persons had serum liver function tests available. To optimise the statistical power for studying the effects of serum iron indices on liver function, a total of 342 age and sex stratified subjects were randomly selected within the six genotype groups based on *HFE* C282Y and H63D. These groups were defined as those without any mutation (wild type homozygotes, Wt/Wt), H63D heterozygotes

(H63D/Wt), C282Y heterozygotes (C282Y/Wt), H63D homozygotes (H63D/H63D), compound heterozygotes (C282Y/H63D) and C282Y homozygotes (C282Y/C282Y). In these samples we assessed iron indices relevant to hemochromatosis, i.e. serum transferrin saturation, serum iron and serum ferritin.

### **Measurements**

Genomic DNA was extracted from a frozen buffy coat using the salting out protocol as described elsewhere.<sup>13</sup> The *HFE* H63D and C282Y mutation analyses were performed as described previously.<sup>5</sup>

We measured serum alanin aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total bilirubin, albumin and total protein according to the protocols of the International Federation of Clinical Chemistry.<sup>14-19</sup> Serum ferritin was measured by calorimetric assay using the Elecsys kits from Roche. Serum iron was measured by immuno(chemiluminescence) assay using Roche/Hitachi 717 Kit. Serum transferrin was measured by immunoturbidimetric assay using Tina-quant kit (Roche) and the transferrin saturation derived as follows:

Serum iron ( $\mu\text{mol/l}$ ) / [2\*transferrin ( $\mu\text{mol/L}$ )]/100.

### **Statistical Methods**

The normality test was carried out on all variables using the Kolmogorov-Smirnov test. We used logarithm transformations for highly skewed variables including ALT, AST, alkaline phosphatase, total bilirubin, ferritin, age at the time of entry to the study and follow up time. Medians were estimated by means of Tukey's biweight test for variables with skewed distribution. Independent t-statistics, Mann-Whitney and  $\chi^2$  tests were used for comparisons of means and frequencies between groups. We fitted statistical models using multivariate linear regression to predict variability in liver function tests by genotypes and levels of serum iron indices. The effect of compound

heterozygotes was introduced in these models as interaction between C282Y and H63D mutations. We adjusted our analyses for important factors associated with liver function tests such as age (years), gender, body mass index (BMI, kg/m<sup>2</sup>), history of chronic use of medications and alcohol consumption (g/day).

The Kaplan Meier method of survival analysis was used to estimate the cumulative survival rates. Breslow's test were used to compare the survival probability curves and Cox proportional hazard regression analysis that controlled for left truncation and afore mentioned covariables was used to assess the significance level of the *HFE* associated liver function tests with overall mortality which is reported as hazard ratio. Continuous variables by genotypes are reported as means with 95 percent confidence interval (95%CI), unless otherwise specified. The magnitude of the association between two continuous variables is reported as regression coefficient ( $\beta$ ) that is the increment in liver function tests for one unit increase in a serum iron indice. A two tailed p-value less than 0.05 was considered as significant. Statistical analysis was performed with SPSS version 10 for Windows software except for survival analysis that was performed with S-Plus 2000 for Windows.

## **Results**

The characteristics of studied subjects are presented in table 1. Mean ( $\pm$ standard deviation) age was 66 ( $\pm$ 6.6) years in men and 66.2 ( $\pm$ 6.9) years in women. Age adjusted means of liver function tests, serum iron indices and red blood cell parameters differed significantly between men and women. Baseline characteristics and genotype prevalence did not differ significantly between participants with available liver function test compared to participants without liver function tests.

**Table 1.** Characteristics of participants

	Women (n=1098)		Men (n=997)	
	n	Mean (95% CI)	n	Mean (95% CI)
Age (yr)	859	66.10 (65.65-66.55)	708	66.40 (65.91-66.89) <sup>†</sup>
BMI (kg/m <sup>2</sup> )	849	26.80 (25.53-27.07)	699	25.70 (25.48-25.92) <sup>†</sup>
Alcohol intake (g/day)	764	6.90 (6.14-7.66)	609	17.50 (15.93-19.07) <sup>†</sup>
ALT (u/l) <sup>§</sup>	846	16.20 (15.70-16.70)	693	18.00 (17.40-18.60) <sup>†</sup>
AST (u/l) <sup>§</sup>	746	19.70 (19.30-20.10)	693	20.30 (19.90-20.70) <sup>‡</sup>
Alkaline phosphates (u/l) <sup>§</sup>	840	76.40 (75.10-77.80)	693	73.00 (71.50-74.60) <sup>†</sup>
Total bilirubin (μmol/l) <sup>§</sup>	762	7.80 (6.10-8.00)	624	9.50 (9.20-9.70) <sup>†</sup>
Total protein (g/l)	855	71.90 (71.57-72.23)	700	71.70 (71.37-72.03)
Albumin (g/l)	850	42.90 (42.74-43.06)	705	43.30 (43.10-43.50) <sup>†</sup>
RBC (10 <sup>12</sup> /l)	750	4.60 (4.57-4.63)	478	4.80 (4.77-4.83) <sup>†</sup>
Hemoglobin (mmol/l)	631	8.60 (8.55-8.65)	478	9.30 (9.23-9.37) <sup>†</sup>
Hematocrite (l/l)	631	40.35 (40.14-40.57)	607	43.52 (43.24-43.81) <sup>†</sup>
Iron (μmol/l)	143	17.03 (16.15-17.91)	104	18.91 (17.85-19.96) <sup>†</sup>
Transferrin saturation (%)	143	28.90 (27.25-30.55)	104	32.20 (34.08-34.32) <sup>‡</sup>
Ferritin (ng/l) <sup>§</sup>	143	129.00 (114.00-146.60)	103	158.60 (135.00-186.70)

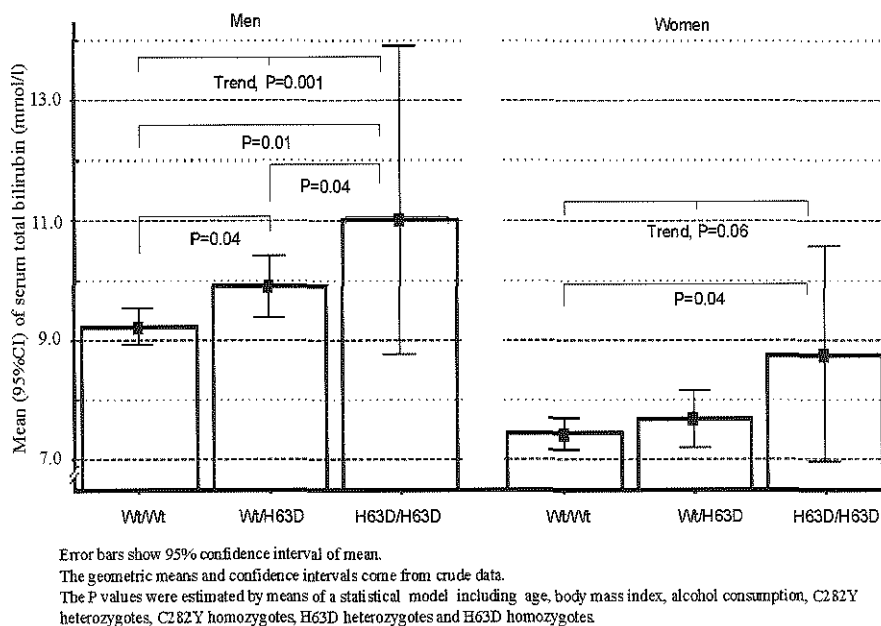
ALT: serum alanin aminotransferase; AST: serum aspartate aminotransferase.

Significance men versus women: ‡ P=0.05; † P=0.001; ‡ P=0.02.

§: Given as geometric mean (95% CI).

Distributions of baseline parameters were not different among genotypes except for BMI that was higher in women heterozygous for C282Y (data not shown). The prevalence of genotypes was 61.9% for Wt/Wt, 23.4% for H63D/Wt, 9.8% for C282Y/Wt, 2.8% for H63D/H63D, 1.7% for H63D/C282Y and 0.3% for

C282Y/C282Y. There were no statistically significant differences in genotype frequencies between men and women. Genotype and allele frequencies were in Hardy-Weinberg equilibrium proportions. Distribution of liver function tests showed high variability. Median (range) of liver function tests was 17.0 (2.0-151.0) for ALT, 19.7 (7.0-217.0) for AST, 76.0 (27.0-404.0) for alkaline phosphatase, 8.0 (1.0-61.0) for total bilirubin, 43.0 (22.0-60.0) for albumin and 72.0 (51.0-114.0) for total protein. Age adjusted means (95%CI) of liver function tests by *HFE* genotypes for men and women are given in table 2. Mean serum total bilirubin was found to be significantly increased in men heterozygous (8%) and homozygous (21%) and women homozygous (14%) for H63D compared to Wt/Wt. Men homozygous for the H63D mutation had an 11% increase in mean serum total bilirubin compared to men H63D heterozygous ( $P=0.04$ ). Bilirubin increased significantly with the number of H63D alleles (Trend test,  $P$  men=0.001,  $P$  women=0.056, figure 1).



**Figure 1.** Geometric mean of serum total bilirubin level by H63D mutation status

**Table 2.** Means (95%CI) of serum liver function tests by *HFE* mutations ‡

<i>HFE</i> Genotype (n=1567)	Men				Women			
	ALT* (u/l)	AST* (u/l)	Total Bilirubin* (μmol/l)	Albumin (g/l)	ALT * (u/l)	AST * (u/l)	Total Bilirubin* (μmol/l)	Albumin (g/l)
Wt/Wt (970)	18.0 (17.3-18.8)	20.0 (19.7-20.9)	9.1 (8.8-9.4)	43.3 (43.1-43.5)	16.0 (15.4-16.7)	19.7 (19.3-20.1)	7.7 (7.5-7.9)	43.1 (42.9-43.3)
Wt/H63D (367)	18.3 (17.2-19.6)	21.0 † (20.1-21.9)	9.9 † (9.4-10.5)	43.3 (42.9-43.7)	15.8 (14.9-16.8)	19.2 (18.4-19.9)	7.9 (7.5-8.2)	42.6 † (42.2-43.0)
Wt/C282Y (158)	16.6 (14.7-8.6)	18.7 (17.5-19.8)	10.0 (9.2-10.9)	43.1 (42.5-43.7)	18.2 (15.6-19.0)	19.7 (18.6-20.9)	7.9 (7.2-8.5)	42.7 (42.1-43.3)
H63D/H63D (45)	18.0 (15.5-21.0)	20.5 (17.1-23.7)	11.1 ¶ (8.8-13.9)	43.7 (42.7-44.7)	16.3 (13.6-20.5)	20.4 (17.4-24.0)	8.8† (7.4-10.5)	42.6 (41.6-43.6)
H63D/C282Y(27)	16.4 (11.6-23.0)	20.9 (16.0-27.2)	9.4 (7.9-11.1)	43.9 (42.3-45.5)	15.6 (12.2-19.9)	19.8 (17.7-22.0)	8.0 (6.8-9.4)	43.0 (42.2-43.8)
C282Y/C282Y(5)	16.9 (3.8-75.8)	19.7 (0.6-650)	9.2 (0.3-281)	42.6 (39.3-45.9)	23.1 (10.0-53.5)	22.4 (17.4-23.0)	8.6 (5.4-13.5)	41.1 (39.7-42.5)

Wt: Wild type variant, ALT: alanin aminotransferase, AST: aspartate aminotransferase.

‡: Statistical models were adjusted for age, BMI and alcohol consumption (for total bilirubin, serum albumin level was also included). Dependent variables were transformed values, except serum albumin.

\*: Given as geometric mean (95%CI).

Significance compared to Wt/Wt: † P= 0.05; ¶ P=0.004.

Women heterozygous for the H63D mutation had a significantly lower mean of serum albumin compared to Wt/Wt. AST levels were significantly ( $P=0.05$ ) increased in men heterozygous for H63D compared to Wt/Wt but this was not observed in H63D homozygotes. The H63D mutation was not associated with serum total protein and alkaline phosphatase in men or women (data not shown). No significant relation was found between the C282Y mutation and liver function tests.

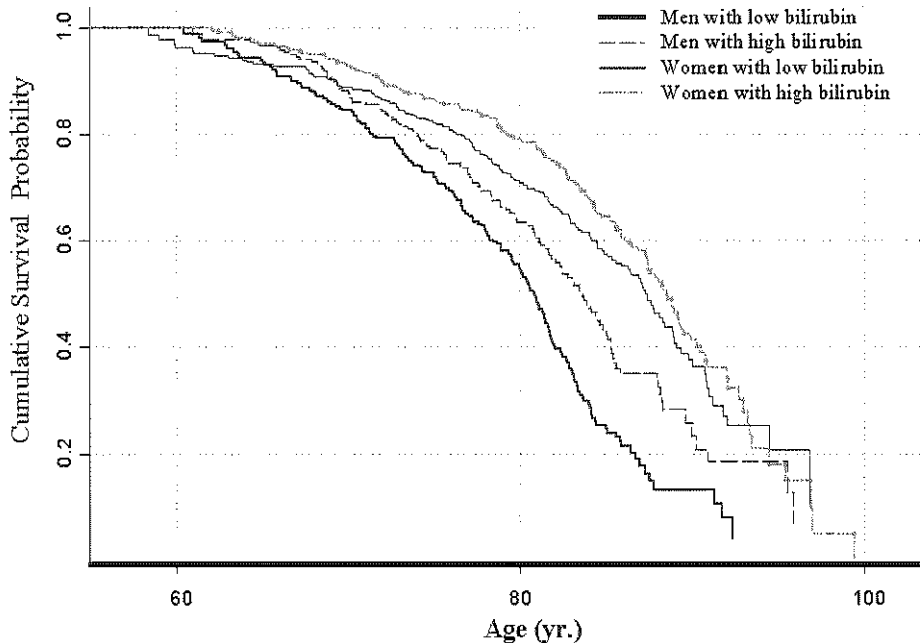
The relationships between levels of serum iron indices and liver function tests in men and women are summarised in table 3. In men, serum ferritin, serum iron and serum transferrin saturation were associated with higher levels of ALT. In women, serum ferritin was significantly associated with raised ALT, AST and serum total bilirubin. In both men and women, serum iron and transferrin saturation were significantly related to bilirubin. In men serum ferritin was also related to albumin. There were very small changes in the magnitude and significance of serum iron indices on liver function tests when we adjusted for *HFE* genotypes.

In the statistical models fitted with both *HFE* genotypes and serum iron parameters, the effect of the genotype turned to be non significant when the models were adjusted for serum iron or serum transferrin saturation levels, except for total bilirubin.

Men heterozygous for the H63D mutation had significantly ( $P=0.001$ ) higher mean ( $\pm$ SE) level of hemoglobin ( $9.50\pm0.06$ ) compared to Wt/Wt ( $9.22\pm0.04$ ). In men, heterozygosity for the H63D mutation was associated with a significantly higher mean hematocrite level ( $44.00\pm0.29$ ,  $P=0.002$ ) and red blood cell count ( $4.96\pm0.03$ ,  $P<0.05$ ) compared to Wt/Wt ( $43.00\pm0.18$  and  $4.88\pm0.02$ , respectively). In women, hemoglobin level was higher ( $P=0.06$ ) in H63D homozygotes and in compound heterozygotes ( $P<0.05$ ) compared to those homozygous for the wild type allele. Men

heterozygous for C282Y had significantly ( $P=0.016$ ) higher hemoglobin levels compared to Wt/Wt.

The Kaplan Meier method of survival analysis estimated that survival probability for men was 0.62 (95%CI 0.57-0.67) and 0.50 (95%CI 0.36-0.67) in participants with higher and lower serum bilirubin level, respectively. In women, these estimates were 0.66 (95%CI 0.62-0.69) and 0.69 (95%CI 0.65-0.72) respectively (figure 2). Further investigation with Cox proportional hazard regression analysis indicated that higher serum total bilirubin level was significantly ( $P=0.0005$ ) associated with lower mortality rate in men (hazard ratio=0.66, 95%CI 0.52-0.83), but this association was not significant ( $P=0.27$ ) in women (hazard ratio=0.88, 95%CI 0.70-1.11).



**Figure 2.** The effect of total bilirubin on survival by gender



**Table 3.** Regression coefficient (95%CI)×10<sup>2</sup> of serum iron indices effects on liver function tests

	Men				Women			
	ALT	AST	Total	Albumin	ALT	AST	Total	Albumin
	(u/l)	(u/l)	Bilirubin	(g/l)	(u/l)	(u/l)	Bilirubin	(g/l)
			(μmol/l)				(μmol/l)	
Ferritin	0.09 †	-7.2e-04	0.04	0.51 ¶	0.12 ‡	0.12 ‡	0.08 †	0.20
	(0.01;0.17)	(-0.05;0.05)	(-0.04;0.11)	(0.13;0.89)	(0.05;0.18)	(0.07;0.17)	(0.01;0.15)	(-0.13;0.53)
Iron	2.71 ¶	0.96	2.95 *	-1.47	0.25	0.82	2.99 *	0.25
	(0.66;4.74)	(-0.19;2.12)	(1.35;4.55)	(-11.52;8.59)	(-1.52;2.03)	(-0.59;2.22)	(1.47;4.51)	(-8.37;8.88)
Transferrin	1.16 †	0.50	1.37 ‡	-1.14	-0.14	0.17	1.70 *	-1.61
saturation	(0.09;2.23)	(-0.09;1.10)	(0.54;2.20)	(-6.295;4.02)	(-1.08;0.81)	(-0.58;0.92)	(0.91;2.49)	(-6.21;2.98)

ALT: alanin aminotransferase, AST: aspartate aminotransferase.

Regression models were adjusted for age, BMI, alcohol consumption (total bilirubin also adjusted for serum albumin level).

Dependent variables were natural logarithm transformed values, except for serum albumin.

Significance: † P<0.05; ¶ P<0.01; ‡ P<0.001; \* P<0.0001.

## Discussion

We found no association between liver function test and *HFE* mutations except for total bilirubin. We observed that H63D heterozygotes and H63D homozygotes had higher means of serum total bilirubin which were for a large part explained by the increase in serum transferrin saturation and serum iron level in carriers. Although the clinical implications of these relationships need more investigations, we found that higher total bilirubin was significantly associated with longevity. Also serum ALT, AST and bilirubin levels increased significantly with increasing levels of serum iron indices in men and women.

Possible biases that could affect the reliability of our findings include genotyping and measurements errors. However, measurements errors in LFT are not expected to be related to *HFE* mutations. We studied a sub-sample of the Rotterdam Study for which liver function tests as well as *HFE* genotypes were available. No differences in baseline characteristics and genotype frequencies were found between this sub-sample and the full cohort suggesting this sub-sample is representative of the full cohort. The small number of participants with extreme genotypes (compound heterozygotes and C282Y homozygotes) resulted in a low statistical power to examine the possible relation of these genotypes with liver function tests.

