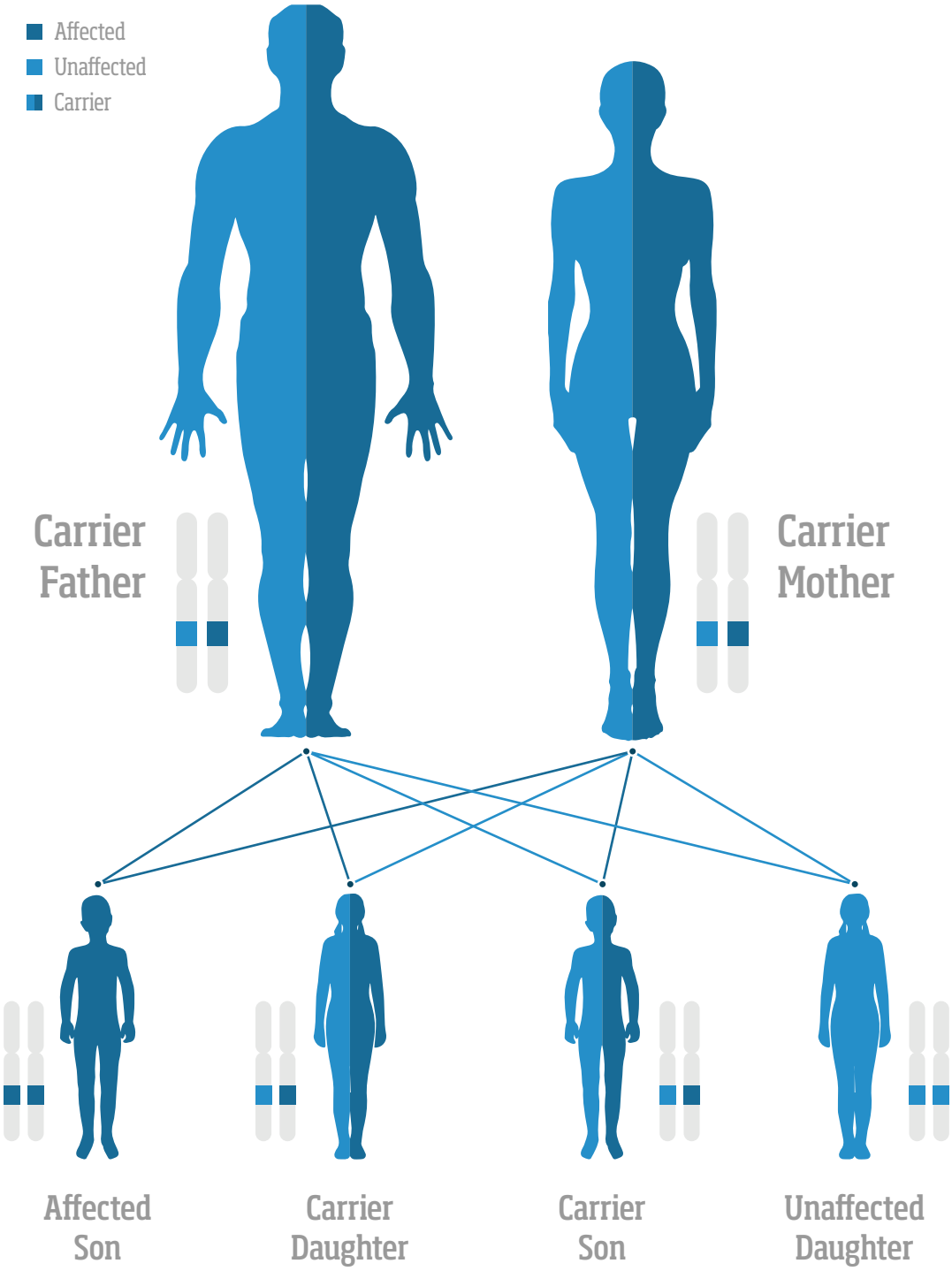
Doctor of Medical Science Thesis by
Christina Ellervik MD, PhD

HEMOCHROMATOSIS GENOTYPES AND ELEVATED TRANSFERRIN SATURATION

- risk of diabetes mellitus, hypertension,cancer, and total mortality



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The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation, which consists of the already published dissertations listed below, for public defence for the doctoral degree in medicine.

Copenhagen, October 11th 2015

Ulla M. Wewer

Head of Faculty

Place and time for defence:

St. Auditorium at Herlev Hospital, June 22nd 2016 at 2pm

Table of Contents

Papers on which the thesis is based

(According to PubMed style)

1. Ellervik C, Mandrup-Poulsen T, Nordestgaard BG, Larsen LE, Appleyard M, Frandsen M, Petersen P, Schlichting P, Saermark T, Tybjærg-Hansen A, Birgens H. Prevalence of hereditary haemochromatosis in late-onset type 1 diabetes mellitus: a retrospective study. Lancet 2001; 358:1405-9.

2. Ellervik C, Mandrup-Poulsen T, Andersen HU, Tybjærg-Hansen A, Frandsen M, Birgens H, Nordestgaard BG. Elevated transferrin saturation and risk of diabetes: three population-based studies. Diabetes Care 2011; 34:2256-8.

3. Ellervik C, Tybjærg-Hansen A, Appleyard M, Ibsen H, Nordestgaard BG. Haemochromatosis genotype and iron overload: association with hypertension and left ventricular hypertrophy. J Intern Med 2010; 268:252-64

4. Ellervik C, Tybjærg-Hansen A, Nordestgaard BG. Risk of cancer by transferrin saturation levels and haemochromatosis genotype: population-based study and meta-analysis. J Intern Med 2012; 271:51-63.

5. Ellervik C, Tybjærg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: two general population studies and a metaanalysis. Clin Chem 2011; 57:459-66.

6. Ellervik C, Andersen HU, Tybjærg-Hansen A, Frandsen M, Birgens H, Nordestgaard BG, Mandrup-Poulsen T. Total Mortality by elevated transferrin saturation in patients with diabetes. Diabetes Care 2013; 36: 2646-2654.

7. Ellervik C, Mandrup-Poulsen T, Tybjærg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes – two general population studies. Diabetes Care 2014; 37: 444-452.

Preface

My interest in hemochromatosis began as a medical student, when I met *Henrik Birgens, Thomas Mandrup-Poulsen, Børge Nordestgaard* and *Anne Tybjærg-Hansen*. Since then, they have encouraged me to continue research within this field. Their contribution to my scientific education has been very valuable and has lead to a fruitful and rewarding collaboration during the years. The present thesis is based on studies carried out within this collaboration over the past 10 years. I owe thanks to all the collaborators for always constructive comments and criticism. I am also grateful to other co-authors of the presented works, from whom I have learnt much.

The assistance of the technical staff and medical doctors at Steno Diabetes Centre (*Sonja Bigand, Merete Frandsen, Henrik Ullits Andersen*) and at the departments of Clinical Biochemistry (Hanne Dam) and Hematology at Herlev Hospital is greatly appreciated. I appreciate the generous and free working conditions created by the head of Clinical Biochemistry at Herlev Hospital, DMSc *Niels Fogh-Andersen*. I appreciate the hospital directors at Næstved and Nykøbing Falster Hospitals, Department of Clinical Biochemistry at Næstved Hospital, the Heads of Research at Næstved Hospital (*Sten Boesby, Jan Kvetny*) and at Region Sjælland (*Steffen Groth, Knud Rasmussen*) for generously supporting my research.

I also owe thanks to the secretariat of the Copenhagen City Heart Study (*Merete Appleyard, Jacob Marrot, Connie Haugaard*), who have always been ready to assist in every conceivable way, and also to the participants and to the initiators of the Copenhagen City Heart Study and the Copenhagen General Population Study.

Finally, I want to thank family and friends for their support, love, and always good mood, especially my husband, *Ulrik Juul Christensen*, and our children, *Caroline Juul Ellervik* and *Elisabeth Juul Ellervik*, my parents in law Bente and *Flemming Christensen*, and *Maren Weischer.*

Scope and delimitation of the thesis

The scope of this thesis is on transferrin saturation, as a measure of iron overload, and the homozygous hemochromatosis genotype C282Y/C282Y for the association with diabetes, hypertension, cancer and total mortality; risk of other C282Y compound and heterozyogous genotypes will also be shortly discussed. Other measures of iron overload exist, e.g. ferritin, and other genetic variants exist for hereditary hemochromatosis as this is a genetically heterogeneous disease. However, elevated transferrin saturation ≥50% has a sensitivity of 75%, a specificity of 95%, and a positive predictive value of 3.5% for detecting HFE C282Y homozygotes[1], whereas ferritin is elevated in many other diseases than hereditary hemochromatosis (e.g. inflammation, high alcohol intake, fatty liver disease)[1, 2]. Thus, transferrin saturation is usually the first screening test for hereditary hemochromatosis, followed by genetic testing and ferritin measurement[3].

The thesis focuses on adult white individuals of Danish descent, as hereditary hemochromatosis is primarily a disease in individuals of Northern European origin[4]. Also, the thesis focuses on Type 1 hereditary hemochromatosis[3], but does not cover other types of hereditary hemochromatosis or secondary hemochromatosis. The thesis does not cover heart disease apart from hypertension, nor does it cover liver disease, arthritis, or pituitary hypogonadism, also features of hemochromatosis[3].

In a previous PhD thesis by Christina Ellervik in 2007, the focus was solely on hemochromatosis genotypes and risk of ischemic heart disease[5], stroke[6], and a meta-analysis[7] of the existing literature at that time including 31 other disease end-points, but not including mortality. It was evident from the meta-analysis in the PhD thesis that most of the studies in the literature were cross-sectional and case-control studies. In contrast, the articles on which the present doctorial thesis is based are primarily follow-up studies. Also, the meta-analysis revealed areas of research that had not previously been investigated: association of late-onset type 1 diabetes with hemochromatosis genotypes (paper 1)[8] and survival of

these patients (paper 6)[9], risk of diabetes mellitus according to transferrin saturation in a follow-up study (paper 2)[10], risk of hypertension according to transferrin saturation and hemochromatosis genotypes (paper 3)[11], and risk of any cancer according to hemochromatosis genotypes in a follow-up study (paper 4)[12]. Hereditary hemochromatosis is a disease in individuals primarily of Northern European descent; the research questions on total mortality in the general population (paper 5)[13] and in individuals with diabetes (paper 7)[14] had been conducted previously in an American study[15] and in an Australian study[16] of mixed ethnicities, respectively, but never in a Northern European white Population. Thus, this thesis is independent of the previous PhD thesis, none of the papers in this thesis formed any part of the PhD thesis, and the articles on which this thesis is based add new research areas not previously covered.

The genotypings used in the articles in this thesis were done in 1999 for the Copenhagen City Heart Study (papers 1-7) and the Steno Diabetes Center (paper 1 and 6), in 2001-2007 for a new cohort at the Steno Diabetes Center (paper 2 and 6), and in 2007 for patients from Losartan Intervention for Endpoint Genetic Sub-study (paper 3) and for Copenhagen General Population Study (paper 2, 3, 5 and 7). The same genotypings of some of the participants were also used for two papers[5, 6] on ischemic heart disease and stroke in the PhD thesis.

The transferrin saturation measurements we used in the articles in this thesis were done in 1999 for the Steno Diabetes Center (paper 1 and 6), in 2001-2007 for a new cohort at the Steno Diabetes Center (paper 2 and 6), in 2007 for the Copenhagen City Heart Study, and in 2003-2007 for the Copenhagen General Population Study (paper 2, 3, 5 and 7). The transferrin saturation measurements did not form part of any of the articles in the PhD thesis.

Introduction

Hereditary hemochromatosis

Hereditary hemochromatosis is an iron-overload disorder characterized by iron accumulation throughout life in various organs, such as the endocrine pancreas, heart, liver, pituitary gland, joints, and skin[3, 17]. Iron overload results in progressive tissue damage and organ failure, including diabetes mellitus, heart failure, liver cirrhosis, liver cancer, pituitary hypogonadism, arthritis, and bronze colour of the skin due to hyperpigmentation[3, 17]. However, several years of iron accumulation are needed before tissue damage leads to clinical signs of organ failure. The earliest detectable biochemical anomaly is increased transferrin saturation[3], which generally represents an increased intestinal iron absorption; this may be followed by an increased ferritin concentration indicating accumulation of cellular iron[3, 17].

Non-hereditary hemochromatosis or secondary hemochromatosis may arise from chronic use of iron supplements, chronic liver disease, and especially chronic red blood cell (RBC) transfusion therapy for anemia in patients with ineffective erythropoiesis such as patients with beta-thalassemia major or myelodysplastic syndrome, or in patients with chronic hemolytic disorders or chronic bone marrow failure[18]. The complications associated with transfusional iron overload are in principle similar to hereditary hemochromatosis, but with earlier onset[19], and require iron chelation therapy[18].

In 1996, the *HFE* gene was identified, with homozygosity for C282Y being responsible for 84% of hereditary hemochromatosis in Europeans and with compound heterozygosity for C282Y/H63D explaining an additional 4%[20]. The locus for *HFE* is on chromosome 6p21.3[20]. A G –> A substitution at nucleotide 845 changes cysteine to tyrosine in codon 282 (C282Y), and a C –>G substitution at nucleotide 187 changes histidine to aspartate in codon 63 (H63D). The genotypes C282Y/C282Y, C282Y/H63D, C282Y/wild type and, in some studies, H63D/H63D, but not H63D/wild type, are associated with evidence of iron overload[21, 22]. The risk of developing the clinical hereditary hemochromatosis phenotype is debated[23, 24], with penetrance estimates ranging from less than 1%[23] to almost 30%[24] for individuals with

Table 1. Comparative overview of genetics in hereditary hemochromatosis (HH).

	Classic HH	Juvenile HH	Juvenile HH	TfR2-related HH	Ferroportin-related HH
OMIM Classification	Type 1	Type 2, subtype A	Type 2, subtype B	Type 3	Type 4
Gene name	HFE	HJV	HAMP	TfR2	SLC40A1
Gene locus	6p21.3	1q21	19q13.1	7q22.1	2q32
Gene product	HFE	Hemojuvelin	Hepcidin	Transferrin receptor 2	Ferroportin
Pattern of inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal dominant
Predominant cell distribution of iron	Parenchymal	Parenchymal	Parenchymal	Parenchymal	Reticulo-endothelial
Main organs accumulating iron	Liver, endocrine glands, heart	Liver, endocrine glands, heart	Liver, endocrine glands, heart	Liver, endocrine glands, heart	Liver, spleen
Decade of onset of symptomatic disease	4th or 5th	2nd or 3rd	2nd or 3rd	4th or 5th	4th or 5th

Table is modified from A. Pietrangelo NEJM 2004;350:2383-97.
OMIM: Online Mendelian Inheritance in Man.
HFE: High iron Fe
HJV: Hemojuvelin
HAMP: Hepcidin Antimicrobial Peptide
TfR2: Transferrin receptor 2 gene
SLC40A1: Solute Carrier Family 40 member A1

C282Y/C282Y and with the least penetrance in women and the highest penetrance in men. The reason for the lower penetrance in women is that women bleed during the reproductive period. Thus, onset in women is usually after the menopause, whereas in men onset is usually in the 30-50ies[3].

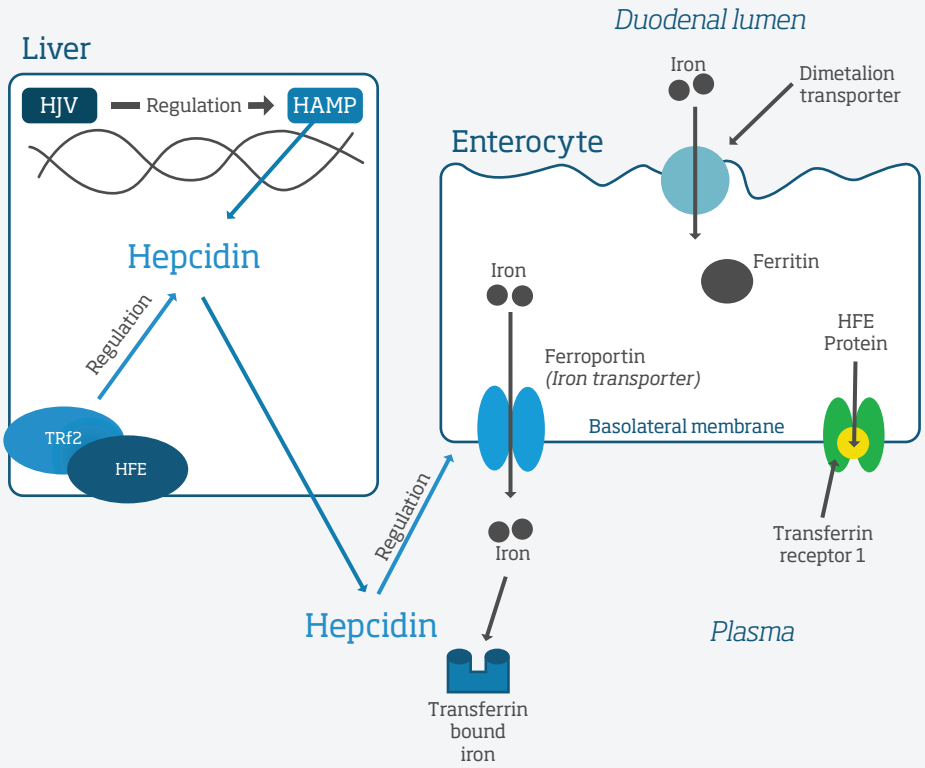
The autosomal recessive form of hereditary hemochromatosis with homozygosity for C282Y is also termed classic or Type 1 hereditary hemochromatosis; however, other genetic and phenotypic variants exist[3] (Table 1). This thesis is focusing on classical hereditary hemochromatosis with homozygosity for C282Y/C282Y.

Population studies have provided information on allele frequencies of C282Y and H63D[25]. There is a north to south gradient in Europe of the C282Y allele frequencies with the highest reported in Northern Europe and in Northern European emigrants (5-11%) and the lowest in Southern Europe (0-5%), and vice versa for H63D with allele frequencies of 10-20% in the south and 5-10% in the north of Europe. It is believed that C282Y originated by chance in a single Celtic[26, 27] or Viking[28] ancestor in North Western Europe about 2000-4000 years ago[3, 25], and a joint hypothesis is that C282Y originated in the Celts but was spread by the Vikings[29]. The reasons why the allele frequencies of C282Y are higher in Northern Europe than in Southern Europe can be explained both by the founder effect, but it may also be speculated that heterozygotes have a survival advantage or that heterozygotes are better protected against anemia[30], which could be beneficial in the reproductive period for younger women; however, there is no difference in parity status among heterozygous women compared to wild types[30]. As will also be seen later in this thesis in the Results section, risk of mortality in individuals with C282Y/wild type is not different from that in wild type/wild type individuals; however, mortality as studied here in later life will not affect reproduction.

Most individuals homozygous for C282Y have elevated levels of transferrin saturation and ferritin[23, 31-34]. Biochemically, cut-off values for transferrin saturation and ferritin in the diagnosis of hemochromatosis have varied across studies[35], but a transferrin saturation, reflecting plasma iron, above 50% and a ferritin concentration, reflecting tissue iron, above 1000 µg/L are generally accepted criteria, although there are gender differences[35].

Figure 1. Hepcidin and iron influx.

The normal infux of iron from enterocytes to the blood stream is mediated through the transporter ferroportin on the basolateral side of the enterocyte. The hormone hepcidin, which is secreted by hepatocytes, is the central regulator of iron homeostasis. Hepcidin transcription is upregulated in response to iron overload and downregulated in response to iron deficiency. In conditions with iron overload, hepcidin downregulates the ferroportin-mediated release of iron from enterocytes and macrophages to the blood. Except for inherited defects of hepcidin itself *(HAMP)* and ferroportin *(SLC40A1)*, all forms of iron-storage disease appear to arise from hepcidin dysregulation. Hepcidin is regulated by various proteins, such as HFE *(HFE)* and transferrin receptor 2 *(TfR2)*, and mutations in the hemojuvelin gene *(HJV)*. See also Table 1.



There is now substantial evidence that the liver plays a central role in determining, how much iron is absorbed from the intestinal tract and in influencing the release of iron from sites of storage[36]. The normal flux of iron from enterocytes to the blood stream is mediated through the transporter ferroportin on the basolateral side of the enterocyte (Figure 1), while the hormone hepcidin, which is secreted by hepatocytes, is the central regulator of iron homeostasis. Hepcidin transcription is up-regulated in response to iron overload and down-regulated in response to iron deficiency[37]. In conditions with iron overload, hepcidin down regulates the ferroportin-mediated release of iron from enterocytes and macrophages to the blood[36]. Iron-storage disease may arise due to mutations in the genes coding for the HFE protein (*HFE*; Type 1), hemojuvelin (*HJV*; Type 2A)[38], hepcidin(*HAMP*; Type 2B), transferrin receptor 2(*TfR2*; Type3), and ferroportin (*SCL40A1*; Type 4)[3, 36] (Table 1).

The underlying mechanism of action for the iron-induced cellular damage involves formation of free radicals through the Fenton reaction resulting in oxidative stress to the cells[39, 40]. Depending on which cells are damaged, different diseases in different organs develop. Clinically, hereditary hemochromatosis is thought of as rare[41, 42]; however, genotyping

studies suggest that it potentially is one of the most common genetic diseases in people of Northern European descent[4], since the prevalence of the genotype C282Y/C282Y is 0.25% and the prevalence of the genotype C282Y/wild type is 9%[8] .

Diabetes mellitus (paper 1 and 2)

In vitro rat beta-cells are prone to reactive oxygen species (ROS)-mediated damage by cytokine-dependent up-regulation of cellular iron import by the divalent metal ion transporter (DMT-1)[43], and inducible beta-cell specific knock-out of DMT-1 protects against inflammatory and high fat diet induced diabetes. Also, introduction of *HFE* mutations in mice results in beta-cell oxidative stress and decreased insulin secretory capacity due to glucose desensitization and to beta-cell apoptosis[44]. Further, increased hepatic glucose production and decreased skeletal muscle glucose oxidation may contribute to diabetes in iron overload in an animal model of Type 2 diabetes[45]. Accordingly, iron restriction or iron chelation protects from diabetes and loss of beta-cell function in obese mice[46].

The defects in both insulin-producing and insulin sensitive tissues are most likely caused by iron-dependent catalysis via the Fenton reaction of ROS, which impair insulin signalling in skeletal muscle and liver, and which cause beta-cell destruction due to insufficient beta-cell antioxidant defence[47, 48].

In autopsies of patients with hereditary or non-hereditary hemochromatosis and diabetes, impaired glucose tolerance, or impaired fasting glucose, iron was found deposited in the beta-cells[49-54]. Also, there is an inverse relationship between beta-cell iron deposits and insulin secretory granules[50, 55], and the number of beta-cells with hemosiderin increases proportionally with blood transfusion volume[51]. Furthermore, the degree of impaired fasting glucose and glucosuria increases with increasing iron overload and with increasing beta-cell iron staining[51]. In patients with only mild hemosiderosis of the pancreas iron staining is confined to acinar cells and the stroma[51, 53], and control autopsies of patients without a history of hemochromatosis, blood transfusions or diabetes do not show any iron staining in the pancreas[51]. In pancreatic tissue from autopsies of patients with hemochromatosis the histological appearance of the islets is normal with their shape and size being unchanged (unlike Type 1 diabetes)[49], amyloid deposits are absent (unlike Type 2 diabetes) [49, 53], and there is no iron staining in the alpha or delta cells[49-52]. Thus, pancreatic involvement in iron overload seems to be a continuum from mild hemosiderosis involving acinar cells and the stroma to severe hemosiderosis with beta-cell involvement.

Functionally, diabetes secondary to hereditary or non-hereditary hemochromatosis is characterised by both insulin resistance[56-60] and insulin deficiency[48], and may therefore mimic both Type 2 diabetes and idiopathic Type 1 diabetes. Patients with hereditary hemochromatosis are more prone to develop diabetes if insulin resistant, since their insulin secretory capacity is decreased but usually not absent[48, 61].

In 2001 (paper 1), we postulated that the most likely explanation for the discrepancy that hemochromatosis was thought of as rare but the frequency of the *HFE* C282Y homozygosity was 0.25% with an estimated penetrance rate of 50%, was that hereditary hemochromatosis was often overlooked[8]. Our aim was to test this hypothesis in patients with late-onset Type 1 diabetes mellitus, a hypothesis that had not been investigated before at that time. Previous studies of risk of diabetes conferred by the C282Y/C282Y genotype had primarily focused on Type 2 diabetes or any diabetes with contradicting results[7, 23, 62-82]. The evidence now in 2014 from epidemiological studies is that hereditary or non-hereditary hemochromatosis is associated with increased risk of developing or dying from diabetes[7-10, 14, 83-86]. It is also now known that high serum ferritin correlates inversely with serum insulin, and proportionally with blood glucose concentrations[87]. Also, high serum ferritin, body iron, haem intake, and dietary iron intake are associated with increased risk of diabetes[83-86, 88-91]. Finally, phlebotomy may improve insulin secretory capacity [92, 93] and insulin sensitivity[94] if instituted early, and it reverses impaired glucose tolerance in patients with hereditary hemochromatosis[93, 95].

In the context of the existing literature, three studies[86, 96, 97] have used transferrin saturation as the primary independent variable (without considering hemochromatosis genotype) for the study of the risk of any diabetes. However, these studies are heterogeneous with regard to ethnicity, study design, size, transferrin saturation threshold, diabetes diagnosis, source of diabetes diagnosis, and conclusions as to whether elevated transferrin saturation is associated with risk of diabetes (one study in favour[96] and two studies not in favour[86, 97]).

Therefore, we undertook another study (paper 2) of whether elevated transferrin saturation conferred increased risk of any type of diabetes mellitus, type 1 diabetes mellitus, and type 2 diabetes mellitus in three independent Danish white population-based studies[10] from the same geographical area.

Hypertension (paper 3) and other heart disease (cardiomyopathy, ischemic heart disease, cardiac arrhythmias)

In asymptomatic subjects with C282Y/C282Y genotype there appears to be an early detectable echocardiographic manifestation of abnormal diastolic function[98], which may be associated with oxidative stress caused by the iron overload[99], but systolic function seems to be preserved[100]. The echocardiographic features of patients with symptomatic hemochromatosis are varying degrees of cardiomyopathy and left ventricular hypertrophy (LVH)[101], the latter also a feature of hypertension[102]. Increased thickness of the ventricular wall is probably the first, and still reversible, cardiac alteration due to iron deposition in the myocardium[103]. Later, with increasing iron overload, left ventricular function becomes impaired and dilated cardiomyopathy develops[103]. In support, accumulating evidence suggests that oxidative stress may alter the modulation of vascular tone[104, 105], thereby affecting blood pressure leading to hypertension. Furthermore, it has been shown that arterial wall thickness was increased before the onset of cardiovascular complications in hemochromatosis patients and that this alteration was reversed by iron depletion[106]. Also, in men with hypertension increased serum ferritin has been shown to be more frequent than in controls[107]. It is therefore possible that individuals with hemochromatosis genotypes and/ or iron overload are overrepresented amongst patients with hypertension and/or LVH[11], a hypothesis that was tested in paper 3.

Meta-analyses have not shown any association of iron overload [108] or HFE genotypes[7, 109] with ischemic heart disease or myocardial infarction; also, HFE genotypes are not associated with oxidized LDL[5].

In single case-study of patients with symptomatic hereditary hemochromatosis, severe cardiac arrhythmias have been described[110, 111]; however, in a larger case-study of symptomatic patients with hereditary hemochromatosis, ECG abnormalities were non-specific compared to controls[103]. In asymptomatic patients with C282Y/C282Y genotype self-reported arrhythmias were not different from that in wild-type subjects[112], but ECG-measurements suggested a marginal but significant difference in non-life-threatening ECG abnormalities[113]. However, it is estimated that in male patients with pacemaker treated atrioventricular block of second or third degree, that the prevalence of patients with hereditary hemochromatosis is 1.3%[114], i.e. 4-5 times the prevalence of C282Y homozygosity in the general population.

Cancer (paper 4)

Iron-induced free radical damage to DNA may be important for the development of cancer[115], and cancer cells grow rapidly in response to iron[116, 117]. Thus, iron overload, either in general or genetically via hemochromatosis genotype C282Y/C282Y, may lead to increased risk of cancer. The most severe outcome of hereditary hemochromatosis is liver cancer[3], also associated with hemochromatosis genotype C282Y/C282Y in case-control studies[7]. However, a meta-analysis of the association of any other cancer than liver cancer with hemochromatosis genotype C282Y/C282Y in case-control studies had insufficient power to exclude an association[7]. Risk of cancer in individuals with iron overload has previously been studied in

various prospective[118-129] and case-control studies[130], but with contradicting results. However, it has been shown that therapeutic iron reduction in a randomized trial[131] and blood donation[132] both seem to reduce cancer risk. No population-based follow-up study of cancer risk as a function of hemochromatosis genotypes has previously been conducted. Therefore, we recently conducted such a study along with a meta-analysis of elevated transferrin saturation and risk of any cancer[12] in paper 4.

Mortality (paper 5, 6 and 7)

Paper5

The 4th and 5th decade is usually the onset of symptomatic organ disease in individuals with hereditary hemochromatosis, with onset for men not uncommon in their thirties and for women after menopause[3]. There is even evidence for increased mortality in patients with clinically overt hereditary hemochromatosis[133-137]. Early diagnosis and instigation of appropriate treatment with repeated venesections can prevent the consequences of hereditary hemochromatosis and restore normal life expectancy[134, 138, 139]. Total mortality according to increased transferrin saturation, a biochemical proxy for iron overload, was examined in a single previous population-based study (NHANES)[15]; this study examined 10,000 individuals including 10% non-whites, who potentially could have a very low chance of having iron overload, since iron overload is a disease most often seen in individuals of Northern European origin[140]. Furthermore, this study did not examine a dose-response relationship from low iron loads to iron overload, did not examine extreme phenotype (iron overload above 70%) but only above 45-60%, and did not show gender-stratified results.

Due to these weaknesses in the NHANES study and since significant results can either arise by chance, confounding, bias, misclassification, or reflect a true association, we therefore conducted a similar study[13] (paper 5). However, our study was larger (45,000 individuals, all whites of Northern European Danish descent); and as a novel idea and finding, in this paper, as was not presented in the NHANES paper, we added a semi-graded relationship of transferrin saturation(%) with cut-points of <20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, and above 80, in order to see if there was a dose-response relationship, which there was[13]. Also, we looked into more extreme phenotypes than the NHANES. The reason for choosing this semi-graded relationship was to find out if there was another lower or higher cut-off that would associate better with overall mortality. Off-course, this semi-graded relationship could have been refined to include other regression models to explore the continuous relationship between transferrin saturation and mortality; however, we wanted to use the terminology of cut-off's at the time this paper was written, largely because results are more easy to use clinically if applicable. However, in the Results section of this thesis is now also included a continuous model for the relationship between transferrin saturation and mortality.

Paper 6

At the time the data for paper 1 was collected and later published in 2001, as well as now in 2014, it was not part of clinical guidelines to assess for hemochromatosis in specialised diabetes clinics or among general practitioners. It was our aim with paper 1 to assess whether there was a higher prevalence of patients with hemochromatosis among these patients than seen in the general population. The data confirmed this hypothesis (see Results and Discussion in this thesis). At that time, it was known from studies of patients with hemochromatosis that early detection[134, 138, 139] of the disease would halter or reduce co-morbidities. A recent study in patients with hereditary hemochromatosis demonstrated a decline in diabetes prevalence in patients diagnosed after determining that they carried the *HFE* gene compared to those diagnosed before[141], suggesting that awareness of *HFE* in general and development of diabetes in those patients in particular possibly will translate into a better life expectancy.

In paper 1 we recommended measurement of transferrin saturation in all diabetic patients with diabetes onset after 30 years of age followed by genetic testing for HFE in cases with a transferrin saturation above 50%, to improve life expectancy in patients with diabetes secondary to hereditary hemochromatosis[8]. Therefore, as part of the Discussion and in the Conclusion in the abstract in paper 1, we hypothesised/speculated that assessing this group of patients even earlier (i.e. at entry to the Steno Diabetes Center) could in the future result in the earlier diagnosis of hemochromatosis and in reduced premature death, but we did not have the data to justify that in paper 1. To assess the recommendation of early measurement of transferrin saturation to prevent premature death, in paper 6 we followed up patients with late-onset type 1 diabetes from paper 1 (Diabetes Cohort 1) compared with another group of patients (Diabetes Cohort 2) with late-onset type 1 diabetes diagnosed at entry to the Steno Diabetes Center, and not at some random time point as in the previous Paper 1. Furthermore, we investigated total mortality in patients with type 2 diabetes and other diabetes but diagnosed at entry to the Steno Diabetes Center, and not at some random time point as in the previous Paper 1; however, for this group of patients, we did not have a "control group" from the Steno Diabetes Center that would have had a random measurement, since this was thought of as unethical due to the results in paper 1 (even though these results only related to late-onset type 1 diabetes). Finally in paper 6, we investigated whether mortality in patients with diabetes was driven by *HFE* genotypes. Only one previous Australian study had investigated that before[16] and could not show evidence of increased mortality according to iron overload or *HFE* genotype in patients with type 2 diabetes[16]; however, that study consisted of only 1,265 patients of mixed ethnicity and had a shorter follow-up and less power, and thus not comparable to our study, which was larger, of homogenous ethnicity, with longer follow-up, and more statistical power. It has indeed previously been shown that ethnicity matters in terms of the risk conferred by iron overload and *HFE* genotype[7].