The *HFE* gene could not explain liver function tests variability within our population-based study. Our findings support those of George et al, (1999)<sup>10</sup> who reported that the hepatic iron concentration is the most important determinant of chronic liver disease and mutations in the *HFE* gene are only a co-factor for liver damage by increasing iron levels. Although a relation between *HFE* and liver diseases was reported recently,<sup>12</sup> our findings and those of others suggest that C282Y

heterozygosity does not appear to lead to significant hepatic iron overload<sup>20</sup> and development of liver fibrosis<sup>3</sup> or cirrhosis.<sup>11,21</sup> It has been reported that major hepatic toxicity of iron overload leads to damage of multiple cell types and to multiple sub-cellular organelles and is a contributory factor in the development of other liver diseases.<sup>22</sup> It is also proposed that minor elevation of body iron stores indicated by increase in serum ferritin or transferrin saturation levels may not be sufficient to result in increased hepatic iron concentration and subsequent hepatocyte injury.<sup>20,21,23</sup> Although the mean serum ALT was not abnormal in our population, our findings suggest that limited increase in serum iron levels may result in hepatic injury associated with increase in serum ALT. The fact that we did not find any association between liver function tests and *HFE* mutations but observed significant associations with serum iron indices, supports earlier reports that the *HFE* genotypes studied explain a limited extent of body iron indices among populations.<sup>7,24</sup>

We found that higher serum bilirubin levels were associated specifically with the *HFE* H63D mutation. This may be explained by the reduced uptake of bilirubin by the liver or influence of this mutation on erythrocyte parameters particularly hemoglobin, in carriers. Barton et al, (2000)<sup>25</sup> and Rossi et al, (2001)<sup>26</sup> reported the association of the *HFE* mutations with increase in peripheral blood erythrocyte parameters such as mean value of hemoglobin, hematocrite, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. Also in our study we found that subjects heterozygous for the H63D mutation have significantly increased mean levels of hemoglobin, hematocrite and red blood cell count. Therefore, the higher bilirubin most likely reflects higher heme turn over because of a higher homeostatic hemoglobin level.

Overall, these evidences along with the above mentioned findings of a functional study, may point towards an effect of the *HFE* mutation product on iron handling in the erythron or reticuloendothelial system, rather than an effect of this mutation on duodenal iron transport. This effect of the H63D mutation on red blood cells, bilirubin and iron metabolism may serve as a model to explain secondary hemochromatosis disorders and their relationship with the *HFE* gene. High bilirubin levels may have clinical and functional implications. Recently, it was shown that lower plasma bilirubin is associated with coronary heart diseases<sup>27</sup> and that the production of bilirubin is increased in patients with Alzheimer's disease.<sup>28</sup>

In practice, the high serum bilirubin level associated with the H63D mutation may have a potentially protective effect against these diseases or their complications which counteract the potential oxidative stress induced by higher iron levels. Our data show that a higher bilirubin level is associated significantly with lower mortality especially in men. Thus, bilirubin may have a protective effect, preventing damage induced by iron excess. In future research these opposite effects of the *HFE* H63D mutation with regards to vessel wall damage have to be evaluated.

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## References

1. Bacon BR, Powell LW, Adams PC, Kresina TF, Hoofnagle JH. Molecular medicine and hemochromatosis: at the crossroads. *Gastroenterology* 1999;116:193-207.
2. Parkkila S, Niemela O, Britton RS, Fleming RE, Waheed A, Bacon BR, Sly WS. Molecular aspects of iron absorption and *HFE* expression. *Gastroenterology* 2001;121:1489-96.
3. Powell LW, Leggett BA, Crawford DHG. Hemochromatosis and other Iron Storage Disorders. In: Schiff ER, Sorrel MF, Maddrey WC (eds.) *Schiff's Diseases of the Liver*. Philadelphia: Lippincott-Raven, 1999:1107-30.
4. Worwood M. What is the role of genetic testing in diagnosis of haemochromatosis? *Ann Clin Biochem*. 2001;38:3-19.
5. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nature Genet* 1996;13:399-408.
6. Moirand R, Jouanolle AM, Brissot P, Le Gall JY, David V, Deugnier Y. Phenotypic expression of *HFE* mutations: a French study of 1110 unrelated iron-overloaded patients and relatives. *Gastroenterology* 1999;116:372-7.
7. Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population-based study of the clinical expression of the hemochromatosis gene. *New Engl J Med* 1999;341:718-24.
8. Beard J. Iron status of free-living elderly individuals. *Am J Clin Nutr* 2001;73:503-4.
9. Powell LW, Yapp TR. Hemochromatosis. *Clin Liver Dis* 2000;4:211-28.
10. George D, Powell L, Losowsky M. The hemochromatosis gene: A co-factor for chronic liver disease? *J Gastroenterol & Hepat* 1999;14:745-9.

11. Hohler T, Leininger S, Kohler HH, Schirmacher P, Galle PR. Heterozygosity for the hemochromatosis gene in liver diseases prevalence and effects on liver histology. *Liver* 2000;20:482-6.
12. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. *Lancet* 2002;359:211-8.
13. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
14. Bergmeyer HU, Horder M, Rej R. International Federation of Clinical Chemistry (IFCC) Scientific Committee, Analytical Section: approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2). *J Clin Chem Clin Biochem* 1986;24:481-95.
15. Bergmeyer HU, Horder M, Rej R. International Federation of Clinical Chemistry (IFCC) Scientific Committee, Analytical Section: approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 2. IFCC method for aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1). *J Clin Chem Clin Biochem* 1986;24:497-510.
16. Tietz NW, Rinker AD, Shaw LM. International Federation of Clinical Chemistry. IFCC methods for the measurement of catalytic concentration of enzymes. Part 5. IFCC method for alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1). IFCC Document Stage 2, Draft 1, 1983-03 with a view to an IFCC Recommendation. *Clin Chim Acta* 1983;135:339F-67F.
17. Bartels H, Bohmer M. [Automated determination of serum bilirubin] Automatische Bestimmung von Serum-Bilirubin. *Z Klin Chem Klin Biochem* 1971;9:133-5.
18. Pinnell AE, Northam BE. New automated dye-binding method for serum albumin determination with bromocresol purple. *Clin Chem* 1978;24:80-6.
19. Sunderman FJ, Sunderman F. Studies of the serum proteins. *Amer J Clin Path* 1958;30:112-9.
20. Adams PC. Prevalence of abnormal iron studies in heterozygotes for hereditary hemochromatosis: an analysis of 255 heterozygotes. *Am J Hematol* 1994;45:146-9.
21. Adams P, Brissot P, Powell LW. EASL International Consensus Conference on Haemochromatosis. *J Hepatol* 2000;33:485-504.
22. Bonkovsky HL, Lambrecht RW. Iron-induced liver injury. *Clin Liver Dis* 2000;4:409-29.
23. George D, Fletcher L, Monk C, Walker N, Do K, Powell L. Is hepatic iron the cause of raised serum transaminases in hemochromatosis. *Hepatology* 1998;28:421A,1034.
24. Hanson EH, Imperatore G, Burke W. *HFE* gene and hereditary hemochromatosis: a HuGE review. Human Genome Epidemiology. *Am J Epidemiol* 2001;154:193-206.
25. Barton JC, Bertoli LF, Rothenberg BE. Peripheral blood erythrocyte parameters in hemochromatosis: evidence for increased erythrocyte hemoglobin content. *J Lab Clin Med* 2000;135:96-104.

26. Rossi E, Bulsara MK, Olynyk JK, Cullen DJ, Summerville L, Powell LW. Effect of hemochromatosis genotype and lifestyle factors on iron and red cell indices in a community population. *Clin Chem* 2001;47:202-8.
27. Hunt SC, Kronenberg F, Eckfeldt JH, Hopkins PN, Myers RH, Heiss G. Association of plasma bilirubin with coronary heart disease and segregation of bilirubin as a major gene trait: the NHLBI family heart study. *Atherosclerosis* 2001;154:747-54.
28. Takahashi M, Dore S, Ferris CD, Tomita T, Sawa A, Wolosker H, Borchelt DR, Iwatsubo T, Kim SH, Thinakaran G, Sisodia SS, Snyder SH. Amyloid precursor proteins inhibit heme oxygenase activity and augment neurotoxicity in Alzheimer's disease. *Neuron* 2002;28:461-73.





## The role of hemochromatosis C282Y and H63D gene mutations in type 2 diabetes

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### Summary

Hemochromatosis is considered to be a risk factor for diabetes mellitus. We studied mutations in the hemochromatosis gene (*HFE*) as a risk factor for diabetes. We estimated the frequencies of the *HFE* C282Y and H63D mutations in 220 patients with diabetes, 254 subjects with impaired glucose tolerance and 595 normoglycemic individuals, all derived from a cohort study, the Rotterdam Study. Because of the low frequency of the C282Y mutation, we re-analysed all published association studies between the *HFE* mutations and type 2 diabetes in a meta-analysis. There was no evidence that the C282Y or H63D mutation is associated with impaired glucose tolerance. There was no significant difference in the frequency of carriers of the C282Y and H63D mutations in patients with type 2 diabetes compared to controls. From the meta-analysis, the overall odds ratio for diabetes was 1.0 (0.8-1.4) in carriers of the C282Y mutation and 1.1 (1.0-1.3) in carriers of the H63D mutation. There is no evidence for an increased frequency of the C282Y or H63D mutation in patients with impaired glucose tolerance or diabetes in our population-based sample or in the meta-analysis. These findings suggest that the role of *HFE* mutations in the pathogenesis of diabetes in the general population is limited.

## **Introduction**

Diabetes mellitus is a disease more commonly found in patients with hemochromatosis.<sup>1-3</sup> Patients with diabetes have a two fold mobilizable iron compared to non diabetics and the stage of diabetes is related to the amount of iron deposited in the pancreas.<sup>4</sup> Hepatic cirrhosis and damage to the pancreatic beta-cells have been suggested as the major factors responsible for the clustering of these two diseases.<sup>5-7</sup> Increased iron stores have been associated with abnormal glucose tolerance and insulin resistance.<sup>8</sup> Also animal experiments have shown that glucose intolerance and diabetes mellitus occur after intraperitoneal or oral iron administration.<sup>9</sup> Two major mutations in the hemochromatosis gene (*HFE*) are identified, the C282Y and the H63D mutations. The C282Y mutation explains up to 80% of Caucasian patients with hemochromatosis.<sup>10</sup> Whether mutations in the *HFE* gene play an important role in the pathogenesis of type 2 diabetes is a matter of controversy. Some studies have reported an increased frequency of the C282Y and H63D mutations in patients with type 2 diabetes.<sup>11,12</sup> Other studies have suggested that there was no over-representation of these mutations in patients with diabetes.<sup>13-15</sup> A problem hampering the interpretation of results is the fact that the C282Y mutation in particular is relatively rare. This results in a high probability of both false positive and false negative findings. We have studied type 2 diabetes and glucose intolerance in relation to *HFE* mutations in a sample of elderly people. In addition, to increase the statistical power of our study of the C282Y mutation, we carried out a meta-analysis of published literature on the association between the C282Y and H63D mutations and type 2 diabetes.

## **Material and Methods**

### **Study population**

Participants in the present investigation were part of a population-based study, the Rotterdam Study, which is a cohort study of subjects aged 55 years and over living in a suburb of Rotterdam, The Netherlands. The design and objective of this study have been previously reported<sup>16</sup> and has been approved by the medical ethics committee of the Erasmus Medical Centre, Rotterdam. Among the 7983 participants, 2083 subjects were available for the diabetes study. These subjects did not differ in their main characteristics from the non-available subjects. From this group, 1200 individuals were randomly selected to undergo a fasting 75 gram oral glucose tolerance test (OGTT). Results were available for 1069 participants. They were thereafter grouped into 3 groups. Group 1, persons with normal glucose tolerance ( $n=595$ ) if fasting glucose was below 6.1 mmol/L and 2 hours post-load glucose below 7.8 mmol/L, group 2, persons with impaired glucose tolerance ( $n=254$ ) if fasting glucose was between 6.1 and 7.0 mmol/L and/or a 2 hours post-load glucose between 7.8 and 11.1 mmol/L, and group 3, individuals with diabetes mellitus ( $n=220$ ) were those with a fasting glucose level of 7.0 mmol/L or above and/or a 2 hours post-load glucose of 11.1 mmol/L or above, or subjects treated for diabetes. Persons treated for diabetes did not undergo a glucose tolerance test. Information on current health status and medical history was obtained by means of a structured interview using a standardised questionnaire. Height and weight were measured and body mass index (BMI in kg/m<sup>2</sup>) was calculated. Glucose levels were measured by the glucose hexokinase method in fasting and post-load serum samples. Insulin levels were measured using a commercially available assay (IRMA, Medgenix Diagnostics, intra-assay and inter-assay variation of 3-6 percent and 5-12 percent respectively).

### **Genotyping of *HFE* mutations**

Genomic DNA was extracted from frozen buffy coat using the salting out protocol. Fragments of DNA were amplified by the Polymerase Chain Reaction (PCR) using oligonucleotides primers as described previously.<sup>7</sup> The 25µl PCR reaction tube contained 100ng of each primer, 2.5µl of 10x PCR buffer, 2.5mM dNTP, 50 mM MgCl<sub>2</sub>, 5 U/µl Ampli Taq Gold and 2 µl (~ 50 ng) DNA. We carried out 15 minutes of initial denaturation at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1.5 minutes at 72°C. Restriction digestion was performed directly on the PCR products by addition of 0.1 U/µl Sna BI (Perkin-Elmer) for codon 282 or 0.1 U/µl Dpn II (Perkin-Elmer) for codon 63 and incubating overnight at 37°C. The products were electrophoresed on a 3 % agarose gel containing ethidium bromide in presence of a 1Kb DNA standard. Control samples of known status (normozygous, heterozygous and homozygous) for each mutation were included at the PCR stage. Amplification with the primer for codon 282 produces a PCR product of 390 bp, which will not digest the wild type and will digest the mutant to produce 276 bp and 113 bp pieces. Amplification with the primer for codon 63 produces a PCR product of 208 bp, that digests the wild types to produce 138 bp and 70 bp pieces but will not digest in the mutant.

### **Meta-analysis**

A Public Medline search was constructed to include all English language publications indexed in the Index Medicus from 1996 (year of *HFE* gene cloning) up to 20 March 2002, which included the terms *HFE*, C282Y, H63D, hemochromatosis, haemochromatosis and diabetes. Most of the retrieved studies focussed on the association between the C282Y mutation and type 2 diabetes. Out of the 23 studies found, 13 (77 %) studies which compared the prevalence of *HFE* mutations in

patients with type 2 diabetes and a (non-hospitalized) control group were selected.<sup>12-15,17-25</sup> Out of these 13 studies, 11 (85%) studies which were all case-control studies provided sufficient details on the frequency of the C282Y allele (number of heterozygotes or homozygotes), to be included in a meta-analysis.<sup>12-15,17-23</sup> These studies included 10 population-based studies and 2 clinic-based studies leading to a total (including the present study) of 2630 patients with type 2 diabetes and 3437 control individuals. Among the 11 studies considered for the meta-analysis, only 7 studies have reported on the frequency of the H63D mutation,<sup>12-14,19-22</sup> leading to a total (including the present study) of 1889 patients with type 2 diabetes and 2524 controls for the study between the H63D mutation and type 2 diabetes. Heterogeneity between studies was tested using the  $\chi^2$  test, and the random effect model which is valid when between studies variation exists was considered.<sup>26</sup> The meta-analysis was done with the Review manager version 4.

### **Statistical analysis**

Genotype frequencies were estimated by gene counting and estimation of sample proportions. We used a multiple logistic regression model to estimate first the odds ratio for diabetes and glucose intolerance with 95% confidence interval (95% CI) and subsequently adjusted our analysis for age, gender and BMI. Analyses were done using the SPSS for Windows software, version 9.

### **Results**

Table 1 shows the baseline characteristics of the study population. There were significantly more men among subjects with impaired glucose tolerance and diabetes ( $P<0.05$ ). Mean age, BMI, fasting glucose and insulin concentrations were significantly ( $P<0.005$ ) higher in individuals with impaired glucose tolerance and

**Table 1.** Baseline characteristics of the study population

	Normal glucose metabolism (n=595 )	Impaired glucose metabolism (n=254)	Diabetes mellitus (n=220)
Men (%)	273 (45.9)	130 (51.2)*	124 (56.4)*
Age in years	64.1 (5.2)	64.7 (5.4)	65.9 (5.4) <sup>#</sup>
body mass index in kg/m <sup>2</sup>	25.9 (3.2)	27.2 (3.7) <sup>#</sup>	27.5 (3.5) <sup>#</sup>
waist hip ratio	0.90 (0.09)	0.93 (0.08) <sup>#</sup>	0.94 (0.09) <sup>#</sup>
Fasting glucose in mmol/l	5.5 (0.4)	6.2 (0.5) <sup>#</sup>	8.5 (2.5) <sup>#</sup>
Fasting insuline in mU/l	11.6 (5.7)	15.5 (8.8) <sup>#</sup>	19.2 (20.5) <sup>#</sup>

Values are numbers of subjects (%) or means (SD).

\* P<0.05 versus persons with normal glucose tolerance.

<sup>#</sup> P<0.005 versus persons with normal glucose tolerance.

diabetes compared to normoglycemic subjects. Genotypes and allele frequencies were in Hardy-Weinberg equilibrium proportions.

The frequencies of *HFE* mutations and odds ratio for impaired glucose tolerance and diabetes are shown in table 2. For the C282Y mutation, 26 (10.5%) subjects with glucose intolerance and 61 (10.6%) controls were carriers, yielding an adjusted odds ratio of 0.9 (0.6-1.5). For the H63D mutation, 65 (26.0%) subjects and 168 (28.5%) controls were carriers, yielding an adjusted odds ratio of 0.9 (0.6-1.2). Twenty-four (11.0%) patients with diabetes were carriers of the C282Y mutation compared to 61 (10.6%) of the control subjects, odds ratio; 1.0 (0.6-1.7). For the H63D mutation, 56 (25.7%) were carriers compared to 168 (28.5%) of the control subjects, yielding an odds ratio of 0.8 (0.6-1.1). There were too few homozygotes for the C282Y mutation in our population to yield reliable findings.

**Table 2.** Comparison of the frequency of *HFE* mutations in subjects with impaired glucose tolerance, diabetes and in controls

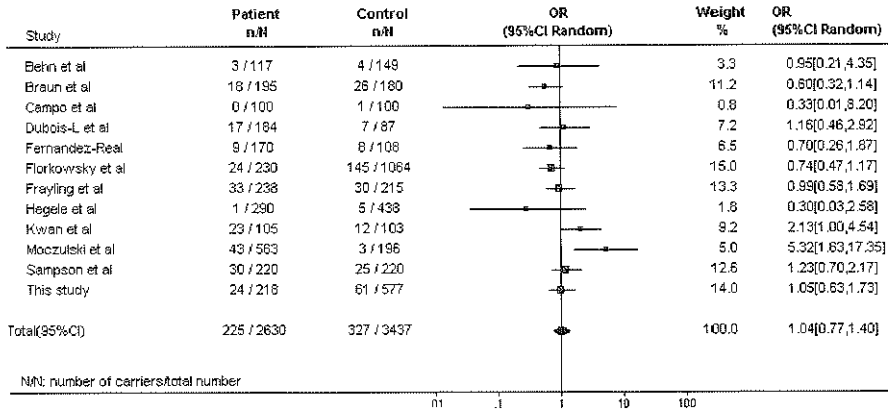
	IGT	Controls	Odds ratios (95% CI)	
			Unadjusted	Adjusted
C282Y				
Wild types (%)	221 (89.5)	516 (89.4)	Reference	
Carriers (%)	26 (10.5)	61 (10.6)	1.0 (0.6-1.6)	0.9 (0.6-1.5)
H63D				
Wild types (%)	185 (74.0)	421 (71.5)	Reference	
Carriers (%)	65 (26.0)	168 (28.5)	0.9 (0.6-1.2)	0.9 (0.6-1.2)

	Diabetes	Controls	Odds ratios (95% CI)	
			Unadjusted	Adjusted
C282Y				
Wild types (%)	194 (89.0)	516 (89.4)	Reference	
Carriers (%)	24 (11.0)	61 (10.6)	1.1 (0.6-1.7)	1.0 (0.6-1.7)
H63D				
Wild types (%)	162 (74.3)	421 (71.5)	Reference	
Carriers (%)	56 (25.7)	168 (28.5)	0.9 (0.6-1.2)	0.8 (0.6-1.1)

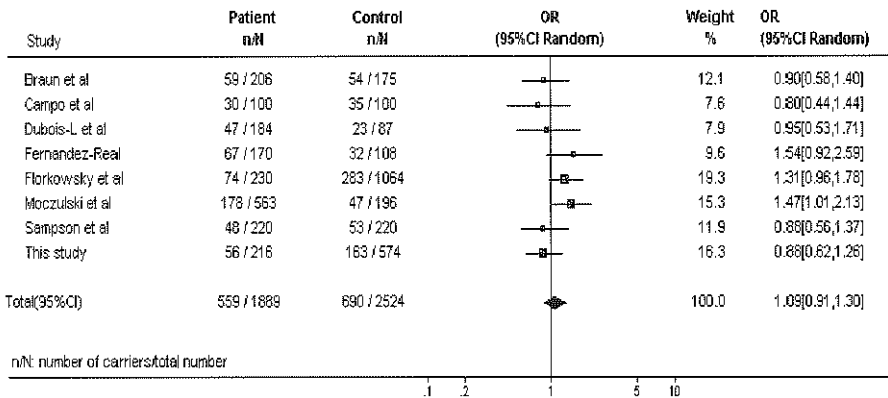
IGT: Impaired glucose tolerant subjects.

Odds ratios were adjusted for age, gender and BMI.

Figure 1 shows the meta-analysis of the C282Y mutation in patients and controls and the odds ratios for diabetes. There was no evidence for heterogeneity ( $\chi^2=18.5$ ,  $df=11$ ,  $P=0.07$ ) between studies. From the 11 studies included, two studies showed an increased frequency of the C282Y mutation in diabetes patients compared to controls and one study showed a reduced frequency. However, the overall odds ratio for



**Figure 1.** Meta-analysis of the frequency of the C282Y mutation in patients with type 2 diabetes



**Figure 2.** Meta-analysis of the frequency of the H63D mutation in patients with type 2 diabetes



diabetes in carriers of the C282Y mutation was 1.0 (0.8-1.4), suggesting no association between C282Y and the risk of diabetes. When studying C282Y homozygosity, there was no significant association to diabetes (odds ratio; 1.1 (0.6-2.3). However, this meta-analysis included only 8 patients and 7 controls homozygous for the C282Y mutation.

The meta-analysis of the frequency of the H63D mutation in patients with type 2 diabetes and control subjects is shown in figure 2. There was no significant ( $\chi^2=9.8$ ,  $df=7$ ,  $P=0.20$ ) evidence for heterogeneity between studies. Although there were three studies showing a slightly increased frequency of the H63D mutation in patients with type 2 diabetes, the overall odds ratio was 1.1 (1.0-1.3). The frequency of H63D homozygosity was 1.2 (1.1-2.3) fold increased in type 2 diabetes patients, suggesting no major effect of the H63D mutation on type 2 diabetes.

## Discussion

We found no increased frequency of the C282Y or H63D mutation in patients with diabetes. Our meta-analysis, including the present study, shows that the C282Y, the main mutation involved in hemochromatosis, and the H63D mutation are not more frequent in type 2 diabetes patients.

One limitation of our study is the low number of subjects homozygous for the C282Y mutation. To overcome this problem, we conducted a meta-analysis of all studies on the C282Y mutation and type 2 diabetes. Our findings in the Rotterdam Study were similar to that of the meta-analysis. A second problem in our study is the fact that our type 2 diabetes patients were prevalent cases. These series may be biased by mortality. We tried to overcome this problem by including subjects with impaired glucose tolerance which is a strong risk factor for diabetes. In contrast to prevalent

patients, series of patients with impaired glucose tolerance are not likely biased by selection due to mortality.

In the Rotterdam Study, we found that the C282Y and H63D mutations are not more frequent in patients with type 2 diabetes. This finding is in line with several other reports<sup>13-15, 17-22</sup> and was confirmed in our meta-analysis in which 2630 patients were tested for the C282Y mutation and 1889 patients were tested for the H63D mutation. In the meta-analysis, there was no evidence for heterogeneity between studies. This suggests that the various studies were homogeneous. The random effect and the fixed effect models gave almost similar results. Two studies found an increased frequency of the C282Y mutations in patients with type 2 diabetes.<sup>12,23</sup> The study of Kwan et al, (1998)<sup>23</sup> is a relatively small study (n=105 type 2 diabetes patients), and uses type 1 diabetes patients (n=103) as controls. The study of Moczulski et al, (2001)<sup>12</sup> showing a significant association between the C282Y mutation and type 2 diabetes was a relatively large study of 563 diabetes patients and 196 controls. Patients in this study were derived from an out-patient clinic. Not only is the frequency of the C282Y mutation in patients high compared to other studies, more unexpected, the frequency of the C282Y mutation in controls (1.5%) is very low compared to the average in the overall sample (9.5%). Thus, the increased frequency observed may be explained by difference in ethnic composition. Basically, the control group consisted of workers from a local factory and are likely to include individuals from different countries. Even the 2630 patients studied in the meta-analysis yielded only 225 (9%) C282Y heterozygotes. This corroborates with the findings of Beutler et al, (2002)<sup>25</sup> in a very large study of 23065 subjects. This study was not included in the meta-analysis because the authors did not provide data on the number of heterozygotes for the H63D and C282Y mutations.

Also for the H63D mutation, the meta-analysis did not show evidence for an increased frequency of the H63D mutation in type 2 diabetes patients. Again the study of Moczulski et al, (2001)<sup>12</sup> together with that of Fernandez-Real et al, (1999)<sup>22</sup> found an increased frequency in patients with type 2 diabetes. The test for heterogeneity was not significant ( $P=0.20$ ), suggesting that pooling all studies is justified. Although there is a proven effect of the H63D mutation on serum iron, ferritin and transferrin saturation, there is no evidence this mutation plays an important role in the etiology of type 2 diabetes.

In conclusion, we could not show a relation between carriership of *HFE* C282Y and H63D mutations and glucose intolerance or type 2 diabetes in our study. The meta-analysis showed no increased frequency of the C282Y or H63D mutations in patients with type 2 diabetes. The role of these mutations in the pathogenesis of type 2 diabetes is therefore very limited.

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### **References**

1. Phelps G, Hall P, Chapman I, Braund W, Mackinnon M. Prevalence of genetic hemochromatosis among diabetic patients. *Lancet* 1989;8657:233-4.
2. Adams PC, Speechley M, Kertesz AE. Long-term survival analysis in hereditary hemochromatosis. *Gastroenterology* 1991;101:368-72.

3. Niederau C, Fischer R, Purschel A, Stremmel W, Haussinger D, Strohmeyer G. Long-term survival in patients with hereditary hemochromatosis. *Gastroenterology* 1996;110:1107-19.
4. Yaouanq J, Poirier JY, Maugendre D, Brissot P, Alannic H. Genetic hemochromatosis and diabetic association: a study in 474 hemochromatosis patients. *Diabetologia* 1990;33(SupplA):142.
5. Saddy R, Feingold J. Idiopathic hemochromatosis and diabetes mellitus. *Sem Med* 1896;16:514.
6. Saudek CD. In: Podolsky S, Viswanathan M, eds. Secondary diabetes: the spectrum of the diabetic syndrome. New York: Raven Press; 1980. Pp.257-68.
7. Niederau C, Berger M, Stremmel W, Starke A, Strohmeyer G, Ebert R, et al. Hyperinsulinemia in cirrhotic haemochromatosis: impaired hepatic insulin degradation? *Diabetologia* 1984;26:546-54.
8. Tuomainen TP, Nyyssönen K, Salonen R, Tervahauta A, Korpela H, Lakka T, Kaplan GA, Salonen JT. Body iron stores are associated with serum insulin and blood glucose concentrations: population study in 1,013 eastern Finnish men. *Diabetes Care* 1997;20:426-8.
9. Rahier J, Loozen S, Goebbels RM, Abraham M. The haemochromatosis human pancreas: a quantitative immunochemical and ultrastructural study. *Diabetologia* 1987;30:5-12.
10. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
11. Salonen JT, Tuomainen TP, Kontula K. Role of C282Y mutation in hemochromatosis gene in development of type 2 diabetes in healthy men: prospective cohort study. *BMJ* 2000;320:1706-7.
12. Moczulski DK, Grzeszczak W. Role of hemochromatosis C282Y and H63D mutation in *HFE* gene in development of type 2 diabetes and diabetic nephropathy. *Diabetes Care* 2001;24:1187-91.
13. Florkowski CM, George PM, Willis JA, Stott MK, Burt MJ, Upton JD, Nesbit J, Walmsley TA, Scott RS. Haemochromatosis gene mutations Cys282Tyr and His63Asp are not increased in type 2 diabetic patients compared with the Canterbury (New Zealand) general population. *Diab Res Clin Pract* 1999;43:199-203.
14. Dubois-Laforgue D, Caillat-Zucman C, Djilali-Saiah I, Larger E, Mercadier A, Boitard C, Bach JF, Timsit J. Mutations in *HFE*, the hemochromatosis candidate gene, in patients with NIDDM. *Diabetes Care* 1998;21:1371-2.
15. Frayling T, Ellard S, Grove J, Walker M, Hattersley AT. C282Y mutation in *HFE* (haemochromatosis) gene and type 2 diabetes. *Lancet* 1998;351:1933-4.
16. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of diseases and disability in the elderly: The Rotterdam Elderly Study. *Eur J Epidemiol* 1991;4:403-22.
17. Behn PS, Glaser B, Permutt MA. Contribution of C282Y hereditary mutation to type 2 diabetes mellitus in Ashkenazi Jews. *Diabetes* 1998;47(Suppl1):1523.
18. Hegele RA, Harris SB. Hemochromatosis and diabetes mellitus. *Ann Intern med* 1998;129:587.
19. Braun J, Donner H, Plock K, Rau H, Ysadel KH, Badenhoop K. Hereditary hemochromatosis mutations (*HFE*) in type 2 diabetes mellitus. *Diabetologia* 1998;41:983-4.
20. Campo S, Restuccia T, Villari D, Raffa G, Cucinotta D, Squadrito G, Pollicino T, Raimondo G. Analysis of haemochromatosis gene mutations in a population from the Mediterranean Basin. *Liver* 2001;21:233-6.

21. Sampson MJ, Williams T, Heyburn PJ, Greenwood RH, Temple RC, Wimperis JZ, Jennings BA, Willis GA. Prevalence of *HFE* (hemochromatosis gene) mutation in unselected male patients with type 2 diabetes. *J Lab Clin Med* 2000;135:170-3.
22. Fernandez-Real JM, Vendrell J, Baiget M, Gimferrer E, Ricart W. C282Y and H63D mutations of the hemochromatosis candidate gene in type 2 diabetes. *Diabetes Care* 1999;22:525-6.
23. Kwan T, Leber B, Ahuja S, Carter R, Gerstein HC. Patients with type 2 diabetes have a high frequency of the C282Y mutation of the hemochromatosis gene. *Clin Invest Med* 1998;21:251-7.
24. Ellervik C, Mandrup-Poulsen T, Nordestgaard BG, Larsen LE, Appleyard M, Frandsen M, et al. Prevalence of hereditary hemochromatosis in late-onset type 1 diabetes mellitus: a retrospective study. *Lancet* 2001;358:1405-9.
25. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. *Lancet* 2002;359:211-8.
26. Normand SL. Meta-analysis: formulating, evaluating, combining, and reporting. *Stat Med* 1999;18:321-59.



*Chapter 3.5***Mutations in the hemochromatosis gene (*HFE*) and stroke**

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**Summary**

Increased serum iron is found to be a risk factor for stroke. Carriers of *HFE* C282Y and H63D mutations have elevated serum iron levels and may have an increased risk for stroke. We studied the association between *HFE* gene mutations, carotid atherosclerosis and stroke. We compared the frequency of the *HFE* C282Y and H63D gene mutations in 202 prevalent and incident cases of stroke to that of 2730 controls from a population-based study, the Rotterdam Study. The influence of *HFE* mutations on the relationship between hypertension, smoking and stroke was studied using a logistic regression model. In the analyses of hypertension, we used non carriers and non hypertensives as reference while in the analysis of smoking, we used non carriers and never smokers as reference group. Further, we studied the mean intima-media thickness of the common carotid artery in relation to the hypertension, smoking and *HFE* genotype in subjects without stroke. The percentage of both C282Y and H63D carriers in cases (43.7%, n=87) did not differ significantly ( $P=0.09$ ) from that of controls (37.6%, n=986). The odds ratio for stroke (95% CI) for *HFE* carriers who also suffered from hypertension was 3.0 (1.9-4.6) and for *HFE* carriers who were also smokers, the odds ratio for stroke was 2.6 (1.4-5.0). The mean (SD) intima-media thickness of the carotid artery was 0.77 (0.14) for non carriers without a history of hypertension or smoking compared to 0.81 (0.17) for *HFE* carriers who were smokers ( $P<0.004$ ) and 0.84 (0.20) for *HFE* carriers who were hypertensives ( $P<0.001$ ).

Mutations in the *HFE* gene were not significantly related to stroke or atherosclerosis in the carotid artery. The *HFE* gene may modify the relationship between smoking and stroke.

## Introduction

Studies of the role of mutations in the hemochromatosis (*HFE*) gene and the risk of atherosclerosis and stroke have yielded controversial results.<sup>1-4</sup> Two major mutations are known in the *HFE* gene, i.e. the C282Y and the H63D mutation. Although these mutations may determine only minor (<5%) variation, they are associated with significant lifetime increase in levels of serum iron, ferritin and transferrin saturation.<sup>5,6</sup> This elevation may be clinically relevant particularly in the elderly due to lifetime accumulation of iron. Rossi et al, (2000)<sup>7</sup> reported that the C282Y mutation does not influence the formation of plaques or the mean intima-media thickness (IMT) of the carotid artery. However, they found that serum ferritin levels were independently associated with the formation of plaques in the carotid artery of female carriers of the C282Y mutation. Mortality from cerebrovascular disease was found to be significantly related to the C282Y mutation in women heterozygous for the C282Y mutation.<sup>3</sup> The association was strongest in women with a history of hypertension and/or smoking, which are important risk factors for stroke. Up until now, the influence of the H63D mutation on the pathogenesis of atherosclerosis and stroke has been given little attention. We studied the association between the C282Y and H63D mutations in the *HFE* gene in relation to atherosclerosis and stroke in a population-based sample of elderly people aged 55 years and over.

## Methods

### Study population

This study was conducted within the Rotterdam Study, an ongoing population-based cohort study for which all inhabitants aged 55 years or over, living in a suburb of Rotterdam, The Netherlands, were invited. The rationale and design of the Rotterdam



Study have been described elsewhere.<sup>8</sup> Baseline data collection was performed between 1990 and 1993. Written informed consent and permission to retrieve information from medical records were obtained from every participant. The study has been approved by the medical ethics committee of the Erasmus Medical Centre. A total of 7983 subjects participated in the study (response rate 78%) which includes individuals from the general population and those living in nursing homes. At baseline interview, information on current medication, alcohol intake and smoking habits was obtained. People who smoked were asked for the age at first smoking, for the duration of interval periods without smoking, and for the average daily number of cigarettes smoked. For the purpose of this study, only current smokers (n=761, 26%) and those who never smoked (n=792, 27%) were considered. Former smokers (n=1353, 46%) were excluded for two reasons; (1) the majority of patients may have quit smoking after the stroke and (2) in non-affected, smoking in the past may have irreversible effects on the process of atherosclerosis. By contrasting never smokers to current smokers, we maximised both this contrast and the statistical power. Former smokers were excluded only in the analysis of smoking.

Two blood pressure measurements were taken with a random zero sphygmomanometer with the subject in sitting position and the average of these two measurements was taken. Hypertension was defined as a systolic blood pressure  $\geq 160$  mmHg or a diastolic blood pressure  $\geq 95$  mmHg on two consecutive measurements or current use of blood pressure lowering drugs for indication of hypertension.

#### **Assessment of stroke and atherosclerosis at the carotid artery**

During the interview at baseline, a previous stroke was assessed by asking the question, "did you ever suffer from stroke, diagnosed by a physician?" Medical

records of subjects who answered 'yes' were checked and a previous stroke was considered to have occurred if confirmed by medical records.<sup>9</sup> Once subjects enter the Rotterdam Study they are continuously monitored for major events through automated linkage with files from the general practitioners. When an event or death had been reported, additional information was obtained by interviewing the general practitioner and by scrutinizing information from hospital discharge records in case of admittance or referral. Information from reports on all possible strokes was reviewed by two research physicians and a neurologist who classified the stroke as definite, probable or non-stroke. The stroke was definite if the diagnosis was based on typical clinical symptoms and neuro-imaging excluded other diagnoses. The stroke was considered probable in case typical clinical symptoms were present but neuro-imaging was not performed. For fatal strokes, other causes of death, especially cardiac, should have been excluded. Since a mixture of multiple genetic and environmental factors may determine stroke at late age, the present study focussed on early stroke (age at onset  $\leq$  75). In total 202 stroke cases (110 stroke cases at baseline, 92 newly identified cases by January 1, 1998) were considered. The controls consisted of a group of 2730 subjects (aged  $\leq$  75 years) without any history of stroke and selected randomly from the total cohort.

The IMT and the presence of atherosclerotic plaques of the common carotid artery (CCA) were assessed with ultrasound.<sup>10</sup> For each subject, the mean IMT ((left + right)/2) was taken as measure of wall thickness of the distal CCA.

### **Laboratory procedures**

From all the subjects, blood samples were collected by venepuncture and kept frozen until analysis. Genomic DNA was extracted from frozen buffy coat using the salting out

procedure. Fragments of DNA were amplified by the Polymerase Chain Reaction (PCR) and genotyped using oligonucleotides primers as described elsewhere.<sup>11</sup>

### Statistical analysis

Given the small number of patients studied (n=202), there were only one homozygote for the C282Y and 2 homozygotes for the H63D mutation. We therefore pooled the homozygotes and heterozygotes in the analysis. To increase the statistical power of the study, data of incident and prevalent cases were pooled resulting in a series of 202 patients. Based on this number of patients and the 2730 controls, the statistical power was 80% to find a two-fold increase in frequency of C282Y heterozygosity (percentage carriers in controls: 12.2%). For H63D (percentage carriers in controls: 26.5%), the power was above 80% to find a 1.5 increase in frequency. Earlier (chapter 3.2), we studied the relation of *HFE* to iron parameter in a subset of 335 subjects based on genotype (74 wild type, 73 H63D heterozygotes, 71 C282Y heterozygotes, 60 H63D homozygotes, 51 compound heterozygotes and 6 C282Y homozygotes. Similar to the findings of Whitfield et al, (2000),<sup>6</sup> this study showed that the relation of the two mutations in heterozygote state was similar for both mutations (serum iron: 17.1 umol/l for C282Y versus 17.3 umol/l for H63D, serum ferritin: 189.9 ug/l for C282Y versus 172.2 ug/l for H63D and serum transferrin saturation: 29.5 % for C282Y versus 28.5 % for H63D. The presence of either mutation was significantly associated with serum iron ( $P<0.001$ ), ferritin ( $P<0.003$ ) and transferrin saturation ( $P<0.001$ ). The two mutations were pooled yielding a frequency of 37.6% in controls. This further improved the statistical power for our analysis of interaction with smoking and hypertension.