Paper 7

As mentioned before, a study in patients with hereditary hemochromatosis demonstrated a decline in diabetes prevalence in those patients diagnosed after determining that they carried the HFE gene compared to those diagnosed before[141], suggesting that awareness of hemochromatosis in general and development of diabetes in those patients in particular could translate into an improved life expectancy. Furthermore, we demonstrated a decline in mortality in patients with late-onset type 1 diabetes offered targeted screening for transferrin saturation[9] (results from paper 6). Thus, we have recommended targeted screening for transferrin saturation in specialised diabetes clinics[8, 9] (in paper 6). Whether mortality was also increased in white individuals of Northern European descent ascertained from the general population with diabetes and increased transferrin saturation or hemochromatosis genotype C282Y/C282Y was unknown. If this was the case, however, recommendation on targeted screening could also cover individuals with diabetes in the general population, when these individuals see their general practitioner for regular diabetes check-ups, that is, if they have not already previously been diagnosed with hemochromatosis. Therefore, in paper 7, we investigated total and cause-specific mortality according to increased transferrin saturation or hemochromatosis genotype C282Y/C282Y in white Danish individuals with diabetes ascertained from two general population studies.

Other diseases associated with hereditary hemochromatosis, but not part of the thesis

Liver disease

Hereditary hemochromatosis is associated with liver cirrhosis and liver cancer[2, 142]; likewise, in a previous meta-analysis we showed that C282Y/C282Y was also associated with liver cancer[7]. But iron overload and C282Y/C282Y genotype also seem be to risk factors for other

non-hemochromatotic liver diseases[7, 142]. Iron overload and C282Y/C282Y is associated with increased risk of porphyria cutanea tarda, a metabolic disorder of the hem-pathway[7, 142]. Also, in patients with hepatitis C carrying the C282Y mutation, fibrosis seems to be more advanced than in patients without the C282Y mutation[142]. There seems to be evidence that alcohol increases liver injury in patients with hereditary hemochromatosis[142;] however, whether iron overload and C282Y/C282Y genotype are also associated with non-alcoholic fatty liver disease is disputed[7, 143].

Arthritis

One third of patients with hemochromatosis have joint pain and it is typically the second and third metacarpophalangeal joint that is affected, but any joint can be affected[144]. The arthritis is a non-inflammatory condrocalcinosis[144]. A meta-analysis of risk of arthritis according to C282Y/C282Y genotype did not show any association[7]; however, the studies were all small case-control studies. But a recent nationwide follow-up study has shown that patients with hereditary hemochromatosis have increased risk of arthropathies and joint replacement surgery[145.]

Pituitary hypogonadism

Men with hereditary hemochromatosis may experience pituitary hypogonadism[146] and loss of libido[147]. In support of this, the C282Y polymorphism has been associated with higher sex hormone binding globulin (SHBG) than wild types[148], although this could also be attributed to liver disease.

Porphyria cutanea tarda

In a previous meta-analysis we showed that H63D/wild type, H63D/H63D, C282Y/wild type, C282Y/H63D, and C282Y/C282Y genotypes were associated with porphyria cutanea tarda in a dose-dependent manner with the largest risk conferred by C282Y/C282Y genotype[7].

Classification of diabetes mellitus

Until the 1970'ies, the classification by the World Health Organisation (WHO) of diabetes was by either age or type[149] (Table 2). However, it became clear that in some patients with diabetes insulin was life-saving, whereas in others it was not. In the WHO 1980[150]/1985[151] classification system, diabetes was then divided into insulin-dependent diabetes mellitus(IDDM) and non-insulin dependent diabetes mellitus (NIDDM). Thereafter, studies showed that islet cell auto-antibodies were discriminative of some NIDDM patients (5-10%)[152], and that these patients carrying such antibodies showed resemblance of IDDM; this group of patients were termed "latent auto-immune diabetes of the adult (LADA)[153]". Today the islet cell auto-antibody glutamic acid decarboxylase (GAD-65) is the first choice of test, because of its high sensitivity, specificity, and positive predictive value and it is the best standardized and characterized type 1 diabetes analysis[154.]

In 1999 on the recommendation by the American Diabetes Association[155](ADA), WHO reclassified the diabetes criteria[156] into the etiological types of primarily type 1 diabetes (autoimmune and idiopathic) characterised by beta-cell destruction usually leading to absolute insulin deficiency, and type 2 diabetes characterised by a range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance.

In the 1965 WHO-report hereditary hemochromatosis is acknowledged as a pancreatic disorder that may lead to diabetes; in the WHO-1980 report, diabetes secondary to hemochromatosis was described in the following way: "In a very few societies, hemochromatosis is a major cause of diabetes. This occurs when iron consumption is high". In the WHO-1999 report hemochromatosis became part of the classification system as a subgroup to the group:

Table 2. WHO expert committee classifications of diabetes in 1965, 1980, and 1999 and by ADA in 2006

WHO-1965	WHO-1980	WHO-1999 and ADA-2006
(Data derived from the expert committee report)	(Data derived from the expert committee report, **table 2:** *Classification of diabetes mellitus and other categories of glucose intolerance*)	(Data derived from the expert committee report, **Table 2:** *Aetiological classification of disorders of glycaemia* **and Table 3** *Other specific types of diabetes*) & **ADA 2006** (*Table 1*)
A classification by age: **1)** Infantile or childhood diabetes *(0-14 years)* **2)** young diabetes *(15-24 years)* **3)** adult diabetes *(25-64 years)* **4)** elderly diabetes *(above 65 years)* **A classification by type:** ***-Juvenile diabetes*** ***-Brittle Diabetes*** ***-Insulin-resistent diabetes*** ***-Gestational diabetes*** ***-Pancreatic diabetes*** *(eg. hemochromatosis)* ***-Endocrine diabetes*** ***-Iatrogenic diabetes***	**A. Clinical classes** *Diabetes Mellitus:* **-Insulin-dependent** *(Type 1)* **-Non-insulin dependent** *(Type 2)* **a)** *Non-obese* **b)** *Obese* **Other types including diabetes mellitus associated with certain conditions and syndromes:** **1)** *pancreatic disease,* **2)** *disease of hormonal etiology,* **3)** *drug- or chemical-induced condition,* **4)** *insulin receptor abnormalities* **5)** *certain genetic syndromes,* **6)** *miscellaneous.* **Impaired glucose tolerance** **a)** *Non-obese* **b)** *Obese* **c)** *Impaired glucose tolerance associated with certain conditions and syndromes* **Gestational diabetes** **B. Statistical risk classes** **(subjects with normal glucose tolerance but substantially increased risk of developing diabetes)** **-previous abnormality of glucose tolerance** **-potential abnormality of glucose tolerance**	**Etiologic classification of diabetes mellitus** **I. Type 1** (*beta-cell destruction, usually leading to absolute insulin deficiency*) **-Autoimmune** **-Idiopathic** **II. Type 2** (*may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance*) **III. Other specific types** **A.** *Genetic defects of beta-cell function* **B.** *Genetic defects of insulin action* **C.** *Diseases of the exocrine pancreas* -Fibrinocalculous pancreatopathy -Pancreatitis -Trauma/pancreatectomy -Neoplasia -Cystic Fibrosis -Hemochromatosis -Others **D.** *Endocrinopathies* **E.** *Drug- or chemical induced* **F.** *Infections* **G.** *Uncommon forms of immune-mediated diabetes* **H.** *Other genetic syndromes* **IV. Gestational diabetes**

Data in the table are derived from the WHO expert Committee reports and ADA, see text for references. Not all details are shown due to limited space.

***WHO:** World Health Organization; **ADA:** American Diabetes Association.*

"exocrine pancreatic disorders". However, as described above, this is a misclassification, as the pancreatic diabetes likely originates from destruction of beta-cells. In hemochromatosis with end-organ damage, histopathological deposition of hemosiderin in the exocrine pancreas can be seen in the acinar cells[50, 157], but destruction of lobular architecture and significant fibrosis, both features of chronic pancreatitis, are lacking[157], and the effect on exocrine pancreatic function is controversial[158, 159]. Although older studies have suggested that exocrine pancreatic secretion facilitated iron absorption[160, 161], these studies have been disproven[159, 162, 163]. Furthermore, hemochromatosis genotypes are not associated with aggravation of alcoholic, idiopathic or familial pancreatitis, or with pancreatic adenocarcinoma[164].

Danish registries for ascertainment of diagnoses used in the thesis

The national Danish Civil Registration System[165] (CRS) was established in 1968 and contains current and historical information on individuals living in Denmark. The CRS includes individual information on the unique personal identification number, name, gender, date of birth, place of birth, citizenship, identity of parents and continuously updated information on vital status, place of residence and spouses, and on immigration and emigration as well[165]. Use of CRS with other Danish registries is a unique opportunity to link information to data obtained in epidemiological studies.

The National Danish Patient Registry[166] was established in 1977. Until 1995 it contained information on all inpatient hospitalisations, from 1995 it covers all hospital inpatient, outpatient, emergency care and other hospitalisations. Until 1994 the registry used World Health Organisation International Classification of diseases (ICD) edition 8 (ICD-8), and from 1994 and onwards the registry has used edition 10 (ICD-10).

The National Danish Cancer Registry contains data on the incidence of cancer in the Danish population since 1943[167]. The information recorded in the registry covers personal information (at date of diagnosis) and tumor information (e.g. morphology, topography, stage, grade etc.). The registry used ICD-7 coding until 1977, and from 1978 it has used ICD-10 coding.

The National Danish Causes of Death Registry (NDCDR)[168] was established in 1875 and covers all deaths among citizens dying in Denmark. Classification of causes of death is done according to the ICD-system and since 1994 using the ICD-10 edition. The NDCDR contains information on all underlying and contributing causes of death; until 2007, the coding was done by the Danish National Board of Health based on paper-based death certificates completed by physicians in hospitals, general practice, or forensic medicine; after 2007, it is the physician who verifies the death and issues the electronic death certificate and who also classifies the causes of death according to the ICD coding[168].

Objectives

To summarise from the introduction, the following hypotheses were tested in this thesis:

1. Hereditary hemochromatosis is a disease often overlooked in patients with late-onset type 1 diabetes mellitus, a late manifestation of untreated iron overload[8] (paper 1).

2. Elevated transferrin saturation (a proxy for iron overload) is associated with increased risk of any form of diabetes, as well as type 1 and type 2 diabetes separately[10] (paper 2).

3. The hemochromatosis genotype C282Y/C282Y and/or extreme elevation in transferrin saturation (proxies for iron overload) are associated with hypertension and/or left ventricular hypertrophy[11] (paper 3).

4. Elevated transferrin saturation levels and hemochromatosis genotype C282Y/C282Y (proxies for iron overload) are associated with an increased risk of cancer[12] (Paper 4).

5. Elevated transferrin saturation levels (a proxy for iron overload) are associated with increased risk of total mortality[13] in the general population (Paper 5).

6. Elevated transferrin saturation and hemochromatosis genotype C282Y/C282Y are associated with total and cause-specific mortality, and early measurement of transferrin saturation improves life expectancy in patients with diabetes ascertained in a specialized diabetes clinic[9] (paper 6).

7. Elevated transferrin saturation and hemochromatosis genotype C282Y/C282Y are associated with total and cause-specific mortality in patients with diabetes ascertained in the general population (paper 7)[14].

Methods

Study populations

The studies were approved by Herlev Hospital and by Danish and Scandinavian ethics committees. Written informed consent was obtained from participants. The studies complied with the Declaration of Helsinki.

General Population studies (papers 1, 2, 3, 4, 5, and 7)

We examined two similar but independent white Danish population-based follow-up studies, the Copenhagen City Heart Study(CCHS) 1991–1994 and 2001–2003 examinations[8, 10-13, 169] and the Copenhagen General Population Study (CGPS) 2003–2007 examination[11, 13, 169]. Individuals in the 2 studies were ascertained and examined similarly (Table 3).

The Copenhagen City Heart Study (papers 1, 2, 3, 4, 5, and 7)

This is a prospective general population study of individuals from Copenhagen (*Østerbro and Nørrebro*) randomly selected on the basis of the Danish Civil Registration System Code to reflect the adult general population. The participants, age-stratified within 5-year age groups from 20-80 years were examined in 1976-1978[170], 1981-1983, 1991-1994[171], and in 2001-2003. In 1976-1978, 19329 individuals were invited and 14223 participated (74%). In 1981-1983, the previous cohort was invited plus an additional 500 individuals aged 20-25 years; 12698 participated (70%). In 1991-1994, those who previously were invited in the first or the second examination were invited (N=13560) and an additional 3000 individuals aged 20-49 years were invited; 10135 individuals participated (61%). In 2001-2003, 12599 were invited including an additional 2464 individuals aged 20-29 years old; 6237 partipated (50%). At each examination, participants filled out a questionnaire (e.g. physical activity, smoking, diet, alcohol, reproductive history (women), and medication) and attended a health examination including blood samples. Participants were not asked about a previous diagnosis of hemochromatosis. DNA was isolated in the 1991-1994 examination (N=9259), and data in the thesis are based on those individuals who attended the 1991-1994 examination (mean age 60 years). Depending on the specific selection criteria for the study objectives in the thesis, 8740-9174 individuals were included from the 1991-1994 CCHS examination.

The Copenhagen General Population Study (paper 2, 3, 4, and 7)

This is a prospective general population study with enrolment ongoing; the study was initiated in 2003 and is still recruiting. Individuals were randomly selected on the basis of the Danish Civil Registration System Code to reflect the adult general population in the Greater Copenhagen Area (Region Hovedstaden). Inclusion criteria were age 20-100 years, white and of Danish descent; Danish descent is defined in the Danish Civil Registration System as a person who is born in Denmark with Danish citizenship with both parents also born in Denmark with Danish citizenship. Data in this thesis are based on those who attended from

2003-2007 (mean age 58 years). The secretariat from CGPS could not provide me data on non-participants, but they have roughly estimated the response rate to be 45% (personal communication with BG Nordestgaard), although this number has some uncertainty as the study is recruiting continuously. Participants filled out a questionnaire (e.g. physical activity, smoking, diet, alcohol, reproductive history (women), and medication) and attended a health examination including blood samples. Participants were not asked about a previous diagnosis of hemochromatosis. Depending on the specific selection criteria for the study objectives, 24195-36480 individuals were included; but since CGPS is an ongoing study with continuous enrolment, number of individuals also varied across the papers. It was not possible to get additional information from the Secretariat for CGPS to supply the diagnoses for total mortality or co-morbidities on the non-participants in CGPS, as this part of the data is only planned to be organised later.

Patients

Steno Diabetes Center Cohort 1999 (Diabetes Cohort 1) (paper 1 and 6)

We recruited all patients from Copenhagen and the former Copenhagen county (Københavns Amt) who attended Steno Diabetes Center between April, 1999, and November, 1999, and who had late-onset type 1 diabetes mellitus diagnosed[8] (i.e. after 30 years of age) according to WHO-1980 criteria[150]. Since the centre is a first-line referral clinic for individuals more than 16 years of age with diabetes from the Greater Copenhagen Area, this cross-sectional sample is representative of a population of about 600 000 inhabitants. More than 90% of those diagnosed with type 1 diabetes were referred to the Steno Diabetes Center. Because diabetes first manifests in late adulthood in patients with hereditary hemochromatosis, we enrolled only those who developed type 1 diabetes after age 30 years. We asked 792 patients to participate, 716 (90%) accepted and were genotyped; 708 (99%) of these were of Danish descent. Mean age was 51 years. None of the patients were diagnosed with hemochromatosis at recruitment. At study entry in 1999, both clinicians and patients were unaware of the patients' iron and genotype status, and patients had a blood sample for measurement of transferrin saturation and genotyping for hereditary hemochromatosis. This study is retrospective in the sense that in 1999 we examined the relation between hemochromatosis genotype C282Y/C282Y and late-onset type 1 diabetes. Table 4 shows characteristics of the participants. We did not have data on the non-participants.

Steno Diabetes Center Cohort 2001-2007(Diabetes Cohort 2)(paper 2 and 6)

Based on results in Diabetes Cohort 1 (shown in the Results section), a decision was made at the Steno Diabetes Centre to introduce routine measurement of transferrin saturation during the first meeting with an endocrinologist for all patients attending the Steno Diabetes Center; increased transferrin saturation above 50% was followed by a genotyping for *HFE*. We therefore consecutively included population-based white Danish patients with diabetes (N=6129) for paper 2[10] (median age 56 years) from Copenhagen and the former Copenhagen County who attended the Steno Diabetes Center between November 2001 and November 2007[10]. None of the patients were diagnosed with hemochromatosis at recruitment. There was no overlap between patients from the Diabetes Cohort 1 and Diabetes Cohort 2. In paper 6[9], comparing these two cohorts in order to examine the effect of early measurement of transferrin saturation, 9 patients were lost to follow-up due to emigration, and 6120 patients were included. Table 4 shows characteristics of the participants from Diabetes Cohort 1 and Diabetes Cohort 2 from paper 6.

Patients with diabetes ascertained in CCHS and CGPS (paper 7)

We included 3346 individuals with diabetes (375 from CCHS, 2971 from CGPS) from a total population size of 84,865 (8740 from CCHS and 76,125 from CGPS); 118 had type 1 diabe-

tes and 3328 had type 2 diabetes. Patients with diabetes were those who answered yes to either one of the following questions a) Do you have diabetes (yes/no), b) are you treated with insulin?, c) are you treated with other antidiabetic drugs?, d) or those who answered no to the questions but had a non-fasting blood glucose above 11 mmol/L. At the time this study was performed, the CGPS had included more individuals in the study, than those used for the previous papers.

Losartan Intervention for End-Point Reduction in Hypertension Genetic Substudy (LIFEGEN) (paper 3)

Losartan Intervention for End-Point Reduction in Hypertension (LIFE) was a double-blind, prospective, parallel group study designed to compare the effects of losartan with those of the b-blocker atenolol on the reduction of cardiovascular morbidity and mortality[172]. 33 000 screening ECGs were received, and approximately 19 000 ECGs were approved as meeting the study ECG-LVH criteria. 9124 patients with essential hypertension, aged between 55 and 80 years, and ECG-documented left ventricular hypertrophy (LVH) were included and randomized. It is not clear from the protocol how many patients that were asked for participation at the time of screening[172].

Losartan Intervention for End-Point Reduction in Hypertension Genetic Substudy (LIFEGEN) included 3815 Scandinavian patients with hypertension and electrocardiogram (ECG)-verified LVH[172-174] who were all genotyped for C282Y and H63D[11]. All patients were white and were recruited between June 1995 and April 1997 in Denmark (N = 904), Finland (N = 1262), Norway (N = 641), and Sweden (N =1008). Median age was 66 years (Table 3). Patients were not asked about a previous diagnosis of hemochromatosis.

Genotyping

Genotyping of CCHS and patients with late-onset type 1 diabetes (Diabetes Cohort 1) was done in 1999 as part of paper 1. Genotyping of patients with diabetes in Diabetes Cohort 2 was done consecutively from 2001-2007 as part of paper 2. Genotyping of the CCHS participants and diabetes patients[8] for C282Y (dbSNP: rs1800562), a G/A nucleotide change at position 845 in *HFE*[20], and H63D (dbSNP: rs1799945), a C/G nucleotide change at position 187 in *HFE*[20], was by allele specific amplification[175], with restriction enzyme digestion to confirm genotyping[8, 20]. The amplification refractory mutation system (ARMS) simultaneously detects both hereditary hemochromatosis mutations C282Y and H63D, including sense and antisense primers for C282Y, H63D and human growth hormone as an internal amplification control[175]. Genotyping of the CGPS and LIFEGEN participants was done in 2007 and was by a TaqMan assay (Applied Biosystems, Foster City, CA, USA)[11]. Each run included a known non-carrier, a heterozygous, and a homozygous control verified by sequencing. After 2 reruns, call rates for genotypes where above 99.9% for all assays. CCHS, CGPS, and LIFEGEN populations were in Hardy–Weinberg equilibrium (HWE) for C282Y and H63D. In diabetes Cohort 1, H63D was in HWE, but C282Y was in Hardy-Weinberg disequilibrium (p=0.002). It was not possible to estimate HWE in Diabetes Cohort 2 as only those patients with transferrin saturation above 50% were genotyped. The data on genotyping in the papers 3, 4, 6 and 7 thus rely on the genotyping data from the previous papers.

Measurement of transferrin saturation

CCHS, CGPS, diabetes patients: A non-fasting transferrin saturation level of ≥50% was chosen as suggestive of increased transferrin saturation, in accordance with accepted clinical practice[32, 176, 177]. Transferrin saturation(%) was determined as iron levels (in µmol/L) divided by 2*transferrin levels (in µmol/L) *100. Transferrin was measured by turbidimetry and iron levels by absorption photometry ((Konelab autoanalyzer (ThermoFisher Scientific; Waltham, MA, USA) and Hitachi 912 (Roche)). Coefficient of variation (CV%) of iron was 2.3 % at the level of 20.6 µmol/L and of transferrin 5.7% at the level of 25.9 µmol/L.

Table 3. Characteristics of participants in CCHS, CGPS and LIFEGEN

(from paper 3)

	CCHS	CGPS	LIFEGEN
Numbers, N	8992	36480	3815
Age,	60(47-70)	58(48-67)	66(60-72)
Men, %	44	46	46
Diastolic blood pressure, mmHg	84(75-92)	83(75-90)	99(94-104)
Systolic blood pressure, mmHg	136(122-152)	140(126-155)	174(165-185)
Pulse pressure, mmHg	51(42-65)	55(45-68))	76(66-87)
BMI, kg/m²			
1st tertile	22(20-23)	22(21-23)	24(23-25)
2nd tertile	25(24-26)	26(25-27)	27(26-28)
3rd tertile	30(28-32)	30(29-32)	32(31-34)
Smokers, %			
Current smokers, yes/no (%)	49	23	16
Alcohol consumption, units*/week			
None, %	21	14	40
1-4, %	23	20	43
5-7, %	14	14	9
8-10, %	11	12	4
More than 10, %	31	40	4
Cholesterol, mmol/L			
1st tertile	4.9(4.5-5.3)	4.7(4.3-5.0)	5.1(4.7-5.4)
2nd tertile	6.1(5.8-6.4)	5.7(5.5-5.9)	6.1(5.9-6.4)
3rd tertile	7.4(7.0-8.0)	6.7(6.4-7.2)	7.2(6.9-7.8)
HDL cholesterol, mmol/L			
1st tertile	1.1(1.0-1.2)	1.2(1.0-1.3)	7.1(1.0-1.2)
2nd tertile	1.5(1.4-1.5)	1.6(1.5-1.7)	1.5(1.4-1.6)
3rd tertile	2.0(1.9-2.3)	2.2(2.0-2.4)	2.0(1.8-2.2)
Diabetes mellitus, %			
	4	4	11

BMI: body mass index.
CCHS: Copenhagen City Heart Study.
CGPS: Copenhagen General Population Study.
HDL: high-density lipoprotein.
LIFEGEN: Losartan Intervention For Endpoint Reduction in Hypertension Genetic Substudy.
Values are frequencies (%) or medians (interquartile ranges).
*one unit of alcohol equals 12 g.

The transferrin measurements we used in the articles in this thesis were done in 1999 for Steno Diabetes Center (Diabetes Cohort 1) (paper 1 and 6) on fresh plasma samples; consecutively from 2001-2007 for a new cohort at the Steno Diabetes Center (Diabetes Cohort 2) (paper 2 and 6) on fresh plasma samples; in 2007 for the Copenhagen City Heart Study on biobank plasma samples that had been stored for approximately 15 years at -80 degress; and consecutively from 2003-2007 for the Copenhagen General Population Study (paper 2, 3, 5 and 7) on fresh plasma samples.

Reasons for different cut-offs of transferrin saturation in the papers: in 2001 (paper 1) a cut-off of 50% was generally accepted as a clinical screening cut-off for iron overload. However, as other articles included more extreme[15, 123, 128] or less extreme values of transferrin saturation[121], we were inspired to explore other cut-off levels of transferrin saturation and to explore a semi-continuous relationship of transferrin saturation in categories ranging from <20% to above 70 or 80% in other articles (paper 3, 5, and 7) with larger sample sizes and larger statistical power due to the inclusion of the CGPS. But in paper 4 using only CCHS we did not have enough statistical power to include more categories than three (<50%, 50-60%, and above 60%); another reason for including the category above 60% in that study was that two other studies of transferrin saturation had used a cut-off of 60%[123, 128], and in order to perform a comparable meta-analysis with these studies, we chose to explore the category above 60% even though power was small. In paper 2 (risk of diabetes according to transferrin saturation), we used only a cut-off of 50% since this was the cut-off previously used in paper 1 and since neither the case-control study nor the CCHS in these analyses had enough statistical power to extend transferrin saturation groups beyond 50%.

Ascertainment of diagnoses

The diagnosis of late-onset type 1 diabetes in paper 1[8] was based on the WHO-1980[150] definition of type 1 diabetes as insulin-dependent diabetes mellitus and late-onset was defined by the authors as diabetes occurring after 30 years of age. Diagnoses of diabetes mellitus (paper 2[10], 6[9], and 7[14]), cancer (paper 4[12]) and total and cause-specific mortality(paper 5[13], 6[9], and 7[14]) were obtained from the National Danish Patient Registry[166], the National Danish Cancer Registry[167], the national Danish Civil Registration System[165] , and National Danish Causes of Death Registry (NDCDR)[168]. Furthermore, information on diabetes mellitus[8, 10] and hypertension[11] was also obtained from the questionnaire and physical examination, and in addition, if diabetes mellitus was not registered with any of these methods, a non-fasting blood sugar level above 11 mmol/L[10, 14] was taken as evidence of diabetes mellitus. There were no losses to follow-up on individuals in CCHS and CGPS. 9 patients with diabetes were lost to follow-up in paper 6.

An audit (August 2013, Steno Diabetes Center) was made for this thesis on the patients with late-onset type 1 diabetes who participated in paper 1. Since these patients' diagnoses were based on WHO-1980 classification[150], these patients could potentially have been re-classified using the more updated version WHO-1999[156] or ADA-2006[178] definitions, since diabetes classification has changed over time (Table 2). A random computer-generated sample of 12 wild type/wild type patients and all patients with C282Y/C282Y were selected. Data on C-peptide levels, plasma glucose, auto-antibodies (GAD-65), therapy (insulin or other anti-diabetic medication), body mass index(BMI), age in 1999, diabetes onset (year), date of blood sampling, and transferrin saturation were retrieved from the patients' electronic and manual records at Steno Diabetes Center. It was not possible to retrieve a complete dataset for the patients, since some data were not available in the records. ICD-8 and ICD-10 diagnoses on diabetes were retrieved from the Steno Diabetes Center, the national Danish Patient Registry, and NDCDR. Results of the audit are presented in the Results section.

Statistics

Papers 1, 2, 3, 4, 5, 6, and 7

We used the statistical programme Stata/SE 9.0-11.0, except for power calculations for which we used NCSS PASS software. Two-sided P-values <0.05 were considered significant. Mann–Whitney U-test and Kruskal-Wallis test were used for continuous comparisons, and Pearson chi-square test was used for categorical comparisons.

For cross-sectional[11] and case-control[8, 10] studies either matched[10] or unmatched[8, 11] logistic regression analyses were performed.

In follow-up studies[10, 12, 13, 169], cumulative incidences of disease (diabetes, hypertension and

left ventricular hypertrophy, cancer) and cumulative survival rates (total and cause-specific mortality) were plotted as a function of age and levels of transferrin saturation or *HFE* genotype (Kaplan-Meier curves), and with log rank tests as measures of significance. Cox regression analyses were used to estimate hazard ratios for disease as a function of levels of transferrin saturation and *HFE* genotype. For Cox regression analyses to produce valid risk estimates, the assumption of proportionality of hazards must be met, meaning that the ratio of the hazards, comparing different exposure groups, must remain constant over time[179]. This was tested for using Schoenfeld residuals, and no violations were observed. Continuous variables entered in Cox regression models must display linearity on the log risk scale. Linearity exists when a change in the dependent variable is constant for a one-unit change in the independent variable, irrespective of the value of the independent variable. Another possibility is to categorise all continuous variables, which is what we did.

Analyses were tested for interaction in both logistic and Cox regression models by inserting two-factor interaction terms using -2loglikehood test for significance, one at the time, in the models. Covariates were entered as categorical variables.

Absolute risks by transferrin saturation levels or genotypes were estimated using the regression coefficients from a Poisson regression model[11, 12]. Absolute risks are presented as estimated incidence rates (events/10 years) in percentages.

Population-attributable risk[180] was estimated as [f(HR-1)]/[1+f(HR-1)], where f is the frequency of the exposure in the population (C282Y/C282Y genotype or transferrin saturation ≥50%), and HR is the hazard ratio or odds ratio for the endpoint[8, 10-13]. The population-attributable risk estimate is an approximation of the excess fraction of cases that would not have occurred had the exposure been absent.

Imputation of missing values were done in CCHS, CGPS, and Diabetes Cohorts for missing values on confounders in papers 4-7, but not for missing values on exposure (transferrin saturation, genotype) or outcome. The missing data in CCHS and CGPS are not reported in the papers, but biochemical data had an average of 0.1% missing values, questionnaire data 1%, and health examination data 1%. In CCHS and CGPS (paper 4, 5, 7) imputations were based on replacing the missing value with the median in the dataset for continous values and the most frequent categorical value for categorical data. In the Diabetes Center Cohorts, the missing data on BMI ranged from 4%-9% and missing data on medication ranged from 7-27%; missing data were imputed using simple imputation depending on age and sex for both continous and categorical variables.

Meta-analyses[10, 12, 13] were performed with the Stata "Meta" command calculating both fixed and random effect measures from reports of effect measures and confidence intervals[181]. Statistical heterogeneity was assessed by the Q statistic with a corresponding P-value, although the power of this statistic is low with only a few studies[182]. Owing to very few studies in the meta-analyses[12, 13], it was not possible to assess publication bias. Methodological heterogeneity was assessed *a priori* by stratification according to sex.

Supplementary statistics for the thesis

As a supplement to the papers in the thesis, a calculation of the hazard ratios in non-participants vs. participants in the Copenhagen City Heart Study third examination are carried out for total mortality overall, total mortality among patients with diabetes, incident diabetes, incident cancer, and incident ischemic heart disease. A crude Cox regression model with age as time scale and delayed entry is used. The secretariat for the CGPS did not have data on non-participants organised as of 2014. Neither did we have data on non-participants for the Steno Cohorts or LIFEGEN.