The chi-square statistic was used to compare categorical variables and the two-sample t test to study normally distributed and continuous variables. The carrier frequencies

for the C282Y and H63D mutations were estimated by counting gene and calculating sample proportions. We used logistic regression methods to estimate the odds ratios for stroke with 95% confidence interval adjusted for age and sex. Effect modification of the relation between smoking, hypertension and stroke by *HFE* was explored by stratifying the data into four categories. For hypertension, the first category consisted of non *HFE* carriers and non hypertensives (reference group), the second category of non *HFE* carriers with a history of hypertension, the third category of *HFE* carriers with no history of hypertension and the last category of *HFE* carriers with a history of hypertension. For smoking, the first category consisted of non carriers that never smokers, the second category of non carrier that smoked, the third category of *HFE* carriers who never smoked and the fourth category of carriers who were smokers. Interaction was evaluated according to an additive model and using the synergy index<sup>12,13</sup> which is defined as the ratio of the relative risk of both measurements indicating severe outcome minus 1 divided by the sum of the risk of each exposure minus 2. A synergy index of 1 indicates no synergy. Further, the significance level of the interaction was evaluated by adding a product term of the two factors studied in the regression model.

## Results

Table 1 shows the baseline characteristics of the study population. Mean age of cases (69.3 years) was significantly ( $P<0.001$ ) different to that of controls (65.1 years). There were significantly more men, hypertensives and smokers among the cases compared to the controls ( $P<0.05$ ). The percentage of both C282Y and H63D carriers

**Table 1.** Baseline characteristics of the study population

	Stroke (n=202)	Controls (n=2730)
Mean age in years	69.3 (6.5)	65.1 (5.4)†
Men (%)	114 (56.4)	1323 (48.5)*
Hypertensives (%)	128 (64.0)	1004 (38.2)†
Smoking		
Current (%)	62 (57.4)	699 (48.4)*
Past (%)	94 (30.7)	1259 (46.6)*
Never (%)	46 (22.8)	746 (27.6)
Mean IMT in mm	0.873 (0.218)	0.771 (0.144)
<i>HFE</i> carriers		
C282Y (%)	28 (14.1)	323 (12.2)
H63D (%)	62 (31.2)	707 (26.5)
<i>HFE</i> (%)	87 (43.7)	986 (37.6)

Values are means (SD) or number (%) based on all available data.

†:  $P < 0.005$  \*:  $P < 0.05$ .

in cases (43.7%,  $n=87$ ) did not differ significantly ( $P=0.09$ ) from that of controls (37.6%,  $n=986$ ).

The effect of *HFE* on the relation between stroke, hypertension and smoking is shown in table 2. Hypertension was significantly associated with stroke in the absence of the *HFE* mutations (adjusted odds ratio: 2.3, 95% CI 1.5-3.4). By themselves, the mutations in the *HFE* gene showed only a weak association with stroke (odds ratio: 1.3, 95% CI: 0.8-2.2). Patients with hypertension who were also carriers of *HFE* mutations showed a significant relationship with stroke (adjusted odds ratio: 3.0, 95% CI: 1.9-4.6). The synergy index was 1.25, while the  $P$  value for interaction was 0.36. Neither smoking nor *HFE* mutations were significantly associated with stroke if the

other factor was not present. But in those subjects who smoked and who were also *HFE* carriers, there was a significant relationship observed (odds ratio: 2.6, 95% CI: 1.4-5.0). The synergy index was 2.67, and the P value for interaction was 0.18. We obtained similar findings when comparing men and women and when we analysed prevalent and incident cases of stroke separately (data not shown).

**Table 2.** Interaction between *HFE* C282Y and H63D mutations, hypertension, smoking and stroke

*HFE* and hypertension

<i>HFE</i> carrier	Hypertensive	Stroke	Controls	Odds ratios (95% CI)	
				Unadjusted	Adjusted*
No	No	41 (21.0)	968 (39.5)	Reference	
No	Yes	71 (36.4)	601 (24.5)	2.8 (1.9-4.2)	2.3 (1.5-3.4)†
Yes	No	30 (15.4)	552 (22.5)	1.3 (0.8-2.1)	1.3 (0.8-2.2)
Yes	Yes	53 (27.2)	332 (13.5)	3.8 (2.5-5.8)	3.0 (1.9-4.6)†

*HFE* and smoking

<i>HFE</i> carrier	Smoker	Stroke	Controls	Odds ratios (95% CI)	
				Unadjusted	Adjusted*
No	No	24 (22.9)	422 (31.4)	Reference	
No	Yes	29 (27.6)	435 (32.4)	1.2 (0.7-2.1)	1.3 (0.7-2.3)
Yes	No	22 (21.0)	261 (19.4)	1.5 (0.8-2.7)	1.3 (0.7-2.4)
Yes	Yes	30 (28.6)	224 (16.7)	2.4 (1.3-4.1)	2.6 (1.4-5.0)†

Values are number of individuals (percentage).

\*: Adjusted for age and sex.

†: P<0.05.

Table 3 shows the effect of *HFE* mutations and its interaction with hypertension and smoking on the mean IMT. There was no significant difference in the mean IMT

**Table 3.** Interaction between *HFE* C282Y and H63D mutations, hypertension, smoking and intima-media thickness*HFE* and hypertension

<i>HFE</i> carrier	Hypertensive		Mean intima-media thickness in mm (SD)	P value
No	No	(n=904)	0.761 (0.136)	Reference
No	Yes	(n=671)	0.831 (0.163)	<0.001
Yes	No	(n=530)	0.774 (0.151)	0.10
Yes	Yes	(n=386)	0.836 (0.190)	<0.001

*HFE* and smoking

<i>HFE</i> carrier	Smoker		Mean intima-media thickness in mm (SD)	P value
No	No	(n=458)	0.771 (0.143)	Reference
No	Yes	(n=419)	0.796 (0.150)	0.01
Yes	No	(n=280)	0.784 (0.151)	0.24
Yes	Yes	(n=226)	0.807 (0.173)	0.004

between *HFE* carriers (0.761mm, SD: 0.136) and non carriers (0.774mm SD: 0.151).

In the absence of smoking or hypertension, the *HFE* mutations were not significantly associated to IMT. Hypertension was associated with a significant increase in mean IMT ( $P=0.001$ ) both in the presence and absence of *HFE* mutations. Among hypertensives, there was a minor difference in the mean IMT between those with and without a *HFE* mutation (0.005,  $P=0.12$ ). Smoking was also significantly associated with an increased mean IMT in the presence ( $P<0.004$ ) or absence ( $P=0.01$ ) of *HFE* mutations. In smokers, the difference in the mean IMT between those with and

without a *HFE* mutation was 0.011,  $P=0.06$ , suggesting an additive effect of smoking and *HFE*.

## Discussion

In our study, the C282Y and H63D mutations were not significantly associated with stroke or carotid atherosclerosis by themselves. The classical risk factors for stroke, hypertension and smoking were associated to the disease in the absence of *HFE* mutations. However, the presence of *HFE* mutations modified this association particularly the relation between smoking and stroke.

When studying atherosclerosis of the carotid artery, a major risk factor for stroke, there was no evidence for an effect of *HFE* on atherosclerosis nor was there evidence for modification of the relationship between hypertension and stroke. Although *HFE* was not significantly associated to IMT in the absence of smoking, there was evidence for an additive effect of smoking and *HFE*.

Our data are based on a mixture of prevalent and incident cases. The use of prevalent patients imposes limitations in that, patients may change (smoking) habits after the stroke and risk factors studied may be related to rather survival. We aimed to overcome this problem here by studying atherosclerosis of the carotid artery, a major risk factor for stroke as a proxy for early disease pathology. However, as atherosclerosis of the carotid artery and stroke are only partly equivalent, our data remain to be confirmed preferably in a series of incident cases. In particular, the study of interaction with smoking is in need of replication in larger patient series. Although the synergy index was high (2.67), the test for interaction was not statistically significant ( $P=0.18$ ). The a-priori statistical power to find interaction was low based on the 202 patients studied. In an observational study, misclassification is difficult to



exclude but such mishaps are not likely to be related to the genotype of the person and are therefore less of a problem in genetic studies than in studies of environmental factors. The main advantage of our study is its population-based design, which is less susceptible to selection bias. However, a point of concern with regard to the generalisability of our data is that only Caucasians were studied, limiting the extrapolation to other ethnic groups. Also, the difference in effects of H63D and C282Y found across studies<sup>5,6,14-16</sup> in Caucasians asks for caution in generalisation of findings.

Our findings are compatible with those of Roest et al, (1999)<sup>3</sup> who found that in women carrying the C282Y mutation, mortality for cerebrovascular disease was 2.4 times increased. Roest et al, (1999)<sup>3</sup> found a strong effect modification by hypertension and smoking. In our study, the odds ratios for stroke (95% CI) in *HFE* carriers who were also hypertensives was 3.0 (1.9-4.6) and for *HFE* carriers who were also smokers, the odds ratio for stroke was 2.6 (1.4-5.0). The evidence for effect modification of hypertension, smoking and *HFE* reported by Roest et al, (1999)<sup>3</sup> was only partially confirmed in our study. The synergy index for hypertension and *HFE* was modestly increased (1.25) and the P value for interaction was not significant (P=0.36). However, for smoking, the synergy was 2.67 times increased and the P value for interaction was low (0.18) given the sample size, suggesting that the effect of smoking and *HFE* combined was more than additive based on the multiplication of risks. This observation only concerned the outcome of stroke. When studying IMT as a risk factor for stroke, the effect of *HFE* and smoking were additive. The finding of an additive effect of smoking and *HFE* on IMT suggests that smoking and *HFE* are both involved in stroke in a common pathway i.e. atherosclerosis. This observation is compatible with high iron concentration found in human atherosclerotic lesions.<sup>17</sup>

Although it is still a matter of controversy in the literature as to whether *HFE* mutations are associated with coronary heart diseases,<sup>1,4,18</sup> experimental studies have shown that iron overload contributes to atherogenesis.<sup>19</sup> It has also been reported that iron overload increases the risk of cardiovascular diseases.<sup>20</sup> The mechanism through which the *HFE* gene may modify the risk of stroke is not known. Increased blood iron concentration may lead to an increase in the viscosity of blood, which may result in thrombosis. We and others have indeed found that carriers of *HFE* mutations do have significantly increased levels of iron.<sup>5,6,16</sup> Although not replicated in all studies, at least in our population, we found the C282Y and H63D were significantly associated to serum iron, ferritin and transferrin saturation. However, since we have data on serum iron, ferritin and transferrin in only a very limited sub-sample of this population, we cannot verify this hypothesis in the statistical analysis.

The modification of the relationship between smoking and *HFE* in relation to stroke and the additive effect of the two factors on atherosclerosis opens another possible mechanism. Smoking and *HFE* mutations may both result in increased oxidative stress and thus cause damage to the vessel walls. Adding the oxidative effect of smoking to that of high iron levels in *HFE* carriers may increase the risk of atherosclerosis according to an additive model. Interestingly, with regard to the risk of stroke, our data suggest that the effect of these two risk factors may be more than additive. Further research is needed on the role of the *HFE* mutations in stroke.

Our data suggest that the C282Y and H63D mutations by themselves are not strongly related to stroke or atherosclerosis. In the presence of smoking, these mutations increase the risk of carotid atherosclerosis and stroke in carriers of *HFE* mutations.

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## References

1. Franco RF, Zago MA, Trip MD, ten Cate HEA, Prins MH, Kastelein JJ, et al. Prevalence of hereditary hemochromatosis in premature atherosclerotic vascular disease. *Br J Haematol* 1998;102:1272-5.
2. Roest M, van der Schouw YT, de Valk B, Marx JJM, Tempelman MJ, de Groot PG, Sixma JJ, Banga DJ. Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation* 1999;100:1268-73.
3. Gillum RF, Sempos CT, Makuc DM, Looker AC, Chien CY, Ingram D. Serum transferrin, stroke incidence and Mortality in women and men. The NHANES I epidemiologic follow-up study. *Am J Epidemiol* 1996;144:59-68.
4. Calado RT, Franco RF, Pazin-Filho A, Simoes MV, Marin-Neto JA, Zago MA. *HFE* gene mutations in coronary atherothrombotic disease. *Braz J Med Biol Res* 2000;33:301-6.
5. Datz C, Haas T, Rinner H, Sandhoffer F, Patsch W, Paulweber B. Heterozygosity for the C282Y mutation in the hemochromatosis gene is associated with increased serum iron, transferrin saturation, and hemoglobin in young women: a protective role against iron deficiency? *Clinical Chemistry* 1998;44:2429-32.
6. Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL and Martin NG. Effects of *HFE* C282Y and H63D polymorphism and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000;66:1246-58.
7. Rossi E, McQuillam BM, Hung J, Thompson PL, Kuek C, Beilby JP. Serum ferritin and C282Y mutation of the hemochromatosis gene as predictor of asymptomatic atherosclerosis in a community population. *Stroke* 2000;31:3015-20.
8. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of diseases and disability in the elderly: The Rotterdam Elderly Study. *Eur J Epidemiol* 1991;4:403-22.
9. Bots ML, de Jong PT, Hofman A, Grobbee DE. Left, right, near or far wall common carotid intima-media thickness measurements: associations with cardiovascular disease and lower extremity arterial atherosclerosis. *J Clin Epidemiol* 1997;50:801-7.

10. Bots ML, Looman SJ, Koudstaal PJ, Hofman A, Hoes AW, Grobbee DE. Prevalence of stroke in the general population. The Rotterdam Study. *Stroke* 1996;27:1499-501.
11. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
12. Rothman KJ. Modern epidemiology. Boston, litle, Brown, 1986.
13. Hosmer DW, Lemeshow S. Confidence interval estimation of interaction. *Epidemiology* 1992;3:452-6.
14. Gochee PA, Powell LW, Cullen DJ, Du Sart D, Rossi E, Olynyk JK. A population-based study of the biochemical and clinical expression of the H63D hemochromatosis mutation. *Gastroenterology* 2002;122:646-51.
15. Rossi E, Bulsara MK, Olynyk JK, Cullen DJ, Summerville L, Powell LW. Effect of hemochromatosis genotype and lifestyle factors on iron and red cell indices in community population. *Clin Chem* 2001;47:202-8.
16. Burt MJ, George PM, Upton JD, Collett JA, Frampton CMA, Chapman TM, Walmsley TA, Chapman BA. The significance of hemochromatosis gene mutations in the general population: implications for screening. *Gut* 1988;43:830-6.
17. Swain J, Gutteridge JM. Prooxidant iron and copper, with ferroxidase and xanthine oxidase activities in human atherosclerotic material. *FEBS Lett* 1995;368:513-5.
18. Miller M, Hutchins GM. Hemochromatosis, multiorgan hemosiderosis, and coronary artery disease. *JAMA* 1994;272:231-3.
19. Araujo JA, Romano EL, Brito BE, Parthe V, Romano M, Bracho M, Montano RF, Cardier J. Iron overload augments the development of atherosclerotic lesions in rabbits. *Arteriosclerosis Thrombosis and Vascular Biolog* 1995;15:1172-80.
20. Sullivan JL. Iron and the genetics of cardiovascular disease. *Circulation* 1999;100:1260-3.

## **Chapter 4**

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**A search for new genes for hemochromatosis**



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## Chapter 4.1

### Introduction

Hemochromatosis is for a large part explained by mutations in the *HFE* gene. Over 80% of the Caucasian patients with the hereditary form of the disease are homozygous for the *HFE* C282Y mutation. Yet, there is a considerable number of patients and families in which the disease segregates for which the mutation is unknown. We have identified a family with a non classical form of hemochromatosis segregating as an autosomal dominant trait. In chapter 4.2, the clinical and phenotypic characteristics of patients from this family are described and their relation to *HFE* and other known mutations investigated. The search for the genetic cause of the disease is described in chapter 4.3. In chapter 4.4, the prevalence of this new gene in patients with diabetes is assessed.





## **A family with a hereditary form of iron overload distinct from classical hemochromatosis**

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### **Summary**

Hereditary hemochromatosis is classically inherited as a recessive trait but is genetically heterogeneous. Mutations in the *HFE* and the *TFR2* genes account for about 85% of Caucasian patients and a third locus on chromosome 1q is responsible for juvenile hemochromatosis. We describe here the clinical and biological characteristics of a large Dutch family with an autosomal dominant form of iron overload. Clinical signs of iron overload in patients include joint pains, cardiomyopathies, liver fibrosis and hormonal disorders including diabetes mellitus. The main and most common clinical symptoms in this family were joint complaints and early signs of arthrosis. Serum ferritin levels in iron overloaded subjects varied from 31 to 2179 ng/ml and the transferrin saturation from 13 to 88.6%. The iron overload is moderate compared to patients with type 1 hemochromatosis but the deferoxamine test was normal in all patients. The disease in this family segregated as a dominant trait. None of the patients was homozygous or compound heterozygous for any known mutation in the *HFE* or *TFR2* genes. The disease in this family represents a non-classical form of iron overload. Our genome screen suggests that the disease is associated with a mutation in the gene encoding the metal transporter called *SLC11A3*.

## Introduction

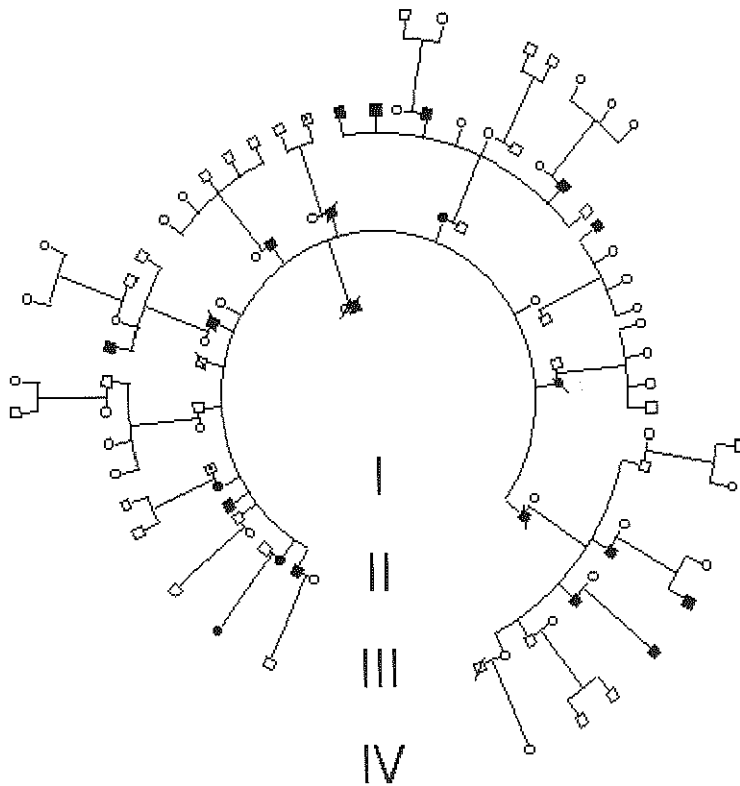
Genetic heterogeneity is a well-recognised feature in hereditary hemochromatosis.<sup>1-3</sup> Hemochromatosis is a disorder of iron overload, which is usually inherited as a recessive trait. The disease is characterised by excessive iron storage in tissues resulting in multi-organ damage and pathologies such as liver diseases, diabetes, arthritis, cardiomyopathy, hypogonadism, stroke and cancer.<sup>4</sup> In most patients the disease becomes clinically manifest in the fourth decade of life with unspecific symptoms including weakness and lethargy. Several genes involved in iron metabolism have been implicated in the pathology of hemochromatosis, viz. a locus for juvenile hemochromatosis on chromosome 1q, mutations in the *HFE* gene on chromosome 6p21.3 and the transferrin receptor gene (*TFR2*) on chromosome 7q.<sup>5-7</sup> Still not all cases of hereditary iron overload can be explained by these mutations. Recently, we have identified a mutation in the gene encoding the metal transporter termed *SLC11A3* alias ferroportin that is responsible for this type of hemochromatosis.<sup>8</sup> In this paper, we describe the clinical characteristics of patients with a non-classical form of iron overload segregating as a dominant trait.