A fractional polynomial model was run in Stata SE 13.0 using the *fracpoly* command[183] for the continuous relationship of transferrin saturation(%) and hazard ratio for total mortality in the general population (additional model for paper 5) and for patients with diabetes (additional model for paper 7). The model is a more advanced and refined method than

Table 4. Characteristics of participants in Diabetes Cohort 1 and Diabetes Cohort 2 (modified from Ellervik et al. Total mortality by elevated transferrin saturation in patients with diabetes. *Diabetes Care 2013;36(9):2646-54*)

	Cohort 1		Cohort 2			
	Late-onset type 1	All	Type 1	Late-onset type 1	Type 2	Other
Men, N(%)	401(56.0)	3450(56.4)	1415(53.4)	632(56.7)	1868(58.8)	167(58.0)
Women, N(%)	315(44.0)	2670(43.6)	1241(46.7)	483(43.3)	1308(41.2)	121(42.0)
Total N	716	6120	2656	1115	3176	288
Recruited, date	1999	2001-2007	2001-2007	2001-2007	2001-2007	2001-2007
Age, years	58(49-68)	56(43-66)	45(35-56)	58(50-65)	63(55-71)	57(43-68)
BMI>=25, N(%)	313(47.7%)	3590(60.8%)	991(37.8%)	429(39.1%)**	2524(81.6%)	75(38.7%)
Missing data BMI, N(%)	60(8.4)	213(3.5)	36(1.4)	18(1.6)	83(2.6)	94(32.3)
Diabetes onset, age, years	46(38-56)	47(28-59)	27(18-38)	41(35-50)**	57(49-66)	51(38-62)
age<30years, N(%)	0	1669(27.3)	1541(58.0)	0	89(2.8)	39(13.5)
age>=30 years, N(%)	716(100)	4451(72.7)	1115(42.0)	1115(100)	3087(97.2)	249(86.5)
Diabetes duration, years	20(16-26)	15(8-25)	26(17-31)	22(13-29)	10(6-15)	8(0-16)
Diabetes complications, N(%)	342(48%)	2829(46%)	1411(53%)	567(51%)	1321(42%)	97(34%)
Renal	10(1.4%)	331(5.6%)	31(1.2%)	29(2.6%)	286(9.2%)	14(6.5%)
Ophtalmological	29(4.0%)	430(7.2%)	72(2.7%)	65(5.9%)	347(11.2%)	11(5.1%)
Neurologica	31(4.3%)	523(8.8%)	47(1.8%)	45(4.1%)	462(14.9%)	14(6.5%)
Circulatory	28(3.9%)	226(3.8%)	113(4.3%)	40(3.6%)	104(3.4%)	9(4.2%)
Other	294(41.1%)	2116(34.6%)	1298(48.9%)	495(44.4%)	736(23.2%)	82(28.5%)
Anti-diabetic medication						
Missing data, N(%)†	48(6.7)	1652(27.0)	567(21.4)	266(23.8)	913(28.7)	172(59.7)
Insulin only, N(%)	664(92.7)	2828(46.2)	2060(77.6)	840(75.3)	673(21.2)	95(33.0)
Oral antidiabetics only, N(%)	0	1375(22.5)	19(0.7)	0	1343(42.3)	13(4.5)
Insulin+oral, N(%)	4(0.6)	258(4.2)	10(0.4)	9(0.8)	240(7.6)	8(2.8)
Diet only, N(%)	0	7(0.1)	0	0	7(0.2)	0
Iron, umol/L	16(13-20)	16(12-20)	17(13-21)	16(13-20)	16(12-20)	14(10-20)
Transferrin, umol/L	28(26-32)	31(27-34)	29(26-32)	28(26-31)	32(29-35)	31(27-34)
Transferrin saturation, %	28(22-36)	26(20-34)	29(22-38)	28(22-35)	24(19-31)	23(16-31)
<50%, N(%)	670(93.7)	5847(95.5)	2463(92.7)	1049(94.1)	3108(97.9)	276(95.8)
Transferrin saturation, %	27(21-34)	26(20-32)	28(21-35)	28(21-34)	24(18-30)	23(15-30)
>=50%, N(%)	45(6.3)	273(4.5)	193(7.3)	66(5.9)	68(2.3)	12(4.2)

Table 4. continued

	Cohort 1		Cohort 2			
	Late-onset type 1	All	Type 1	Late-onset type 1	Type 2	Other
Transferrin saturation, %	56(52-62)	56(52-64)	56(52-63)	56(52-63)	55(52-66)	57(52-71)
Hemochromatosis genotype, N(%)						
All						
Wild type/wild type	474(66.2)	-	-	-	-	-
H63D/wild type	143(20.0)	-	-	-	-	-
H63D/H63D	15(2.1)	-	-	-	-	-
C282Y/wild type	67(9.4)	-	-	-	-	-
C282Y/H63D	8(1.1)	-	-	-	-	-
C282Y/C282Y	9(1.3)	-	-	-	-	-
Transferrin saturation>=50%						
Wild type/wild type	24(53.3)	129(51.8)	100(54.6)	35(56.5)	25(43.1)	4(50.0)
H63D/wild type	6(13.3)	55(22.1)	40(21.7)	14(22.6)	13(22.4)	2(25.0)
H63D/H63D	1(2.2)	8(3.2)	3(1.6)	2(3.2)	5(8.6)	0
C282Y/wild type	4(8.9)	33(13.3)	27(14.8)	6(9.7)	5(8.6)	1(12.5)
C282Y/H63D	1(2.2)	13(5.2)	9(4.9)	4(6.5)	4(6.9)	0
C282Y/C282Y	9(20.0)	11(4.4)	4(2.2)	1(1.6)	6(10.3)	1(12.5)
Total**	45	249	183	62	58	8

Continuous variables are median and interquartile range. Mann-Whitney U-test for continuous variables and Pearson Chi-Square for categorical variables *(for the comparison of late-onset type 1 diabetes in Cohort 1 vs. Cohort 2)*:*, p<0.01, **p<0.001.

Type 1 diabetes *(WHO E10)*, Type 2 diabetes *(E11)*, other diabetes *(E13, E14)*.
Diabetes complications *(ICD10-codes)*: renal*(.2)*, ophtalmological*(.3)*, neurological*(.4)*, circulatory*(.5)*, other*(.0,.1,.6,.7,.8)*.

See article for explanation of anti-diabetic medication.
In Cohort 2, HFE genotypes were only available for patients with transferrin saturation above 50%.
† Due to the difference in missing values for late-onset type 1 diabetes in Cohort 1 and Cohort 2, no p-value is calculated.

splines and linear models[183-185]. Deviance was used as a measure of model fit, the larger the deviance the less fit, and vice verca; however, the deviance is not interpreted directly, but it is the difference in deviance between the models, that is useful: again the larger the difference of model-1 to model-0 (with the least deviance), the less likely model-1 is to fit the data; a p-value for the deviance difference is given.

For a mediation analysis from genotype through transferrin saturation to outcome to be meaningful, 1) the genotype should be related to the outcome, 2) the genotype should be related to the mediator transferrin saturation, 3) the mediator transferrin saturation should be related to the outcome, 4) and to establish if transferrin saturation completely mediates the effect of genotype on outcome, the effect of genotype on outcome controlling for transferrin saturation should be zero. If all steps are fulfilled, transferrin saturation completely mediates the effect[186]. If 4) is different from zero, transferrin saturation partially mediates the effect of genotype on outcome.

A mediation analysis for cancer was done using the following equation[187] giving a raw estimate of the Percentage of excess risk mediated (*PERM*)=
(RR(genotype)-RR(genotype and transferrin saturation))*100%/((RR genotype)-1)

Another, more refined and advanced method was used in *Stata* SE 13.0 using the command: *sgmediation*, with genotype as the independent variable, transferrin saturation as the mediator, sex as a covariate, and first event of any cancer as the dependent variable. Following the command, a bootstrap-estimation of the confidence intervals for the direct and indirect effects was performed, with 1000 repetitions, to calculate for the uncertainty of PERM. If the confidence interval of the coefficients includes zero, then the effect of the mediator is not statistically significant.

Results

Total mortality and incident diabetes, hypertension, cancer, and ischemic heart disease in non-participants vs. participants

In Table 5 is shown total mortality and incident diabetes, cancer, and ischemic heart disease in non-participants vs. participants in the Copenhagen City Heart Study third examination. Hazard ratios for total mortality in non-participants vs. participants was 1.6(1.5-1.7; p<0.001) in the general population, and 1.6(1.4-1.8; p<0.001) in patients with diabetes in the general population. Hazard ratios for incident diabetes, hypertension, cancer, and ischemic heart disease in non-participants vs. participants were 1.2(1.1-1.4; p<0.001), 0.9(0.9-1.0; p=0.03), 1.0(0.9-1.1; p=0.6), and 1.1(1.0-1.2; p=0.008), respectively.

Table 5. **Total mortality and incident diabetes, hypertension, cancer, and ischemic heart disease in non-participants vs. participants in the Copenhagen City Heart Study 1991-1994 examination.**

Non-participants, N=6,425 Participants, N=10,135 Years of follow-up (mean(SD)): 14(7)	Crude hazard ratio (95% CI)
Total mortality in the general population	1.6(1.5-1.7), p<0.001
Total mortality in patients with diabetes	1.6(1.4-1.8), p<0.001
Incident diabetes mellitus	1.2(1.1-1.4), p<0.001
Incident hypertension	0.9(0.9-1.0), p=0.03
Incident cancer	1.0(0.9-1.1), p=0.6
Incident ischemic heart disease	1.1(1.0-1.2), p=0.008

Table 6. Odds ratio for hemochromatosis and mean transferrin saturation by *HFE* genotype in patients with late-onset type 1 diabetes mellitus compared to the general population.

	Patients with late-onset Type 1 diabetes mellitus		General population			Patients with late-onset type 1 diabetes mellitus
Genotype	No.	Frequency (%)	No.	Frequency (%)	Odds ratio (95% confidence-interval)	Mean transferrin saturation (SD)
Wild type/ wild type	474	66.2	6135	66.9	1.0	0.28 (0.11)
H63D/ wild type	143	20.0	1881	20.5	1.0 (0.8-1.2)	0.30 (0.11)
H63D/H63D	15	2.1	158	1.7	1.2 (0.7-2.1)	0.31 (0.11)
C282Y/ wild type	67	9.4	846	9.2	1.0 (0.8-1.3)	0.32 (0.11)*
C282Y/ H63D	8	1.1	131	1.4	0.8 (0.4-1.7)	0.40 (0.09)**
C282Y/ C282Y	9	1.26	23	0.25	4.6 (2.1-10.1)	0.84 (0.16)***
All	716	100	9174	100		

(Table modified from Paper 1; Ellervik et al. Lancet 2001; 358: 1405-1409).
Distribution of genotype frequencies differed between patients with diabetes and the general population (p=0.001).
Odds ratio by logistic regression adjusted for age and sex.
*Mean transferrin saturation: *** ANOVA, p=0.001 between all six genotypes;*
*post-hoc t-test vs. wild type/wild type: *p=0.007, ** p=0.005, *** p<0.0001.*

Table 7. Transferrin saturation in patients with late-onset type 1 diabetes mellitus.

	No. of patients		
Transferrin saturation	C282Y/C282Y	Other genotypes	All
Above 50%	9	26	35
Less than 50%	0	680	680
All	9	706	715*
Positive predictive value:		0.26	
Negative predictive value:		1.00	
Sensitivity:		1.00	
Specificity:		0.96	

(Table modified from Paper 1; Ellervik et al. Lancet 2001; 358: 1405-1409).
**Blood sample from one patient was hemolysed and could not be tested.*

Table 8a. Audit of patients with late-onset type 1 diabetes and genotype C282Y/C282Y from paper 1

						Status: date of blood sampling			
Patient	Lancet-pt.nr*	sex	Hemochroma-tosis Genotype	Diabetes Onset	Date of blood sampling	Glucose mmol/L	C-peptide pmol/L	GAD-65** Units/ml	Fasting
1	5	M	C282Y/C282Y	1989	5/31/1990		190		
2	1	F	C282Y/C282Y	1986	9/4/1992	10.1	605		
3	1	F	C282Y/C282Y	1981	5/13/2002	13.7	10		
4	6	M	C282Y/C282Y	1995	5/26/1995		477		y
4	6	M	C282Y/C282Y	1995	6/9/1997	16.3	509		
5	9	M	C282Y/C282Y	1995	7/28/1995	11.5	137		y
6	3	F	C282Y/C282Y	1989	2/19/2007	9.4	10	27	
6	3	F	C282Y/C282Y	1989	2/19/2007	9.4	< 10	27	
6	3	F	C282Y/C282Y	1989	4/23/2007	9.1	< 10		
6	3	F	C282Y/C282Y	1989	4/23/2007	9.1	10		
6	3	F	C282Y/C282Y	1989	4/12/2010	4.9	34		
6	3	F	C282Y/C282Y	1989	4/12/2010	4.9	34		
7	8	M	C282Y/C282Y	1990	5/2/1995	18	591		
7	8	M	C282Y/C282Y	1990	8/18/2003	12.7	391		
7	8	M	C282Y/C282Y	1990	8/18/2003	12.7	391		
8	7	M	C282Y/C282Y	1993					
9	4	F	C282Y/C282Y	1992	12/1/1997		212		
9	4	F	C282Y/C282Y	1992	5/4/2006	7.1	452	0	
9	4	F	C282Y/C282Y	1992	5/4/2006	7.1	452	0	y

*From Lancet Table 3 in Thesis-paper 1
**GAD-65: Glutamatdecarboxylase antibody
Missing values: data were not available in the electronic system or manual files.
M: male, F: female

Table 8a. continued

Status: 1999						Status: 2013
Body mass index	age (yr)	Transferrin saturation (%)	Oral antidiabetic medication	Receiving insulin	World Health Organisation Diagnoses retrieved from The Danish National Patient Registry ICD-8 codes and ICD-10 codes	ICD-10 codes at Steno Diabetes Center
-	55	94	no	yes	24900(1989), DE10.9(doddiag 1999)	
-	57	85	no	yes	250.09(1992), 249(1992), DE10(2008)	DE10/DE13
27	49	85	no	yes	250.00(1981), 249(1993)	
-	62	90	no	yes	DE11(1995), DE14.9(1997),DE10(1995)	
-	62	90	no	yes	DE11(1995), DE14.9(1997),DE10(1995)	
26	52	103	no	yes	DE10.9(1995),DE11(1995)	DE10
26	47	84	no	yes	249(1989), 250.09(1989)	
26	47	84	no	yes	249(1989), 250.09(1989)	
26	47	84	no	yes	249(1989), 250.09(1989)	
26	47	84	no	yes	249(1989), 250.09(1989)	
26	47	84	no	yes	249(1989), 250.09(1989)	
26	47	84	no	yes	249(1989), 250.09(1989)	
23	44	89	no	yes	250.09(1990)/DE10(1995), DE11(1995),(E10.9 dod 2004)	DE11
23	44	89	no	yes	250.09(1990)/DE10(1995), DE11(1995),(E10.9 dod 2004)	DE11
23	44	89	no	yes	250.09(1990)/DE10(1995), DE11(1995),(E10.9 dod 2004)	DE11
26	52	95	no	yes	249.09(1993), 250(1993)	DE13
28		57	no	yes	250.09(1978), DE10(2001)	DE13
28	43	57	no	yes	250.09(1978), DE10(2001)	DE13
28	43	57	no	yes		

*From Lancet Table 3 in Thesis-paper 1
**GAD-65: Glutamatdecarboxylase antibody
Missing values: data were not available in the electronic system or manual files.
M: male, F: female

Table 8b. Audit of patients with late-onset type 1 diabetes and genotype wild type/wild type from paper 1

						Status: date of blood sampling			
Patient	Lancet-pt.nr*	sex	Hemochroma-tosis Genotype	Diabetes Onset	Date of blood sampling	Glucose mmol/L	C-peptide pmol/L	GAD-65** Units/ml	Fasting
10		F	wild type	1978	9/4/2000	11.1	10		y
10		F	wild type	1978	9/4/2000	11.1	< 10		y
11		F	wild type	1988	2/18/2008	7.8	10	0	
12		M	wild type	1986					
13		M	wild type	1971					
14		M	wild type	1974	11/27/2006	17.2	< 10		
14		M	wild type	1974	11/27/2006	17.2	10		
14		M	wild type	1974	4/30/2012	14.2		250	
15		F	wild type	1992	8/3/2009	21.1	10		
15		F	wild type	1992	8/3/2009	21.1	< 10		
16		F	wild type	1996	3/26/1996	12.1	224		
16		F	wild type	1996	8/27/2002	6.2	32		
16		F	wild type	1996	8/27/2002	6.2	32		
17		M	wild type	1976					
18		M	wild type	1988					
19		M	wild type	1990	9/17/2002	20.3	10		
20		F	wild type	1996	6/12/1996		323***		y
21		F	wild type	1976	5/21/2002	21.8	10		

*These did not have numbers in the Lancet publication
**GAD-65: Glutamatdecarboxylase antibody
***Glucagon-simulated CPEP: 219 pmol/L
Missing values: data were not available in the electronic system or manual files.
M: male, F: female

Table 8b. continued

Status: 1999						Status: 2013
Body mass index	age (yr)	Transferrin saturation (%)	Oral antidiabetic medication	Receiving insulin	World Health Organisation Diagnoses retrieved from The Danish National Patient Registry ICD-8 codes and ICD-10 codes	ICD-10 codes at Steno Diabetes Center
18	81	16	no	yes	249(1979), 249(1987),250(1988)	
18	81	16	no	yes	249(1979), 249(1987),250(1988)	
25	60	25	no	yes	250.09(1988), 249(1990)	
31	45	31	no	yes	250.09(1986), 249(1989)	DE10
21	72	52	no	yes	250.09(1982), 249(1990)	DE10
26	64	18	no	yes	DE11.9(1993), DE10(1993)	
26	64	18	no	yes	DE11.9(1993), DE10(1993)	
26	64	18	no	yes	DE11.9(1993), DE10(1993)	
27	71	24	no	yes	250.00(1993), 249(1993)	
27	71	24	no	yes	250.00(1993), 249(1993)	
24	35	21	no	yes	DE14.0(1996), DE10(1996)	
24	35	21	no	yes	DE14.0(1996), DE10(1996)	
24	35	21	no	yes	DE14.0(1996), DE10(1996)	
24	79	25	no	yes	250.09(1978), DE10(2008)	DE11
28	48	34	no	yes	249(1988)	DE10
26	45	33	no	yes	249.09(1990)	
23	51	33	no	yes	DE10.9(1996), DE11(2010)	
26	52	58	no	yes	DE10.3(1993)	

*These did not have numbers in the Lancet publication
**GAD-65: Glutamatdecarboxylase antibody
***Glucagon-simulated CPEP: 219 pmol/L
Missing values: data were not available in the electronic system or manual files.
M: male, F: female

Risk of diabetes mellitus (paper 1 and 2)

Paper 1

First, we performed a retrospective study[8] in which we genotyped for the C282Y and H63D mutations in the hemochromatosis gene in 716 unselected Danish patients who developed type 1 diabetes mellitus after age 30 years and in 9174 controls from the Danish general population. We also screened for hereditary hemochromatosis by assessment of transferrin saturation in the patients with diabetes.

More patients with diabetes (n=9 (1.26%)) than controls (n=23 (0.25%)) were homozygous for C282Y with a corresponding odds ratio of 4.6 (95% confidence interval (CI): 2.1-10.1)[8] (Table 6). These patients had unrecognised signs of hemochromatosis. Transferrin saturation and ferritin concentrations ranged from 57% to 102% and 17µg/L to 8125µg/L, respectively. The positive and negative predictive values of transferrin saturation greater than 50% for identification of C282Y homozygosity were 0.26 and 1.00, respectively (Table 7). A saturation of less than 50% therefore excluded C282Y homozygosity, whereas a saturation of more than 50% suggested C282Y homozygosity.

Patient-audit

Table 8a and 8b show the results of the patient-audit of late-onset type 1 diabetes.
Patients with C282Y/C282Y (N=9, 4 women and 5 men) all had transferrin saturation above 50% and generally had higher C-peptide levels (10-605pmol/L) than patients with wild type/wild type (compare Tables 8a and 8b). In 1999 at recruitment, all patients (both C282Y/C282Y and wild type/wild type) received insulin therapy and none received oral anti-diabetic medication. Patient number 2 with C-peptide level of 605 pmol/L at a non-fasting glucose of 10 mmol/L may be compatible with type 1 diabetes secondary to hemochromatosis due to some residual beta-cell function. Patient number 6 may have two diagnoses (either auto-immune type 1 diabetes and hemochromatosis) or type 1 diabetes secondary to hemochromatosis and then by chance have auto-antibodies as 2% of the population have without developing diabetes[154]. The auto-antibody titer of 27 U/ml is however not very high, and the measurement is 18 years after diagnosis, thus the patient may likely both have auto-immune type 1 diabetes and hemochromatosis. Only 2 patients had a measurement of auto-antibodies. Body mass index ranged from 26-28 in 5 patients and was 23 in one patient; 3 patients had missing values. There was no clear pattern in the ICD-diagnoses of the patients other than a mixture of diagnoses of type 1 diabetes (ICD-8: 249, ICD-10: DE10), type 2 diabetes(ICD-8: 250, ICD-10: DE11), and secondary diabetes (ICD-10: DE13).
In wild type/wild type patients (N=12, 6 women and 6 men)(Table 8b) all but one had transferrin saturation below 50%. All patients either had a non-stimulatory or stimulatory C-peptide below 200 pmol/L or close to 200. Only two patients had auto-antibodies measured and one of them was positive; the rest of the patients had missing values. Body mass index ranged from 25-31 in 7 patients, and from 18-24 in 5 patients. These patients also had a mixture of ICD-diagnoses (type 1: ICD-8: 249, ICD-10: DE10; type 2 diabetes: ICD-8: 250, ICD-10: DE11), but none had DE13.

Paper 2

Secondly, we examined two general population studies, The Copenhagen City Heart Study (CCHS, N=9121) and The Copenhagen General Population Study (CGPS, N=24195), and a 1:1 age and gender matched population-based case-control study with 6129 patients with diabetes and 6129 controls, totalling 8535 patients with diabetes mellitus and 37039 controls[10].

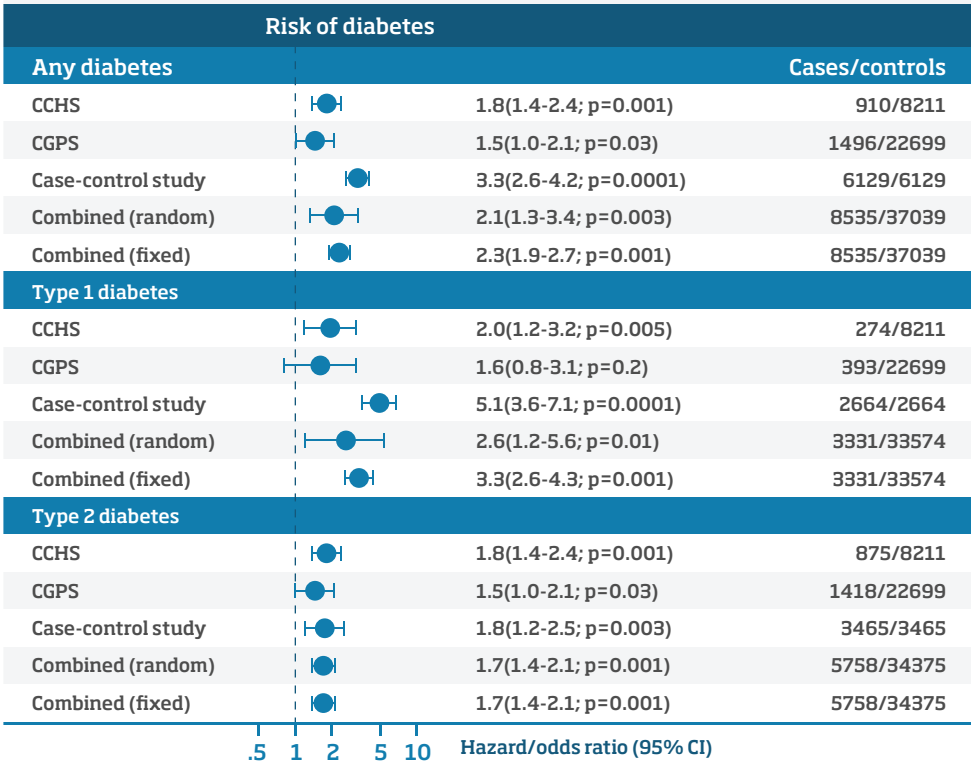
Cumulative incidence by age of diabetes mellitus increased with transferrin saturation ≥50% vs. <50% in the CCHS (log-rank: p=0.0001) and the CGPS (p=0.03). Corresponding hazard ratios were 1.8 (1.4-2.4) in the CCHS and 1.5 (1.0-2.1) in the CGPS while the corresponding odds ratio in the case-control study was 3.3 (2.6-4.2) (Figure 2). In the combined

Figure 2.

Risk of diabetes mellitus according to transferrin saturation ≥50% vs. <50% in the Copenhagen City Heart Study(CCHS), the Copenhagen General Population Stud (CGPS) and in a case-control study, individually and combined.

The case-control study comprised patients with diabetes from Steno Diabetes Centre, Copenhagen, and controls ascertained like in the CGPS, but from a different sample than those included in the CGPS only study. There was no overlap of individuals between the three studies, thus allowing independent confirmation of findings. Heterogeneity for the combined results for men and women was: **Q=17** and **p=0.001** (*any diabetes*), **Q=15** and **p=0.001** (*type 1 diabetes*), and **Q=0.7** and **p=0.7** (***Type 2 diabetes***). CI=confidence interval.

(Paper 2: Ellervik et al. Diabetes Care 2011)



studies, odds ratios in individuals with transferrin saturation of ≥50% versus <50% were 2.1 (1.3-3.4) for any diabetes, 2.6 (1.2-5.6) for type 1 diabetes, and 1.7 (1.4-2.1) for type 2 diabetes.

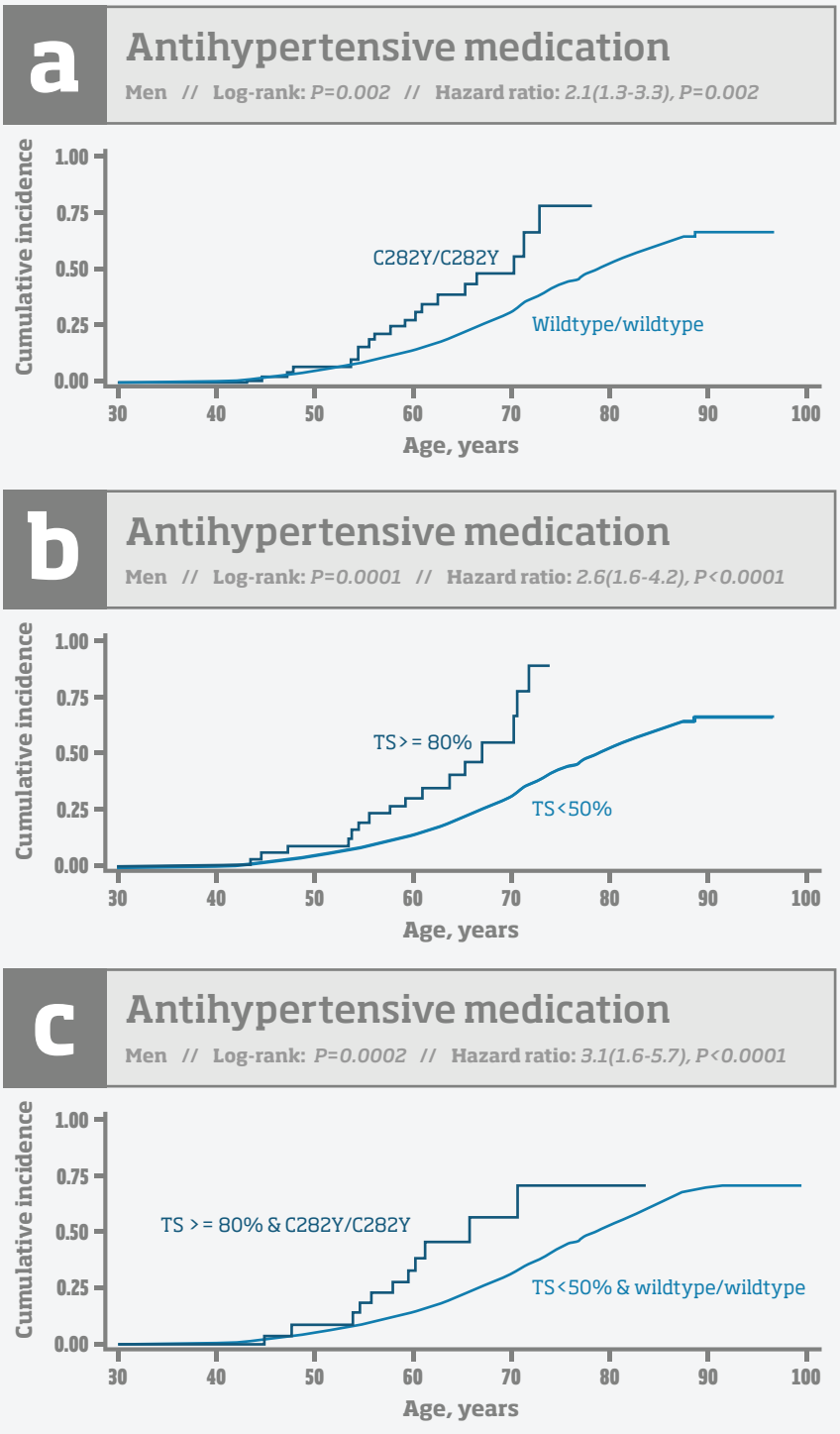
Risk of hypertension (paper 3)

We analysed data from a cross-sectional study of the general population including 8992 individuals from the Copenhagen City Heart Study (CCHS), a follow-up study of 36480 individuals from the Copenhagen General Population Study (CGPS), and a case-only study of 3815 Scandinavians from the Losartan Intervention for End-Point Reduction in Hypertension Genetic Substudy (LIFEGEN) with LVH and hypertension[11].

In the CCHS, individuals with C282Y/C282Y versus wild type/ wild type had an odds ratio for antihypertensive medication use of 4.8 (95% CI: 1.8–13). In the CGPS, the corresponding hazard ratio was 1.7 (1.0–2.3). Also, hazard ratios for antihypertensive medication use in the CGPS were 1.6 (1.0–2.6) for transferrin saturation ≥80% vs. <50%, and 2.3 (1.3–4.2) for C282Y/C282Y + transferrin saturation ≥80% vs. wild type/ wild type + transferrin saturation <50%. These results were most pronounced in men (Figure 3). We did not find any

Figure 3. Cumulative incidence of use of antihypertensive medication by HFE C282Y genotype **(a)**, iron overload **(b)** or the combination of genotype and iron overload **(c)** in men in the Copenhagen General Population Study (CGPS).

TS= transferrin saturation. *(Paper 3: Ellervik et al. JIM 2010)*



association between C282Y/C282Y or iron overload and LVH or hypertension (measured as blood pressure at a single occasion or continuous blood pressure), or LVH with hypertension in the CCHS or with severity of LVH in LIFEGEN.

Risk of cancer (paper 4)

We conducted a population-based study[12] of 8763 individuals from the CCHS and a meta-analysis. In the CCHS 1417 individuals developed a first cancer during 15 years of follow-up. Elevated transferrin saturation was not associated with any cancer overall or in men. In women, transferrin saturation above 60% versus below 50% was associated with a hazard ratio of 3.6 (95% CI: 2.0–6.5) for any cancer with a corresponding absolute 10-year risk of any cancer of 34% and 30% in smokers and non-smokers, respectively (Figure 4). Overall, hemochromatosis genotype C282Y/C282Y versus wild type/wild type was associated with a hazard ratio of 2.4 (1.1–5.3) for any cancer. In men, hemochromatosis genotype C282Y/C282Y versus wild type/wild type was associated with a hazard ratio of 3.7 (1.2–12) for any cancer with a corresponding absolute 10-year risk of cancer of 39% and 27% in smokers and non-smokers, respectively (Figure 4). In women, hemochromatosis genotype C282Y/C282Y versus wild type/wild type was not associated with any cancer.

Results for exploratory analyses among cancer subgroups had limited statistical power; however, in women, we observed a multifactorially adjusted hazard ratio for female cancer of 3.2 (1.3–7.7) for transferrin saturation ≥60% vs. <50%. Also, risk of liver can-

Figure 4. **Absolute 10-year risk of any cancer by transferrin saturation levels and hemochromatosis genotype C282Y/C282Y.**

Based on 8763 individuals from the Copenhagen City Heart Study followed for 15 years, during which time 1417 cancers developed.

(Paper 4: Ellervik et al. Journal of Internal Medicine 2011)

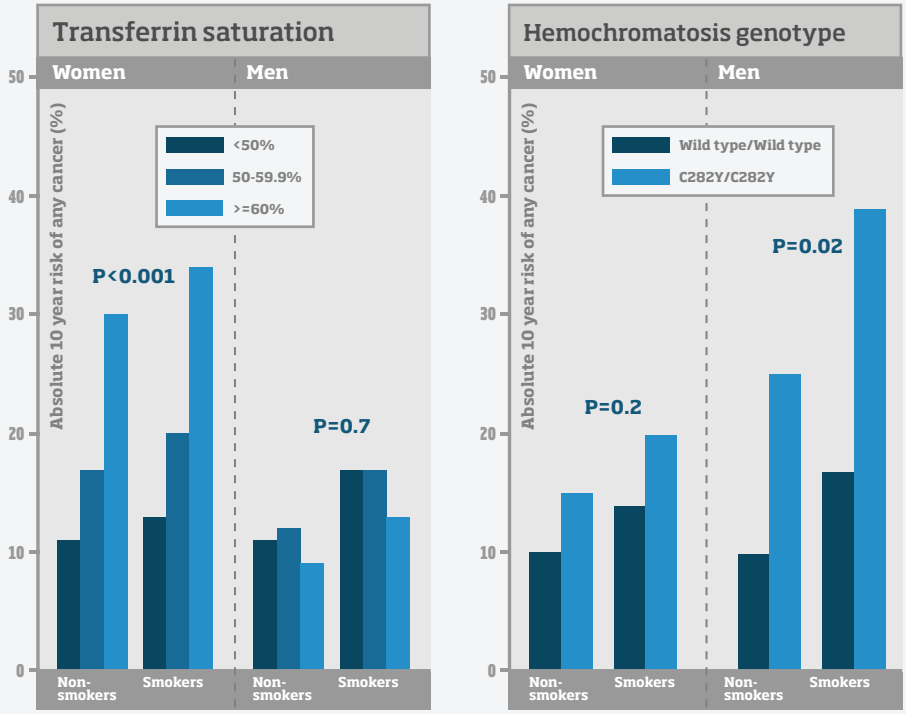


Figure 5. Flow-diagram of articles for meta-analysis of cancer.

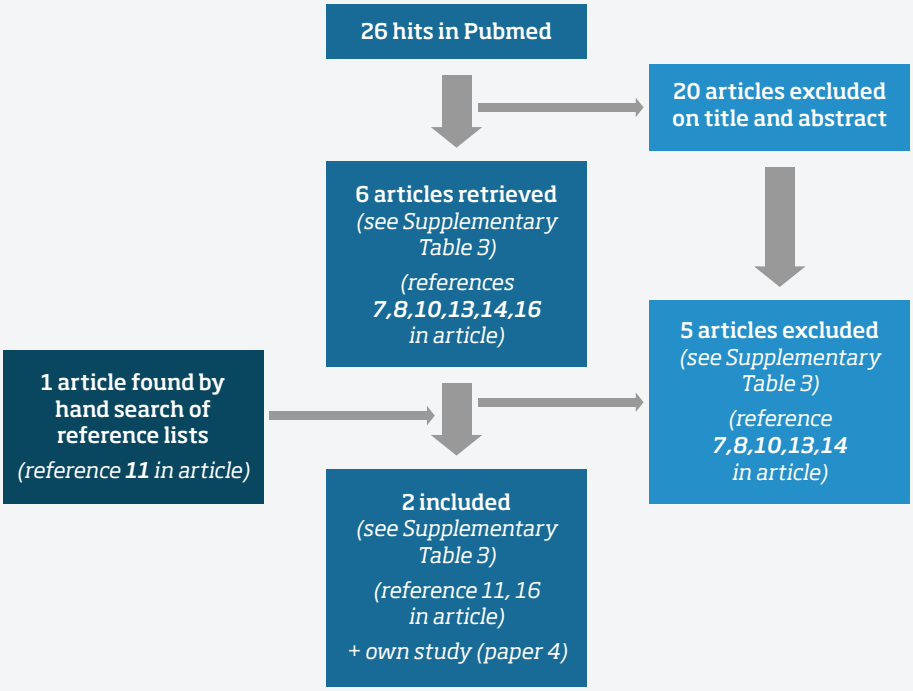
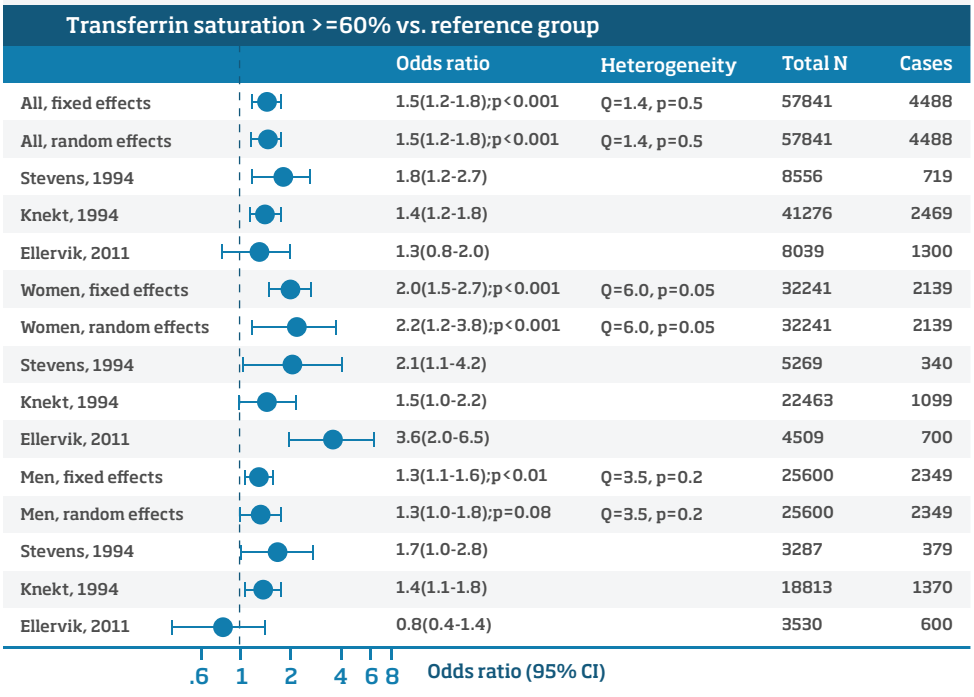


Figure 6. Meta-analysis of risk of any cancer in prospective studies - transferrin saturation ≥60% vs. reference group.

The reference groups varied slightly across studies (transferrin saturation ≤30% to <60%). CI=confidence interval.

(Paper 4: Ellervik et al. Journal of Internal Medicine 2011)



cer was increased in both women and men with transferrin saturation ≥60% vs. <50%. From the meta-analysis (flow diagram shown in Figure 5), the odds ratio of any cancer for transferrin saturation ≥60% versus a reference group was 1.5(1.2-1.8) for women and men combined (Figure 6).

For this survey, a mediation analysis for the risk of cancer according to C282Y/C282Y genotype via transferrin saturation was performed. The hazard ratio for cancer according to genotype C282Y/C282Y adjusted for sex was 2.3(1.0-5.1), and the hazard ratio for cancer according to genotype C282Y/C282Y adjusted for sex and transferrin saturation was 2.1(0.9-1.6); thus, the PERM was 15%. Using the more advanced sgmediation command in Stata revealed an indirect effect coefficient of 0.017 and a total effect coefficient of 0.082; thus, the proportion of the effect that was mediated was 21%, but this effect was insignificant as the confidence interval for the indirect effect included zero: 0.017 (-0.003 to 0.04).

Risk of total mortality (paper 5, 6 and 7)

Paper 5

We examined total mortality (i.e. death of any cause) according to baseline transferrin saturation[13] and hemochromatosis genotype[169] in two Danish population-based follow-up studies (CGPS and CCHS) comprising a total of 45,159 individuals, of whom 4568 died during up to 18 years of follow-up, and in a meta-analysis comprising the present studies and an additional general population study[15].

In the combined studies (CGPS+CCHS), the cumulative survival was reduced in individuals with transferrin saturation ≥50% vs. <50% (log-rank P<0.0001). Multifactorially adjusted hazard ratios for total mortality for transferrin saturation ≥50% vs. <50% were 1.4 (95% CI: 1.2-1.6) overall, 1.3 (1.1-1.6) in men, and 1.5 (1.1-2.0) in women (Table 9). Results were similar if the two studies were considered separately (this stratification was suggested by a reviewer; p for interaction was 0.1). A stepwise increased risk of total mortality

Table 9. **Sex-specific and study-specific total mortality according to transferrin saturation in two Danish population-based follow-up studies combined*.**

Gender	Transferrin saturation	Participants N	Events N	HR 95% CI	P-value
All	<50%	44306	4391	1.0	
	≥50%	853	177	1.4(1.2-1.6)	<0.001
Sex-specific:					
Men	<50%	20146	2237	1.0	
	≥50%	569	126	1.3(1.1-1.6)	0.003
Women	<50%	24160	2154	1.0	
	≥50%	284	51	1.5(1.1-2.0)	0.005
Study-specific:					
CGPS	<50%	35900	1097	1.0	
	≥50%	519	28	1.8(1.2-2.6)	0.003
CCHS	<50%	8406	3294	1.0	
	≥50%	334	149	1.2(1.0-1.4)	0.02

*Based on the Copenhagen General Population Study (CGPS) and the Copenhagen City Heart Study (CCHS).
CGPS: median follow-up of 4 years (interquartile range 3-4). CCHS: median follow-up of 15 years (interquartile range 10-16).
HR: hazard ratio with 95% CI (confidence interval) adjusted for age, sex (not for sex stratified analyses), body mass index, tobacco consumption, smoking habits, cholesterol, antihypertensive medication, alcohol consumption, and physical activity.
(Paper 5: Ellervik et al. Clinical Biochemistry 2011)

Figure 7. Meta-analysis of risk of total mortality by transferrin saturation in prospective studies of the general population.

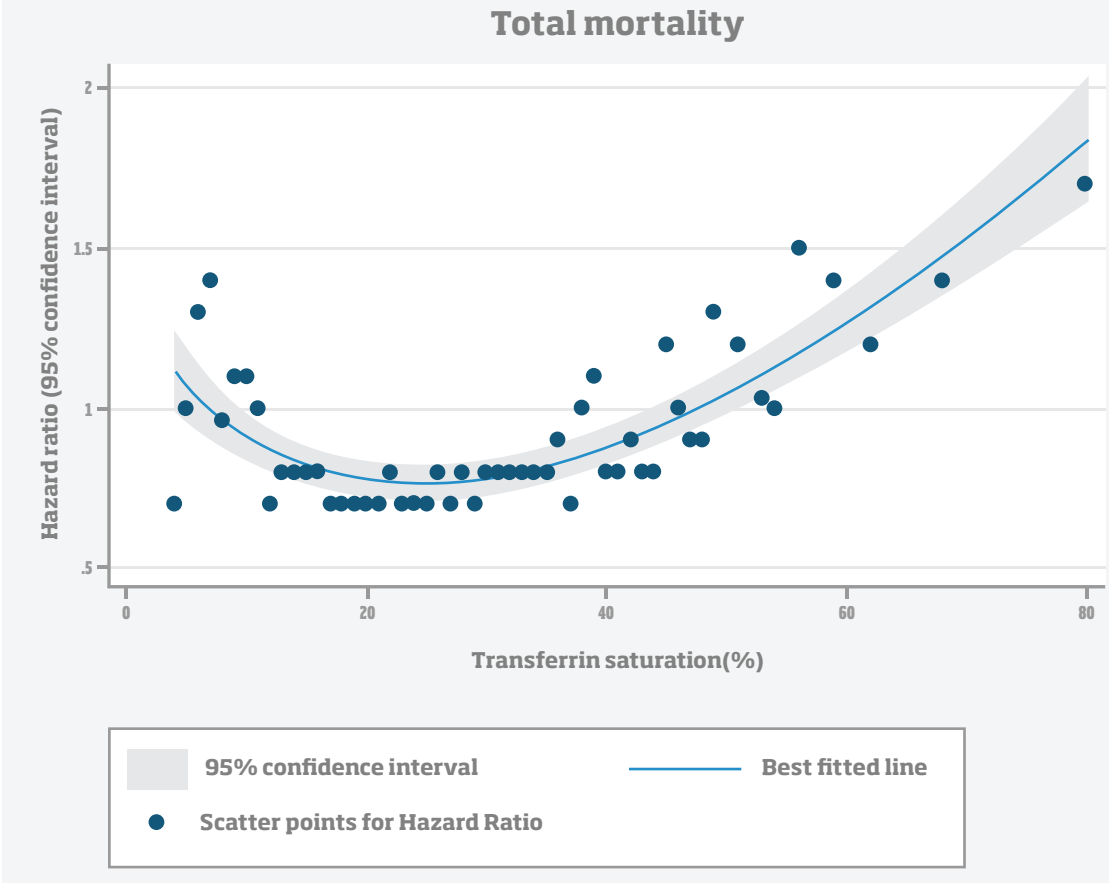
Transferrin saturation approximately ≥50% vs. <50%. Heterogeneity Q=5.1, P=0.08. CI:Confidence Interval

(Paper 5: Ellervik et al. Clinical Chemistry 2011)

Meta-analysis		N Total
Copenhagen General Population Study, Denmark	1.8(1.2-2.6)	36419
Copenhagen City Heart Study, Denmark	1.2(1.0-1.4)	8740
NHANES, USA	1.6(1.2-2.2)	10714
Combined risk, fixed effects model	1.3(1.2-1.5; p<0.001)	55873
Combined risk, random effects model	1.4(1.1-1.9; p=0.005)	55873

Odds ratio (95% CI)

Figure 8. Hazard ratio (95% confidence interval) for total mortality by transferrin saturation(%) in two general population studies combined: The Copenhagen City Heart Study and The Copenhagen General Population Study (new results for paper 5).R^2=0.65.



was observed for stepwise increasing levels of transferrin saturation (log-rank P<0.0001), with the highest risk conferred by transferrin saturation ≥80% vs. transferrin saturation <20% with a hazard ratio of 2.2 (1.4 -3.3). The population-attributable risk for total mortality in the combined studies in individuals with transferrin saturation ≥50% vs. <50% was 0.8%. In a meta-analysis, the odds ratio for total mortality for transferrin saturation ≥50% vs. <50% was 1.3 (1.2-1.5) under the fixed effects model (Figure 7). In response to a letter to this article, we showed that total mortality according to C282Y/C282Y hemochromatosis genotype[169] was 1.3(0.7-2.4) in the combined studies; thus, risk of premature death according to transferrin saturation was not driven by hemochromatosis genotype.

For this thesis, we also explored the continuous relationship between transferrin saturation and hazard ratio for total mortality using a fractional polynomial model. Figure 8 shows the continuous relationship of hazard ratio for total mortality according to transferrin saturation. Figure 8 shows the best fitted polynomial model (deviance: -57) compared to a cubic spline regression (deviance: -36, p<0.001), and* a linear model (deviance=-15, p<0.001); thus the polynomial model (R^2=0.65) is a better fit, than both the cubic spline and the linear model. Figure 8 is J-shaped, showing that for very low levels of transferrin saturation (indicative of anemia) risk of total mortality is larger than 1.0., in the range of 10-50% risk is below 1.0, and above 50% risk is larger than 1.0. Thus, the polynomial model gives a better prediction than just simple cut-offs. However, like using cut-off values this model illustrate that risk of total mortality increases with increasing transferrin saturation, like that shown in paper 5[13].

Paper 6

In paper 1, we found that C282Y/C282Y genotype was more prevalent among patients with diabetes than in the general population, and that measurement of transferrin saturation had a 100% negative predictive value, meaning that transferrin saturation below 50% would rule out a patient being C282Y/C282Y in patients with late-onset type 1 diabetes. We concluded that measurement of transferrin saturation followed by genetic testing, could improve life expectancy. However, at that time, we did not have the evidence to justify that conclusion, it was only speculative. But the results from paper 1 changed clinical practice at the Steno Diabetes Center in that screening for iron overload was introduced in all prevalent and newly-referred patients to secure early diagnosis of hemochromatosis at the Steno Diabetes Center. Thus, from 2001-2007 all patients were screened for iron overload. In paper 6, we therefore investigated total and cause-specific mortality according to increased transferrin saturation (≥50% vs.<50%), whether mortality is driven by *HFE* genotype, and whether early measurement of transferrin saturation helps to predict mortality outcome in these cohorts. Cohort 1 consisted of the patients with late-onset type1 diabetes (N=716) recruited at the Steno Diabetes Center in 1999 with a cross-sectional measurement of transferrin saturation and *HFE* genotype performed in 1999[8]. Cohort 2 consisted of consecutively recruited patients with any diabetes (N=6120) recruited at the Steno Diabetes Center from 2001-2007, and with a transferrin saturation measurement at referral and HFE genotype if transferrin saturation was above 50%.

In patients with late-onset type 1 diabetes (Cohort 1), age- and gender adjusted hazard ratio for total mortality was 2.3(95% CI: 1.3-3.9; p=0.002) overall, and 2.4(1.3-4.2; p=0.003) in men but the result for women was not significant (Figure 9). Mortality due to neoplasms was increased in patients with late-onset type 1 diabetes and transferrin saturation≥50% vs. <50% with an age- and gender-adjusted hazard ratio of 5.8(2.4-14; p=0.00007); two of seven cases of neoplasms among patients with transferrin saturation≥50% were liver cancers, whereas no liver cancers were observed among patients with transferrin saturation<50% (Fishers exact p=0.003). Mortality due to endocrinological or cardiovascular causes was not different; excluding C282Y/C282Y and C282Y/H63D gave similar results. Hazard ratio for total mortality was 4.0(1.2-13;p=0.01) and cause-specific mortality by neo-

Figure 9.

Total mortality(A) and cause-specific mortality(B) in Cohort 1 and Cohort 2. Crude hazard ratio is age- and gender-adjusted. Adjusted is multifactorially adjusted for age, gender, body mass index, diabetes onset and complications. (from paper 6)

A. Total Mortality

Cohort 1	Crude	Adjusted	Cases Total/exp/not exp	Controls Total/exp/not exp	Power 80%
All	2.3(1.3-3.9);p=0.002	2.2(1.3-3.8);p=0.003	178 / 16 / 162	538 / 29 / 509	2.1
Men	2.4(1.3-4.2);p=0.003	2.3(1.3-4.1);p=0.005	103 / 14 / 89	298 / 20 / 278	2.3
Women	2.0(0.5-8.5);p=0.4	1.9(0.4-8.4);p=0.4	75 / 2 / 73	240 / 9 / 231	3.7
Cohort 2					
All	1.1(0.8-1.5);p=0.4	1.1(0.8-1.5);p=0.6	1293 / 44 / 1249	4827 / 229 / 4598	1.3
Men	1.1(0.7-1.6);p=0.7	1.0(0.7-1.5);p=0.9	793 / 30 / 763	2657 / 158 / 2499	1.4
Women	1.3(0.7-2.2);p=0.4	1.3(0.7-2.1);p=0.4	500 / 14 / 486	2170 / 71 / 2099	1.7
Early onset	1.3(0.7-2.5);p=0.4	1.2(0.6-2.2);p=0.7	104 / 11 / 93	1565 / 118 / 1447	1.9
Late-onset	1.1(0.8-1.6);p=0.5	1.1(0.7-1.5);p=0.7	1189 / 33 / 1156	3262 / 111 / 3151	1.4
Type 1 diabetes	1.1(0.7-1.7);p=0.7	1.0(0.6-1.6);p=0.9	342 / 20 / 322	2314 / 173 / 2141	1.6
Type 2 diabetes	1.2(0.8-1.9);p=0.3	1.2(0.8-1.9);p=0.4	836 / 20 / 816	2340 / 48 / 2292	1.7
Other diabetes	1.1(0.4-3.0);p=0.9	3.4(0.7-17);p=0.1	115 / 4 / 11	173 / 8 / 165	2.9
Late-onset type 1 diabetes	0.9(0.4-1.7);p=0.7	0.8(0.4-1.6);p=0.5	250 / 9 / 241	865 / 57 / 808	1.8

Crude hazard ratio (95% CI)

exp=*exposed*
not exp= *not exposed*

B. Cause-specific mortality

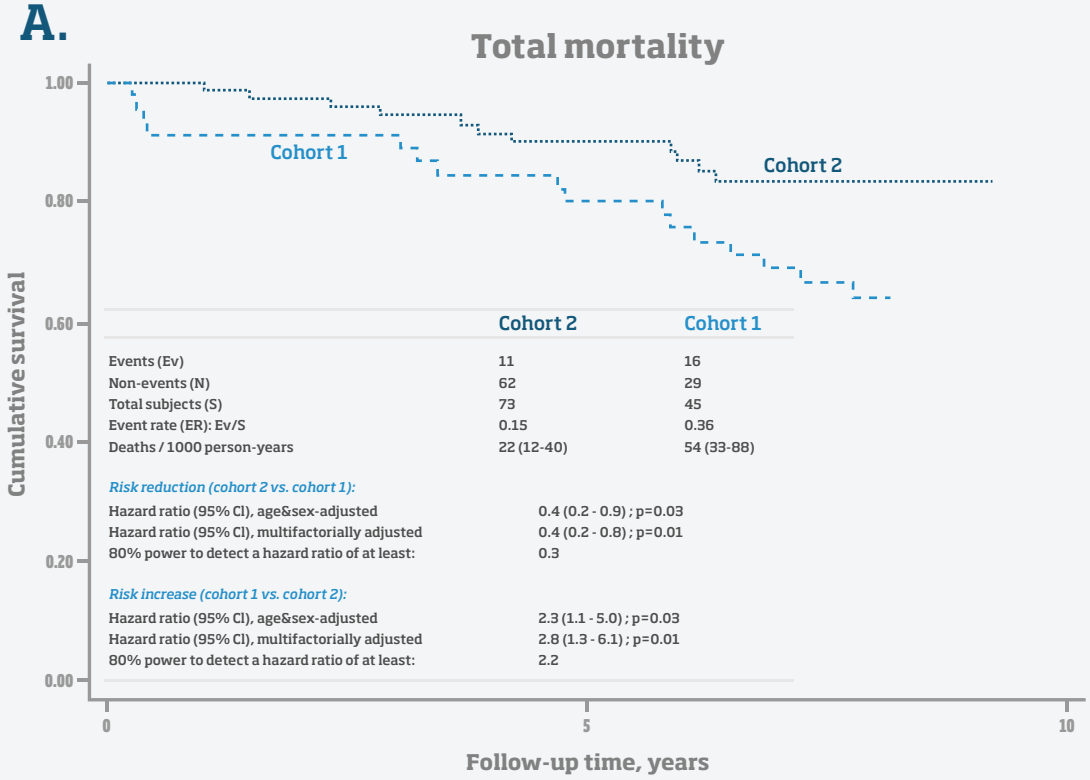
Cohort 1	Crude	Adjusted	Cases Total/exp/not exp	Controls Total/exp/not exp	Power 80%
Neoplasms	5.8(2.4-14);p=0.00007	5.9(2.5-14);p=0.00005	46 / 7 / 39	538 / 29 / 509	3.6
Liver cancer	Fishers exact;p=0.003		2 / 2 / 0	538 / 29 / 509	10.0
Endocrinological	2.6(0.8-8.8);p=0.1	2.7(0.8-9.1);p=0.1	52 / 3 / 49	538 / 29 / 509	3.5
Cardiovascular	2.6(0.8-8.8);p=0.1	2.2(0.6-7.6);p=0.2	41 / 3 / 38	538 / 29 / 509	3.6
Cohort 2					
Neoplasms	1.7(0.8-3.4);p=0.2	1.6(0.8-3.3);p=0.2	150 / 8 / 142	4827 / 229 / 4598	2.3
Liver cancer	3.5(0.8-15);p=0.1	3.2(0.8-14);p=0.1	18 / 2 / 16	4827 / 229 / 4598	3.5
Endocrinological	0.7(0.3-1.7);p=0.4	0.6(0.3-1.6);p=0.3	191 / 5 / 186	4827 / 229 / 4598	2.1
Cardiovascular	0.6(0.2-1.8);p=0.3	0.6(0.2-1.8);p=0.3	178 / 3 / 175	4827 / 229 / 4598	2.1
Late-onset type 1 diabetes					
Neoplasms			19 / 0 / 19	865 / 57 / 808	5.2
Liver cancer			0 / 0 / 0	865 / 57 / 808	7.0
Endocrinological	0.6(0.1-2.4);p=0.3	0.3(0.1-2.2);p=0.2	59 / 1 / 58	865 / 57 / 808	3.0
Cardiovascular			23 / 0 / 23	865 / 57 / 808	4.3

Crude hazard ratio (95% CI)

exp=*exposed*
not exp= *not exposed*

Figure 10. (A+B) Total mortality in late-onset type 1 diabetes and elevated transferrin saturation (≥50%) in Cohort 2 vs. Cohort 1. *(from paper 6)*

A.



	Cohort 2	Cohort 1
Events (Ev)	11	16
Non-events (N)	62	29
Total subjects (S)	73	45
Event rate (ER): Ev/S	0.15	0.36
Deaths / 1000 person-years	22 (12-40)	54 (33-88)

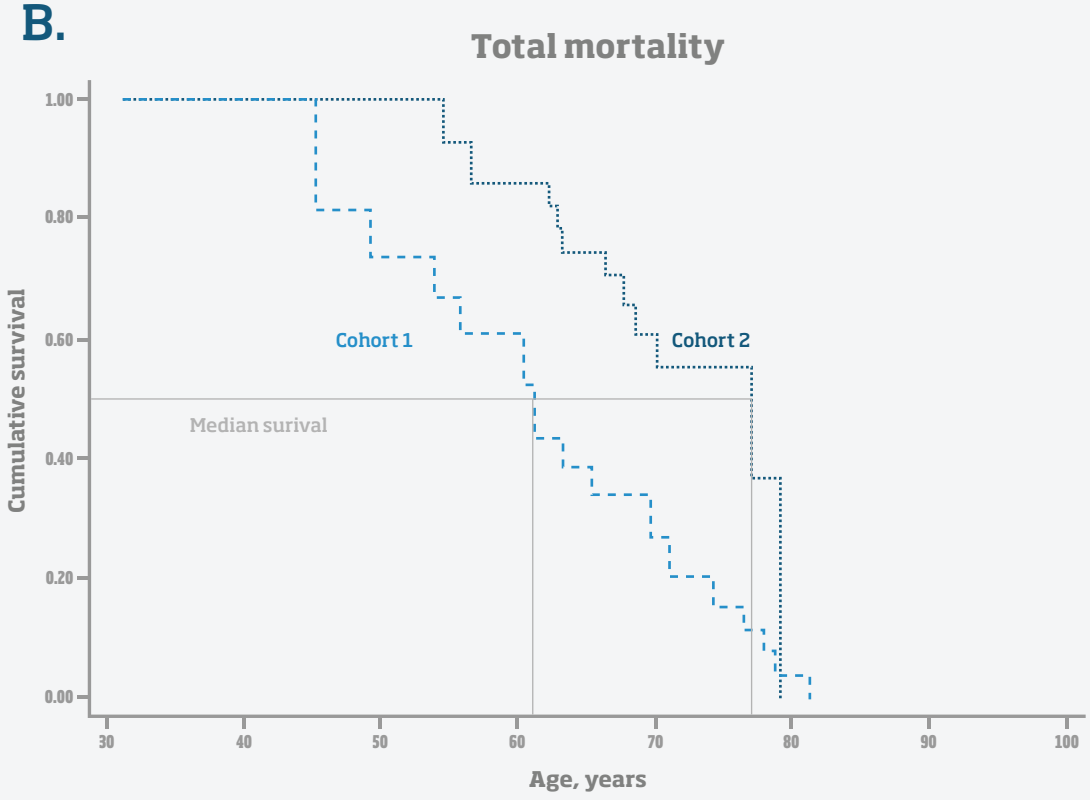
Risk reduction (cohort 2 vs. cohort 1):

Hazard ratio (95% CI), age&sex-adjusted	0.4 (0.2 - 0.9) ; p=0.03
Hazard ratio (95% CI), multifactorially adjusted	0.4 (0.2 - 0.8) ; p=0.01
80% power to detect a hazard ratio of at least:	0.3

Risk increase (cohort 1 vs. cohort 2):

Hazard ratio (95% CI), age&sex-adjusted	2.3 (1.1 - 5.0) ; p=0.03
Hazard ratio (95% CI), multifactorially adjusted	2.8 (1.3 - 6.1) ; p=0.01
80% power to detect a hazard ratio of at least:	2.2

B.



plasms 13(3.6-49;p=0.0001) in patients with C282Y/C282Y vs. wild type. Thus, risk was both conferred by C282Y/C282Y genotype but also from other sources of iron overload that we were not able to detect, as we did not have any supplemental information on iron supplements, food intake, or co-existing liver disease.

In Cohort 2, total mortality and cause-specific mortality was not different in patients with transferrin saturation ≥50% vs. <50% (Figure 9). It was not possible to assess risk according to genotype, as only those patients with transferrin saturation ≥50% were genotyped.

In order to examine whether early measurement of transferrin saturation influences the ability to predict mortality outcome, Cohort 2 (early measurement of transferrin saturation) was compared with Cohort 1 (random measurement of transferrin saturation). In patients with late-onset type 1 diabetes and transferrin saturation ≥50%, hazard ratio for total mortality was 0.4 (0.2-0.9;p=0.03) in Cohort 2 vs. Cohort 1(Figure 10A); , corresponding to a median survival age of 77 years (Cohort 2) and 61 years (Cohort 1) (Figure 10B); thus, early measurement of transferrin saturation in these patients leading to early intervention may improve life expectancy.

Paper 7

In paper 6, we showed that early measurement of transferrin saturation improved life expectancy in patients with late-onset type 1 diabetes in a specialised diabetes clinic. Whether mortality was also increased in individuals with diabetes and increased transferrin saturation ascertained from the general population was unknown. From two general population studies (CCHS and CGPS) we included 3346 patients with diabetes (type 2 diabetes N=3328,

Figure 11. **Total and cause-specific mortality by transferrin saturation ≥50% vs. <50%. in patients with diabetes in the general population**

Power is 80% to detect a given hazard ratio.
***Individuals with C282Y/C282Y and C282Y/H63D excluded from analyses. (from paper 7)**

TS: transferrin saturation, unexp: unexposed, exp: exposed, CCHS: Copenhagen City Heart Study, CGPS: Copenhagen General Population Study.

Mortality *by TS>=50% vs. <50%*

Total mortality	Person-time *(yr)* Unexp/exp	Failures *(N)* Unexp/exp	Incidence rate (95% CI) Events/1000 person-years Unexp/exp	Adjusted hazard ratio *(95% CI)*	Power 80%
All	14521/452	511/30	35(32-38)/66(46-95)	1.7(1.2-2.5);p=0.004	2.2
Men	8011/370	328/26	41(37-46)/71(48-103)	1.5(1.0-2.3);p=0.05	2.4
Women	6510/82	183/4	28(24-32)/49(18-130)	3.8(1.4-11);p=0.01	4.3
CCHS	3681/266	264/21	72(64-81)/79(51-121)	1.1(0.7-1.7);p=0.7	5.2
CGPS	10840/187	247/9	23(20-26)/48(25-92)	2.5(1.2-4.9);p=0.01	3.6
Type 2, only	14077/453	504/30	36(33-39)/66(46-95)	1.7(1.2-2.5);p=0.004	2.2
Genotypes excluded*	11428/326	431/25	38(34-41)/77(52-113)	1.9(1.2-2.8);p=0.003	2.4
Cause-specific mortality					
Neoplasms	12200/302	120/8	9.8(8.2-12)/26(13-53)	2.8(1.3-6.0);p=0.007	3.2
Cardiovascular death	12433/304	135/6	11(9.2-13)/20(9-44)	1.3(0.6-3.1);p=0.5	3.1
Endocrinological death	12504/306	149/14	12(10-14)/38(23-65)	4.2(1.7-11);p=0.002	3.4

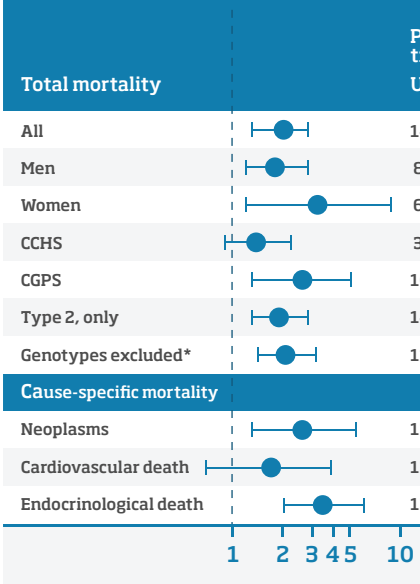
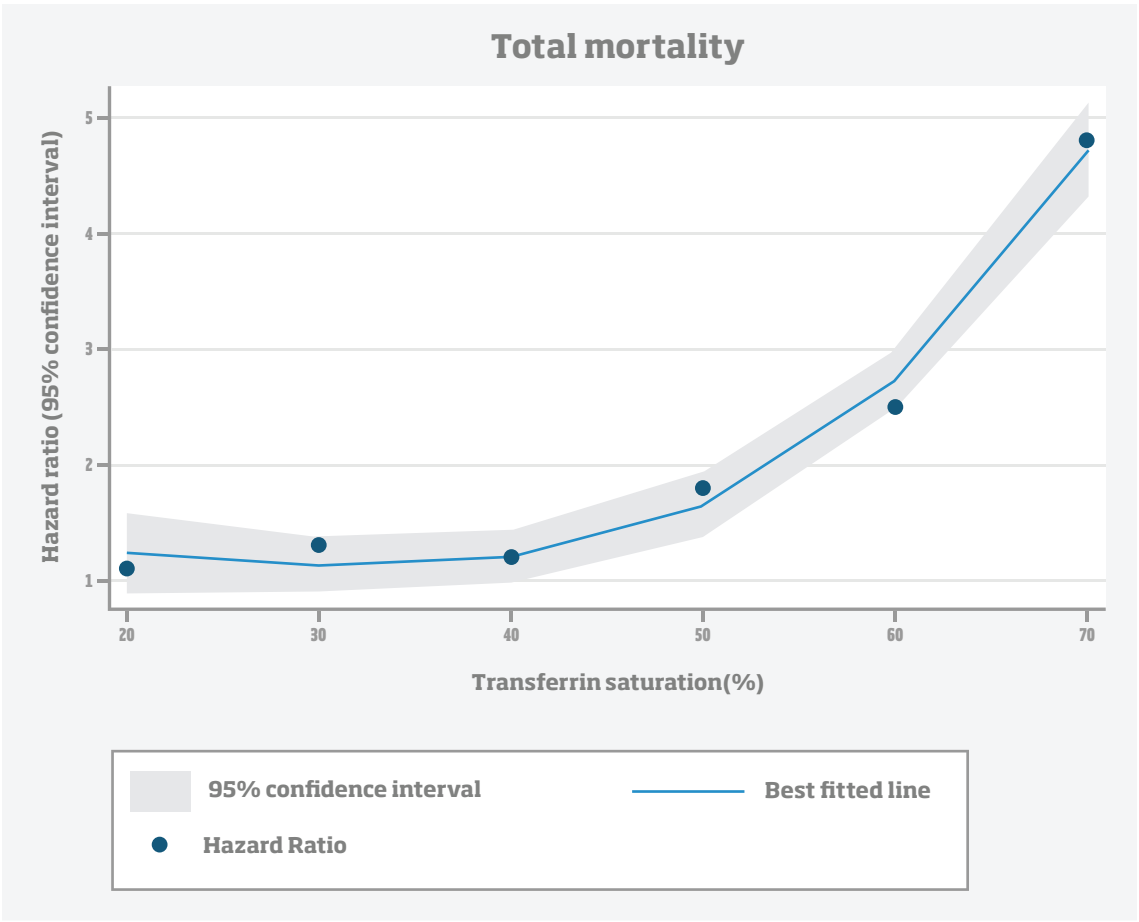


Figure 12. Hazard ratio (95% confidence interval) for total mortality by transferrin saturation(%) in patients with diabetes (new results for paper 7). R^2=0.98.



type 1 diabetes N=118) and a baseline measurement of transferrin saturation. The cumulative survival was reduced in individuals with diabetes with transferrin saturation ≥50% vs.<50% (log-rank;P<0.0001), with median survival age of 66 and 79 years, respectively. The unadjusted hazard ratio for transferrin saturation ≥50% vs.<50% was 2.0(95%CI:1.3-2.8;P=0.0004) for total mortality overall (and similar for men and women separately); 2.6(1.3-5.4;P=0.008) for neoplasms and 3.4(2.0-6.0;P=0.00002) for endocrinological death (Figure 11); death of cardiovascular disease was not significantly different. A stepwise increased risk of total mortality was observed for stepwise increasing levels of transferrin saturation (log-rank:P=0.0001), with a hazard ratio for transferrin saturation ≥70% vs. transferrin saturation <20% of 4.8(2.0-12;P =0.0006). The unadjusted hazard ratio for total mortality in individuals with diabetes for C282Y/C282Y vs. wildtype/wildtype was 3.3(1.04-10;P=0.04), and for (C282Y/C282Y& transferrin saturation ≥50%) vs. (wildtype/wildtype& transferrin saturation <50%) 6.0(1.5-24;P=0.01). Adjusted analyses and analyses excluding C282Y/C282Y and C282Y/H63D showed similar results. Six percent of these premature deaths can possibly be avoided by early screening for transferrin saturation or *HFE* genotype according to calculation of population atributable risk.