## Methods

### Subjects

A family with multiple patients with hereditary iron overload was identified during a study of type 2 diabetes in a genetically isolated community. The pedigree is shown in the figure. Affected individuals underwent extensive clinical and laboratory investigations including biopsy of the liver and serum iron, ferritin and transferrin saturation measurements.<sup>9,10</sup> In 1999, this family (including all new affected individuals) was re-investigated as part of the program Genetic Research in Isolated

Populations (GRIP). Serum iron, ferritin and transferrin saturation were re-measured using conventional methods and the deferoxamine test was performed. Magnetic resonance imaging (MRI) of the brain and liver were done. Patients were considered to have iron overload if they consecutively had a serum ferritin level over 450 ng/ml or a serum transferrin saturation over 50% or if they showed evidence of iron accumulation on MRI. We examined medical records to obtain information about patient conditions and hemochromatosis related disorders such as liver disease, hematological, endocrine and cardiovascular disorders and joint complaints.



**Figure.** Pedigree of the Dutch family with autosomal dominant hemochromatosis

Information on alcohol intake, smoking behaviour and use of medication was collected using a standardised questionnaire. All subjects gave written informed consent and the GRIP program is approved by the ethics committee of the Erasmus Medical Centre.

#### **Laboratory studies.**

After the collection of liver biopsy, each specimen was divided into two parts. One half was stained with Perls' Prussian blue to detect iron and in the other half the amount of iron (mmol per 100 grams of dry liver weight) was calculated and the hepatic iron index (HII) derived. At the follow-up in 1999, genomic DNA was obtained from peripheral white blood cells. All patients were tested for the presence of the C282Y or H63D mutations in the *HFE* gene as described elsewhere<sup>5</sup>. Individuals from this family with increased iron levels (12) were then genotyped for common mutations described as causing hemochromatosis. We checked if the disease in this family was linked to described genes on chromosome 1q, 6p and 7q or any gene in linkage disequilibrium with the causative gene by testing microsatellite markers: D1S252, D1S498, D6S1610, D6S422, D7S477 and D7S647 and carrying out genetic linkage analysis. Analyses were performed using the MLINK and ILINK programs of the LINKAGE package (version 5.1) which gives the odds of the disease being linked to a given marker on a chromosomal region. To exclude involvement of diseases with similar expression as hemochromatosis, serum ceruloplasmin and copper levels were measured.

## Results

In 1972, 4 generations were studied including 58 subjects of whom 6 were diagnosed with iron overload. In 1999, as part of the GRIP study, we identified 6 new patients yielding a total of 12 patients (10 men and 2 women). The youngest patient was 25 years and the oldest patient 80 years old. The table summarises the outcome of the clinical and laboratory investigations in the 12 patients. Serum ferritin levels ranged from 31 to 2179 ng/ml and the serum transferrin saturation from 17 to 88.6%. Six patients had a serum ferritin above 450 ng/ml and 3 patients had transferrin saturation above 50%. These patients had not been treated. Six patients had low serum ferritin or transferrin saturation on re-examination but had phlebotomy treatment previously. Three young patients (25-38 years) showed high transferrin saturation but a moderate increase in serum ferritin level. Blood copper and ceruloplasmin levels were within normal range in all patients.

Perl's Prussian blue staining of liver biopsies showed pronounced iron accumulation in the parenchymal cells, Kupffer cells and portal macrophages of all these patients. There was minimal fibrosis in the specimens although most cases had steatosis. Only the two oldest patients had liver cirrhosis on biopsy, but no clinical sign of hepatic insufficiency. Five of the 6 patients had hepatomegaly and five had hemosiderosis. MRI scanning showed iron accumulation in patients' liver. With regard to morbidity, seven patients (58%) reported joints complaints and three (25%) had disturbed glucose metabolism. High cortisol levels were recorded in three patients (25%) and one patient (8%) had cardiomyopathy. Three (25%) patients reported fatigue, four (33%) had abdominal pains and four others (33%) had psychiatric disturbances. Medical records revealed one (8%) patient aged 55 years with colon carcinoma, osteoarthritis and obesity. Another patient had alcohol and smoking abuse.

Table. Characteristics of patients

Patient	Age (Years)	Ferritin (mmol/l)	Transferrin saturation (%)	Hepatic iron index	Liver pathologies	Phlebotomy	Clinical findings
II:3	76	219	40.4	---	Hemosiderosis, Steatosis	No	Smoking and drinking abuse
II:7	64	223	34.4	---	Hepatomegaly, hemosiderosis	---	Joint complaints, fatigue, abdominal pain, obesity, hypercortisolism
II:8	63	113	17.0	27.5	Hepatomegaly, hemosiderosis steatosis	Yes	Vascular disease, obesity, skin pigmentation, abdominal pain
II:10	80	133	27.9	12.3	Hepatomegaly, hemosiderosis, periportal steatosis	Yes	Osteoarthritis
II:12	REFUSED TO PARTICIPATE IN THE STUDY						
II:17	69	35	34.0	12.8	Hepatomegaly, hemosiderosis periportal and sternal	Yes	Diabetes mellitus, colon carcinoma
II:18	66	---	---	---	Liver steatosis without hemosiderin, arthritis	Yes	Hemosiderosis, diabetes mellitus
III:1	58	105	33.5	---	No liver biopsy performed	---	---
III:3	57	2179	82.8	---	No liver biopsy performed	No	Osteoarthritis, diabetes mellitus, skin pigmentation
III:5	55	1238	41.6	---	No liver biopsy performed	No	Osteoarthritis, hypertrophic cardiomyopathy, arrhythmia, burn out
III:8	43	382	16.0	---	No liver biopsy performed	---	---
III:11	53	31	24.1	---	Periportal hemosiderosis	Yes	Obesity, high cortisol
III:12	46	770	42.8	---	No liver biopsy performed	No	Diabetes mellitus
III:14	45	180	23.1	---	Hepatomegaly, Fe-stained periportal midzonal parenchymes	Yes	Joint complaints, fatigue, abdominal pains, obesity high cortisol
III:18	47	45	30.4	---	No liver biopsy	Yes	Treated for hemochromatosis
III:19	38	1279	88.6	---	No liver biopsy performed	No	Lumbago, abdominal pains, obesity
III:21	33	396	28.7	---	No liver biopsy performed	---	---
IV:1	32	1738	85.3	---	No liver biopsy performed	No	Joint complaints
IV:2	25	480	33.8	---	No liver biopsy performed	No	---
IV:3	24	109	19.6	---	No liver biopsy performed	---	---

---: No information available

The deferoxamine test was normal in all patients. The disease in this family segregated as a dominant trait with affected individuals in each generation and only one parent affected. None of the 12 patients was homozygous nor compound heterozygous for the C282Y or H63D mutation of the *HFE* gene. Negative lod scores were obtained for markers flanking loci on chromosome 1q, 6p or 7q suggesting that none of these loci were associated with the disease.

## Discussion

In this particular family, clinical manifestations of a long-standing and clear iron overload in parenchymal and non-parenchymal tissues is limited mainly to osteoarthritis of relatively late onset, and few patients with concomitant type 2 diabetes. The clinical picture also included hypertrophic cardiomyopathy without left ventricular outflow which is not typical in classical genetic hemochromatosis. The deferoxamine test was negative and iron overload in members of this family did not have very high levels of ferritin or transferrin saturation as seen in classical hemochromatosis patients. Serum ferritin and transferrin saturation levels were very high in patients who were not treated. Patients who underwent a phlebotomy responded very slowly to this treatment.

A very early onset of hemochromatosis was noticed in two patients (25 and 38 years) from this family. These patients did not show evidence for juvenile hemochromatosis.<sup>11</sup> Another hereditary form of iron overload disorder, the hyperferritinemia cataract syndrome,<sup>12</sup> is associated with a different clinical phenotype. The *HFE* C282Y or H63D gene mutations were not detected in this family. Iron overload in this pedigree segregated as an autosomal dominant trait. There have been some reports of families in which hemochromatosis segregated as a

dominant trait.<sup>2</sup> A pseudo-dominant mode of inheritance can not be excluded. Our genome screen reported in the next chapter shows evidence that the N144H mutation in the *SLC11A3* gene is associated with autosomal dominant hemochromatosis in this family.<sup>8</sup>

In conclusion, we have described a 5 generations family in which several members have an iron overload condition. The clinical chemistry is somewhat different and the ferritin and transferrin saturation are not as high as in classical adult hemochromatosis. Most strikingly the deferoxamine test in these patients is normal. The clinical findings include mainly joint pains and arthrosis, cardiomyopathies, liver fibrosis and diabetes mellitus.

### Acknowledgement

We thank the patients and relatives from the Genetic Research in Isolated Populations (GRIP) areas, the local health care centres and the municipality for making this study possible. We are grateful to J Janssens, and local physicians TWC Snieders, G Droge, P van Wouw, M Kraanen, C Van Broeckhoven, WHA Bettink and LJ Dronkers for their help during the study, E Wauters and J Houwing-Duistermaat for technical Assistance.

### References

1. Papanikolaou G, Politou M, Terpos E, Fourlemadis S, Sakellaropoulos N, Loukopoulos D. Hereditary hemochromatosis: *HFE* mutation analysis in Greeks reveals genetic heterogeneity. *Blood Cells Mol Dis* 2000;26:163-8.
2. Pietrangelo A, Montosi G, Totaro A, Garuti C, Conte D, Cassanelli S, Fraquelli M, Sardini C, Vasta F, Gasparini P. Hereditary hemochromatosis without pathogenic mutations in the hemochromatosis gene. *New Engl J Med* 1999;341:725-32.
3. Camaschella C, Fargion S, Sampietro M, Roetto A, Bosio S, Garozzo G, Arosio C, Piperno A. Inherited *HFE*-unrelated hemochromatosis in Italian families *Hepatology* 1999;29:1563-4.



4. Edwards CQ, Cartwright GE, Skolnick MH, Amos DB. Homozygosity for hemochromatosis: Clinical manifestations. *Ann Intern Med* 1980;93:519-25.
5. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
6. Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P. The gene *TFR2* is mutated in a new type of haemochromatosis mapping to 7q22. *Nature Genet* 2000;25:14-5.
7. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, Carella M, Zelante L, Kelly AL, Cox TM, Gasparini P, Camaschella C. Juvenile hemochromatosis locus maps to chromosome 1q. *Am J Hum Genet* 1999;64:1388-93.
8. Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JWF, Breuning MH, Snijders PJLM, Rutten WPF, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P. A mutation in *SLC11A3* is associated with autosomal dominant hemochromatosis. *Nature Genet* 2001;28:214-5.
9. Goossens JP. Idiopathic hemochromatosis: Juvenile and familial type-endocrine aspects. *Neth J Med* 1975;18:161.
10. Goossens JP, Schreuder I, Went LN. Inheritance of idiopathic hemochromatosis *Lancet* 1977;1:1106-7.
11. Camaschella C, Roetto A, Cicilano M, Pasquero P, Bosio S, Gubetta L, et al. Juvenile and adult hemochromatosis are distinct genetic disorders. *Eur J Hum Genet* 1997;5:371-5.
12. Girelli D, Piccoli P, Corrocher R. Hyperferritinemia-cataract syndrome: Description of a new hereditary disease, from anamnesis to molecular diagnosis. *Minerva Med* 1997;88:405-10.



## **A mutation in *SLC11A3* (ferroportin) is associated with autosomal dominant hemochromatosis**

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### **Summary**

Hereditary hemochromatosis (HH) is a very common disorder characterised by increased absorption and progressive storage of iron in body tissues resulting in multi-organ damage and widespread pathology. Several genes involved in iron metabolism have been implicated in the pathology of hemochromatosis<sup>1,2</sup>, but not all patients can be explained by mutations in these genes. A large Dutch family has been described in which the disease segregates as a dominant trait<sup>3,4</sup> that does not map to any known locus. We performed a systematic genome scan for linkage in this family and mapped the disease gene to chromosome 2q. In the critical region, the *SLC11A3* gene (ferroportin) is localised.<sup>5-8</sup> Subsequent mutation analysis identified a heterozygous base change in all patients but not in healthy individuals from the family or the general population. This A to C transversion at position 734 (A734C) leads to a substitution of asparagine by histidine (N144H) that is predicted to result in excessive *SLC11A3* mediated iron transport.

## **Introduction**

Hemochromatosis is one of the most common genetic disorders in populations of Caucasian origin with a prevalence of 2-3/1000.<sup>9-12</sup> In most patients, hemochromatosis is inherited as an autosomal recessive trait and mutations in several genes have been identified; homozygosity for the C282Y mutation in the *HFE* gene on chromosome 6p accounts for about 80% of Caucasian patients<sup>13</sup> and compound heterozygosity for other less severe mutations in the *HFE* gene is found in 10% of cases.<sup>14</sup> A mutation in a transferrin receptor (*TFR2*) on chromosome 7q has been identified in a single family, and a form of juvenile hemochromatosis maps to chromosome 1q.<sup>15</sup> Approximately 10% of patients can not be explained by mutations in these genes. Moreover, families with a dominant or pseudo-dominant mode of inheritance are reported for whom the genetic defect is unknown.<sup>3,4,16-18</sup>

We used a large Dutch family in which hemochromatosis segregates as a dominant trait (figure), for a genome wide scan for linkage.

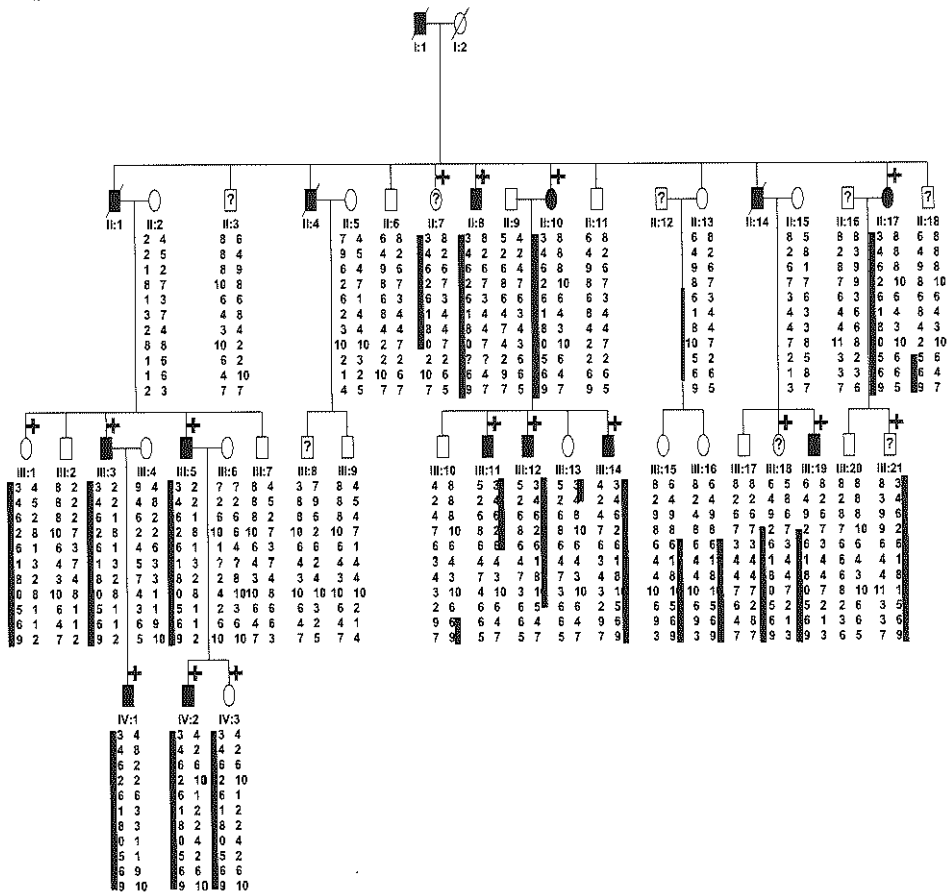
## **Methods**

This study is part of the Genetic Research in Isolated Population (GRIP) study, which is approved by the Erasmus University Rotterdam medical ethics committee.

## **Diagnosis of hemochromatosis**

The clinical symptoms in patients from this family were similar to complaints in other hemochromatosis patients and include joint pains, osteoarthritis, fatigue, cardiomyopathies and endocrine disorders like diabetes mellitus. Patients were suspected to have hemochromatosis if they had a blood ferritin level above 450 ng/μl and/or transferrin saturation >50%. A medical specialist further confirmed this diagnosis through liver biopsy and Magnetic Resonance Imaging (MRI) of the liver. Some subjects were called possibly affected because they had iron overload but did

not show iron accumulation in liver biopsy or on MRI. These were either very young males or females. The latter usually have a late age of hemochromatosis onset due to iron loss during menstruation. In the pedigree (figure 1), the possibly affected individuals are marked with a ? sign. Diseases with similar expression such as Hereditary Hyperferritinemia Cataract Syndrome (HHCS) and aceruloplasminemia were excluded (clinical details will be described elsewhere). Patients were treated by putting them on a regular phlebotomy regime.



**Figure 1.** Pedigree of the family with autosomal dominant hemochromatosis with haplotypes ordered from centromere to telomere. Black symbols represent affected individuals. White symbols represent unaffected individuals. Question mark indicates individuals who may be affected. + indicates confirmed carriers of N144H mutation. Only relevant family members are shown

### **DNA isolation and PCR:**

Genomic DNA was isolated from peripheral blood as described.<sup>25</sup> For the systematic genome scan 400 short tandem repeat polymorphism (STRPs) from the Genethon markers set were used (<http://www.genethon.fr>). Genomic DNA (25 ng) was amplified in 10 µl PCR reactions containing 1X GeneAmp PCR Gold Buffer; 1.5 mM MgCl<sub>2</sub>; 25 ng of fluorescent forward primer; 25 ng unlabelled reverse primer and 0.4 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Initial denaturation was 15' at 95 °C followed by 32 cycles of 30" denaturation at 95 °C, 30" annealing at 55 °C and 90" extension at 72 °C. PCR products were pooled and loaded on an ABI377 automated sequencer (filterset D; 5% denaturing FMC LongRanger acrylamide gel), data were analysed using ABI GeneScan3.1 and ABI Genotyper2.1 software.