For this thesis, we also explored the continuous relationship between transferrin saturation and hazard ratio for total mortality in patients with diabetes ascertained in the general population using a fractional polynomial model. Due to the low number of patients, it was

however not possible to perform a very detailed continuous relationship (in comparison to the continuous relationship for paper 5); thus, the continuous relationship is made from the same cut-points as the semigraded relationship in the article, the difference is just that a line is drawn from point to point. Figure 12 shows the best fitted polynomial model (deviance: -5.7) compared to a cubic spline regression (deviance: 4.4, p=0.075), and a linear model (deviance=12, p=0.005); thus the polynomial model (R^2=0.98) is a better fit, than both the cubic spline and the linear model, even though the spline model was not statistically different from the fractional polynomial model. Figure 12 is slightly J-shaped with an almost flat line from 20-30% at 1.0 and thereafter a steep rise from transferrin saturation 40-70% with a hazard ratio rising from 1.2 to almost 5 with the fitted line and confidence intervals above 1.0. Thus, the polynomial model gives a better visual prediction than just the cut-offs. However, like using cut-off values this model illustrates that risk of total mortality in individuals with diabetes from the general population increases with increasing transferrin saturation, like that in paper 7[14].

Results on hemochromatosis genotypes C282Y/H63D and C282Y/wild type in paper 1, 3, 4, 6 and 7

Risk of diabetes, hypertension, cancer, or premature death was not increased in individuals or patients with diabetes with C282Y/H63D or C282Y/wild type compared to wild type/ wild type.

Key findings

Diabetes mellitus (paper 1 and 2):

The results of this work lend support to the hypothesis that hereditary hemochromatosis is a disease generally overlooked in patients with late-onset type 1 diabetes mellitus; furthermore, the results suggest an association between C282Y homozygosity and type 1 diabetes[8]. Secondly, transferrin saturation ≥50% is associated with a 2-3 fold increased risk of developing any form of diabetes mellitus, as well as type 1 diabetes and type 2 diabetes, separately, independently of hemochromatosis genotype[10]; thus, increased transferrin saturation ≥50% is an independent risk marker of diabetes mellitus. This was the first and largest population based study that consistently demonstrated transferrin saturation as a risk marker of any form of diabetes, and of type 1 and type 2 diabetes separately, in three independent cohorts.

Hypertension (paper 3):

Hemochromatosis genotype C282Y/C282Y and extremely elevated transferrin saturation either separately or combined are associated with increased risk of use of antihypertensive medication[11]. These are novel findings.

Cancer (paper 4):

Elevated transferrin saturation levels are associated with increased risk of cancer in women in the CCHS and in men and women combined in the meta-analysis. A novel finding is that hemochromatosis genotype C282Y/C282Y overall and in men separately is associated with increased risk of cancer[12]. This is the first population-based follow-up study of transferrin saturation levels and hemochromatosis genotype including estimation of both relative and absolute risks of any cancer. This is also the first meta-analysis estimating association of elevated transferrin saturation with risk of cancer.

Total mortality (paper 5, 6, and 7):

Paper 5: Individuals in the general population with a transferrin saturation ≥50% vs. <50% have an increased risk of premature death[13], shown independently in two Danish studies and a meta-analysis combining the present studies and a single previous American study. Our study is the largest and most comprehensive study to date estimating risk of total mortality by increased transferrin saturation. Risk was not driven by hemochromatosis genotype[169].

Paper 6: Increased transferrin saturation and hemochromatosis genotype C282Y/C282Y predict total mortality in patients with late-onset type 1 diabetes in patients ascertained in a specialized diabetes clinic, and post-diagnosis increased transferrin saturation is an independent risk factor[9]. Early measurement of transferrin saturation in these patients leading to early intervention may improve life expectancy. These are novel findings.

Paper 7: Individuals with diabetes, ascertained in the general population, with increased transferrin saturation or hemochromatosis genotype have a 2-6-fold increased risk of premature death[13]. Results for transferrin saturation were novel.

Discussion - Methodological considerations

Errors in sampling

1) Selection bias: Non-participants vs. participants

Selection bias is a systemic error in the sampling of individuals. Participants' decision on participation in a general population study is an example of selection bias. The data in this thesis from the CCHS examination were from the third examination, as DNA was not extracted

Table 10. Factors affecting participation in population-based studies .

1. Design options
1. Short distance to survey location increases participation
2. Location of health study (home visits have higher degree of participation than field centers)
3. Good transportation possibilities
4. Length of questionnaire (the longer, the higher risk of non-participation)
5. Reminders increases participation
6. Time of the day (may depend on the job situation)
7. Season of the year (snow may delay transportation and thus decrease partipation)
8. Kind of survey (case-studies tend to have higher participation rates than general health examinations)
9. Return of results increases participation
2. Study focus
3. Participant background
1. Social class (low social class, low income, low educational level increase risk of non-participation)
2. Living in a relationship with a partner increases participation
3. Sex (women tend to attend more than men)
4. Age (very young and old age groups usually have lower participation rates)
5. Morbidity status (the more diseased the individual is, the lower the participation rate)

until this examination; thus, those who attended the third examination were survivors from the second, participants from the second, and some newly recruited participants; thus, the participants in the third examination may be selected introducing selection bias in the results with probably less cases than would have been expected from a new study. Furthermore, those eligible to invite at the third examination were the survivors which potentially could introduce survivor selection bias; thus, all the genetic analyses and analyses of transferrin saturation in this cohort is based on those who survived and were willing to attend. However, an argument against major selection survivor bias is the fact that the genotypes in the general population studies were in Hardy-Weinberg Equilibrium. The original cohort was supplemented with younger participants, in order to minimize survivor selection bias. If survivor selection bias existed, it would tend to draw the results against the null hypothesis, but it cannot likely explain the significant results.

Non-participants in third CCHS had higher total mortality and increased risk of diabetes and ischemic heart disease compared to participants. Risk of hypertension was decreased in non-participants; this most likely reflects that non-participants are less likely to seek a medical doctor to get a diagnosis of hypertension, or it is a chance finding. Risk of cancer was not different in non-participants compared to participants. Two of 23 participants with C282Y/C282Y in the CCHS third examination had diabetes mellitus; the reason for this prevalence could be that there were more individuals with hemochromatosis and diabetes among non-participants than among participants; however, as later studies have shown, a cross-sectional estimation of diabetes mellitus[23, 188] in individuals with hemochromatosis underestimates the risk compared to follow-up studies (paper 2)[10].

Participation rates have been declining over the years in the CCHS with 61% response rate at the 1991-1994 examination compared to those who participated in examination 1976-1978 or 1981-1983. In the CGPS (2003-2007), the response rate was estimated to be 45%. In accordance with these results, participation rates in general population studies in Europe vary from 10%[189] to 72%[190], and have been declining during the past 30 years[191]. In population based health studies many factors influence participation rate[192-195], some of which are listed in Table 10. A low participation rate may limit the external validity of the results, but it will not affect the estimated association between the exposure of interest and disease, unless non-response is associated with both endpoint and exposure. It is not possible to estimate whether non-participants would have had a different transferrin saturation than those who participated, since there were no information on non-participants on transferrin saturation. However, non-participation was associated with higher risk of total mortality and diabetes; thus, the risk estimates in the papers in this thesis may be conservative and may underestimate or hide a true risk in those analyses that did not show increased risk. Overall, the participation rate is generally high among people aged 40-70 years, which is the population of interest in most general population studies[193].

In some of the papers (paper 2, 5, and 7) we pooled the data in CCHS and CGPS, and the two cohorts were analyzed as one cohort to obtain maximal statistical power as the cohorts were ascertained alike with similar ethnicity, geographical location, questionnaires, health examinations, and biochemical data; also, there were no duplicate individuals in the two cohorts. However, such pooling could magnify associations as well as errors.

The participation rate in LIFEGEN is not clear from the protocol[172]. In Diabetes Cohort 1, participation rate was 90%, and thus higher than in the general population studies, maybe because the motivation to contribute to research is higher when you actually have a disease than before you get diseased; the deviation of C282Y from HWE could be explained by sampling bias, mistyping of genotypes, or a spurious association due to population stratification; however, the most likely explanation for Hardy-Weinberg disequilibrium in a diseased cohort is that the marker locus is in association with the disease, provided that the control group is in HWE, which was the case for CCHS in paper 1[196]. Diabetes Cohort 2 was all patients attending the Steno Diabetes Center from 2001-2007 and who all had a routine measurement of transferrin saturation.

2) Recall bias

It is well known that information on smoking and alcohol consumption is often under-reported, a general problem in epidemiological studies. A confounder is a factor that affects both the exposure and the outcome, and must not be part of the pathway. Because of Mendelian randomization, confounding by conventional risk factors is rarely a problem in studies of genetic factors[197]. However, in analyses of transferrin saturation reflecting iron status as exposure for outcome, alcohol consumption, liver disease, iron supplementation, and menopausal status may be confounders. Information on iron supplementation was not available in any of the studies, and information on alcohol intake was so poorly reported at the Steno Diabetes Center so it could not be used in the analyses.

3) Misclassification

Misclassification of exposure (i.e. transferrin saturation or genotype) or outcome may be non-differential or differential. Non-differential misclassification is when all categories of a variable have the same error rate or probability of being misclassified for all study subjects resulting in an underestimate of the hypothesized relationship between exposure and outcome. Differential misclassification occurs when the error rate or probability of being misclassified differs across groups of study subjects. For example, if disease status impedes exposure status. The effect(s) of such misclassification usually results in an overestimation or an underestimation of the true value.

When examining for genetic traits, it is important to test for Hardy-Weinberg equilibrium (HWE). All genotypes in the general population studies and LIFE were in HWE, whereas C282Y was in Hardy-Weinberg disequilibrium in Diabetes Cohort 1 (as described above). If the observed genotype frequencies differ from those predicted, a laboratory misclassification error is often the cause, and more rarely it is caused by selection, migration, mutation, or genetic drift[198]. Misclassification of genotype could have occurred from at least two pre-analytical sources: 1) Contamination of the sample by foreign DNA. 2) Mistaken identity of samples. The only way to exclude these kinds of errors would be to analyse a new blood sample. Due to the large size of this study, such re-analysis would however be very costly. Analytical errors, e.g. incomplete restriction enzyme digestion, are unlikely to have lead to incorrect results, since we included a DNA marker, an uncut control, a positive control, and water control for each gel electrophoresis; in the case of incomplete digestion, the sample was re-analysed. Furthermore, 2 independent genotyping analyses were performed. For TaqMan analyses, each run included a known noncarrier, a heterozygous, and a homozygous control verified by sequencing; after 2 reruns, call rates for genotypes where above 99.9% for all assays. Thus, these misclassifications would be non-differential. In order to avoid post-analytical errors and differential misclassification, genotype diagnosis and data base entry were performed blind to outcome variables and scrutinized by two different laboratory technicians.

Transferrin saturation was a single non-fasting measurement; thus, it cannot be excluded that some individuals with high transferrin saturation could have a bone marrow disorder with reduced or high iron turnover such as hypoplastic anemia or hemolytic disease, hepatocellular injury, or a transient non-specific rise in transferrin saturation rather than increased iron stores (this would be differential misclassification). However, a significant contribution of such conditions is unlikely because of the rarity of these diseases. A study of the circadian variation of transferrin saturation levels in iron-overloaded patients showed that determination of transferrin saturation may be performed at any time during the day[199]; thus, if misclassification occurs, it would be non-differential. Misclassification of outcomes is discussed in "Ascertainment and misclassification of diagnoses" below.

4) Problems with population stratification

"..Population stratification is the distortion of the relationship between a genotype of interest and disease due to the effect of a true risk factor that is related to the genotype"[200]. Allele frequencies may vary within and between populations as each population have its unique social ancestral patterns of geographical migration, ethnicity, mating practices, reproductive history and accidental variations[200, 201]; if the allele frequencies vary between cases and controls due to differences in these factors, or if disease prevalence varies between cases and controls, population stratification is existent. Population stratification may be a reason for non-replication in genetic association studies[201]. However, all the participants in the thesis were white and of Danish descent (CCHS, CGPS, Diabetes Cohort 1 and 2) or white from mixed Scandinavian countries (LIFEGEN) and consequently we did not control for ethnicity in the analyses. Apart from LIFEGEN study (paper 3) the rest of the papers relies on populations ascertained from the same geographical area in Denmark, and thus social ancestral patterns of geographical migration, ethnicity, mating practices, reproductive history and other accidental variations[200, 201] and consequently genetic variation must be assumed to be similar in these cohorts and within subgroups of the cohorts as the source of the populations are similar. However, there are more advanced strategies to control for population stratification[200, 201]: controlling for stratification with families (using relatives as controls), and controlling for stratification with genetic markers (using genetic markers as controls); we did not apply any of these controlling strategies in the analyses. Thus, we do not know if these strategies would have either unmasked true associations or the reverse. However, we believe that since the source of the populations is the same, a potential problem with population stratification is small. Also, since the genotype distributions across the populations were in HWE, and genotype distribution did not change with age, we did not have reasons to believe that population stratification would be a problem.

Health examinations

1) Definitions of hypertension and LVH in paper 3

We decided for the general population studies CCHS and CGPS to take prescription of antihypertensive medication as the best indicator of hypertension in the risk association studies, as we did not have data on 24h-blood pressure measurements; furthermore, blood pressure measured in the ambulatory under non-ideal conditions carries a risk of reflecting white-coat hypertension or the fact that the individual have not rested long enough before the measurement. Continuous blood pressure measurements were used in CCHS, CGPS, and in LIFEGEN. In CCHS and CGPS, there may be a risk that increased blood pressure reflects white-coat hypertension due to the circumstances above. LIFEGEN participants were phenotypically well-characterised cases with hypertension defined as a diastolic blood pressure reading of 95-115 mmHg or a systolic blood pressure reading of 160-200 mmHg; thus for the continuous measurements, the study groups were not ascertained alike introducing heterogeneity in the study. Left ventricular hypertrophy was diagnosed by a standard 12-lead ECG in both CCHS and LIFEGEN but by the Minnesota code and Sokolow-Lyon index, respectively, which make the two studies not completely comparable. Though, the best estimate of LVH would have been an echocardiography, which was not performed in either study.

Other factors influencing study validity

Missing values in studies may impact study validity. Missing values may be due to partial response in questionnaires[193, 202], missing laboratory data[203], or missing information on follow-up status[204]. Methods for handling missing data are required to correct for inconsistencies that may impact validity of results[202]. Missing values may be imputed in several ways[203, 205]. In this thesis, the missing values were imputed using single imputation depending on age and sex in paper 6, and in CCHS and CGPS (paper 4, 5, 7) imputations were based on replacing the missing value with the median in the dataset for continous values and the most

frequent categorical value for categorical data. The imputation method in paper 6 is more accurate than that in the CCHS and the CGPS, but in the general population studies missing values were low compared to data from the Steno Diabetes Center, which rely on clinical data and not systematically collected research data. Follow-up of participants was 100% complete for CCHS, CGPS and LIFEGEN, but 9/6129 (0.1%) of patients in Diabetes Cohort 2 were lost to follow-up in the Danish registries and at Steno Diabetes center; however, this is not different from well-conducted trials[206].

Patient audit: Classification of diabetes

The patient audit of late-onset type 1 diabetes showed that patients with C282Y/C282Y had C-peptide levels ranging from 10-605 pmol/L, thus resembling patients with almost absent beta-cell function and patients with residual beta-cell function. The data are in accordance with the evidence that diabetes secondary to hemochromatosis is characterised by both insulin resistance[56] and beta-cell destruction[49], and may therefore mimic both type 2 diabetes[49,56] and non-autoimmune type 1 diabetes[49]. Furthermore, a study has shown that loss of secretory capacity in patients with late-onset type 1 diabetes is less pronounced[207], thus supporting the C-peptide values among these patients. However, in patients that were wild type/wild type, 6 individuals had low C-peptide values, 2 patients had beta-cell residual function, and 4 patients lacked a measurement. All C282Y/C282Y and wild type/wild type patients received insulin and none received other anti-diabetic medication. At the time of the study patients were classified according to WHO-1980 classification. Only four patients had a measurement of auto-antibodies (two positive (one C282Y/C282Y, and one wild type/wild type) which may be explained by the period in which the study was conducted in 1999 and at that time auto-antibody measurement was not a routine measurement[154] at Steno Diabetes Center; thus, it cannot be ruled out from the scarcity of data on auto-antibodies that patients in a WHO-1999 classification would have been auto-immune (either type 1 from the beginning or latent autoimmune diabetes); however, there is no evidence to date to either support or contrast that speculation in the literature for patients with hemochromatosis, but a genome-wide association study of auto-antibody positivity in patients with type 1 diabetes did pick up on *HFE* as a possible association[208]. But since the etiology of diabetes in patients with hereditary hemochromasis is different from patients with auto-immune type 1 diabetes, it will not be expected that auto-antibodies will be abundantly prevalent in these patients; however, it may be expected due to the population prevalence that 2% of patients with C282Y/C282Y have auto-antibodies[154]. A limitation to the scarce data on auto-antibodies is also that only GAD-65 auto-antibody was measured; but since the 4 measurements were done 14-38 years after diagnosis, GAD-65 is the autoantibody with the highest sensitivity, specificity and positive predictive value[154] even many years after diagnosis in comparison to other islet cell auto-antibodies[154,209]. Two of the four patients with auto-antibody measurement had serial measurements, thus these results may be valid, but the one wild type type/wild type patient with a single positive auto-antibody test should ideally have had a serial measurement in order to confirm auto-immune type 1 diabetes[152].

The patients with late-onset type 1 diabetes all had a mixture of diagnoses. For patients with wild type/wild type, this could reflect that these patients were latent autoimmune diabetes of the adult but also type 2 diabetics[178,210]. For patients with hereditary hemochromatosis as the cause, the ICD-codings may reflect the current confusion on how to classify diabetes mellitus secondary to diabetes; the different ICD codings could also reflect the nature of disease, in that patients with hemochromatosis usually develop insulin resistance before beta-cell destruction[48]. In the current American Diabetes Association (ADA)[178] and World Health Organisation (WHO)[210] classifications of diabetes mellitus, hereditary hemochromatosis is listed under the heading "Diseases of the exocrine pancreas"; however, this classi-

fication does not comply with the current evidence of the cellular and pathophysiological mechanisms in hereditary hemochromatosis and other iron overload diseases or the clinical phenotype of patients with these diseases as described in the introduction.

Ascertainment and misclassification of diagnoses

The National Danish Civil Registration System[165] provides data on total mortality, which is a more unbiased measure than disease-specific mortality[211] with diagnoses from the registries.

The diagnoses in *The National Danish Patient Registry(NPR)*[166] and The *National Danish Causes of Death Registry (NDCDR)*[168] are not precise; thus, there may be diagnoses from hospitalisations and death that are not registered, and some diseases may be misclassified. The validity of the administrative data in *NPR* is high ranging from 85%-99% (date, referral etc.)[212] and the completeness is high due to the civil registration number[212]. Older patients tend to get less valid diagnoses than younger patients, probably due to the more multimorbidity, and surgical diagnoses has a higher correctness (86%) than internal medicine diagnoses (66%)[212]. Differences in coding may also be due to different coding practises at the hospitals[212]. A study has shown that diabetes diagnoses in NPR has 64% sensitivity (i.e. finds correctly 64% of those diagnosed with diabetes, but misses 36%), and 97% positive predictive value (i.e. 97% of the patients with a diabetes diagnosis in NPR, has in fact diabetes)[213]. Combining the NPR and The Danish National Health Service Register[213, 214] increases the sensitivity of the NPR diagnosis of diabetes to 86% but decreases the positive predictive value to 89%. If the Danish National Health Service Register[213, 214] is combined with data from the hospitals laboratory databases, the sensitivity of a diabetes diagnosis is 96% and the positive predictive value 89%. Thus, due to unsatisfactory sensitivity of diabetes diagnoses in NPR, The National Diabetes Register was established in 2006[215]; this register includes NPR diagnoses of diabetes, registration of chiropody, glucose measurements, purchase of oral antidiabetic drugs and insulin. The findings of sensitivity for a diabetes diagnosis corresponds well with a similar Scottish study with a sensitivity of 59% and a positive predictive value of 99%[216]; the Scottish study also demonstrated that completeness of recording of diabetes as a co-morbidity also varied by primary diagnosis such that 70% and 41% of admissions with coronary heart disease and cancer as the primary diagnosis mentioned co-existing diabetes, respectively[216]. Another limitation of the registries is that the ICD-coding (type 1 diabetes (ICD8: 249, ICD10: E10), and type 2 or other or unspecified diabetes (ICD8:250, ICD10: E11, E13, E14)) is not mutually exclusive; however a "true" type 1 diabetes ICD-10 coding would most likely be one with only an E10 code; those, who have both E10 and E11 are most likely type 2 diabetics; however, just exactly patients with hemochromatosis in a more advanced stage usually only get insulin, and as was discussed above, these patients may not belong to E10 but to E13. Thus, we used only diabetes diagnoses from NPR for incidence, but we combined them with glucose measurements and information on diabetes medication for prevalence in the population studies. Consequently, the number of cases with diabetes in the follow-up studies is most likely less than the "true" incidence.

The National Danish Cancer Registry[167] has a very high completeness of 95-98%[167, 217]. Although, the registry contains source of information on how the diagnosis was made (microscopy, autopsy, clinical etc.), the proportion of morphologically verified tumors is 89%[167]; it is estimated that this proportion can get higher even though it internationally is a high proportion[167]. Validity of diagnoses are secured through quality control routines and linkage to NPR and The Danish Pathology Register[167, 218]. Thus, the use of the cancer registry in paper 4 most likely covers all the diagnoses.

The National Danish Causes of Death Registry (NDCDR)[168]: The correctness of the underlying and contributing causes of death relies on the classification of the codes and on the physicians who have filled in the death certificates[168]. Before 2007, it was the National Board of Health in Denmark who interpreted the written information on underlying and contributing causes of death on paper-based death certificates issued by physicians and translated

this information to ICD-codes; this practice may have resulted in misinterpretations[168]. After 2007, electronic coding is done by the physician who issues the electronic death certificate and thus the coding relies on the diagnostic accuracy by the attending physicians[168]. However, both systems share some general limitations. Differences in the causes of death may be due to new diagnostic techniques, increased focus on special diseases, and less focus on ill-defined diseases[168]. Furthermore, in 1990 the legislation on autopsies on persons who died a natural death in Denmark was changed from a practice where a previous consent from the person who died or consent from the family was not needed to a practice where either of these consents was required. Thus, autopsy rates in Denmark has since declined and is now low (below 10%)[168]. A recent meta-analysis on the discrepancy between clinical and autopsy diagnoses estimates that 30% of the diagnoses on the death certificates are incorrect[219]. Furthermore, in Denmark, causes of death are not regularly validated[168] as opposed to e.g. Finland where a validation report estimated that of 7% questionable death certificates, half of them were re-assigned to a different ICD-code[220]. Thus, correctness of causes of death is crucial for mortality statistics and health surveillance but also for research purposes. However, total mortality in Denmark is based on the Danish Civil Registration System which updates vital status continuously and is thus considered complete for Danish residents[165]; however, for those persons who have emigrated or disappeared, death is only registered if the Danish authorities are informed about their death or the death occurred in Denmark[165].

Discussion - results in perspective

Diabetes mellitus

Paper 1

The study of late-onset type 1 diabetes in 2001 was the first of its kind and is still unique. Our results supported the hypothesis, that hereditary hemochromatosis is a disease generally overlooked in patients with late-onset type 1 diabetes, and they also suggested an association of C282Y/C282Y with late-onset type 1 diabetes. We recommended that patients with late-onset type 1 diabetes mellitus should be assessed for hereditary hemochromatosis to reduce morbidity and mortality, and that screening programmes should measure transferrin saturation initially, and, if the value was greater than 50%, genetic tests should be done[8]. Extrapolation of the data to the entire general population suggests that 15-20 individuals in every million would not develop type 1 diabetes and hereditary hemochromatosis if they were investigated for hemochromatosis early in life. Whether one calculates the frequency of diabetes in C282Y homozygotes or the frequency of homozygosity in those with diabetes, the degree of attributable risk seems small, at least from the perspective of a busy general clinician. To a specialist in diabetes mellitus, however, screening for hemochromatosis might be useful in the differential diagnosis of secondary causes of diabetes, allowing them to secure the appropriate treatment for this subgroup of patients[8] that have a serious prognosis, if the hemochromatosis diagnosis is overlooked.

Men with C282Y/C282Y had biochemically higher iron overload (tranferrin saturation and plasma ferritin), and were phenotypically more severely affected than women in accordance with other studies[134, 136]; however, unexpectedly, women had earlier onset of diabetes than men and with less iron overload than men. The reason for the earlier onset in women is not apparently clear from the study as men usually have earlier onset of hemochromatosis compared to women who present with symptoms after menopause[3].

Previous studies of risk of diabetes according to C282Y/C282Y genotype have primarily focused on type 2 diabetes or any diabetes with contradicting results[7, 23, 62-82]; to solve this question, I and co-authors undertook a meta-analysis in 2007[7], showing that the risk of any diabetes among Northern Europeans according to C282Y/C282Y genotype was 3.4-fold (95% CI: 1.1-11).

In a recent study[221], diabetes mellitus secondary to pancreatic disorders accounted for 9% (172) of 1868 patients with diabetes mellitus of whom only 12(6%) had hemochromatosis (equal to 0.6% of all the diabetes patients). In our own study[8], 1.26% of patients with late-onset type 1 diabetes with onset above 30 years of age were homozygous for the C282Y mutation (the prevalence of C282Y homozygosity in the Danish general population is 0.25%), corresponding to the prevalence found in another similar study[222]. We estimated that the population attributable risk of diabetes given iron overload is 3%, meaning that 3% of diabetes could be avoided if iron overload was eliminated[10].

We used the CCHS 1991-1994 examination as a comparison population to the patients with late-onset type 1 diabetes. In the CCHS, only 23 had C282Y/C282Y, and of those, only two had type 2 diabetes, and none had type 1 diabetes. Since the background population represents those who participated in the population study, these participants may be healthier or less diseased than those who did not participate, and furthermore also reflects the population that survived from the second examination to the third. Thus, as is speculated in this thesis, if iron overload leads to increased morbidity and mortality, those severely affected with iron overload may not participate, and thus the prevalence of C282Y/C282Y in the CCHS could be underestimated. The diabetes diagnoses from the CCHS were collected from The National Danish Patient Registry[166] and The National Danish Causes of Death Registry (NDCDR)[168]. As described above, these registers are not precise. In NDCDR, diabetes mellitus is very seldom used as a diagnosis, instead the co-morbidity to diabetes i.e., ischemic heart disease is often given as the cause of death. Also in relation to hospital discharge register the diagnose type 1 diabetes is not always part of the diagnosis. Consequently, the number of cases with type 1 diabetes in the background population could be less than the "true" prevalence.

At the time the data in this paper was collected and later published in 2001 as well as now in 2014, it was not part of clinical guidelines to assess for hemochromatosis in specialised diabetes clinics or among general practitioners. It was our aim with this paper to assess whether there was a higher prevalence of patients with hemochromatosis among these patients than seen in the general population. The data confirmed this hypothesis. At that time, it was known from studies of patients with hemochromatosis that early detection of the disease would halter or reduce comorbidities.

Therefore, as part of the discussion and in the Conclusion in the abstract in paper 1, we hypothesised/speculated using the hypothetical words "could"/"might" that assessing this group of patients even earlier (i.e. at entry to Steno Diabetes Center) could in the future result in the earlier diagnosis of hemochromatosis and reduced premature death. However, we did not have the data to justify this in paper 1, hence the hypothetical wording. Therefore, in paper 6, we followed up these patients as well as another group of patients with late-onset type 1 diabetes and patients with type 2 diabetes and other diabetes but diagnosed at entry to the Steno Diabetes Center, and not at some random time point as the patients in Paper 1. Our hypothesis, that premature death among patients with late-onset type 1 diabetes would be reduced if iron overload was detected at entry rather than at random or later, was confirmed in paper 6. Thus, we hope that early assessment of iron overload status with measurements of iron overload parameters and genotyping will be integrated as part of future clinical guidelines in specialised diabetes clinics; however, for this to be a reality, cost-effective analyses must be carried out.

Paper 2

We demonstrated that transferrin saturation ≥50% was associated with a 2-3 fold increased risk of developing any form of diabetes mellitus, as well as type 1 diabetes and type 2 diabetes, separately. This was the first and largest population-based study that consistently demonstrated transferrin saturation as a risk marker of any form of diabetes, and of type 1 and type 2 diabetes separately, in three independent and ethnically and geographically ho-

Table 11. Studies of the association of transferrin saturation and risk of diabetes.

Author	Mainous	Thomas	Acton	Ellervik
Publication year	2002	2004	2006	2011
Ethnicity	Mix	Not specified	Mix	White
Study design	Restrospective cohort	Case-study with cohort	Cross-sectional	2 follow-up studies 1 case-control
Study size	9000	820	97000	45574
Transferrin saturation cut-off	45%	35%	Mean values, no cut-off	50%
Diabetes diagnosis	Any	Type 1, Type 2	Any	Type 1, Type 2, any
Source of diabetes	Self-report, ICD-9	Patients in a diabetes clinic	Self-report	ICD-8, ICD-10, blood glucose, self-report, medication
Result	No association	Association	No association	Association
Statistics	Logistic regression	Logistic regression	Analysis of variance	Hazard ratio

Mainous AG, III, et all. Is an elevated serum transferrin saturation associated with the development of diabetes? *J Fam Pract 2002 November;51(11):933-6.*

Thomas MC et al. Elevated iron indices in patients with diabetes. *Diabet Med 2004 July;21(7):798-802.*

Acton RT et al. Relationships of serum ferritin, transferrin saturation, and HFE mutations and self-reported diabetes in the emochromatosis and Iron Overload Screening (HEIRS) study. *Diabetes Care 2006 September;29(9):2084-9.*

Ellervik C et al. Elevated Transferrin Saturation and Risk of Diabetes: Three population-based studies. *Diabetes Care 2011 October;34(10):2256-8.*

mogenous studies. The risk of increased transferrin saturation observed in our study was not secondary to an increased risk conferred by the C282Y genotype. As the risk of developing diabetes mellitus increased with the degree of iron overload[10], for which there is a simple treatment (phlebotomy), 1 to 3% of cases of diabetes mellitus in the general population and 7% of cases in a diabetes population, corresponding to approximately 300 patients per million in Denmark, could likely be resolved if patients received such a treatment earlier in life[10]. These findings reinforce the importance of investigating for iron overload in the differential diagnosis of secondary causes of diabetes, and they support the arguments in favour of at least a targeted screening approach for iron overload in symptomatic patients and their families[223].

In epidemiological studies, hereditary or non-hereditary hemochromatosis is associated with increased risk of developing or dying from diabetes[7-10, 14, 83-86]. High serum ferritin correlates inversely with serum insulin and proportionally with blood glucose concentrations[87]. Phlebotomy may improve insulin secretory capacity [92, 93] and insulin sensitivity[94] if instituted early and reverses impaired glucose tolerance in patients with hereditary hemochromatosis[93, 95]. Likewise, in patients with beta-thalassemia major and tissue iron overload due to enhanced iron absorption and multiple transfusions, iron chelation may improve glucose metabolism by reducing insulin resistance and improving beta-cell function[224-226].