### **Genetic linkage analysis**

Simulation studies using the SLINK program<sup>26</sup> showed that the family was sufficient for finding significant evidence for linkage with an average maximum lod score of  $Z=3.24$  by using the following linkage analysis model: hemochromatosis was assumed to be an autosomal dominant disorder with a disease gene frequency of 0.001. To account for differences in age at onset of male and female patients three (age and sex dependent) liability classes were defined with penetrances of 0.02, 0.6 and 0.8 for class 1 (male < 30, female < 40 years), class 2 (male 30-60 and female 40-70 years) and class 3 (male >60, female >70 years), respectively. Two point linkage analysis was performed using the MLINK and ILINK programs of the LINKAGE package (version 5.1).<sup>27</sup> Recombination frequency ( $\theta$ ) was assumed to be equal for males and females. Equal allele frequencies were used. For haplotype analyses, phase was assigned on the basis of the minimum number of recombination events.

## Mutation analysis

*SLC11A3* has been described under several aliases; iron regulated transporter gene 1 (*IREG1*), metal transport protein 1 (*MTP1*) or Ferroportin1 (*FPN1*). (GENBANK accession numbers AF226614, AF231121 and AF215636).

The genomic structure of *SLC11A3* was determined by aligning cDNA (AF231121) and genomic (RP11-270G18, AC013439) sequences. *SLC11A3* exons (1-8) were amplified from genomic DNA using primers designed to flank intronic sequence (at least 50 bp intronic sequence on both sides of each exon). The following primers were used:

Exon	Forward primer 5'-3'	Reverse primer 5'-3'
1	CCCCGACTCGGTATAAGAG	TTCTCCAGAACTCG TGT
2	TGGATAAGCATTCTGCCCTC	TAAAGCATGTGTACTTGGATG
3	AATGTAGCCAGGAAGTGCC	AGAGGTGGTGCCATCTAAG
4	GGATAAGAACAGTCTCACTG	TTCATCCTTTACCACTACCAG
5	TTAAACTGCCTTGTTTAGTG	GCCTCATTATCACCACCG
6	TTGTGTAAATGG GCAGTCTC	CCTCGTCTACCAAAGCGATA
7part1	GCTTTTATTCTACATGTCC	GCTGTGCCAATCCTGAGATC
7part2	GAGCATCAGCTATAACTGG	TAATGGATTCTCTGAACCTAC
8part1	TTGAAATGTATGCCTGTAAAC	TTCCATGCCTCAACATAAGG
8part2	GTTTTTACCACAGCTGTGCC	ATACCTAAGATCAATAGGATC

25 µl PCR reactions contained 2.5 mM dNTP, 1mM MgCl<sub>2</sub>, 1.25 unit of Taq polymerase (Gibco BRL) and 25 ng DNA. Amplification was done using 10' initial denaturation at 94 °C, 35 cycles of 30" at 94 °C, 30" at 55 °C and 1' at 72 °C with a final extension of 5' at 72 °C. PCR products were purified using the Millipore Multiscreen®-PCR Plates, and their approximate concentrations estimated using a DNA size standard (BRL).

Mutation detection was done by direct sequencing of both strands of PCR products on an ABI377 automated sequencer using BigDye Terminator chemistry (Applied Biosystems). Analysis was done with Factura software and Sequence Navigator (Applied Biosystems) for heterozygous base calls and sequence alignment.

Testing of the base change in exon 5 in remaining family members and controls was done using Allele Specific Oligonucleotide Hybridisation (ASO). PCR products containing exon 5 were amplified as described under sequencing. PCR products were blotted onto positively charged membranes. The blots were hybridised at 37°C for 45' with either the normal (TATTGCA~~A~~ATTGGC) or mutated sequence (TATTGCACATTGGC). Filters were washed until a final stringency of 0.3 x SSC/0.1% SDS was obtained for 15' at 37°C.

#### **Protein structure prediction and amino acid alignment:**

Sequences were found by homology searches of the human protein sequence to the non-redundant database using the BLAST algorithm. Aligning of the sequences was done using the program Vector NTI which implemented the Clustal-W algorithm.

The protein structure was predicted using PEPTIDE STRUCTURE and PEPLOT programs from Genetics Computer Groups (GCG) to obtain hydropathy plots. Transmembrane domains were identified and their topography predicted using TopPred, PHDhtm, SOSUI, HMMTOP, TMHMM and Tmpred programs. These programs predicted 9 or 10 transmembrane domains and all predicted the N144H mutation to be in a transmembrane domain.

#### **Northern blot analysis**

Northern blots containing multiple adult human tissues (2 µg of polyA mRNA per lane) (Clontech), and multiple adult human digestive system tissues (1 µg of polyA mRNA per lane) (Clontech) and a Northern blot containing human monocytes and



lymphoblast cells (10 µg total RNA) were hybridised with a genomic 431 bp PCR product, from exon 7 of *SLC11A3*, using ExpressHyb™ hybridisation solution (Clontech) according to the manufacturers instructions. Labelling of the probe was done using the Strip-EZ DNA™ kit (AMBION).

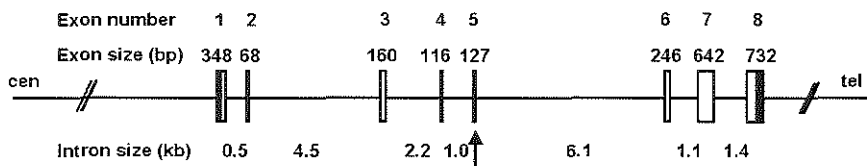
## Results and Discussion

For several markers on chromosome 2q, two-point linkage analyses yielded positive lod scores (table 1). The highest was obtained with marker D2S389, ( $Z_{\max}=3.01$  at  $\theta=0.0$ ). We tested additional markers from the region and constructed haplotypes (figure1). All patients share a common allele for markers D2S389 and D2S2167 but recombination events were identified for D2S2273 on the centromeric site and D2S117 on the telomeric site, defining the critical region to 9 cM on the sex average genetic map. This is the fourth locus identified for hemochromatosis and we propose to name it *HFE4*.

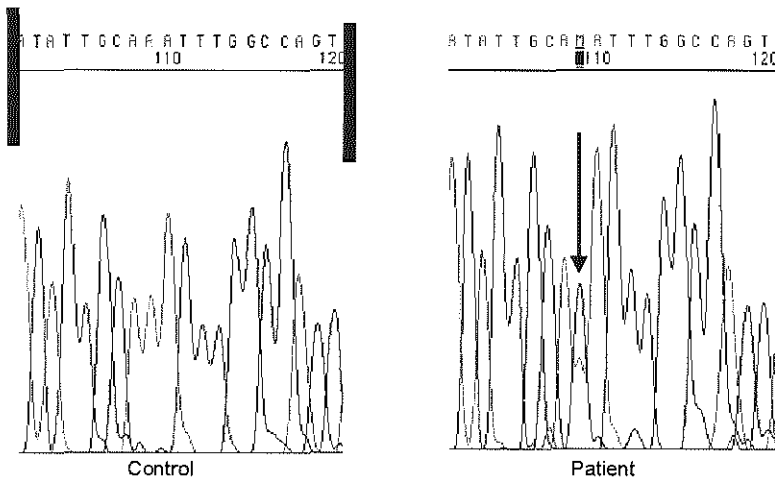
**Table.** Lod scores for markers on chromosome 2q

Marker	Recombination fraction ( $\theta$ )						
	0.00	0.01	0.05	0.10	0.20	0.30	0.40
D2S335	0.77	1.36	1.75	1.74	1.40	0.89	0.32
D2S2314	1.90	1.87	1.72	1.54	1.14	0.71	0.27
D2S2273	0.69	1.13	1.50	1.51	1.22	0.78	0.29
D2S389	3.01	2.96	2.73	2.44	1.80	1.09	0.38
D2S2167	0.45	0.44	0.38	0.31	0.21	0.13	0.07
D2S117	1.60	1.64	1.70	1.62	1.27	0.78	0.27
D2S311	0.99	1.04	1.14	1.13	0.91	0.55	0.16
D2S2392	1.59	1.55	1.41	1.23	0.86	0.48	0.17
D2S2289	1.06	1.11	1.18	1.15	0.91	0.57	0.22
D2S325	0.90	0.90	0.94	0.95	0.81	0.52	0.19
D2S2382	1.12	1.09	1.00	0.87	0.61	0.36	0.13

A search for candidate genes in public databases and literature identified a large number of expressed sequence tags and genes with known functions. The most obvious candidate gene was the recently described *SLC11A3* gene encoding the metal transporter also called ferroportin1 (*FPN-1*), metal transporter protein (*MTP1*) or iron regulated gene (*IREG1*) which has been mapped to human chromosome 2q.<sup>5-8</sup>



**Figure 2.** Genomic structure of *SLC11A3* spanning 20KB. Exons are indicated by boxes, number and sizes are shown above

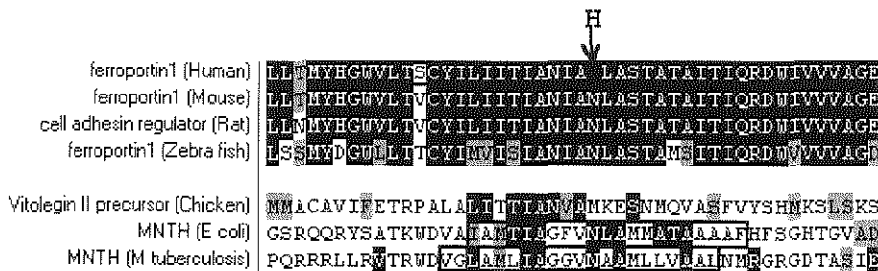


**Figure 3.** Mutation analysis: control (left) and patient (right) sequences depicting the mutation

On genomic DNA, the human *SLC11A3* gene encompasses 20kb and consists of 8 exons (figure 2). The open reading frame of 1716bp starts at position 305 of exon 1 and ends at position 314 of exon 8 encoding a protein of 571 amino acids with 9 or 10 transmembrane domains. Since there has been conflicting evidence in the literature, we confirmed that human *SLC11A3* is expressed in most tissues involved in iron metabolism. Expression is highest in the digestive tract, liver, placenta, kidneys and monocytes. *SLC11A3* is a basolateral membrane protein implicated in the movement of iron across the enterocytes to the circulation.<sup>6-8,19-21</sup> *SLC11A3* mRNA contains the stem-loop structure characteristic of an iron responsive element (IRE) in its 5' untranslated region (UTR). This IRE binds to iron responsive proteins 1 and 2 (IRP1 and IRP2) indicating that expression of SLC1A3 is probably regulated by intracellular iron levels.<sup>11</sup>

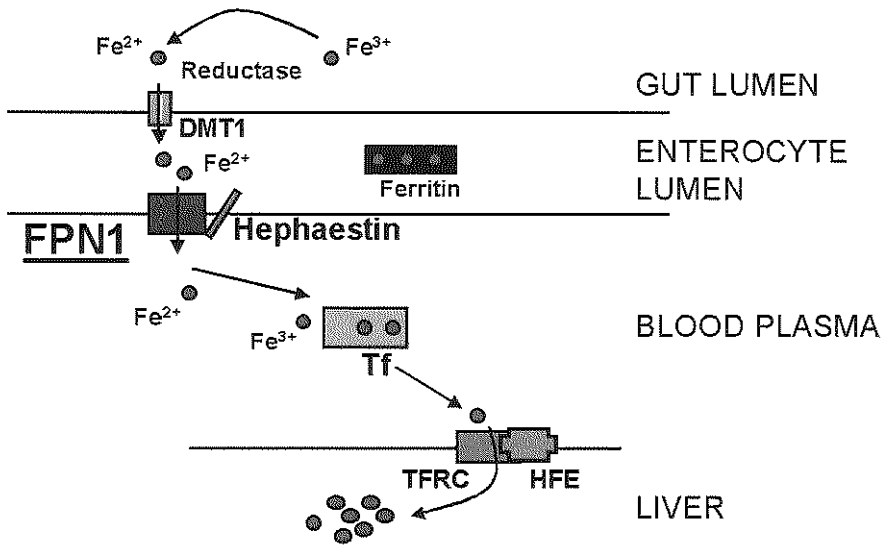
Mutation analysis of all exons including intron-exon boundaries revealed a heterozygous A to C at position 734 in exon 5 in all patients but not in healthy individuals from the family (figure 3) or 200 healthy controls from the Dutch general population, suggesting that this base change is the causative mutation. The mutation leads to an amino acid substitution of asparagine by histidine (N144H). Linkage disequilibrium of this mutation with another yet unidentified locus is possible but this is not likely for a number of reasons. First, the entire coding region plus intron-exon boundaries were sequenced and no other sequence alteration segregating with the disease was found. Moreover, the substituted asparagine is a highly conserved amino acid in vertebrates suggesting that this is an important amino acid for *SLC11A3* function. The mutation is predicted to reside in a transmembrane domain by all

protein structure prediction programs used. Asparagine is a neutral amino acid and is replaced by histidine, which is polar, reducing the hydrophobicity of the transmembrane domain suggesting an effect on the structure (folding) or function of the protein. Interestingly, a search for homology of the regions directly adjacent to the mutation revealed other divalent metal transporter that otherwise show little homology to *SLC11A3* (figure 4) suggesting that the mutation resides within a region of the protein that is important for metal ion binding or transport. Ferrous iron (Fe<sup>2+</sup>) is transported through the apical surface of the enterocytes by the divalent metal transporter *DMT1* (figure 5).



**Figure 4.** Sequence homology analysis of the region around the N144H mutation

Iron can then be stored as ferritin or transported across the basolateral membrane by *SLC11A3* with the aid of haephaestin (Hp). In the blood plasma, iron is then loaded onto ferrotransferrin as ferric iron (Fe<sup>3+</sup>) and subsequently taken up by body tissues such as the liver by transport over a transferrin receptor (*TFR2*) that is associated with *HFE*.<sup>22</sup> Mutated *SLC11A3* in *HFE4* patients may cause disruption of the normal regulation of *SLC11A3* activity and lead to transport of excessive amounts of iron out of the enterocytes towards the circulation. This suggests that the N144H is an activating mutation. As a result, iron levels in the enterocytes are expected to be depleted. Intracellular iron levels control activity of *DMT1*.



**Figure 5.** Model for iron transport from the gut lumen to body through enterocytes into the circulation

Low iron levels will lead to increased iron uptake by *DMT1*. The excessive uptake of iron from the lumen of the gut and transport to the circulation then leads to accumulation of iron in blood plasma and body tissues. How the mutation reported here results in overactivation of *FPN1* is unknown but modelling the effect of the mutation in functional experiments will allow us to understand the effect of this protein on iron metabolism in more detail.

We have described an heterozygous mutation in the *SLC11A3* gene that can explain autosomal dominant hemochromatosis and which may also have important implications for explaining hemochromatosis in 10% of patients who do not carry the *HFE* and *TFR2* gene mutations. In addition, it was recently reported that a mutation in the *DMT1* gene leads to anemia in rodents.<sup>23-24</sup> Since this activating mutation in *SLC11A3* may result indirectly in activation of *DMT1* activity, as described above, *SLC11A3* may be an attractive target for developing therapeutic approaches for anemia as well.

## Acknowledgements

We thank the patients and relatives from the Genetic Research in Isolated Populations (GRIP) areas, the local health care centres and the municipality for making this study possible. We are grateful to JP Goossens, J Janssens, and local physicians TWC Snieders, G Droge, P van Wouw, M Kraanen, C Van Broeckhoven, WHA Bettink and LJ Dronkers for their help during the study, E Wauters and J Houwing-Duistermaat for technical Assistance.

## References

1. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
2. Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P. The gene *TFR2* is mutated in a new type of hemochromatosis mapping to 7q22. *Nature Genet* 2000;25:14-5.
3. Goossens JP. Idiopathic hemochromatosis: juvenile and familial type-endocrine aspects. *Neth J Med* 1975;18:161.
4. Goossens JP, Schreuder I, Went LN. Inheritance of idiopathic hemochromatosis. *Lancet* 1977;1:1106-7.
5. Haile DJ. Assignment of *SLC11A3* to mouse chromosome 1 band 1B and *SLC11A3* to human chromosome 2q21 by in situ hybridization. *Cytog Cell Genet* 2000;88:328-9.
6. Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt JS, Moynihan J, Paw BH, et al. Positional cloning of zebrafish ferroportin 1 identifies a conserved iron exporter. *Nature* 2000;403:776-81.
7. McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, et al. A novel duodenal iron-regulated transporter ferroportin1 implicated in the basolateral transfer of iron to the circulation. *Molecular Cell*. 2000;5:200-9.
8. Abboud S, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *The Journal of Biochem* 2000;26:19906-12.
9. MacSween RNM, Scott AR. Hepatic cirrhosis: a clinico-pathological review of 520 cases. *J Clin Path* 1973;26:936-42.
10. Beaumont C. Serum ferritin as a possible marker of the hemochromatosis allele. *New Eng J Med* 1979;301:169-74.
11. Cartwright GE. Hereditary hemochromatosis: phenotypic expression of the disease. *New Eng J Med* 1979;301:175-9.

12. Lalouel JM. Genetic analysis of idiopathic hemochromatosis using both qualitative (disease status) and quantitative (serum iron) informations. *Am J Hum Genet* 1985;37:700-18.
13. Rochette J. Multicentric origin of hemochromatosis gene (*HFE*) mutations. *Am J Hum Genet* 1999;64:1056-62.
14. de Villiers JN, Hillerman R, Loubser L, Kotze JM. Spectrum of mutations in the *HFE* gene implicated in haemochromatosis and porphyria. *Hum Mol Genet* 1999;8:1517-22.
15. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, Carella M, Zelante L, Kelly AL, Cox TM, Gasparini P, Camaschella C. Juvenile hemochromatosis locus maps to chromosome 1q. *Am J Hum Genet* 1999;64:1388-93.
16. Eason RJ, Adams PC, Aston CE, Searle J. Familial iron overload with possible autosomal dominant inheritance. *Aust N Z J Med* 1990;20:226-30.
17. Camaschella C, Fargion S, Sampietro M, Roetto A, Bosio S, Garozzo G, Arosio C, Piperno A. Inherited *HFE*-unrelated hemochromatosis in Italian families. *Hepatology* 1999;29:1563-4.
18. Pietrangelo A, Montosi G, Totaro A, Garuti C, Conte D, Cassanelli S, Fraquelli M, Sardini C, Vasta F, Gasparini P. Hereditary hemochromatosis without pathogenic mutations in the hemochromatosis gene. *New Engl J Med* 1999;341:725-32.
19. Cairo G, Pietrangelo A. Iron regulatory proteins in pathobiology. *Biochem J* 2000;352:241-50.
20. Kaplan J, Kushner P. Mining the genome for iron. *Nature* 2000;403:711-2.
21. Lieu PT, Heiskala M, Peterson PA, Yang Y. The roles of iron in health and disease. *Mol Aspects Med* 2001;22:1-87.
22. Gross CN, Irrinki A, Feder JN, Enns CA. Co-trafficking of *HFE*, a non classical major histocompatibility complex class I protein with the transferrin receptor implies a role in intracellular iron regulation. *J Biol Chem* 1998;273:22068-74.
23. Flemming MD. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genet* 1997;19:383-6.
24. Flemming MD. Nramp2 is mutated in the anaemic Belgrade rat: evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci USA* 1998;95:1148-53.
25. Miller, SA Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 1988;16:1215.
26. Weeks DE, Ott J. SLINK: a general simulation program for linkage analysis. *Am J Hum Genet* 1990;47:204.
27. Lathrop GM, Lalouel JM. Easy calculations of lod scores and genetic risks on a small computer. *Am J Hum Genet* 1984;36:460-5.