In the context of the existing literature, three other studies[86, 96, 97] besides our own[10] have used transferrin saturation as the primary independent variable (without considering hemochromatosis genotype) for the study of the risk of any diabetes (Table 11). However, these studies are heterogeneous concerning ethnicity (two of the studies used mixed populations[86, 97], another did not report ethnicity[96]), study design (cross-sectional[86], retrospective cohort[97], and case-study with historical controls[96]), size (44000 whites/53000 other ethnicities[86], 9000[97], and 820[96]), transferrin saturation threshold (no cut-off but only mean values[86], cut-off of at least 45%[97], cut-off of 35%[96]), diabetes diagnosis (any diabetes[86, 97],

type 1[96], type 2[96]), source of diabetes diagnosis (self-report[86, 97], use of ICD-9 code[97], diabetes patients in an endocrinology department[96]), and finally also results (one in favour[96] and two not in favour[86, 97] of association of elevated transferrin saturation and risk of diabetes). In comparison, our study consisted of three individual studies consistent in study design (two large general population studies similarly ascertained, and a case-control study using patients with diabetes ascertained from a similar population as the general population studies and compared to controls ascertained like the population studies with no overlap of individuals in the three individual studies), ethnicity (only white individuals of Northern European Danish descent), transferrin saturation cut-off value of 50% in accordance with accepted clinical practice[32, 176, 177], diabetes diagnosis (any diabetes, type 1 and type 2 for all three studies), source of the diabetes diagnosis (self-report, medication, ICD-coding, and a non-fasting glucose above 11 mmol/L), and finally also consistent in results.

It is a limitation of our study that only approximately 45% participated in the CGPS; thus, as discussed above, the participating population may be healthier than the non-participating population. And as described above, the CCHS suffer from survivor bias, which is also reflected by the fact that non-participants had a higher incidence of diabetes than participants. Age was higher in CCHS and CGPS than in the Steno Diabetes Center Cohort 2, but age was adjusted for in the follow-up analyses of diabetes in CCHS and CGPS, and the case-control study was matched on age and gender 1:1. As discussed above, The National Danish Patient Registry[166] and The National Danish Causes of Death Registry (NDCDR)[168] may underestimate the true incidence of diabetes; however, it must be anticipated that this applies to both participants as well as non-participants, and thus the relative difference would be expected to be the same. Another limitation of the registries is that the ICD-coding (type 1 diabetes (ICD8: 249, ICD10: E10), and type 2 or other or unspecified diabetes (ICD8:250, ICD10: E11, E13, E14)) is not mutually exclusive. Thus, the numbers of type 1 diabetes in the population studies may seem high compared to type 2 diabetes. In CCHS and CGPS, 910 and 1496 were classified with diabetes mellitus, 274 and 393 were classified with type 1 diabetes, and 875 and 1418 were classified with type 2 diabetes or other or unspecified diabetes mellitus, respectively. However, in the National Danish Patient Registry, 239 and 315 had diagnoses for both type 1 and type 2 diabetes in the CCHS and the CGPS, respectively; thus, the stratification on type 1 and type 2 for the general population studies is not mutually exclusive. In other words, type 1 diabetes reflects those treated with insulin and includes a large proportion of patients with type 2 diabetes and only a smaller proportion of those with autoimmune type 1 diabetes. However, in light of the discussion above, this may be relevant, as most patients with diabetes secondary to hemochromatosis need insulin. But of course, the wording could have been otherwise chosen (insulin dependent and non-insulin dependent diabetes).

It could be questioned, that with a mean age of about 60 years not all cases of type 2 diabetes have been developed, while most cases of type 1 diabetes had become overt given that the mean age in Denmark for developing type 2 diabetes is about 55 years. It could also be questioned that the difference in prevalence of diabetes between the two populations is difficult to understand, since the mean age did not differ between the two cohorts at time of investigation. Median ages in paper 2 are those at recruitment=baseline. However, since we performed a follow-up study in both CCHS and CGPS, all individuals in the population studies who later developed diabetes were counted as incident cases irrespective of the age of the individual entering the study. Thus, we have included all the patients with diabetes that had developed during the follow-up period; however, off course the longer the follow-up the more the incident cases you get. The CGPS started recruitment 10 years later than the CCHS, thus the number of participants developing diabetes in the CGPS was only 1.4 times that in the CCHS, even though the CGPS was 2.6 times larger than the CCHS probably because of the difference in the years of follow-up. Therefore, we could have underestimated the effect

in the CGPS; and also because the registries are not complete in the diabetes coding. In the case-control study, cases and controls were matched 1:1, thus the diabetic group is compared to the similar age and gender group among controls.

Hypertension (paper 3)

Given the results, testing for hemochromatosis genotype C282Y/C282Y and extreme transferrin saturation could be considered in patients with essential hypertension. As the susceptibility for hypertension (determined by the use of antihypertensive medication) may be a genetic factor causing iron overload for which there is a treatment (phlebotomy), 0.1–0.4% of cases of hypertension likely could be resolved, if patients received such a treatment. Extrapolating these results to all patients with hypertension in Denmark, 200–600 patients per million could be helped with phlebotomy[11].

Indeed, very few individuals experienced a transferrin saturation above 80%, which is an extreme biochemical phenotype. What is interesting is that it may seem that a much larger iron overload may be needed to develop hypertension, than to develop e.g. diabetes (in comparison to the other results in the thesis); the reason for this is not clear. In support of this, it is known that affection of the heart is much less common in iron overload (especially iron overload resulting from C282Y/C282Y genotype). Only one woman had iron overload above 80% and was treated with antihypertensive medication; women in general have smaller iron stores compared to men, and women only start accumulating iron after the menopause; thus, it is expected that few women have extreme iron overload. Also, risk of hypertension was increased in women with transferrin saturation 70-80%. It may seem peculiar that women experience hypertension at a lower transferrin saturation level than men, but it could be speculated that the acute rise in iron overload after the menopause may be more toxic than a steady and gradual iron increase over the years as is seen in men.

In the CCHS 11% were on antihypertensive medication, whereas in the CGPS 19% were on antihypertensive medication; these differences may reflect differences in medication practice from 1991-1994 (CCHS) compared to 2003-2007(CGPS). In the CCHS 11% and 30% were on antihypertensive medication in wild type/wild type vs. C282Y/C282Y; the corresponding percents in the CGPS were 16% and 23%. These differences may be explained by differences in medication practice in general in the wild type group, but for C282Y/C282Y it could be speculated that these individuals at the 1991-1994 examination of the CCHS already were detected earlier with hypertension; in comparison the CGPS represents a more naive clear baseline cohort compared to the CCHS, and thus the differences between the genotypes are not as big as in the CCHS. Thus, the risk estimate for the CCHS may be overestimated, whereas the risk estimate in the CGPS may be a more conservative estimate.

The absent association of measured blood pressure on a continuous scale may be explained by the individuals already being on antihypertensive medication having a lowered blood pressure.

The reason for a non-significant finding of transferrin saturation and C282Y/C282Y genotype with LVH or LVH combined with hypertension may be due to: limited power in the studies, that ECG's are neither as specific nor as sensitive as an echocardiography for LVH, and that LVH may be caused by other factors increasing afterload (i.e. aortic stenosis, aortic insufficiency) than hypertension. The absent association of risk of LVH may also be explained because LVH represents end-organ damage; thus, longer time may be needed for the hypertension to affect the heart. Another reason for the lack of association is that it is well known that heart disease from classical Type 1 hemochromatosis (i.e. *HFE* hemochromatosis) is seen far less common than in Type-2 hemochromatosis (a juvenile form), where the individuals often die early (i.e in their thirties) from heart failure. Furthermore, the extreme clinical phenotypes with affection of the heart may be too diseased to attend a health study and may be underrepresented in the CCHS. Also, previous evidence of LVH in hemochromatosis comes from case-only studies without any control population.

The association between C282Y/C282Y genotype and/or extreme transferrin saturation and treatment with antihypertensive medication may be caused by extreme iron overload accumulated over a long period. Previous findings have shown an association between hyperferritinemia[89, 107, 227] and features of the metabolic syndrome including hypertension. This study is the first study to investigate and show an association between C282Y/C282Y or transferrin saturation and use of antihypertensive medication. However, it is worth noting that increased transferrin saturation measured in this study (generally thought to represent an increased intestinal iron absorption typical of hemochromatosis) is different from hyperferritinemia without increased transferrin saturation, which is a common presentation of the so called dysmetabolic iron overload syndrome not related to hemochromatosis[228].

A functional explanation for the detected association of transferrin saturation and C282Y/C282Y with hypertension may be that long-term iron overload generates reactive oxygen species by the Fenton reaction, creating oxidative stress that increases vascular tone[104, 229] and consequently this results in hypertension.

Cancer (paper 4)

Risk of any cancer based on increased transferrin saturation was only increased in women in CCHS, but this study may be underpowered for sex-stratified analysis. The meta-analysis, on the other hand, showed increased risk of any cancer overall, in women, and to a lesser degree in men, in accordance with previous studies[123, 128]. This finding is supported by results from a clinical trial of the effect of iron reduction[131] and from a cohort of regular blood donors[132], suggesting an effect of iron reduction on decreased risk of cancer.

Risk of cancer in individuals with iron overload has previously been studied in various prospective[118-129] and case-control studies[130]; the studies are not homogeneous in design making comparisons difficult, despite the many articles written about the subject. The studies have primarily focused on liver cancer[121], gastrointestinal cancer[127, 130], cancer-mortality[125, 126], or a range of cancer subtypes[120], but few previous studies have studied any cancer[123, 128] like our own[12].

An association between liver cancer and increased transferrin saturation is in accordance with a previous finding[121]; however, although liver cancer is part of clinical hemochromatosis, we did not find any C282Y/C282Y homozygous subject with liver cancer in the CCHS, which could indicate the low penetrance of the genotypic disease compared with that of the biochemical disease or survivor bias, but risk of cancer was not different in non-participants vs. participants; it could also indicate a power problem, but since the study was powered in men and they are most prone for hemochromatosis, this is not a likely explanation; though, power was a problem in women. These results underscore the fact that the risk of liver cancer as a function of C282Y/C282Y genotype observed from case-control studies may be overestimated[7] due to ascertainment bias. However, owing to the low number of C282Y homozygous individuals in the CCHS, this study may not be adequately powered for this analysis, and thus may also suffer from ascertainment bias due to less severe disease in individuals attending the study.

Increased risk of cancer outside the liver was found in one cross-sectional study of patients with hereditary hemochromatosis[230]; but C282Y/C282Y genotype was not found to be associated with cancer outside the liver in a our own meta-analysis[7] of case-control studies in 2007 (not part of this thesis); but we did not have enough power to sub-stratify into different cancers for C282Y/C282Y genotype. However, 3 recent meta-analyses of C282Y/C282Y genotype and risk of hepatocellular cancer[231], breast cancer[232], and colorectal cancer[232] showed associations, whereas a meta-analysis of gastric cancer did not show any association[233].

The mediation analysis of transferrin saturation as a mediator on the risk of C282Y/C282Y on cancer was not surprisingly insignificant as in the hazard ratio-analysis of transferrin saturation, the overall effect of transferrin saturation, on cancer was also insignificant.

If a causal association exist between iron overload and C282Y/C282Y and cancer risk, then patients with iron overload and C282Y/C282Y genotype may be candidates for individualized cancer screening programmes[230].Targeted case finding for men of Northern European descent has previously been proposed[234], but it may be worth focusing also on women with iron overload, as they seem to be at a considerably increased risk of cancer.

Total Mortality (Paper5,6 and7)

Paper 5

The finding that increased transferrin saturation was associated with increased risk of premature death in the CCHS and the CGPS[13] confirmed a previous finding[15]; combining these three studies in an aggregated estimate in a meta-analysis showed similar results[13]. Due to a letter[235] questioning our results[13] in terms of regression dilution bias, we showed that correcting for regression dilution bias[169] revealed larger hazard ratios; thus, the results in paper 5 were conservative[169]. We were also questioned if the same results applied if we explored total mortality risk with increasing iron or transferrin concentration; we confirmed the results with both iron and transferrin concentration[13, 169]. The polynomial model explored in this thesis was a more refined analysis of the continuous relationship between hazard ratios for total mortality according to continuous transferrin saturation levels; the model revealed a J-shaped non-linear relationship, with risk larger than 1.0 for very low levels of transferrin saturation (indicative of anemia) and also for levels of transferrin saturation above 50%, in the range from 10-50% risk was below 1.0.; however, risk for iron overload was larger than for anemia.

We estimated total mortality, since this is a more unbiased measure than disease-specific mortality[211]. We focused on total mortality and its relationship with the biochemical marker transferrin saturation, an intermediate step between clinically silent hemochromatosis and clinical hemochromatosis[236]. Mortality among advanced cases of untreated hemochromatosis is high and usually due to liver cirrhosis and diabetes mellitus[134, 136, 237]; however, whether clinically unnoticed increases in transferrin saturation also lead to premature death has hitherto been unknown. In support of the present findings, a recent study of total mortality in hemochromatosis patients compared to normal controls showed a hazard ratio of 2.2 (1.6 -3.0)[137], close to the estimates in our study[13].

None of the individuals in the CCHS with genotypes associated with hereditary hemochromatosis developed overt hemochromatosis[33]; however, we have no specific hemochromatosis follow-up of individuals in the CGPS yet.

Previous studies have not shown any associations between all-cause mortality and hemochromatosis genotype C282Y/C282Y[24, 238, 239]; however, in one study heterozygosity in postmenopausal women for C282Y has been associated with cardiovascular death[240] mainly driven by cerebrovascular death with effect modification by hypertension on hemochromatosis genotype. However, in people diagnosed with phenotypic hemochromatosis, mortality still remains high[133-137]; thus, the mortality from C282Y/C282Y genotype may be higher than observed in the population-based studies due to the fact that population-based studies are subject to selection bias due to more severe disease and/or increased mortality in non-participants.

The data from the CCHS in this article were the data from a third re-examination in 1991-1994[171], with the previous examinations being in 1976-1978[170] and 1981-1983[171]. As commented on above, those eligible to be invited at the 1991-1994 examination were the survivors who potentially could introduce survivor selection bias; in order to minimize survivor selection bias, the original cohort was supplemented with younger participants. As total mortality was increased in non-participants, a survivor selection bias exists drawing the results against the null hypothesis; thus, since we found the significant findings, these findings are conservative. The CCHS estimate for total mortality was accordingly also lower and less significant (though still significant) even though the follow-up for this study was

longer than for the CGPS. The CGPS must then reflect a more true estimate, since the CGPS does not suffer from survivor bias in the sense that it's not a re-examination. However, it was not possible to get the data to explore this in detail.

Confirmation studies, if large and well-conducted, are just as needed as novel studies, in order to find out the robustness and reproducibility of findings[241]. Furthermore, for health prevention issues and clinical decision makers, we need to provide a solid basis for integrating data in order to give precise and consistent estimates on the totality of available evidence. However, a lot of factors may influence the finding of a p-value slightly less than 0.05 as in NHANES[15]: 1) chance finding (i.e. the rejection of the null-hypothesis when the null-hypothesis is in fact true), 2) a true association (i.e. rejection of the null-hypothesis that there is no difference in survival among individuals with transferrrin saturation less than or above 50%), 3) results are due to other factors such as confounding, ascertainment bias, misclassification etc. Thus, confirmation studies are needed in other populations in order to know if the previous finding was by chance. Our study was different from NHANES with respect to country and ethnicity: NHANES had 90% whites of both Southern and Northern European descent, while the two Danish population studies were all white of Northern European Danish descent. It is known that hemochromatosis/iron overload is more prevalent in countries of Northern European origin and it has previously been shown, that ethnicity matters in terms of the risk conferred by iron overload and hemochromatosis genotype; thus, NHANES included 10% non-whites, who potentially could have a very low chance of having iron overload. Furthermore, NHANES comprised approximately 10,000 individuals while the Danish studies added an additional 45,000 individuals. Thus, we confirmed the study by NHANES but in another population, especially for individuals that were white and of Northern European Danish descent. The Achilles heel of meta-analyses is heterogeneity; thus, in fact similar studies are needed in order to confirm or reject previous findings, otherwise the differences can just be attributed to the heterogeneity. Furthermore, as a novel idea and finding, in this paper, as was not presented in the NHANES paper, we added a semi-graded relationship of transferrin saturation (%) with cut-points: <20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, and above 80, in order to explore more extreme values of transferrin saturation. I presume the NHANES study did not have a large enough sample size and power to do that. The reason for choosing this semi-graded relationship was to find out if there was another lower or higher cut-off that would associate more strongly with overall mortality.

Paper 6 and 7

In the conclusion in paper 1, we hypothesized that premature death among patients with late-onset type 1 diabetes would be reduced if iron overload was detected at entry rather than at random or later. In paper 6 we showed that increased transferrin saturation and genotype C282Y/C282Y predicted total mortality in a population of patients with late-onset type 1 diabetes after diagnosis, and that post-diagnosis increased transferrin saturation is a risk factor independent of hemochromatosis genotype. We also showed that early measurement of transferrin saturation in patients with diabetes at referral may improve life expectancy in these patients. Death due to liver cancer may be specific for hereditary hemochromatosis; however, the number of individuals with liver cancer was low. These are novel findings.

The increased risk of death of neoplasms in paper 6 underscore the results in paper 4 (cancer) but to a larger extent. Even though paper 4 measured any incident of cancer (non-fatal as well as fatal), and paper 6 only measured fatal cancer (but both as an underlying and a contributing cause), thus it may seem that when organ damage (late-onset type 1 diabetes) has occurred owing to hereditary hemochromatosis, risk of cancer is higher than compared to individuals from the general population. Also, risk of cancer in patients with late-onset type 1 diabetes was slightly higher for individuals with C282Y/C282Y genotype compared to the general population study; this was also confirmed in men; women with

C282Y/C282Y genotype in either study did not have increased risk of any cancer, but the studies were underpowered in women, and there was not enough power to sub-stratify into different cancer types in paper 6.

It was not possible in paper 6 to examine if early measurement of transferrin saturation in patients with other diabetes than late-onset type 1 was beneficial. We showed that patients with other diabetes than late-onset type 1 diabetes and with an early measurement of transferrin saturation did not have increased risk of premature death. But whether the results could be ascribed to the nature of the diabetes or to the early measurement, was not clear. As a consequence we ascertained patients with any diabetes (mostly type 2 diabetes) from two general population studies, in which a baseline measurement at some random time point was done; the results were presented in paper 7.

In paper 7, we showed that individuals with diabetes ascertained in the general population with the threshold transferrin saturation ≥50% vs. transferrin saturation <50% had an increased risk of premature death overall and that individuals with diabetes had a stepwise increased risk of total mortality for stepwise increasing levels of transferrin saturation, with the highest risk conferred for transferrin saturation ≥70%; results were similar in men and women and for type 2 diabetes alone. Also, individuals with diabetes and transferrin saturation ≥50% vs. transferrin saturation <50% had an increased risk of cause-specific death from neoplasms and endocrinological diseases, which were diabetes related diagnoses. For this thesis, we also explored the continuous relationship of increasing transferrin saturation with hazard ratio for total mortality. The relationship revealed a J-shaped curve almost flat to the left of 30%, and after 40% with a steep rise in the hazard ratio. Thus, the curve was slightly different from the curve that was seen from the general population studies alone. Also the curve for the diabetics were generally much higher located in the diagram, almost with most of the curve in the whole range of transferrin saturation above a hazard ratio of 1.0 for total mortality compared to the curve for the general population study (paper 5) where the curve had a large proportion from 10-50% under a hazard ratio of 1.0.

Our findings that elevated transferrin saturation was associated with increased risk of premature death in individuals with type 2 diabetes underscore the results from our paper 6 in patients with late-onset type 1 diabetes from a highly specialised diabetes clinic[9]. Both of these studies were based on a baseline transferrin saturation test at a random timepoint in life and not an early transferrin saturation test. In paper 6 we also showed that awareness of iron overload in a diabetes clinic with early measurement of transferrin saturation may reduce mortality in patients with late-onset type 1 diabetes relative to that of the background diabetic population[9]; furthermore, we showed that patients with type 2 diabetes in a diabetes clinic who have an early measurement of transferrin saturation have a mortality similar to the background diabetic population. However, for that patient cohort we lacked a control group who had a random transferrin saturation test for comparison, which is provided in the paper 7.

Risk of premature death in patients with diabetes ascertained in the general population (paper 7) was independent of hemochromatosis genotype since risk was still increased when excluding hemochromatosis genotypes C282Y/C282Y. Moreover, elevated transferrin saturation and C282Y/C282Y genotype were both associated with increased risk of premature death independently, but the joint effect of exposure to transferrin saturation and C282Y/C282Y genotype was higher than the sum of both effects. We calculated population attributable risk showing that six percent of premature deaths among individuals with diabetes in the general population could potentially be avoided by early screening for transferrin saturation or *HFE* genotype. Thus, transferrin saturation and C282Y/C282Y genotype independently and in combination was associated with increased risk of premature death 2-6-fold in individuals with diabetes from a general population study. These were novel findings. Taken together, the results from paper 6 and paper 7 showed that unawareness of iron overload and hemochromatosis genotypes in patients with late-onset type 1 diabetes and in patients

with type 2 diabetes is associated with increased risk of premature death, and that patients with late-onset type 1 diabetes possibly will benefit from an early measurement of transferrin saturation and appropriate intervention with a potential gain of 16 years of life; however, such estimates are insecure and needs to be confirmed in independent studies.

There is evidence for increased mortality in patients with clinically overt hereditary hemochromatosis[133-137] and among untreated patients usually due to liver cirrhosis and diabetes mellitus[134, 136.] Our own previous general population study and meta-analysis (paper 5) have shown increased risk of premature death associated with increased transferrin saturation[13], and a recent study showed that diabetic patients on maintenance hemodialysis with serum ferritin levels above 700 ng/mL had increased 1-year mortality[242]. This is in accordance with our results.

A study in patients with hereditary hemochromatosis concluded that awareness of the diagnosis in general will translate into a greater life expectancy[141]. This is also supported by a study of health check-ups and family screening where subjects offered early detection of iron overload had improved survival compared to that of the background population[243].

The reason for the improved survival in subjects offered early transferrin saturation test is likely conferred by early diagnosis and treatment of iron overload or other conditions, but we have no data to demonstrate the reasons for specific health benefits of early measurement of transferrin saturation. To support this, it has been shown that iron depletion in patients with diabetes ameliorates HbA1c levels, insulin secretion, insulin resistance[244], and vascular dysfunction[94].

The limitations in paper 6 and 7 of the use of the different registers are described in detail above. Other limitations in the study included lack of well-known risk factors for liver disease (e.g. history of alcohol use (paper 6), and viral hepatitis (paper 6 and 7)) which could weaken the conclusions drawn on transferrin saturation as a risk factor for liver cancer. Furthermore, the percentage of missing data in Diabetes Cohort 1 and 2 in paper 6 were high for many of the adjusting variables, but missing data were imputed depending on age and gender; and unadjusted and adjusted analyses did not differ. Also, whether the test for iron overload should be transferrin saturation or ferritin, or both, need to be resolved.

Discussion of hemochromatosis genotypes C282Y/H63D and C282Y/wild type (paper 1, 3, 4, 6 and 7)

In 1996, when the *HFE* gene was discovered, it was estimated that in a group of patients with hemochromatosis, the prevalence of C282Y/C282Y, C282Y/H63D, and C282Y/wild type would be 84%, 4%, and 1%, respectively; this has been confirmed by many other studies[2]. A meta-analysis has showed that odds ratios of iron overload in individuals with C282Y/C282Y, C282Y/H63D, and C282Y/wild type is 1000, 36, and 4, respectively[22]. We found no increased risk of diabetes, hypertension, cancer or premature death in individuals from the general population or patients with diabetes with C282Y/H63D or C282Y/wild type, which is in accordance with findings in other studies[23, 188, 245, 246], that documented iron overload disease is rare but most often occur when other co-morbid factors are present such as steatosis and heavy drinking[246]. A recent study in hemochromatosis patients with C282Y/H63D showed that these patients were more often heavy drinkers and overweight compared to patients with C282Y/C282Y. Even though the population studies with unrelated individuals have not been able to provide evidence for increased risk of disease in C282Y/H63D and C282Y/wild type, studies of 1st degree relatives to patients with hereditary hemochromatosis have shown an increased risk of conditions related to hemochromatosis[247]; thus, probably other genetic confounders exist, not yet identified. It could be discussed whether C282Y/wild type would have an advantage compared to wild type/wild type, and these individuals have indeed less iron overload than C282Y/C282Y and C282Y/H63D. In younger women in the reproductive age, C282Y/wild type seems to protect from iron deficiency and iron deficiency anemia[30, 248]. Thus, it is speculated that this advantage in younger women could con-

tribute to the higher frequency of the *HFE* genotypes in white populations[30]; though this could not be explained by differences in number of pregnancies which were the same in C282Y/wild type vs. wild type/wild type[30].

In a meta-analysis from 2007 of 31 end-points including 66,000 cases and 226,000 controls[7] we did not find any association of C282Y/H63D or C282Y/wild type with liver disease, heart disease, diabetes, arthritis, stroke, neurodegenerative disease, or venous disease; however, there was a dose-response relationship with all hemochromatosis genotypes and increased risk of porphyria cutanea tarda (familial and sporadic), a disorder of the hem-production pathway, with C282Y/C282Y showing the highest risk and H63D/wild type the lowest increased risk. Patients with porphyria cutanea tarda may experience iron overload, and respond to iron reduction therapy[142].

Cut-off or dose-response relationship

The relationship between transferrin saturation and hypertension[11] and total mortality[8, 9, 13, 14] speaks against a cut-off effect, but rather suggests a continuum, with the most extreme being transferrin saturation ≥80%. Another study of risk of liver cirrhosis and liver cancer according to transferrin saturation also showed a graded relationship[121]. Some studies have examined risk of cancer[12, 122, 128] and mortality[249] according to quartiles of transferrin saturation; however these studies have not found any associations; the reason may be that the increase in transferrin saturation between quartiles is too small and that the highest quartile is only >35%, and may thus contain individuals without increased risk, which could tend to attenuate the risk estimates. For the clinician, a cut-off is usually more practical; thus, a cut-off greater than 40% could be suggested instead of the previous 50% cut-off as a predictor of diabetes, cancer, hypertension and mortality.

However, for hemochromatosis genotypes (with the most extreme being C282Y/C282Y and then followed by C282Y/H63D, and C282Y/wild type), there does not seem to exist a graded genotype-relationship for risk of diabetes[8], hypertension[11], or cancer[12] in the general population, or total mortality[9, 14] in patients with diabetes, but rather a cut-off with C282Y/C282Y being the only genotype conferring risk.

Screening for iron overload

In paper 1, we tested the possibility of prediction of C282Y/C282Y by a measurement of transferrin saturation above 50%. Sensitivity and specificity were 1.0 and 0.96, respectively; thus, almost ruling out false negatives and false positives. Positive predictive value was 0.26; thus, a transferrin saturation above 50% may have other causes than homozygosity for C282Y/C282Y. Negative predictive value was 1.0 indicating that a transferrin saturation value of less than 50% rules out homozygosity for C282Y/C282Y in patients with late-onset type 1 diabetes. In comparison, in the paper 7 among patients with type 2 diabetes, the positive and negative predictive values of transferrin saturation ≥50% for detecting C282Y/C282Y were 12% and 99%, respectively; and the sensitivity and specificity were 64% and 97%, respectively. Likewise, in the general population studies used in paper 2 and 5, the positive and negative predictive values of transferrin saturation ≥50% for detecting C282Y/C282Y were 12% and 99%, respectively; and the sensitivity and specificity were 70% and 99%, respectively. Thus, the specificities were comparable and high and corresponded to those reported in The HEmochromatosis and IRon overload Screening study (HEIRS)[1]. The sensitivity was highest among patients with late-onset type 1 diabetes; but in the general population overall and in patients with type 2 diabetes sensitivities corresponded to that in HEIRS but were relatively low limiting the role of transferrin saturation as a screening test. The positive predictive values were low like in HEIRS[1]; thus, transferrin saturation ≥50% reflects more than just homozygosity for C282Y/C282Y. Unfortunately, it was not possible to estimate positive and negative predictive values or sensitivity or specificity for patients with other diabetes than late-onset type 1 diabetes in papers 2 and 6, since only patients

with a transferrin saturation above 50% were genotyped.

Screening for iron overload in the general population is not recommended by experts in the field[1, 250, 251] ; the evidence has been reviewed according to the WHO criteria and current evidence. However, targeted screening in families with hereditary hemochromatosis is needed, and targeted screening in men could be considered. But screening in women after menopause was not discussed, only women in general.

Clinical penetrance is essential in considering screening for a disease: penetrance of genotypes sometimes leading to hereditary hemochromatosis is low[23, 24]; however, our results address the broader question of whether individuals having no awareness of common increased iron stores and being at increased risk of diabetes, hypertension, cancer, and premature death should be screened with a simple blood test for transferrin saturation rather than a genotype test. This question naturally needs further scrutiny of the diagnostic value of a transferrin saturation test, studies of whom to screen, and analysis of the cost-effectiveness of transferrin saturation screening strategies. Our present results may be of value if and when the screening issue is re-evaluated. If screening is recommended, an equally important but also difficult question is whether the screening test should be transferrin saturation or ferritin. Indeed, debate exists as to whether transferrin saturation[32, 176, 177] or ferritin[252] is the best first iron overload indicator for hemochromatosis[2]. The present results are unfortunately not able to solve this issue, except to point at elevated transferrin saturation as an important indicator of risk of diabetes, hypertension, cancer and premature death. Future studies will hopefully examine in parallel the predictive value of transferrin saturation and ferritin levels to identify which test is more optimal.

Strengths and limitations

The strengths of the patient-groups with diabetes and hypertension were that they came from specialised clinics, and formed large, unselected, representative, and well defined groups, thereby reducing the possibility of bias. Misclassification of patients was unlikely, since medical specialists diagnosed the patients.

Strength of the general population studies were that they were large, randomly sampled, ethnically homogenous, ascertained alike, and from two different parts of Copenhagen and from two different time periods, and that the ICD-diagnoses were obtained from the national registers making follow-up complete without losses to follow-up, even though some diagnoses may lack in these registers.

The thesis is based on association studies that have lower evidence rating than well-designed double blinded randomized clinical trials; thus, no causal inferences can be made except for the genetic part of our studies which due to Mendelian randomization also offer clues to causality[197, 253]. Hemochromatosis genotype and/or increased transferrin saturation was associated with diabetes, hypertension, cancer, and total mortality with a) p-values mostly well below $p<0.05$, b) high relative risks (>1.5), c) internal study consistency, d) external study consistency (meta-analyses and previous studies showed similar results), e) a temporality where the exposures (genotypes and increased transferrin saturation) preceded disease-outcome, f) a dose-response relationship for total mortality and hypertension, g) and with biological plausibility (speculated to be a common pathway of free radical damage by the Fenton reaction).

One problem with association studies is the risk of false positive results (=chance findings). One way to solve this problem is to repeat the study in another independent sample, as was done in the CGPS in some of the later published articles[10, 13] in this thesis.

Another problem of association studies is false negative results due to limited power. Although, the CCHS has a long period of follow-up, there are still few incident cases with specific cancer subtypes. This is exemplified by the limited power for the cancer subtypes according to hemochromatosis genotypes and elevated transferrin saturation[12], but again could also reflect survivor bias.

Aggregating data from the literature with own results in a meta-analysis, is another way of dealing with both false positive results and false negative results. The advantages of meta-analyses are the derived aggregated odds ratio, the possibility of revealing consistency or inconsistency across studies and to reveal bias and in particular publication bias[181]. The most prominent limitation of meta-analyses is study heterogeneity; this may however be solved by stratification. The meta-analyses for any cancer and total mortality in this thesis were all conducted on the basis of large population-based studies with long follow-up with low study and statistical heterogeneity; still, the total number of studies included was small.

The sub-classification of diabetes and hypertension was not as well-defined in the general population studies as in the case-control studies[8, 10, 11]. Furthermore, we studied whites only and therefore our results may not apply to other races.

Information on alcohol, well-known to increase iron parameters[254-256] (serum ferritin, transferrin saturation), was included in the models; however, information on other factors either increasing (oral iron supplementations[255-257], dietary iron intake[255, 258]) or decreasing (blood donation[255, 259, 260], egg consumption[255], milk consumption[255]) iron parameters was not assessed as part of the general population studies or case-studies for diabetes or hypertension.