## The hemochromatosis N144H mutation of SLC11A3 in patients with type 2 diabetes

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### Introduction

We recently described a mutation (N144H) in the gene *SLC11A3*, encoding the metal transporter also called ferroportin, associated with an autosomal dominant form of hemochromatosis.<sup>1</sup> This mutation is located in a conserved region of the protein and is thought to result in excessive *SLC11A3* mediated iron transport from the enterocyte to the circulation and iron accumulation in body organs. Mutations in the *HFE* gene associated with hemochromatosis have been implicated in the development of type 2 diabetes mellitus<sup>2</sup> and iron storage has been shown to be associated with insulin resistance and abnormal glucose tolerance.<sup>3</sup> Known *HFE* mutations explain up to 11% of the incidence of type 2 diabetes.<sup>4</sup>

### Methods

To evaluate the role of *SLC11A3* in type 2 diabetes, we screened a series of 200 patients with type 2 diabetes and 298 healthy subjects for the newly described N144H mutation. The patients were ascertained in a population-based study of type 2 diabetes. The diagnosis of diabetes was based on the 1998 criteria of the American Diabetes Association. In all patients, diabetes was diagnosed after the age of 30 years and none of the patients was insulin dependent within the first year following diagnosis. All diabetes patients and 98 of the control subjects were derived from the geographic area in which the mutation was identified. The remaining 200 control individuals came from the general Dutch population.

## Results

We observed that four (2%) of patients with diabetes were heterozygous for the N144H mutation but none of the healthy controls ( $P < 0.025$ ; Fisher exact test). These diabetes patients have also been described previously with autosomal dominant hemochromatosis.<sup>1</sup> There was an autosomal dominant parent to child transmission of diabetes and none of these patients was carrier of the *HFE* C282Y or H63D mutation. Serum ferritin level of these patients ranged from 770 to 2179 ug/l (normal; 20-350 ug/l) and the serum transferrin saturation ranged from 43 to 88% (normal; < 45%). The age of onset of diabetes ranged from 55 to 70 years.

## Discussion

Our findings and the fact that *SLC11A3* is located on chromosome 2q where several genome scans in diabetes families localised a susceptibility locus<sup>4</sup> suggest that *SLC11A3* may be an important gene in a sub-group of patients with diabetes. This may be the N144H mutation we found or another mutation in *SLC11A3*. The dominant transmission of hemochromatosis in our families further suggests that this mutation may explain some autosomal dominant forms of diabetes.

Few dominant genes are known that account for diabetes. The frequency of 2% of the *SLC11A3* gene mutation in diabetes patients is higher than the frequency of other dominant mutations in type 2 diabetes.<sup>4</sup> Excess body iron in carriers of the N144H mutation may enhance oxidation of free fatty acids through accelerated production of free radicals<sup>5</sup>. This may lead to diminished glucose utilisation in muscle tissue and increased gluconeogenesis in the liver, leading to insulin resistance.<sup>5</sup>

Although our study suggests a relatively high frequency of the N144H mutation in type 2 diabetes patients, it remains to be determined whether this mutation is specific

to the population we studied and whether the *SLC11A3* gene is involved in type 2 diabetes elsewhere.

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### References

1. Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JWF, Breuning MH, Snijders PJLM, Rutten WPF, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P. A mutation in *SLC11A3* is associated with autosomal dominant hemochromatosis. *Nature Genet* 2001;28:214-5.
2. Moczulski DK, Grzeszczak W, Gawlik B. Role of hemochromatosis C282Y and H63D mutations in *HFE* gene in development of type 2 diabetes and diabetic nephropathy. *Diabetes care* 2001;24:1187-91.
3. Tuomainen TP, Nyysönen K, Salonen R, Tervahauta A, Korpela H, Lakka T, Kaplan GA, Salonen JT. Body iron stores are associated with serum insulin and blood glucose concentrations: population study in 1,013 eastern Finnish men. *Diabetes care* 1997;20:426-8.
4. Froguel P. Genetic determinants of type 2 diabetes. *Recent Prog Horm Res* 2001;56:91-105.
5. Oberley LW. Free radicals and diabetes. *Free radicals Biology & Medicine* 1988;5:113-24.



## Chapter 5

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*General discussion*



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## Chapter 5.1

### Introduction

The goal of genetic epidemiology is to study the genetic etiology of diseases. There were two main aims for the present thesis. The first aim was to study the effects of the hemochromatosis gene (*HFE*) mutations on serum iron levels and disease associated conditions. Secondly, we aimed at identifying other genes involved in hemochromatosis.

Numerous studies have been carried out on the genetics and epidemiology of hemochromatosis. Still there is a lot of controversies in the literature as to what the contributions of *HFE* gene mutations are to liver diseases, diabetes mellitus, and vascular pathology. Most controversies are due to differences in study designs, case-definition of hemochromatosis, ethnic composition of populations studied, risk modifiers, and genetic heterogeneity. In order to translate the results of genetic research into public health policies, population-based studies are necessary to evaluate the impact of the gene on risk of disease, mortality and quality of life. In this thesis, we have used a population-based cohort study of elderly individuals, the Rotterdam Study, to quantify the effect of *HFE* mutations on several disorders. Further, a genomic screen was carried out in a family with an autosomal dominant form of hereditary hemochromatosis from a genetically isolated community in the south-west of The Netherlands.

This chapter focuses on the main findings of this thesis and their relevance. Chapter 5.2 discusses the findings of population-based studies of *HFE* and the genome screen. Some methodological issues as well as suggestions for future research are embedded. In chapter 5.3, future perspectives and final remarks are given.





## **Main findings in population-based and genome studies**

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### **Population-based studies of HFE**

#### **Effect of *HFE* mutations on serum iron levels**

This study was carried out in the framework of the Rotterdam Study, a cohort study of elderly persons aged 55 years and above. We found that both heterozygotes and homozygotes for the C282Y mutation have significantly increased serum iron and ferritin levels as well as transferrin saturation. We observed an allele dose effect for the H63D mutation on serum iron parameters. The effects of *HFE* mutations on serum iron were more pronounced in men than women. These findings are in line with previous reports.<sup>1-5</sup> We next addressed the question whether we can use *HFE* to screen for hemochromatosis. Screening for the most common mutations may be a cheap alternative to screening based on biochemical parameters. Our study suggests that screening for hemochromatosis based on *HFE* mutations has a low sensitivity and specificity and will not be effective. Although the positive predictive value was high (chapter 3.2), many potential patients will remain undetected. This finding is in agreement with the low penetrance observed by us and others.<sup>1,6</sup> Beutler et al, (2002)<sup>7</sup> estimated that less than 1% of homozygotes for C282Y develop frank clinical hemochromatosis and also that the symptoms commonly associated with hemochromatosis are not more prevalent among homozygotes or compound heterozygotes.<sup>7</sup> This report is at odds with the finding that 80% of patients with hereditary hemochromatosis are homozygous for the C282Y mutation. The key issue here is the case definition of hemochromatosis. Since there is no standardised case definition of hemochromatosis, we used a transferrin saturation > 45% to define subclinical hemochromatosis<sup>8-10</sup> in our population-based study, while Beutler et al,

(2002)<sup>7</sup> used a combination of both serum transferrin and clinical signs or symptoms for hemochromatosis. As stated earlier, signs and symptoms of hemochromatosis are very unspecific. As we are unable to detect hemochromatosis in patients who do not have the *HFE* gene mutations and cannot discriminate those with these mutations that will ultimately not develop the disease,<sup>11-14</sup> screening based on transferrin saturation remains the "gold standard" for early detection and treatment of hemochromatosis.

It is important that studies on gene-gene and gene-environment interactions are set up to answer questions like why some people with the C282Y mutation do not develop hemochromatosis, what the precise mechanism of iron loading in each type of hemochromatosis is and what the modifiers of these mechanisms are.

#### ***HFE* mutations and liver functions**

In this population-based study, we did not find significant evidence for an effect of *HFE* mutations on liver function. This finding is compatible with the hypothesis that *HFE* mutations explain only a limited part of iron variance in the population.<sup>1,7</sup> Yet, we observed that serum iron levels were significantly associated with liver function tests. Some studies have indeed suggested that the C282Y mutation does not have a direct effect on liver diseases nor does it have an effect on hepatic iron stores.<sup>15-17</sup> However, this is not confirmed by other studies.<sup>18-20</sup>

Of interest is the finding that the H63D mutation is significantly related to serum bilirubin levels and shows an allele dose response. Importantly, it has been reported that bilirubin may have some protective effects against coronary heart disease<sup>21,22</sup> and neuro-degenerative diseases like Alzheimer's disease.<sup>23,24</sup> Thus, although high iron levels may have toxic effects through oxidation, our findings suggest that this potentially harmful effects may be counter-balanced by bilirubin through its antioxidative effect. A survival analysis in our population shows that higher bilirubin

levels are significantly associated with longevity. Further, our result points more towards an effect of the *HFE* gene product on iron handling in the erythron or the reticulo-endothelial system, rather than on duodenal iron transport. A major problem to overcome in future research is the difficulty to assess liver injury in population studies because damages to the liver can not be assessed solely by measuring serum enzymes and proteins. A point of special interest will be the mechanism by which the H63D mutation leads to higher bilirubin production.

### ***HFE* mutations and type 2 diabetes**

Diabetes is the most frequently studied disorder in relation to *HFE*. In this thesis, two epidemiological research methods were used to assess the role of *HFE* mutations on the development of type 2 diabetes: a meta-analysis of published literature and a nested case-control study within the Rotterdam Study. The meta-analysis suggested that there is no increased frequency of the C282Y mutation in patients with type 2 diabetes. Our case-control study showed that neither the C282Y nor the H63D mutation is a risk factor for end stage diabetes when studying prevalent patients. Our findings agree with those of the large study of Beutler et al, (2002).<sup>7</sup> There appears to be a major flaw in the interpretation of their data. In men, a significantly reduced prevalence of diabetes is seen in those homozygous for C282Y (1.8%) compared to controls (9.6%) (odds ratio (OR):0.34;95% confidence interval (CI):0.12-0.88; P=0.037). In those with high serum ferritin, the prevalence of diabetes is also significantly reduced in subjects homozygous for C282Y (6.3% versus 16.4% in controls; OR 0.34; 95% CI:0.12-0.88; P=0.015) and compound heterozygotes (prevalence 8.8%;OR=0.49; 95%CI:0.25-0.92; P=0.018). In subjects with a transferrin saturation level above 50%, a similar trend is seen (OR=0.38, 95%CI: 0.09-1.37; P=0.11 for homozygotes and OR=0.25, 95% CI: 0.04-1.00; P=0.05 for compound

heterozygotes). Under the hypothesis of no effect of C282Y on the risk of or survival from diabetes, the frequency of disease is expected to be similar across genotypes. Therefore, we do not agree with the conclusion that *HFE* is not associated with diabetes: the prevalence of diabetes is consistently and substantially reduced in carriers in various subsets.

How to interpret a significant decrease in the prevalence of diabetes in homozygotes for C282Y? Is the mutation protective for diabetes in these subsets? We think the findings of Beutler et al, (2002)<sup>7</sup> strongly argue against this unexpected conclusion. Table 3 shows a 2.12 (95%CI 1.86-2.43; P=0.0001) fold increased risk of diabetes in subjects with high ferritin levels (16.4% compared to 8.4% overall), while table 2 shows that the mutation is associated with increased serum ferritin levels. These findings are not compatible with a reduced risk of diabetes for carriers. The low prevalence of diabetes in carriers in this subgroup may be explained by an increased mortality in carriers developing diabetes. Alternatively, selection bias may have occurred based on the genotype. In this respect, it is important that, of the 45 homozygotes diagnosed (30%) with hemochromatosis, only 17 (38%) were included in their study.

Differential mortality and selection bias in patients are two well known phenomena in epidemiology<sup>25</sup> and have important clinical implications. We cannot make any prediction on this study of prevalent patients with regard to risk of disease or mortality. Given the possible mortality of patients with diabetes carrying the C282Y mutation as well as their finding of an increased risk of liver pathology,<sup>7</sup> we think their report provides a strong argument to screen for carriers of *HFE* mutations and prevent iron related pathology by phlebotomy.<sup>26</sup>

### ***HFE* mutations and stroke**

A recent study carried out in The Netherlands has shown that the C282Y mutation is associated with increased mortality from cerebrovascular diseases. We studied *HFE* mutations as risk factors for stroke and carotid atherosclerosis in a case-control study nested within the Rotterdam Study. Our study in chapter 3.2 showed similar effects of heterozygosity for the C282Y and H63D mutation on serum iron levels. We observed no increased frequency of *HFE* mutations in patients with stroke. The intima-media thickness (IMT) of the common carotid artery did not differ between *HFE* carriers and non carriers. However, we found that the *HFE* mutations were an effect modifier of the relationship between stroke and its classical risk factor, smoking. Roest et al, (1999)<sup>27</sup> found an interaction between the *HFE* C282Y mutation and smoking. In line with our results, Rossi et al, (2000)<sup>28</sup> reported that the C282Y mutation does not influence the intima-media thickness of the carotid artery. In conclusion, the association between cardiovascular diseases and *HFE* mutations is still a matter of controversy. It is likely that iron overload is associated with vascular diseases independent of *HFE* mutations, but that in the presence of this mutation and classical risk factors, the risk of vascular pathology is increased.

## **Findings in genome screen**

### **Familial autosomal dominant hemochromatosis**

A considerable number of patients suffer from hemochromatosis but have no evidence of mutations in the *HFE* or other genes involved in hemochromatosis.<sup>29-34</sup> Genetic heterogeneity is a well recognised feature of hemochromatosis. Through a study of type 2 diabetes in a genetically isolated population, we came across a family with multiple affected persons with hemochromatosis. This family is part of a population

that has been founded by approximately 150 persons in the middle of the 18<sup>th</sup> century and has now expanded to up to 20000 inhabitants. Although this is not the first family reported with autosomal dominant hemochromatosis,<sup>30</sup> in most families this disease segregates as a recessive trait.<sup>33-35</sup> Also a pseudo-dominant mode of inheritance can not be excluded, pedigree analysis shows that the mode of inheritance is autosomal dominant. As described in chapter 4.2, the clinical symptoms in members of this family are similar to that reported in type 1 hemochromatosis but the arthritis is more severe. There is a relatively early onset of hemochromatosis in males of this family. Of special concern is the fact that phlebotomy in these patients does not lead to a rapid fall in iron levels and has to be repeated several times, which usually leads to anemia in these patients. Surprisingly the deferoxamine test is negative (chapter 4.2). This disease could not be explained by known mutations in the *HFE* and *TFR2* genes described in chapter 2.

### **Genomic screen for autosomal dominant hemochromatosis**

The genome wide screen to identify other genes involved in hemochromatosis was carried out in the large family described in chapter 4.2. We identified a heterozygous mutation segregating with the disease (chapter 4.3) in the gene encoding the metal transporter *SLC11A3*. The substituted amino acid resides in a transmembrane domain and is a highly conserved amino acid in several species. Shortly after the publication of our findings,<sup>36</sup> Montosi et al, (2001)<sup>37</sup> also reported a mutation (A77C) in the same gene that was associated with autosomal dominant hemochromatosis in an Italian family. Our findings point towards a role of *SLC11A3* in iron transport and offer an opportunity for functional studies to be carried out. It is also important that the prevalence of this new mutation in the general population is studied. This newly described mutation has important implications in explaining hemochromatosis in

patients who do not carry the *HFE* or *TFR2* mutations,<sup>34,35</sup> or the occurrence of this disease in other ethnic groups. More importantly, because of its possible activation of the *DMT1* gene which is associated with anemia in rodents, it can be used as a target in the treatment of anemia.<sup>38,39</sup>

### **The N144H mutation in patients with type 2 diabetes**

As hemochromatosis is frequently reported in patients with diabetes, the identification of a mutation associated with autosomal dominant hemochromatosis (chapter 4.3) offered the opportunity to study the prevalence of this mutation in patients with type 2 diabetes in our isolated population. We observed that the up to 2% of patients with type 2 diabetes were carriers of the N144H mutation (chapter 4.4). These patients were derived from the family investigated for hemochromatosis and are not a representative sample of the general Dutch population. Few dominant mutations are reported that account for up to 2% of diabetes.<sup>40</sup> The exact mechanism by which this mutation might lead to type 2 diabetes is currently not known. It is possible that this happens by enhancing the oxidation of free fatty acid through accelerated free radical production leading to gluconeogenesis and insulin resistance.<sup>41</sup>





## **Future perspectives and final remarks**

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### **Future perspectives**

The last decade has witnessed a lot of research into the genetics and epidemiology of iron overload disorders like hemochromatosis. Still there remains a lot of unanswered questions. The first priority in the field of iron overload research is the definition of hemochromatosis. Despite the wealth of informations about this condition and the meeting of an expert panel, there is still no standardized case definition of hemochromatosis. This calls for studies to be carried out on the clinical signs, symptoms and progression of the disease in relation to the genotype and phenotype.

An important issue to address in future research is the effect of genetic or environmental modifiers on the risk of hemochromatosis. Studies on gene-gene and gene-environment interactions on the disease process are necessary to understand for example why some individuals homozygous for the C282Y mutation do not have iron overload and what the effect of other physiological, pathological and environmental factors on the risk of hemochromatosis in genetically susceptible individuals is.

The translation of genetic and epidemiologic advances in hemochromatosis into effective public health policies calls for studies on the cost-effectiveness, cost-benefit and cost-utility analyses to be carried out. From this point of view, all informations critical for the implementation of population screening for hemochromatosis are still lacking and need the input and cooperation of scientists in several fields of research.

**Final remarks**

The discovery of the *HFE* C282Y and H63D gene mutations has been a landmark in the field of iron overload disorder. However, this great discovery has brought about more problems than it intended to solve. First, the description of two mutations led all laboratories to start testing only for these mutations and the definition of hemochromatosis was modified to encompass homozygosity for the C282Y mutation. This is a wrong definition because other genes are known to cause hemochromatosis and moreover hemochromatosis in other ethnic groups than the caucasian are not due to *HFE* mutations. Up to date, the precise mechanism by which these mutations lead to iron overload is still not known. The most important pitfall of the *HFE* gene identification remains the unsolved issue of patent. In a recent commentary by Merz et al, (2002),<sup>42</sup> entitled: Diagnostic testing fails the test, the pitfalls of patents are illustrated by the case of hemochromatosis. The patenting of the *HFE* gene has thus led to restriction in offering genetic testing to patients and to inhibit clinical studies needed to validate the discovery of the gene. This results in limited obvious concerns about test quality, patient access to testing services, the cost of clinical testing, innovation of testing methods, and the potential for placing limitations on clinical research. It will be important that genes are identified in the interest of the patients or the progress of research. Unfortunately, this has not been the case with several genes including the *HFE*. Recent data show that patents have inhibited the validation of clinical assays and testing may be compromised by limiting laboratories to a single kit rather than cheaper or better ones. In delivering patents for scientific discoveries, lawyers must be aware of and take into account, the effects of patents on research in general and public health in particular.