A limitation of the general population studies was the lack of measurement of ferritin levels to estimate the extent of iron overload; however, it remains unclear whether measurement of ferritin or transferrin saturation is the better predictor of hereditary hemochromatosis. A recent meta-analysis[22] showed that homozygosity for C282Y/C282Y confers a very high risk for both provisional (elevated serum iron markers) and documented iron overload (elevated serum iron markers associated with evidence of iron excess based on liver biopsy and/or quantitative phlebotomy), with odd ratios for elevated transferrin saturation alone (transferrin saturation >50%) of 614(95% CI: 12-32000) and with odds ratios for elevated serum ferritin alone (concentration thresholds varying across studies) of 69 (7-684) for provisional iron overload and even higher odd ratios for documented iron overload. Patients with hereditary hemochromatosis[234] with mild iron excess can have values of ferritin within the reference range; on the other hand, patients with increased transferrin saturation may never develop organ damage, and ferritin levels will detect the majority of patients who will be clinically affected[252]. Nevertheless, elevated transferrin saturation is clearly associated with hemochromatosis genotypes[5, 11, 22]. In the present thesis, measuring the future risk of diabetes, hypertension, cancer, and total mortality supports a role for the measurement of transferrin saturation in the assessment of disease risk.

Biological plausibility

A biologically plausible mechanism for the association between elevated transferrin saturation and risk of diabetes, hypertension, cancer and total mortality may be iron-induced increased oxidative stress via the Fenton reaction[47, 115, 261]. Likewise, oxidative stress has been shown to be increased in individuals with C282Y/C282Y genotype[40]. The defects in both insulin-producing and insulin-sensitive tissues are most likely caused by iron-dependent catalysis via the Fenton reaction of reactive oxygen radical species which impair insulin signalling in skeletal muscle and liver and cause beta-cell destruction due to insufficient beta-cell deficient antioxidant defence[47]. It has been shown that oxidative stress increases vascular tone[104] and consequently results in hypertension. Iron-induced free radical damage to DNA may be important for the development of cancer[115, 262], and cancer cells may grow more rapidly in response to increased iron levels[116]. In 1956, Denham Harman wrote a "free-radical theory of aging" about endogenous oxidants resulting in cumulative damage[263-265] and hence premature death. Much of the early evidence was based on a correlation between oxidative stress and aging[265]; however, recent research suggests a more causal relationship between oxidative stress and aging[265, 266]. Thus, we suggest that hemochromatosis and even modest iron overload could provide a model for reactive oxygen species production through the Fenton reaction, thus leading to premature death. It is unclear whether it is the absolute

level of oxidative stress or the response to and the degree of defence against oxidative stress that determines life expectancy[265].

There is now substantial evidence, that the liver plays a central role in determining, how much iron is absorbed from the intestinal tract and in influencing the release of iron from sites of storage[36]. The normal flux of iron from enterocytes to the blood stream is mediated through the transporter ferroportin on the basolateral side of the enterocyte (Figure 1). The hormone hepcidin, which is secreted by hepatocytes, is the central regulator of iron homeostasis. Hepcidin transcription is upregulated in response to iron overload and downregulated in response to iron deficiency[37]. In conditions with iron overload, hepcidin downregulates the ferroportin-mediated release of iron from enterocytes and macrophages to the blood[36]. Except for inherited defects of ferroportin and hepcidin itself, all forms of iron-storage disease appear to arise from hepcidin dysregulation. Hepcidin is regulated by various proteins, such as HFE, hemojuvelin, tranferrin receptor 2, bone morphogenic receptor and its ligand bone morphogenic protein[38]. Today we know, that hemochromatosis may arise due to mutations in the genes of *HFE*, hemojuvelin, hepcidin, transferrin receptor 2, and ferroportin[3, 36] (Table 1).

The role of gender in the regulation of human hepcidin gene expression in the liver is unclear[267]. The causes of iron overload – genetic, lifestyle or endogenous (menopause) – may be different between the two sexes[268], but the final pathway may be common, through oxidative stress and downregulation of hepcidin leading to unlimited iron influx to the plasma from duodenal cells. This may also explain the increased risk of liver cancer in both women and men with elevated levels of transferrin saturation. There might, however, be different pathways leading to cancer in individuals with high iron stores in general and specifically in individuals with hemochromatis genotype, explaining the gender differences. It could be speculated that just after the menopause in some women, the sudden rise in serum iron concentration (because of the lack of the physiological premenopausal blood loss) may mimic a mild iron poisoning with sudden and rapid oxidative stress on the liver resulting in down regulation of hepcidin[269] and leading to an uncontrolled iron overload. Moreover, there is evidence that more women (in any age group) than men take dietary supplements[270] including iron supplements, despite the fact that those taking supplements appear to be least likely to need them because of a more balanced diet[270]; thus, the postmenopausal iron overload may be even more exaggerated.

Taken together, hepcidin is the main regulator of iron homeostasis, with the HFE protein being one of several proteins regulating hepcidin. Thus, defects in the HFE protein arising from the C282Y mutation may result in dysregulated iron homeostasis leading to iron overload.

Penetrance

Penetrance of a genetic disease is the proportion of individuals carrying a specific variation of a gene who also express an associated phenotype, i.e. exhibit clinical symptoms[271]. The definition of nonpenetrance depends upon the accuracy of the clinical phenotype analysis[271]. Penetrance of hereditary hemochromatosis can be difficult to estimate reliably, since the onset of symptoms is age-related and the symptoms may not yet have appeared at the time of examination. In *HFE*-related hereditary hemochromatosis, the penetrance is generally sought of as incomplete or reduced[23, 24, 33, 35, 247, 272-281], but the exact percentage is debated, with estimates ranging from less than 1%[23] to almost 30%[24], with the studies agreeing that the penetrance is lower in women because women bleed regularly before the menopause[17] and higher in men, who don't have biological phlebotomy.

The debate about the penetrance of hereditary hemochromatosis is still ongoing, and apart from one study[33] estimating life-time iron overload progression rate, none of them[23, 24, 33, 247, 272-281] estimated life-time penetrance or covered the whole clinical hemochromatosis spectrum. The life-time penetrance of hereditary hemochromatosis[33] was estimated in CCHS, but the study was prone to ascertainment bias, in the direction that individuals at-

tending the study were those alive at a follow-up from a previous study; furthermore, this study also did not cover the whole hemochromatosis spectrum. The discrepancies for the percentage of penetrance between the studies may be due to discrepancies between the studies in geographical location, study design, the population (ethnicity, age, gender, percentage of attendance), awareness of disease, study period, and statistical methods. Thus, the studies are not homogeneous and comparisons are difficult.

Most importantly in the penetrance discussion is that there is no consensus definition to date on what constitutes all aspects of the clinical hemochromatosis phenotype; thus, how can the penetrance be measured, when this question is still unsolved? Are hypertension[11, 107] and extrahepatic cancers[12, 282, 283], as indicated in this thesis, as well as general fatigue[112, 147, 284, 285] also part of the hemochromatosis spectrum?

Is it ethically sound that a genetic disease is only penetrant when it has become clinically manifest, when we in hereditary hemochromatosis are in fact able to measure two intermediary variables (transferrin saturation and ferritin), which mark increasing risk, before disease becomes manifest and irreversible? Should biochemical iron overload without clinical symptoms also be regarded as a phenotype? Compare the situation with intermediary phenotypes such as increased blood pressure or cholesterol; hundreds of thousands of middle-aged and elderly Danes receive antihypertensive or cholesterol lowering treatment, before these conditions develop into severe heart disease. Why should iron overload not also be treated before clinically manifest disease? Intervention studies are needed to answer this question.

Expressivity

Expressivity is the term that describes the differences observed in the clinical phenotype between two individuals with the same genotype. Penetrance and expressivity are two concepts in genetics that are different, but related. It is not possible to measure the degree of expression of a phenotype if the genotype is not expressed as a phenotype. But what constitutes the phenotype, then? The iron overload or the clinical symptoms? Mutations in the same gene may cause different clinical symptoms and different groups of expressivity may be distinguished[271]. The same genotype may present differences in either the severity of the same clinical phenotype or differences in the clinical presentation. In e.g. Marfan syndrome[286], some people have only mild symptoms such as being tall and having long slender fingers, whereas others have overt disease with life-threatening complications involving the heart and blood vessels. The features of this syndrome are highly variable, although most patients with the disorder carry a mutation in the same gene[286]. Thus, could hereditary hemochromatosis be compared with this syndrome? And should we regard any slightly elevated biochemical iron overload as a phenotype, and thus not talk about the penetrance, but about the expressivity of the disease, accepting that hereditary hemochromatosis is not an all or none phenomenon, but rather a disease continuum? This question has not yet been debated in the literature. As mentioned above, results from our hypertension and mortality studies suggested such a continuum[11, 13]. A recent meta-analysis[22] showed that homozygosity for C282Y/C282Y confers a very high risk for both provisional (elevated serum iron markers) and documented iron overload (elevated serum iron markers associated with evidence of iron excess based on liver biopsy and/or quantitative phlebotomy); the odds ratio for elevated transferrin saturation alone (transferrin saturation >50%) was 614 (95% CI: 12-32000) and the odds ratios for elevated serum ferritin alone (concentration thresholds varying across studies) was 69 (7-684) for provisional iron overload and even higher odd ratios for documented iron overload. Evidence from the CCHS on iron overload progression rate possibly suffered from ascertainment bias, and is not firm evidence against life-time penetrance; furthermore, only 22 patients were studied[33].

The combined influence of environmental and genetic factors may lead to the differences in expressivity[271] and penetrance[271, 287]. Lifestyle factors such as high intake of red meat[255, 258], oral iron supplementations[255-257, 288], and alcohol[254-256] and biological factors such as age

and male sex increase concentrations of iron parameters[255] and may thus potentially be factors that can increase risk of clinical symptoms. On the other hand, blood donations and female sex pre-menopausally with periodically bleeding may decrease risk even further[255, 259, 260]. Furthermore, the hemochromatosis genes[289], and in combination with other genes predisposing to the diseases in the hemochromatosis spectrum, may potentially also increase risk of clinical symptoms. Genetic modifiers[290] and common variants in the hepcidin pathway[291] have been shown to modulate symptomatic expression of *HFE*-associated hemochromatosis, leading to a more severe phenotypic expression of the *HFE* gene. However, the additional risks added by these modifiers are unknown. In light of the combined effects of genetics, environment and lifestyle, and other biological factors may hereditary hemochromatosis then be considered a complex disease, and not just merely a simple Mendelian disease?

In the studies in this thesis, risks of hypertension[11] and cancer[12] according to C282Y/C282Y were increased in men but not in women, and risks of any diabetes and subtypes of diabetes[10], hypertension[11], and total mortality[13] according to increased transferrin saturation were almost equal in men and women. However, for diabetes and hypertension, there are no previous studies alike for comparison, and a previous study of total mortality[15] did not stratify on sex.

Furthermore, hereditary hemochromatosis may be caused by 5 genes[3] with some mutations responsible for a more severe phenotype (Type 2A and Type 2B), whereas other mutations (Type 1, Type 3 and Type 4) in comparison lead to a milder phenotype[3, 271].

In conclusion, it is quite possible that the mechanisms behind penetrance and expressivity are similar; however, the distinction between the two phenomena is still important for genetic counseling. It is important for the patients with mild iron overload to know, whether this condition potentially could progress to severe iron overload with clinical symptoms (and thus the condition is a genetic disease with variable expressivity), or whether they can walk out of the consultation happily knowing that this will never progress or be severe (and thus the condition is a genetic disease with low penetrance). Thus, in light of the above discussion for penetrance and expressivity, I believe, that hereditary hemochromatosis is best regarded as a genetic disease with variable disease expressivity.

Future perspectives and unresolved questions

Future research is needed to decide whether hereditary hemochromatosis is a genetic disease with low penetrance or variable expressivity, whether iron overload is an all or none phenomenon with a diagnostic cut-off value or rather a disease continuum with a dose-response relationship, and whether hereditary hemochromatosis should be regarded as a complex disease with gene-environment interactions rather than a simple Mendelian disease.

A health-economic analysis of the cost benefit ratio of screening for elevated transferrin saturation in the general population and in disease specific populations (diabetes, hypertension, cancer) may help to determine the public health consequences of these studies, e.g. advice against iron supplements.

It has been shown in many studies that phlebotomy and iron chelation improve outcomes in patients with primary and secondary hemochromatosis, respectively[292]. Some few intervention studies with phlebotomy or iron chelation have been initiated in patients that do not suffer from primary or secondary hemochromatosis, and some observational studies have been conducted in blood donors on risk of surrogate cardiovascular endpoints (blood pressure, vascular function, glucose) and of hard endpoints as cardiovascular disease, total and cause-specific mortality, and cancer. The intervention studies have shown improvements in vascular function[94] and insulin sensitivity[244], and decreases in cholesterol[293, 294], HbA1c[94], urinary mean albumin/creatinine ratio[295] and blood pressure[294] in patients with type 2 diabetes or metabolic syndrome. The intervention studies have also shown reduced total mortality (in the age-group 43-61)[296] and reduced fatal[131] and non-fatal cancer[131] in pa-

tients with peripheral arterial disease. Observational studies in blood donors vs. non-donors have also found increased insulin sensitivity[297] and enhanced vascular function[298], whereas risk of cardiovascular disease is not associated, and risk of cancer is disputed[132, 299, 300]. Though few and usually small, these intervention studies clearly show a beneficial effect on the surrogate endpoints and hard endpoints that correspond well with the findings in this thesis. However, even larger multicenter studies are needed in order for these phlebotomy or chelation therapies to be instituted as clinical guidelines.

Further research is needed to understand the role of oxidative stress in hemochromatosis. We have shown that iron overload predicts total mortality in the general population and in patients with diabetes. Oxidative stress due to the Fenton reaction in hemochromatosis has been an a priori known hypothesis for most of the hemochromatosis studies conducted in the past[261]. But not until recently was it shown for patients with hemochromatosis that urinary excreted oxidized RNA[40] was increased, and for lipidemic men that iron contributes to strand-breaks in the DNA[301]. Oxidized RNA has been shown to associate with many chronic diseases[302] and decreased survival in patients with diabetes[303]. Thus is seems reasonable to believe that increased risk of premature death associated with iron overload could be mediated by oxidized RNA. Thus, we have collected 4000 urine samples in a general population study (The General Suburban Population Study (GESUS))[193], and these samples will be the basis for a research study of the association between observational and genetic iron overload and increased RNA oxidation in the general population, and whether normal and modified levels of iron from phlebotomy (blood donors) and menopause are associated with the level of RNA oxidation. Furthermore, we want to investigate if premature death associated with iron overload could be mediated by oxidized RNA.

Summary in English

Hereditary hemochromatosis is an iron-overload disorder characterized by iron accumulation throughout life in various organs, such as the endocrine pancreas, heart, liver, pituitary gland, joints, and skin. This thesis is based on elevated transferrin saturation and the autosomal recessive form of hereditary hemochromatosis with homozygosity for *HFE* C282Y. We estimated risks of diabetes, hypertension, cancer and total mortality according to elevated transferrin saturation or homozygosity for C282Y in two general population studies and in specific patient cohorts with diabetes or hypertension. Risk of diabetes, hypertension, cancer and total mortality were increased for individuals with elevated transferrin saturation or individuals homozygous for C282Y. Also, increased transferrin saturation in patients with late-onset type 1 or type 2 diabetes is associated with decreased survival.

Summary in Danish

Hereditær hæmokromatose er en sygdom med livslang jern-ophobning i organer som endokrine pancreas, hjertet, leveren, hypofysen, led, og hud. Denne afhandling er baseret på forhøjet transferrin mætning og den autosomale recessive form af hereditær hæmokromatose med homozygoti for C282Y i *HFE* genet. Vi estimerede risici for diabetes, hypertension, cancer, og total mortalitet i henhold til forhøjet transferrin mætning eller homozygoti for C282Y i to generelle populations-studier og specifikke patient-cohorter med diabetes eller hypertension. Risiko for diabetes, hypertension, cancer og total mortalitet var øget hos individer med forhøjet transferrin mætning eller homozygoti for C282Y. Øget transferrin mætning hos patienter med sent debuterende type 1 diabetes eller type 2 diabetes er associeret med nedsat overlevelse.

Abbreviation list

ADA: American Diabetes Association
BMI: Body mass index
CCHS: Copenhagen City Heart Study
CGPS: Copenhagen General Population Study
WHO: World Health Organisation
ICD: International Classification of Diagnoses
HDL: High-density lipoprotein
LIFEGEN: Losartan Intervention for End-Point Reduction in Hypertension Genetic Substudy
LVH: Left ventricular hypertrophy
NDCDR: The National Danish Causes of Death Registry
NPR: The National Danish Patient Registry (NPR)

References

(1) Adams P, Barton JC, McLaren GD et al. Screening for iron overload: lessons from the he mochromatosis and iron overload screening (HEIRS) study. Can J Gastroenterol 2009 November;23(11):769-72.

(2) EASL clinical practice guidelines for HFE hemochromatosis. J Hepatol 2010 July;53(1): 3-22.

(3) Pietrangelo A. Hereditary hemochromatosis--a new look at an old disease. N Engl J Med 2004 June 3;350(23):2383-97.

(4) Merryweather-Clarke AT, Pointon JJ, Shearman JD, Robson KJ. Global prevalence of putative haemochromatosis mutations. J Med Genet 1997 April;34(4):275-8.

(5) Ellervik C, Tybjaerg-Hansen A, Grande P, Appleyard M, Nordestgaard BG. Hereditary hemochromatosis and risk of ischemic heart disease: a prospective study and a case-control study. Circulation 2005 July 12;112(2):185-93.

(6) Ellervik C, Tybjaerg-Hansen A, Appleyard M, Sillesen H, Boysen G, Nordestgaard BG. Hereditary hemochromatosis genotypes and risk of ischemic stroke. Neurology 2007 March 27;68(13):1025-31.

(7) Ellervik C, Birgens H, Tybjaerg-Hansen A, Nordestgaard BG. Hemochromatosis genotypes and risk of 31 disease endpoints: meta-analyses including 66,000 cases and 226,000 controls. Hepatology 2007 October;46(4):1071-80.

(8) Ellervik C, Mandrup-Poulsen T, Nordestgaard BG et al. Prevalence of hereditary haemochromatosis in late-onset type 1 diabetes mellitus: a retrospective study. Lancet 2001 October 27;358(9291):1405-9.

(9) Ellervik C, Andersen HU, Tybjærg Hansen A et al. Total mortality by elevated transferrin saturation in patients with diabetes. Diabetes Care 2013;36(9):2646-54.

(10) Ellervik C, Mandrup-Poulsen T, Andersen HU et al. Elevated Transferrin Saturation and Risk of Diabetes: Three population-based studies. Diabetes Care 2011 October; 34(10):2256-8.

(11) Ellervik C, Tybjaerg-Hansen A, Appleyard M, Ibsen H, Nordestgaard BG. Haemochromatosis genotype and iron overload: association with hypertension and left ventricular hypertrophy. J Intern Med 2010 September;268(3):252-64.

(12) Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Risk of cancer by transferrin saturation levels and haemochromatosis genotype: population-based study and meta-analysis. J Intern Med 2011 May 23;271:51-63.

(13) Ellervik C, Tybjaerg-Hansen A, Nordestgaard BG. Total mortality by transferrin saturation levels: two general population studies and a metaanalysis. Clin Chem 2011 March;57(3):459-66.

(14) Ellervik C, Mandrup-Poulsen T, Tybjaerg-Hansen A, Nordestgaard BG. Total and cause specific mortality by elevated transferrin saturation and hemochromatosis genotype in individuals with diabetes - two general population studies. Diabetes Care 2014; 37(2): 444-452.

(15) Mainous AG, III, Gill JM, Carek PJ. Elevated serum transferrin saturation and mor= tality. Ann Fam Med 2004 March;2(2):133-8.

(16) Davis TM, Beilby J, Davis WA et al. Prevalence, characteristics, and prognostic signifcance of HFE gene mutations in type 2 diabetes: the Fremantle Diabetes Study. Diabetes Care 2008 September;31(9):1795-801.

(17) Beutler E, Bothwell TH, Charlton RW, Motulsky AG. Hereditary hemochromatosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Bases of Inherited Disease.New York, NY: McGraw-Hill; 2001. p. 3127-62.

(18) Shander A, Sazama K. Clinical consequences of iron overload from chronic red blood cell transfusions, its diagnosis, and its management by chelation therapy. Transfusion 2010 May;50(5):1144-55.

(19) Cunningham MJ, Macklin EA, Neufeld EJ, Cohen AR. Complications of betathalassemia major in North America. Blood 2004 July 1;104(1):34-9.

(20) Feder JN, Gnirke A, Thomas W et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996 Agust;13(4):399-408.

(21) Beutler E. Hemochromatosis: genetics and pathophysiology. Annu Rev Med 2006; 57: 331-47.

(22) Neghina AM, Anghel A. Hemochromatosis genotypes and risk of iron overload -a metaanalysis. Ann Epidemiol 2011 January;21(1):1-14.

(23) Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G--> A (C282Y) *HFE* hereditary haemochromatosis mutation in the USA. Lancet 2002 January 19;359 (9302): 211-8.

(24) Allen KJ, Gurrin LC, Constantine CC et al. Iron-overload-related disease in HFE hereditary hemochromatosis. N Engl J Med 2008 January 17;358(3):221-30.

(25) Distante S, Robson KJ, Graham-Campbell J, Arnaiz-Villena A, Brissot P, Worwood M. The origin and spread of the HFE-C282Y haemochromatosis mutation. Hum Genet 2004 September;115(4):269-79.

(26) Lucotte G, Dieterlen F. A European allele map of the C282Y mutation of hemochromatosis: Celtic versus Viking origin of the mutation? Blood Cells Mol Dis 2003 September;31(2):262-7.

(27) Ropero P, Briceno O, Mateo M et al. Frequency of the C282Y and H63D mutations of the hemochromatosis gene (HFE) in a cohort of 1,000 neonates in Madrid (Spain). Ann Hematol 2006 March 7.

(28) Milman N, Pedersen P. Evidence that the Cys282Tyr mutation of the *HFE* gene originated from a population in Southern Scandinavia and spread with the Vikings. Clin Genet 2003 July;64(1):36-47.

(29) Whittington CA. Was the C282Y mutation an Irish Gaelic mutation that the Vikings help disseminate? Med Hypotheses 2006;67(6):1270-3.

(30) Datz C, Haas T, Rinner H, Sandhofer 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? Clin Chem 1998 December;44(12):2429-32.

(31) Adams PC, Reboussin DM, Barton JC et al. Hemochromatosis and iron-overload screening in a racially diverse population. N Engl J Med 2005 April 28;352(17): 1769-78.

(32) 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 September 2;341(10):718-24.

(33) Andersen RV, Tybjaerg-Hansen A, Appleyard M, Birgens H, Nordestgaard BG. Hemochromatosis mutations in the general population: iron overload progression rate. Blood 2004 April 15;103(8):2914-9.

(34) Delatycki MB, Allen KJ, Nisselle AE et al. Use of community genetic screening to prevent HFE-associated hereditary haemochromatosis. Lancet 2005 July 23; 366(9482):314-6.

(35) Waalen J, Nordestgaard BG, Beutler E. The penetrance of hereditary hemochromatosis. Best Pract Res Clin Haematol 2005 June;18(2):203-20.

(36) Andrews NC. Forging a field: the golden age of iron biology. Blood 2008 July 15; 112(2):219-30.

(37) Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 2003 August 1;102(3):783-8.

(38) Lee PL, Beutler E. Regulation of hepcidin and iron-overload disease. Annu Rev Pathol 2009;4:489-515.

(39) Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology 2011 May 10;283(2-3):65-87.

(40) Broedbaek K, Poulsen HE, Weimann A et al. Urinary excretion of biomarkers of oxidatively damaged DNA and RNA in hereditary hemochromatosis. Free Radic Biol Med 2009 October 15;47(8):1230-3.

(41) Adams PC. Population screening for hemochromatosis. Hepatology 1999 April;29(4):1324-7.

(42) Powell LW, Summers KM, Board PG, Axelsen E, Webb S, Halliday JW. Expression of hemochromatosis in homozygous subjects. Implications for early diagnosis and prevention. Gastroenterology 1990 June;98(6):1625-32.

(43) Hansen JB, Tonnesen MF, Madsen AN et al. Divalent metal transporter 1 regulates iron-mediated ROS and pancreatic beta cell fate in response to cytokines. Cell Metab 2012 October 3;16(4):449-61.

(44) Cooksey RC, Jouihan HA, Ajioka RS et al. Oxidative stress, beta-cell apoptosis, and decreased insulin secretory capacity in mouse models of hemochromatosis. Endocrinology 2004 November;145(11):5305-12.

(45) Huang J, Jones D, Luo B et al. Iron overload and diabetes risk: a shift from glucose to Fatty Acid oxidation and increased hepatic glucose production in a mouse model of hereditary hemochromatosis. Diabetes 2011 January;60(1):80-7.

(46) Cooksey RC, Jones D, Gabrielsen S et al. Dietary iron restriction or iron chelation protects from diabetes and loss of beta-cell function in the obese (ob/ob lep-/-) mouse. Am J Physiol Endocrinol Metab 2010 June;298(6):E1236-E1243.

(47) Oberley LW. Free radicals and diabetes. Free Radic Biol Med 1988;5(2):113-24.

(48) Simcox JA, McClain DA. Iron and diabetes risk. Cell Metab 2013 March 5;17(3):329-41.

(49) Rahier J, Loozen S, Goebbels RM, Abrahem M. The haemochromatotic human pancreas: a quantitative immunohistochemical and ultrastructural study. Diabetologia 1987 January;30(1):5-12.

(50) Hartroft WS. Islet pathology in diabetes. Diabetes 1956 March;5(2):98-104.

(51) Lu JP, Hayashi K. Selective iron deposition in pancreatic islet B cells of transfusional iron-overloaded autopsy cases. Pathol Int 1994 March;44(3):194-9.

(52) Kishimoto M, Endo H, Hagiwara S, Miwa A, Noda M. Immunohistochemical findings in the pancreatic islets of a patient with transfusional iron overload and diabetes: case report. J Med Invest 2010 August;57(3-4):345-9.

(53) Bell ET. The relation of portal cirrhosis to hemochromatosis and to diabetes mellitus. Diabetes 1955 November;4(6):435-46.

(54) Ellervik C, Birgens H, Mandrup-Poulsen T. Need for Reclassification of Diabetes Secondary to Iron Overload in the ADA and WHO Classifications. Diabetes Care 2014 June;37(6):e137-e138.

(55) McGavran M.H., Hartroft WS. The predilection of pancreatic beta cells for pigment deposition in hemochromatosis and hemosiderosis. Am J Pathol 1956;32:63 (abstract).

(56) Hatunic M, Finucane FM, Brennan AM, Norris S, Pacini G, Nolan JJ. Effect of iron overload on glucose metabolism in patients with hereditary hemochromatosis. Metabolism 2010 March;59(3):380-4.

(57) Messina MF, Lombardo F, Meo A et al. Three-year prospective evaluation of glucose tolerance, beta-cell function and peripheral insulin sensitivity in non-diabetic patients with thalassemia major. J Endocrinol Invest 2002 June;25(6):497-501.

(58) Dmochowski K, Finegood DT, Francombe W, Tyler B, Zinman B. Factors determining glucose tolerance in patients with thalassemia major. J Clin Endocrinol Metab 1993 August;77(2):478-83.

(59) Merkel PA, Simonson DC, Amiel SA et al. Insulin resistance and hyperinsulinemia in patients with thalassemia major treated by hypertransfusion. N Engl J Med 1988 March 31;318(13):809-14.

(60) Cario H, Holl RW, Debatin KM, Kohne E. Insulin sensitivity and beta-cell secretion in thalassaemia major with secondary haemochromatosis: assessment by oral glucose tolerance test. Eur J Pediatr 2003 March;162(3):139-46.

(61) McClain DA, Abraham D, Rogers J et al. High prevalence of abnormal glucose homeostasis secondary to decreased insulin secretion in individuals with hereditary haemochromatosis. Diabetologia 2006 March 15.

(62) Behn PS, Glaser B, Permutt MA. Contribution of the C282Y Hereditary Hemochromatosis Mutation to Type 2 Diabetes Mellitus in Ashkenazi Jews. Diabetes 47 [Suppl.1], 1523. 1998. Ref Type: Abstract

(63) Braun J, Donner H, Plock K, Rau H, Usadel KH, Badenhoop K. Hereditary haemochromatosis mutations (HFE) in patients with Type II diabetes mellitus. Diabetologia 1998 August;41(8):983-4.

(64) Campo S, Restuccia T, Villari D et al. Analysis of haemochromatosis gene mutations in a population from the Mediterranean Basin. Liver 2001 August;21(4):233-6.

(65) 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 March;22(3):525-6.

(66) Florkowski CM, George PM, Willis JA et al. Haemochromatosis gene mutations Cys282Tyr and His63Asp are not increased in Type 2 diabetic patients compared with the Canterbury (New Zealand) general population. Diabetes Res Clin Pract 1999 March;43(3):199-203.

(67) Frayling T, Ellard S, Grove J, Walker M, Hattersley AT. C282Y mutation in HFE (haemochromatosis) gene and type 2 diabetes. Lancet 1998 June 27;351(9120): 1933-4.

(68) Habeos IG, Psyrogiannis A, Kyriazopoulou V, Psilopanagiotou A, Papavassiliou AG, Vagenakis AG. The role of Hemochromatosis C282Y and H63D mutations in the development of type 2 diabetes mellitus in Greece. Hormones (Athens) 2003 January;2(1):55-60.

(69) Halsall DJ, McFarlane I, Luan J, Cox TM, Wareham NJ. Typical type 2 diabetes mellitus and HFE gene mutations: a population-based case - control study. Hum Mol Genet 2003 June 15;12(12):1361-5.

(70) Hegele RA, Harris SB, Zinman B. Hemochromatosis and diabetes mellitus. Ann Intern Med 1998 October 1;129(7):587.

(71) Kankova K, Jansen EH, Marova I et al. Relations among serum ferritin, C282Y and H63D mutations in the HFE gene and type 2 diabetes mellitus in the Czech population. Exp Clin Endocrinol Diabetes 2002 August;110(5):223-9.

(72) Malecki MT, Klupa T, Walus M, Czogala W, Greenlaw P, Sieradzki J. A search for association between hereditary hemochromatosis HFE gene mutations and type 2 diabetes mellitus in a Polish population. Med Sci Monit 2003 February;9(2): BR91-BR95.

(73) 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 July;24(7):1187-91.

(74) Njajou OT, Alizadeh BZ, Vaessen N et al. The role of hemochromatosis C282Y and H63D gene mutations in type 2 diabetes: findings from the Rotterdam Study and meta-analysis. Diabetes Care 2002 November;25(11):2112-3.

(75) Qi L, Meigs J, Manson JE et al. HFE Genetic Variability, Body Iron Stores, and the Risk of Type 2 Diabetes in U.S. Women. Diabetes 2005 December;54(12):3567-72.

(76) Salonen JT, Tuomainen TP, Kontula K. Role of C282Y mutation in haemochromatosis gene in development of type 2 diabetes in healthy men: prospective cohort study. BMJ 2000 June 24;320(7251):1706-7.

(77) Sampson MJ, Williams T, Heyburn PJ et al. Prevalence of HFE (hemochromatosis gene) mutations in unselected male patients with type 2 diabetes. J Lab Clin Med 2000 February;135(2):170-3.

(78) Acton RT, Barton JC, Bell DS, Go RC, Roseman JM. HFE mutations in African-American women with non-insulin-dependent diabetes mellitus. Ethn Dis 2001; 11(4):578-84.

(79) Dubois-Laforgue D, Caillat-Zucman S, Djilali-Saiah I et al. Mutations in HFE, the hemochromatosis candidate gene, in patients with NIDDM. Diabetes Care 1998 August;21(8):1371-2.

(80) Rasmussen S, Echwald S, Hansen L et al. Analysis of the relationship between a common Cys282Tyr variant of the hereditary haemochromatosis gene and NIDDM. Diabetologia 40[S1], 653. 1997.
Ref Type: Abstract

(81) Cauza E, Hanusch-Enserer U, Bischof M et al. Increased C282Y heterozygosity in gestational diabetes. Fetal Diagn Ther 2005 September;20(5):349-54.

(82) Cadet E, Capron D, Perez AS et al. A targeted approach significantly increases the identification rate of patients with undiagnosed haemochromatosis. J Intern Med 2003 February;253(2):217-24.

(83) Kunutsor SK, Apekey TA, Walley J, Kain K. Ferritin levels and risk of type 2 diabetes mellitus: an updated systematic review and meta-analysis of prospective evidence. Diabetes Metab Res Rev 2013 May;29(4):308-18.

(84) Bao W, Rong Y, Rong S, Liu L. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. BMC Med 2012 October 10;10(1):119.

(85) Zhao Z, Li S, Liu G et al. Body iron stores and heme-iron intake in relation to risk of type 2 diabetes: a systematic review and meta-analysis. PLoS One 2012;7(7):e41641.

(86) Acton RT, Barton JC, Passmore LV et al. Relationships of serum ferritin, transferrin saturation, and HFE mutations and self-reported diabetes in the Hemochromatosis and Iron Overload Screening (HEIRS) study. Diabetes Care 2006 September; 29(9): 2084-9.

(87) Tuomainen TP, Nyyssonen K, Salonen R et al. Body iron stores are associated with serum insulin and blood glucose concentrations. Population study in 1,013 eastern Finnish men. Diabetes Care 1997 March;20(3):426-8.

(88) Jiang R, Manson JE, Meigs JB, Ma J, Rifai N, Hu FB. Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. JAMA 2004 February 11;291(6): 711-7.