A point of special concern to a genetic-epidemiologist is, what is hemochromatosis. Is hemochromatosis a risk factor like hypertension or is it a genetic disease with delayed but potentially severe complications. Clearly defined, a genetic disease carries with it the implication that all or most of the individuals carrying the disease-associated genotype will be clinically affected. In this context, a genetic test is assumed to be highly predictive of future disease. The differential risk of disease seen with different genotypes evidence of incomplete penetrance for the genotype conferring the highest risk makes it difficult to define hemochromatosis as a genetic disease in this traditional sense. I suggest that hemochromatosis be considered as a genetic susceptibility rather than as a disease condition.

## References

1. Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, Duffy DL and Martin NG. Effects of *HFE* C282Y and H63D polymorphism and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000;66:1246-58.
2. Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population based study of the clinical expression of the hemochromatosis gene. *New Engl J Med* 1999;341:718-24.
3. Beutler E, Felitti VJ, Gelbart T, Ho NJ. The effect of *HFE* genotypes on measurements of iron overload in patients attending a health appraisal clinic. *Ann Intern Med* 2000;133:329-37.
4. Gochee PA, Powell LW, Cullen DJ, Du Sart D, Rossi E, Olynyk JK. A population-based study of the biochemical and clinical expression of the H63D hemochromatosis mutation. *Gastroenterology* 2002;122:646-51.
5. Datz C, Haas T, Rinner H, Sandhoffer F, Patsch W, Paulweber B. Heterozygosity for the C282Y mutation in the hemochromatosis gene is associated with increased serum iron, transferrin saturation, and hemoglobin in young women: a protective role against iron deficiency? *Clinical Chemistry* 1998;44:2429-32.
6. Adams PC, Brissot P, Pietrangelo A, Powell LW. EASL international consensus conference on haemochromatosis. *Journal of Hepatology* 2000;33:485-504.
7. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. *Lancet* 2002;359:211-8.

8. McLaren CE, McLachlan GJ, Halliday JW, Webb SI, Leggett BA, Jazwinska EC, et al. Distribution of transferrin saturation in an Australian population: relevance to the early diagnosis of hemochromatosis. *Gastroenterology* 1998;114:543-9.
9. Leggett BA, Halliday JW, Brown NN, Bryant S, Powell LW. Prevalence of hemochromatosis among asymptomatic Australians. *Br J Haematol* 1990;74:525-30.
10. Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population based study of the clinical expression of the hemochromatosis gene. *New Engl J Med* 1999;341:718-24.
11. Burt MJ, George PM, Upton JD, Collet JA, Frampton CMA, Chapman TM, Waalsley TA, Chapman BA. The significance of haemochromatosis gene mutations in the general population: implications for screening. *Gut* 1998;43:830-6.
12. Burke W, Thomson E, Khoury MJ, McDonnell SM, Press N, Adams PC, et al. Hereditary hemochromatosis. Gene discovery and its implication for population-based screening. *J Am Med Assoc* 1998;280:172-8.
13. Adams PC, Brissot P, Pietrangelo A, Powell LW. EASL international consensus conference on haemochromatosis. *J of Hepatol* 2000;33:485-504.
14. McCullen M, Crawford DHG, Hickman PE. Screening for hemochromatosis. *Clin Chim Acta* 2002;315:169-86.
15. Deugnier YM, Loreal O, Turlin B, Guyader D, Jouanolle H, Moirand R, et al. Liver pathology in genetic haemochromatosis: a review of 135 homozygous cases and their biochemical correlations. *Gastroenterology* 1992;102:2050-9.
16. Teare J, Mantaounis D, Yallow R, Thursz M, Thomas HC. Increased frequency of the hemochromatosis allele in patients with alcoholic liver diseases. *J Hepatol* 1997;26:A1128.
17. George D, Powell L, Losowsky M. The hemochromatosis gene: A co-factor for chronic liver disease ? *J of Gastroenterol & Hepat* 1999;14:745-9.
18. Grove J, Daly AK, Burt AD, Guzail M, James OF, Bassendine MF, Day CP. Heterozygotes for *HFE* have no increased frequency of advanced alcoholic liver disease. *Gut* 1998;43:262-6.
19. Aldersley MA, Howdle PD, Wyatt JI, Robinson PA, Markham AF. Haemochromatosis gene mutation in liver disease patients. *Lancet* 1997;349:1025-6.
20. Bhavnani M, Lloyd D, Bhattacharyya A, Marples J, Elton P, Morwood M. Screening for genetic haemochromatosis in blood samples with raised alanine aminotransferase. *Gut* 2000;46:707-10.
21. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043-6.
22. Siow RCM, Sato H, Mann G. Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovascular research* 1999;41:385-94.
23. Dore S, Kar S, Quirion R. Insulin-like growth factor 1 protects and rescues hippocampal neurons against beta-amyloid and human amylin-induced toxicity. *Proc Natl Acad Sci USA* 1997;94:4772-7.

24. Dore S, Takahashi M, Ferris CD, Hester LD, Guastella D, Snyder SH. Bilirubin, formed by activation of heme oxygenase-2, protects against oxidative stress injury. *Proc Natl Acad Sci USA* 1999;96:2445-50.
25. Rothman KJ & Greenland S. Modern Epidemiology. 2<sup>nd</sup> ed, Lippincott Williams & Wilkins. Philadelphia 1998; Pp 737.
26. Barton JC, McDonnell SM, Adams PC, Brissot P, Powell LW, Edwards CQ, et al. Management of hemochromatosis. *Am J Hematol* 1998;58:16-9.
27. Roest M, van der Schouw YT, de Valk B, Marx JJM, Tempelman MJ, de Groot PG, Sixma JJ, Dirk BJ. Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation* 1999;100:1268-73.
28. Rossi E, McQuilliam BM, Hung J, Thompson PL, Kuek C, Beilby JP. Serum ferritin and C282Y mutation of the hemochromatosis gene as predictor of asymptomatic atherosclerosis in a community population. *Stroke* 2000;31:3015-20.
29. Camaschella C, Fargion S, Sampietro M, Roetto A, Bosio S, Garozzo G, Arosio C, Piperno A. Inherited HFE-unrelated hemochromatosis in Italian families. *Hepatology* 1999;29:1563-4.
30. Pietrangelo A, Montosi G, Totaro A, Garuti C, Conte D, Cassanelli S, Fraquelli M, Sardini C, Vasta F, Gasparini P. Hereditary hemochromatosis without pathogenic mutations in the hemochromatosis gene. *New Engl J Med* 1999;341:725-32.
31. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, Carella M, Zelante L, Kelly AL, Cox TM, Gasparini P, Camaschella C. Juvenile hemochromatosis locus maps to chromosome 1q. *Am J Hum Genet* 1999;64:1388-93.
32. Eason RJ, Adams PC, Aston CE, Searle J. Familial iron overload with possible autosomal dominant inheritance. *Aust N Z J Med* 1990;20:226-30.
33. Camaschella C, Roetto A, Cicilano M, Pasquero P, Bosio S, Gubetta L, et al. Juvenile and adult hemochromatosis are distinct genetic disorders. *Eur J Hum Genet* 1997;5:371-5.
34. Camaschella C, Roetto A, Cali A, De Gobbi M, Garozzo G, Carella M, Majorano N, Totaro A, Gasparini P. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. *Nature Genet* 2000;25:14-5.
35. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet* 1996;13:399-408.
36. Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JWF, Breuning MH, Snijders PJLM, Rutten WPF, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P. A mutation in *SLC11A3* is associated with autosomal dominant hemochromatosis. *Nature Genet* 2001;28:214-5.
37. Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A. Autosomal dominant hemochromatosis is associated with a mutation in the ferroportin (*SLC11A3*) gene. *The J Clin Invest* 2001;108:619-23.

38. Flemming MD. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genet* 1997;19:383-6.
39. Flemming MD. Nramp2 is mutated in the anaemic Belgrade rat: evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci USA* 1998;95:1148-53.
40. Froguel P. Genetic determinants of type 2 diabetes. *Recent Prog Horm Res* 2001;56:91-105.
41. Oberley LW. Free radicals and diabetes. *Free radicals Biology & Medicine* 1988;5:113-24.
42. Merz JF, Kriss AG, Leonard DGB, Cho MK. Diagnostic testing fails the test. *Nature* 2002;415:577 – 579.

## Summary

Hemochromatosis is the most common recessive disease in populations of caucasian origin. The discovery of the hemochromatosis gene (*HFE*) mutations led to a debate on the use of these mutations for population screening. Besides genotypic screening, the use of transferrin saturation as a screening test has been advocated. However, how the genotype and phenotype correlation and their relationship to morbidities is poorly understood. The first part of this thesis describes the effect of *HFE* mutations on serum iron indices, liver function, diabetes and stroke in a population-based sample, the Rotterdam Study. The second part of this thesis concerns a genome search to identify other genes involved in hemochromatosis. This study was done in a genetically isolated population. In **chapter 1** the definition, clinical characteristics of hemochromatosis and the aims of our study are outlined. There is still no clear and agreed definition of hemochromatosis. The disease is common and leads to multiple pathologies. **Chapter 2** provides a review on the genetic epidemiology of hemochromatosis. There are several genes involved in hemochromatosis, therefore many forms of the disease are recognized. Up to date, four types of hemochromatosis are described. Type 4 hemochromatosis is a novel form we describe in this thesis and it is associated with a mutation in the *SLC11A3* (ferroportin) gene. The most important form remains type 1 hemochromatosis which is due to the C282Y and H63D mutations in the *HFE* gene and accounts for about 80% of hemochromatosis cases. Other forms of the disease are due to rare mutations or to defects in genes involved in iron metabolism. In **chapter 3.1**, we studied the effect of *HFE* mutations on serum iron levels and evaluated the potential for genotypic screening. We found that carriers of the *HFE* mutations had significantly higher levels of serum iron indices compared to non carriers. We observed an additive effect of the H63D

mutation on serum iron and transferrin saturation. The *HFE* genotypes explained about 5% variability in serum iron indices. The positive predictive value for hemochromatosis for C282Y homozygotes was higher in men (100%) than women (67%). Screening for subclinical hemochromatosis based on *HFE* mutations had a sensitivity of 70% for men and 52% for women and a specificity of about 60% for both men and women. These values suggest that genotypic screening is not a powerful tool. **Chapter 3.2** describes the relationship between *HFE* mutations and liver function. No relationship between *HFE* genotypes and serum alanin aminotransferase, alkaline phosphatase or total proteins was observed. We found that serum iron, ferritin and transferrin saturation levels were related to total bilirubin and alanine aminotransferase. There was an allele dose effect of the H63D mutation on total bilirubin. *HFE* mutations are not direct risk factors for liver dysfunction but the iron overload caused by these mutations may be detrimental. In **chapter 3.3**, we studied the role of *HFE* mutations in type 2 diabetes mellitus. We found that there was no increased frequency of the *HFE* C282Y and H63D mutations in patients with type 2 diabetes. In our meta-analysis the odds ratio for type 2 diabetes patients to be carrier of the C282Y mutation was 1.0 (0.8-1.4) and 1.1 (1.0-1.3) to be carrier of the H63D mutation. **Chapter 3.4** reports on the association between *HFE* mutations and stroke. Mutations in *HFE* were not associated with an increased risk of stroke or carotid atherosclerosis. There was an effect modification of the relationship between smoking and stroke by *HFE*. We observed an additive effect of *HFE* mutations and smoking on the risk of stroke. **Chapter 4** deals with the genomic screen for other genes involved in hemochromatosis. In **chapter 4.2**, the clinical characteristics of a large pedigree with autosomal dominant hemochromatosis are described. The complaints of patients from this family were similar to that of classical hemochromatosis patients



and consisted mainly of arthritis and joint pains. Further, these patients responded poorly to treatment and the deferoxamine test was negative. We found no linkage to known hemochromatosis loci. **Chapter 4.3** reports the results of the genomic screen. Using linkage analysis, we mapped the disease locus to chromosome 2q. Mutation analysis of the gene encoding the metal transporter called *SLC11A3* or ferroportin revealed an heterozygous mutation (N144H) segregating with the disease. The substituted amino acid is located in a transmembrane domain of the protein and is very conserved in several species. In **chapter 4.4**, the prevalence of this newly described mutation in patients with type 2 diabetes is reported. We found that 2% of type 2 diabetes patients were carriers of the N144H mutation. There was an autosomal dominant parent to child transmission of diabetes and hemochromatosis. In **chapter 5.2**, a general discussion of the main findings of the studies described in this thesis and their relevance are provided. In **chapter 5.3**, future perspectives and final remarks are given.

## Samenvatting

Hemochromatose is de meest voorkomende recessieve ziekte in populaties van Kaukasische origine. De ontdekking van mutaties in het hemochromatose-gen (*HFE*) heeft tot een debat geleid betreffende het gebruik van deze mutaties voor screening in de algemene bevolking. Naast dit genetisch onderzoek wordt bepaling van de transferrine-verzadiging als een screeningstest bepleit. Over de correlatie tussen het genotype en het fenotype, en hun relatie met diverse ziekten, is echter weinig bekend. Het eerste deel van dit proefschrift beschrijft het effect van de *HFE* mutaties op de ijzerwaarden in het serum, de leverfunctie, diabetes mellitus en het cerebro vasculair accident (CVA) in een populatie onderzoek, namelijk het ERGO (Erasmus Rotterdam Gezondheid en Ouderen) onderzoek ofwel 'The Rotterdam Study'. Het tweede deel van dit proefschrift betreft een zoektocht in het menselijk genoom met als doel de identificatie van andere genen die betrokken zijn bij hemochromatose. Dit gedeelte van het onderzoek werd verricht binnen een genetisch geïsoleerde populatie. **Hoofdstuk 1** belicht de definitie en de klinische karakteristieken van hemochromatose en het doel van deze studie. Er bestaat tot dusverre geen eenduidige en algemeen geaccepteerde definitie van hemochromatose. De ziekte komt vaak voor en leidt tot vele aandoeningen. In **hoofdstuk 2** wordt een overzicht gegeven van de genetische epidemiologie van hemochromatose. Er zijn verschillende genen betrokken bij hemochromatose, met als gevolg dat vele vormen van de ziekte kunnen worden onderscheiden. Tot op heden zijn er vier vormen hemochromatose bekend. Hemochromatose type 4 is een nieuwe vorm die in dit proefschrift wordt beschreven en die is geassocieerd met een mutatie in het *SLC11A3* (ferroportine) gen. De belangrijkste vorm blijft hemochromatose type 1, welke wordt veroorzaakt door de C282Y en H63D mutaties in het *HFE* gen, en die ongeveer 80% van de

hemochromatose gevallen omvat. Andere vormen van de ziekte zijn het gevolg van zeldzame mutaties of defecten in genen die een rol spelen in het ijzermetabolisme. In **hoofdstuk 3.1** onderzoeken wij het effect van *HFE* mutaties op serum-ijzerspiegels en evalueren de mogelijkheid van genotypische screening. Draggers van de *HFE* mutaties blijken significant hogere waarden van de ijzer-indices te hebben dan de niet-dragers. Wij observeren een additief effect van de H63D mutatie op de ijzerspiegel in het serum en de transferrine-verzadiging. De *HFE* genotypes kunnen ongeveer 5% van de variabiliteit in de ijzerwaarden in het serum verklaren. De positief voorspellende waarde voor hemochromatose voor individuen die homozygoot zijn voor C282Y is hoger in mannen (100%) dan in vrouwen (67%). Screening voor subklinische hemochromatose op grond van mutaties in *HFE* heeft een sensitiviteit van 70% voor mannen en 52% voor vrouwen en een specificiteit rond de 60% voor beide geslachten. Deze waarden geven aan dat genetisch screenen in de praktijk niet bruikbaar is. **Hoofdstuk 3.2** beschrijft het verband tussen *HFE* mutaties en leverfunctie. Wij vinden geen verband tussen de *HFE* genotypes en serumspiegels van het alanine aminotransferase, alkalische fosfatase of het totaal eiwit. Het serum-ijzer en de ferritine en transferrine verzadigingsspiegels zijn gerelateerd aan het totaal bilirubine en alanine aminotransferase. Er is een dosis-effect van het aantal allelen met de H63D mutatie op het totale bilirubine. Mutaties in het *HFE* gen zijn geen directe risicofactoren voor leverdysfunctie, maar de overvloed aan ijzer veroorzaakt door deze mutaties kan schadelijk zijn. In **hoofdstuk 3.3** hebben we de rol van mutaties in *HFE* bestudeerd in diabetes mellitus type 2. Wij zien geen verhoogde frequentie van de *HFE* C282Y en H63D mutaties in patiënten met diabetes mellitus type 2. In een meta-analyse vinden we een odds ratio van 1,0 (0,8-1,4) voor diabetes type 2 patiënten om drager te zijn van de C282Y mutatie en 1,1 (1,0-1,3) om drager te

zijn van de H63D mutatie. **Hoofdstuk 3.4** behandelt de associatie tussen *HFE* mutaties en het ischemisch CVA. Mutaties in *HFE* zijn niet geassocieerd met een verhoogd risico op CVA of op atherosclerose van de carotiden. Wij vinden effect modificatie door *HFE* in de relatie tussen roken en CVA. Roken en mutaties in *HFE* hebben een additief effect op het risico op CVA. **Hoofdstuk 4** behandelt de genomische zoektocht naar andere genen betrokken bij hemochromatose. In **hoofdstuk 4.2** worden de klinische kenmerken van een familie met een uitgebreide stamboom van autosomaal-dominante hemochromatose beschreven. De verschijnselen van patiënten binnen deze familie zijn gelijk aan die van de klassieke patiënten met hemochromatose en bestaan voornamelijk uit arthritis en gewrichtspijn. Verder reageren deze patiënten slecht op therapie en is de deferoxamine test negatief. Wij vinden geen linkage met de bekende hemochromatose loci. In **hoofdstuk 4.3** worden de resultaten van de genomische screen gepresenteerd. Door middel van linkage analyse hebben we een ziekte locus geïdentificeerd op chromosoom 2q. Mutatie analyse van het gen dat codeert voor het metaal-transport-eiwit *SLC11A3*, of ferroportine, toont een heterozygote mutatie (N144H) aan die met de ziekte segregiert. Het gesubstitueerde aminozuur is gelocaliseerd in een transmembraan domein van het eiwit en is in hoge mate geconserveerd in verschillende (dier)soorten. In **hoofdstuk 4.4** wordt de prevalentie van deze nieuw beschreven mutatie in patiënten met type 2 diabetes mellitus gerapporteerd. Wij vinden dat 2% van de patiënten met type 2 suikerziekte drager is van de N144H mutatie. Er is een autosomaal dominant ouder-op-kind overervingspatroon van diabetes mellitus en hemochromatose. In **hoofdstuk 5.2** worden de belangrijkste bevindingen van deze studies besproken en wordt hun relevantie beschreven. In **hoofdstuk 5.3** worden mogelijkheden voor toekomstig onderzoek en enkele slotopmerkingen gepresenteerd.

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