(89) Jehn M, Clark JM, Guallar E. Serum ferritin and risk of the metabolic syndrome in U.S. adults. Diabetes Care 2004 October;27(10):2422-8.

(90) Ford ES, Cogswell ME. Diabetes and serum ferritin concentration among U.S. adults. Diabetes Care 1999 December;22(12):1978-83.

(91) Park SK, Ryoo JH, Kim MG, Shin JY. Association of serum ferritin and the develop ment of metabolic syndrome in middle-aged Korean men: a 5-year follow-up study. Diabetes Care 2012 December;35(12):2521-6.

(92) Abraham D, Rogers J, Gault P, Kushner JP, McClain DA. Increased insulin secretory capacity but decreased insulin sensitivity after correction of iron overload by phlebotomy in hereditary haemochromatosis. Diabetologia 2006 November;49(11): 2546-51.

(93) Hatunic M, Finucane FM, Norris S, Pacini G, Nolan JJ. Glucose metabolism after normalization of markers of iron overload by venesection in subjects with heredi-tary hemochromatosis. Metabolism 2010 July 30;59(12):1811-5.

(94) Fernandez-Real JM, Penarroja G, Castro A, Garcia-Bragado F, Lopez-Bermejo A, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on vascular reactivity. Diabetes Care 2002 December;25(12):2249-55.

(95) Hramiak IM, Finegood DT, Adams PC. Factors affecting glucose tolerance in hereditary hemochromatosis. Clin Invest Med 1997 April;20(2):110-8.

(96) Thomas MC, MacIsaac RJ, Tsalamandris C, Jerums G. Elevated iron indices in patients with diabetes. Diabet Med 2004 July;21(7):798-802.

(97) Mainous AG, III, King DE, Pearson WS, Garr DR. Is an elevated serum transferrin saturation associated with the development of diabetes? J Fam Pract 2002 November;51(11):933-6.

(98) Shizukuda Y, Bolan CD, Tripodi DJ et al. Significance of left atrial contractile function in asymptomatic subjects with hereditary hemochromatosis. Am J Cardiol 2006 October 1;98(7):954-9.

(99) Shizukuda Y, Bolan CD, Tripodi DJ et al. Does oxidative stress modulate left ventricular diastolic function in asymptomatic subjects with hereditary hemochromatosis? Echocardiography 2009 November;26(10):1153-8.

(100) Shizukuda Y, Bolan CD, Tripodi DJ et al. Left ventricular systolic function during stress echocardiography exercise in subjects with asymptomatic hereditary hemochromatosis. Am J Cardiol 2006 September 1;98(5):694-8.

(101) Click RL, Olson LJ, Edwards WD et al. Echocardiography and systemic diseases. J Am Soc Echocardiogr 1994 March;7(2):201-16.

(102) Summary of the 2007 European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines for the management of arterial hypertension. Vasc Health Risk Manag 2007;3(6):783-95.

(103) Cecchetti G, Binda A, Piperno A, Nador F, Fargion S, Fiorelli G. Cardiac alterations in 36 consecutive patients with idiopathic haemochromatosis: polygraphic and echocardiographic evaluation. Eur Heart J 1991 February;12(2):224-30.

(104) Cai H. Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. Cardiovasc Res 2005 October 1;68(1):26-36.

(105) Schulz E, Gori T, Munzel T. Oxidative stress and endothelial dysfunction in hypertension. Hypertens Res 2011 June;34(6):665-73.

(106) Failla M, Giannattasio C, Piperno A et al. Radial artery wall alterations in genetic hemochromatosis before and after iron depletion therapy. Hepatology 2000 September;32(3):569-73.

(107) Piperno A, Trombini P, Gelosa M et al. Increased serum ferritin is common in men with essential hypertension. J Hypertens 2002 August;20(8):1513-8.

(108) Danesh J, Appleby P. Coronary heart disease and iron status: meta-analyses of prospective studies. Circulation 1999 February 23;99(7):852-4.

(109) van der AD, Rovers MM, Grobbee DE et al. Mutations in the HFE Gene and Cardiovascular Disease Risk. An Individual Patient Data Meta-Analysis of 53 880 Subjects. Circulation - Cardiovascular Genetics 2008;1(1):43-50.

(110) Yalcinkaya S, Kumbasar SD, Semiz E, Tosun Z, Paksoy N. Sustained ventricular tachycardia in cardiac hemochromatosis treated with amiodarone. J Electrocardiol 1997 April;30(2):147-9.

(111) Demant AW, Schmiedel A, Buttner R, Lewalter T, Reichel C. Heart failure and malignant ventricular tachyarrhythmias due to hereditary hemochromatosis with iron overload cardiomyopathy. Clin Res Cardiol 2007 December;96(12):900-3.

(112) Waalen J, Felitti V, Gelbart T, Ho NJ, Beutler E. Prevalence of hemochromatosis-related symptoms among individuals with mutations in the HFE gene. Mayo Clin Proc 2002 June;77(6):522-30.

(113) Shizukuda Y, Tripodi DJ, Zalos G et al. Incidence of cardiac arrhythmias in asymptomatic hereditary hemochromatosis subjects with C282Y homozygosity. Am J Cardiol 2012 March 15;109(6):856-60.

(114) Rosenqvist M, Hultcrantz R. Prevalence of a haemochromatosis among men with clinically significant bradyarrhythmias. Eur Heart J 1989 May;10(5):473-8.

(115) Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006 March 10;160(1):1-40.

(116) Weinberg ED. The role of iron in cancer. Eur J Cancer Prev 1996 February;5(1):19-36.

(117) Le NT, Richardson DR. The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim Biophys Acta 2002 October 2;1603(1):31-46.

(118) Mainous AG, III, Wells BJ, Koopman RJ, Everett CJ, Gill JM. Iron, lipids, and risk of cancer in the Framingham Offspring cohort. Am J Epidemiol 2005 June 15;161(12):1115-22.

(119) Mainous AG, III, Gill JM, Everett CJ. Transferrin saturation, dietary iron intake, and risk of cancer. Ann Fam Med 2005 March;3(2):131-7.

(120) Herrinton LJ, Friedman GD, Baer D, Selby JV. Transferrin saturation and risk of cancer. Am J Epidemiol 1995 October 1;142(7):692-8.

(121) Ioannou GN, Weiss NS, Kowdley KV. Relationship between transferrin-iron saturation, alcohol consumption, and the incidence of cirrhosis and liver cancer. Clin Gastroenterol Hepatol 2007 May;5(5):624-9.

(122) Stevens RG, Jones DY, Micozzi MS, Taylor PR. Body iron stores and the risk of cancer. N Engl J Med 1988 October 20;319(16):1047-52.

(123) Stevens RG, Graubard BI, Micozzi MS, Neriishi K, Blumberg BS. Moderate elevation of body iron level and increased risk of cancer occurrence and death. Int J Cancer 1994 February 1;56(3):364-9.

(124) Wu T, Sempos CT, Freudenheim JL, Muti P, Smit E. Serum iron, copper and zinc concentrations and risk of cancer mortality in US adults. Ann Epidemiol 2004 March;14(3):195-201.

(125) Wurzelmann JI, Silver A, Schreinemachers DM, Sandler RS, Everson RB. Iron intake and the risk of colorectal cancer. Cancer Epidemiol Biomarkers Prev 1996 July; 5(7):503-7.

(126) van Asperen I, Feskens EJ, Bowles CH, Kromhout D. Body iron stores and mortality due to cancer and ischaemic heart disease: a 17-year follow-up study of elderly men and women. Int J Epidemiol 1995 August;24(4):665-70.

(127) Kato I, Dnistrian AM, Schwartz M et al. Iron intake, body iron stores and colorectal cancer risk in women: a nested case-control study. Int J Cancer 1999 March 1;80(5):693-8.

(128) Knekt P, Reunanen A, Takkunen H, Aromaa A, Heliovaara M, Hakulinen T. Body iron stores and risk of cancer. Int J Cancer 1994 February 1;56(3):379-82.

(129) Cross AJ, Gunter MJ, Wood RJ et al. Iron and colorectal cancer risk in the alpha-tocopherol, beta-carotene cancer prevention study. Int J Cancer 2006 June 15; 118(12):3147-52.

(130) Nelson RL. Iron and colorectal cancer risk: human studies. Nutr Rev 2001 May;59(5):140-8.

(131) Zacharski LR, Chow BK, Howes PS et al. Decreased cancer risk after iron reduction in patients with peripheral arterial disease: results from a randomized trial. J Natl Cancer Inst 2008 July 16;100(14):996-1002.

(132) Edgren G, Reilly M, Hjalgrim H et al. Donation frequency, iron loss, and risk of cancer among blood donors. J Natl Cancer Inst 2008 April 16;100(8):572-9.

(133) Yang Q, McDonnell SM, Khoury MJ, Cono J, Parrish RG. Hemochromatosis-associated mortality in the United States from 1979 to 1992: an analysis of Multiple-Cause Mortality Data. Ann Intern Med 1998 December 1;129(11):946-53.

(134) Niederau C, Fischer R, Purschel A, Stremmel W, Haussinger D, Strohmeyer G. Long-term survival in patients with hereditary hemochromatosis. Gastro-enterology 1996 April;110(4):1107-19.

(135) Adams PC, Speechley M, Kertesz AE. Long-term survival analysis in hereditary hemochromatosis. Gastroenterology 1991 August;101(2):368-72.

(136) Milman N, Pedersen P, Steig T, Byg KE, Graudal N, Fenger K. Clinically overt hereditary hemochromatosis in Denmark 1948-1985: epidemiology, factors of significance for long-term survival, and causes of death in 179 patients. Ann Hematol 2001 December;80(12):737-44.

(137) Crooks CJ, West J, Solaymani-Dodaran M, Card TR. The epidemiology of haemo-chromatosis: a population-based study. Aliment Pharmacol Ther 2009 January 15;29(2):183-92.

(138) Bomford A, Williams R. Long term results of venesection therapy in idiopathic haemochromatosis. Q J Med 1976 October;45(180):611-23.

(139) Davis WD, Jr., ARROWSMITH WR. The effect of repeated phlebotomies in hemochromatosis; report of three cases. J Lab Clin Med 1952 April;39(4):526-32.

(140) Merryweather-Clarke AT, Pointon JJ, Jouanolle AM, Rochette J, Robson KJ. Geography of HFE C282Y and H63D mutations. Genet Test 2000;4(2):183-98.

(141) O'Sullivan EP, McDermott JH, Murphy MS, Sen S, Walsh CH. Declining prevalence of diabetes mellitus in hereditary haemochromatosis--the result of earlier diagnosis. Diabetes Res Clin Pract 2008 September;81(3):316-20.

(142) Alla V, Bonkovsky HL. Iron in nonhemochromatotic liver disorders. Semin Liver Dis 2005 November;25(4):461-72.

(143) Hernaez R, Yeung E, Clark JM, Kowdley KV, Brancati FL, Kao WH. Hemochromatosis gene and nonalcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol 2011 November;55(5):1079-85.

(144) Guggenbuhl P, Brissot P, Loreal O. Miscellaneous non-inflammatory musculoskeletal conditions. Haemochromatosis: the bone and the joint. Best Pract Res Clin Rheumatol 2011 October;25(5):649-64.

(145) Elmberg M, Hultcrantz R, Simard JF, Carlsson A, Askling J. Increased risk of arthropathies and joint replacement surgery in patients with genetic hemochromatosis: a study of 3,531 patients and their 11,794 first-degree relatives. Arthritis Care Res (Hoboken) 2013 May;65(5):678-85.

(146) McDermott JH, Walsh CH. Hypogonadism in hereditary hemochromatosis. J Clin Endocrinol Metab 2005 April;90(4):2451-5.

(147) Dever JB, Mallory MA, Mallory JE, Wallace D, Kowdley KV. Phenotypic characteristics and diagnoses of patients referred to an iron overload clinic. Dig Dis Sci 2010 March;55(3):803-7.

(148) Yeap BB, Beilin J, Shi Z et al. The C282Y polymorphism of the hereditary hemochromatosis gene is associated with increased sex hormone-binding globulin and normal testosterone levels in men. J Endocrinol Invest 2010 September;33(8):544-8.

(149) WHO. WHO technical report series no. 310: Diabetes Mellitus. Report of a WHO expert committee 1965.

(150) WHO Expert Committee on Diabetes Mellitus: second report. World Health Organ Tech Rep Ser 1980;646:1-80.

(151) WHO technical report series no. 727: Diabetes Mellitus report of a WHO study group. Genève: WHO 1985.

(152) Lohmann T, Kellner K, Verlohren HJ et al. Titre and combination of ICA and autoantibodies to glutamic acid decarboxylase discriminate two clinically distinct types of latent autoimmune diabetes in adults (LADA). Diabetologia 2001 August;44(8):1005-10.

(153) Towns R, Pietropaolo M. GAD65 autoantibodies and its role as biomarker of Type 1 diabetes and Latent Autoimmune Diabetes in Adults (LADA). Drugs Future 2011 November;36(11):847.

(154) Mandrup-Poulsen TR. [Auto-antibodies against glutamic acid decarboxylase and diabetes]. Ugeskr Laeger 2007 January 22;169(4):300-4.

(155) Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20(1183):1197.

(156) World Health Organisation. Report of a WHO consultation: Definition, diagnosis and classification of Diabetes Mellitus and its complications. WHO 1999.

(157) Shimizu M, Hirokawa M, Manabe T. Histological assessment of chronic pancreatitis at necropsy. J Clin Pathol 1996 November;49(11):913-5.

(158) Goebell H, Bode C, Lepler U, Martini GA. [Studies of exocrine pancreas function in liver cirrhosis of various etiology, hemochromatosis and portocaval shunt]. Acta Hepatosplenol 1971 November;18(6):437-52.

(159) Simon M, Gosselin M, Kerbaol M, Delanoe G, Trebaul L, Bourel M. Functional study of exocrine pancreas in idiopathic hemochromatosis, untreated and treated by venesections. Digestion 1973;8(6):485-96.

(160) Biggs J, Davis A. Relationship of diminished pancreatic secretion to haemochromatosis. Lancet 1963 October 19;186:814.

(161) Deller DJ. Iron-59 absorption measurements by whole-body counting: studies in alcoholic cirrhosis, hemochromatosis, and pancreatitis. Am J Dig Dis 1965 March; 10:249-58.

(162) Strohmeyer G, Kuni H, Borchers HH, Goebell H. [Quantitative studies on the influence of exocrine pancreas function on human iron absorption]. Dtsch Med Wochenschr 1970 October 9;95(41):2067-75.

(163) Dymock IW, Smith PM, Williams R, Godfrey BE. Iron absorption studies in idiopathic haemochromatosis. Br J Radiol 1970 April;43(508):284.

(164) Hucl T, Kylanpaa-Back ML, Witt H et al. HFE genotypes in patients with chronic pancreatitis and pancreatic adenocarcinoma. Genet Med 2007 July;9(7):479-83.

(165) Pedersen CB. The Danish Civil Registration System. Scand J Public Health 2011 July;39(7 Suppl):22-5.

(166) Lynge E, Sandegaard JL, Rebolj M. The Danish National Patient Register. Scand J Public Health 2011 July;39(7 Suppl):30-3.

(167) Gjerstorff ML. The Danish Cancer Registry. Scand J Public Health 2011 July;39 (7 Suppl):42-5.

(168) Helweg-Larsen K. The Danish Register of Causes of Death. Scand J Public Health 2011 July;39(7 Suppl):26-9.

(169) Ellervik C, Nordestgaard BG. Letter to the Editor: In reply. Clin Chem 2011;57(6): 923-4.

(170) Appleyard M, Tybjærg Hansen A, Jensen G, Schnohr P, Nyboe J. The Copenhagen City Heart Study: Østerboundersøgelsen: a book of tables with data from the first examination (1976-78) and a five year follow up (1981-1983). Scand J Soc Med Suppl 1989;41:1-160.

(171) Schnohr P, Jensen G, Lange P, Scharling H, Appleyard M. The Copenhagen City Heart Study - Tables with data from the third examination 1991-1994. Eur Heart J Suppl 2001;3:1-83.

(172) Dahlof B, Devereux R, de Faire U et al. The Losartan Intervention For Endpoint reduction (LIFE) in Hypertension study: rationale, design, and methods. The LIFE Study Group. Am J Hypertens 1997 July;10(7 Pt 1):705-13.

(173) Dahlof B, Devereux RB, Julius S et al. Characteristics of 9194 patients with left ventricular hypertrophy: the LIFE study. Losartan Intervention For Endpoint Reduction in Hypertension. Hypertension 1998 December;32(6):989-97.

(174) Dahlof B, Devereux RB, Kjeldsen SE et al. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002 March 23;359(9311):995-1003.

(175) Baty D, Terron KA, Mechan D, Harris A, Pippard MJ, Goudie D. Development of a multiplex ARMS test for mutations in the HFE gene associated with hereditary haemochromatosis. J Clin Pathol 1998 January;51(1):73-4.

(176) Cartwright GE, Edwards CQ, Kravitz K et al. Hereditary hemochromatosis. Phenotypic expression of the disease. N Engl J Med 1979 July 26;301(4):175-9.

(177) 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 May 26;318(21):1355-62.

(178) American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 2006;29, supplement 1.

(179) Kirkwood BR, Sterne JAC. Medical Statistics. 2nd ed. Blackwell Science; 2003.

(180) Khoury MJ, Beaty TH, Cohen BH. Fundamentals of Genetic Epidemiology. New York, NY, Oxford University Press; 1993.

(181) Egger M, Smith GD, Altman DG. Systematic Reviews in Health Care. BMJ Books; 2003.

(182) Huedo-Medina TB, Sanchez-Meca J, Marin-Martinez F, Botella J. Assessing heterogeneity in meta-analysis: Q statistic or I2 index? Psychol Methods 2006 June; 11(2):193-206.

(183) Royston P, Ambler G, Sauerbrei W. The use of fractional polynomials to model continuous risk variables in epidemiology. Int J Epidemiol 1999 October;28(5): 964-74.

(184) Royston P. A strategy for modelling the effect of a continuous covariate in medicine and epidemiology. Stat Med 2000 July 30;19(14):1831-47.

(185) Royston P. Choice of scale for cubic smoothing spline models in medical applications. Stat Med 2000 May 15;19(9):1191-205.

(186) MacKinnon DP, Krull JL, Lockwood CM. Equivalence of the mediation, confounding and suppression effect. Prev Sci 2000 December;1(4):173-81.

(187) Metabolic mediators of the effects of body-mass index, overweight, and obesity on coronary heart disease and stroke: a pooled analysis of 97 prospective cohorts with 1.8 million participants. Lancet 2013 November 21.

(188) Barton JC, Acton RT, Leiendecker-Foster C et al. Characteristics of participants with self-reported hemochromatosis or iron overload at HEIRS study initial screening. Am J Hematol 2008 February;83(2):126-32.

(189) Watts G. UK Biobank gets 10% response rate as it starts recruiting volunteers. BMJ 2007 March 31;334(7595):659.

(190) Hofman A, van Duijn CM, Franco OH et al. The Rotterdam Study: 2012 objectives and design update. Eur J Epidemiol 2011 August;26(8):657-86.

(191) Galea S, Tracy M. Participation rates in epidemiologic studies. Ann Epidemiol 2007 September;17(9):643-53.

(192) Drivsholm T, Eplov LF, Davidsen M et al. Representativeness in population-based studies: a detailed description of non-response in a Danish cohort study. Scand J Public Health 2006;34(6):623-31.

(193) Bergholdt HK, Bathum L, Kvetny J et al. Study design, participation and characteristics of the Danish General Suburban Population Study. Dan Med J 2013 September;60(9):A4693.

(194) Tjonneland A, Olsen A, Boll K et al. Study design, exposure variables, and socioeconomic determinants of participation in Diet, Cancer and Health: a population--based prospective cohort study of 57,053 men and women in Denmark. Scand J Public Health 2007;35(4):432-41.

(195) Edwards PJ, Roberts I, Clarke MJ et al. Methods to increase response to postal and electronic questionnaires (Review). The Cochrane Library 2009;3:1-463.

(196) Esser C, Tomluk J. Reporting Hardy-Weinberg tests in case-control studies: reasons for caution but not for panic reactions. J Invest Dermatol 2005 May;124(5):1082-3.

(197) Smith GD, Ebrahim S. Mendelian randomization: prospects, potentials, and limitations. Int J Epidemiol 2004 February;33(1):30-42.

(198) Thomas DC. Statistical Methods in Genetic Epidemiology. 1st ed. Oxford University Press; 2004.

(199) Guillygomarc'h A, Christian J, Romain M, Vincent Q, Veronique D, Deugnier Y. Circadian variations of transferrin saturation levels in iron-overloaded patients: implications for the screening of C282Y-linked haemochromatosis. Br J Haematol 2003 January;120(2):359-63.

(200) Wacholder S, Rothman N, Caporaso N. Population stratification in epidemiologic studies of common genetic variants and cancer: quantification of bias. J Natl Cancer Inst 2000 July 19;92(14):1151-8.

(201) Cardon LR, Palmer LJ. Population stratification and spurious allelic association. Lancet 2003 February 15;361(9357):598-604.

(202) Coste J, Quinquis L, Audureau E, Pouchot J. Non response, incomplete and inconsistent responses to self-administered health-related quality of life measures in the general population: patterns, determinants and impact on the validity of estimates - a population-based study in France using the MOS SF-36. Health Qual Life Outcomes 2013;11:44.

(203) Waljee AK, Mukherjee A, Singal AG et al. Comparison of imputation methods for missing laboratory data in medicine. BMJ Open 2013;3(8).

(204) Akl EA, Briel M, You JJ et al. Potential impact on estimated treatment effects of information lost to follow-up in randomised controlled trials (LOST-IT): systematic review. BMJ 2012;344:e2809.

(205) Rubin DB, Schenker N. Multiple imputation in health-care databases: an overview and some applications. Stat Med 1991 April;10(4):585-98.

(206) Ridker PM, Danielson E, Fonseca FA et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008 November 20;359(21):2195-207.

(207) Sosenko JM, Palmer JP, Greenbaum CJ et al. Patterns of metabolic progression to type 1 diabetes in the Diabetes Prevention Trial-Type 1. Diabetes Care 2006 March;29(3):643-9.

(208) Plagnol V, Howson JM, Smyth DJ et al. Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. PLoS Genet 2011 August;7(8):e1002216.

(209) Ronkainen MS, Savola K, Knip M. Antibodies to GAD65 epitopes at diagnosis and over the first 10 years of clinical type 1 diabetes mellitus. Scand J Immunol 2004 March;59(3):334-40.

(210) WHO. Definition, diagnosis and classification of diabetes mellitus and its complications. 1999.

(211) Black WC, Haggstrom DA, Welch HG. All-cause mortality in randomized trials of cancer screening. J Natl Cancer Inst 2002 February 6;94(3):167-73.

(212) Drivsholm T. Datavaliditet og dækningsgrad i landspatientregistret. Ugeskr Laeger 2002;164(1):33-4.

(213) Kristensen JK, Drivsholm TB, Carstensen B, Steding-Jensen M, Green A. [Validation of methods to identify known diabetes on the basis of health registers]. Ugeskr Laeger 2007 April 30;169(18):1687-92.

(214) Andersen JS, Olivarius NF, Krasnik A. The Danish National Health Service Register. Scand J Public Health 2011 July;39(7 Suppl):34-7.

(215) Carstensen B, Kristensen JK, Marcussen MM, Borch-Johnsen K. The National Diabetes Register. Scand J Public Health 2011 July;39(7 Suppl):58-61.

(216) Anwar H, Fischbacher CM, Leese GP, Lindsay RS, McKnight JA, Wild SH. Assessment of the under-reporting of diabetes in hospital admission data: a study from the Scottish Diabetes Research Network Epidemiology Group. Diabet Med 2011 December;28(12):1514-9.

(217) Storm HH. Completeness of cancer registration in Denmark 1943-1966 and efficacy of record linkage procedures. Int J Epidemiol 1988 March;17(1):44-9.

(218) Bjerregaard B, Larsen OB. The Danish Pathology Register. Scand J Public Health 2011 July;39(7 Suppl):72-4.

(219) Roulson J, Benbow EW, Hasleton PS. Discrepancies between clinical and autopsy diagnosis and the value of post mortem histology; a meta-analysis and review. Histopathology 2005 December;47(6):551-9.

(220) Lahti RA, Penttila A. Cause-of-death query in validation of death certification by expert panel; effects on mortality statistics in Finland, 1995. Forensic Sci Int 2003 January 28;131(2-3):113-24.

(221) Ewald N, Kaufmann C, Raspe A, Kloer HU, Bretzel RG, Hardt PD. Prevalence of diabetes mellitus secondary to pancreatic diseases (type 3c). Diabetes Metab Res Rev 2012 May;28(4):338-42.

(222) Phelps G, Chapman I, Hall P, Braund W, Mackinnon M. Prevalence of genetic haemochromatosis among diabetic patients. Lancet 1989 July 29;2(8657):233-4.

(223) Utzschneider KM, Kowdley KV. Hereditary hemochromatosis and diabetes mellitus: implications for clinical practice. Nat Rev Endocrinol 2010 January;6(1):26-33.

(224) Christoforidis A, Perifanis V, thanassiou-Metaxa M. Combined chelation therapy improves glucose metabolism in patients with beta-thalassaemia major. Br J Haematol 2006 October;135(2):271-2.

(225) Platis O, Anagnostopoulos G, Farmaki K, Posantzis M, Gotsis E, Tolis G. Glucose metabolism disorders improvement in patients with thalassaemia major after 24-36 months of intensive chelation therapy. Pediatr Endocrinol Rev 2004 December;2 Suppl 2:279-81.

(226) Farmaki K, Angelopoulos N, Anagnostopoulos G, Gotsis E, Rombopoulos G, Tolis G. Effect of enhanced iron chelation therapy on glucose metabolism in patients with beta-thalassaemia major. Br J Haematol 2006 August;134(4):438-44.

(227) Vari IS, Balkau B, Kettaneh A et al. Ferritin and transferrin are associated with metabolic syndrome abnormalities and their change over time in a general population: Data from an Epidemiological Study on the Insulin Resistance Syndrome (DESIR). Diabetes Care 2007 July;30(7):1795-801.

(228) Moirand R, Mortaji AM, Loreal O, Paillard F, Brissot P, Deugnier Y. A new syndrome of liver iron overload with normal transferrin saturation. Lancet 1997 January 11; 349(9045):95-7.

(229) Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? Hypertension 2004 September;44(3):248-52.

(230) Geier D, Hebert B, Potti A. Risk of primary non-hepatocellular malignancies in hereditary hemochromatosis. Anticancer Res 2002 November;22(6B):3797-9.

(231) Jin F, Qu LS, Shen XZ. Association between C282Y and H63D mutations of the HFE gene with hepatocellular carcinoma in European populations: a meta-analysis. J Exp Clin Cancer Res 2010;29:18.

(232) Chen W, Zhao H, Li T, Yao H. HFE gene C282Y variant is associated with colorectal cancer in Caucasians: a meta-analysis. Tumour Biol 2013 August;34(4):2255-9.

(233) Agudo A, Bonet C, Sala N et al. Hemochromatosis (HFE) gene mutations and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Carcinogenesis 2013 June;34(6):1244-50.

(234) Brissot P, Moirand R, Guyader D, Loreal O, Turlin B, Deugnier Y. Hemochromatosis after the gene discovery: revisiting the diagnostic strategy. J Hepatol 1998;28 Suppl 1:14-8.

(235) Benyamin B, Montgomery GW, Martin NG, Whitfield JB. Transferrin saturation and mortality. Clin Chem 2011 June;57(6):921-3.

(236) Diagnosis and management of haemochromatosis since the discovery of the HFE gene: a European experience. Br J Haematol 2000 January;108(1):31-9.

(237) Niederau C, Fischer R, Sonnenberg A, Stremmel W, Trampisch HJ, Strohmeyer G. Survival and causes of death in cirrhotic and in noncirrhotic patients with primary hemochromatosis. N Engl J Med 1985 November 14;313(20):1256-62.

(238) Pankow JS, Boerwinkle E, Adams PC et al. HFE C282Y homozygotes have reduced low-density lipoprotein cholesterol: the Atherosclerosis Risk in Communities (ARIC) Study. Transl Res 2008 July;152(1):3-10.

(239) van Aken MO, de Craen AJ, Gussekloo J et al. No increase in mortality and morbidity among carriers of the C282Y mutation of the hereditary haemochromatosis gene in the oldest old: the Leiden 85-plus study. Eur J Clin Invest 2002 October;32(10): 750-4.

(240) Roest M, van der Schouw YT, de Valk B et al. Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. Circulation 1999 September 21;100(12):1268-73.

(241) The Cochrane Collaboration. Cochrane Handbook for Systematic Reviews of Interventions 4.2.6. The Cochrane Collaboration; 2006.

(242) Jenq CC, Hsu CW, Huang WH, Chen KH, Lin JL, Lin-Tan DT. Serum ferritin levels predict all-cause and infection-cause 1-year mortality in diabetic patients on maintenance hemodialysis. Am J Med Sci 2009 March;337(3):188-94.

(243) Aleman S, Endalib S, Stal P et al. Health check-ups and family screening allow detection of hereditary hemochromatosis with less advanced liver fibrosis and survival comparable with the general population. Scand J Gastroenterol 2011 September;46(9):1118-26.

(244) Fernandez-Real JM, Penarroja G, Castro A, Garcia-Bragado F, Hernandez-Aguado I, Ricart W. Blood letting in high-ferritin type 2 diabetes: effects on insulin sensitivity and beta-cell function. Diabetes 2002 April;51(4):1000-4.

(245) Gurrin LC, Bertalli NA, Dalton GW et al. HFE C282Y/H63D compound heterozygotes are at low risk of hemochromatosis-related morbidity. Hepatology 2009 July;50(1):94-101.

(246) Walsh A, Dixon JL, Ramm GA et al. The clinical relevance of compound heterozygosity for the C282Y and H63D substitutions in hemochromatosis. Clin Gastroenterol Hepatol 2006 November;4(11):1403-10.

(247) Bulaj ZJ, Ajioka RS, Phillips JD et al. Disease-related conditions in relatives of patients with hemochromatosis. N Engl J Med 2000 November 23;343(21):1529-35.

(248) Bulaj ZJ, Griffen LM, Jorde LB, Edwards CQ, Kushner JP. Clinical and biochemical abnormalities in people heterozygous for hemochromatosis. N Engl J Med 1996 December 12;335(24):1799-805.

(249) Menke A, Muntner P, Fernandez-Real JM, Guallar E. The association of biomarkers of iron status with mortality in US adults. Nutr Metab Cardiovasc Dis 2011 February 15.

(250) Whitlock EP, Garlitz BA, Harris EL, Beil TL, Smith PR. Screening for hereditary hemochromatosis: a systematic review for the U.S. Preventive Services Task Force. Ann Intern Med 2006 August 1;145(3):209-23.

(251) Nadakkavukaran IM, Gan EK, Olynyk JK. Screening for hereditary haemochromatosis. Pathology 2012 February;44(2):148-52.

(252) Waalen J, Felitti VJ, Gelbart T, Beutler E. Screening for hemochromatosis by measuring ferritin levels: a more effective approach. Blood 2008 April 1;111(7):3373-6.

(253) Smith GD, Timpson N, Ebrahim S. Strengthening causal inference in cardiovascular epidemiology through Mendelian randomization. Ann Med 2008;40(7):524-41.

(254) Wood MJ, Powell LW, Ramm GA. Environmental and genetic modifiers of the progression to fibrosis and cirrhosis in hemochromatosis. Blood 2008 May 1;111(9):4456-62.

(255) Pedersen P, Milman N. Extrinsic factors modifying expressivity of the HFE variant C282Y, H63D, S65C phenotypes in 1,294 Danish men. Ann Hematol 2009 October; 88(10):957-65.

(256) Aranda N, Viteri FE, Montserrat C, Arija V. Effects of C282Y, H63D, and S65C HFE gene mutations, diet, and life-style factors on iron status in a general Mediterranean population from Tarragona, Spain. Ann Hematol 2010 August; 89(8):767-73.

(257) Mursu J, Robien K, Harnack LJ, Park K, Jacobs DR, Jr. Dietary Supplements and Mortality Rate in Older Women: The Iowa Women's Health Study. Arch Intern Med 2011 October 10;171(18):1625-33.

(258) Chan DS, Lau R, Aune D et al. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. PLoS One 2011;6(6):e20456.

(259) Rosvik AS, Ulvik RJ, Wentzel-Larsen T, Hervig T. Blood donors with hereditary hemochromatosis. Transfusion 2010 August;50(8):1787-93.

(260) Crawford DH. Determinants of disease expression in Australian haemochromatosis patients. J Gastroenterol Hepatol 1996 November;11(11):1043-5.

(261) Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem 2005;12(10):1161-208.

(262) Tas F, Hansel H, Belce A et al. Oxidative stress in breast cancer. Med Oncol 2005; 22(1):11-5.

(263) Harman D. Free radical theory of aging: an update: increasing the functional life span. Ann N Y Acad Sci 2006 May;1067:10-21.

(264) Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956 July;11(3):298-300.

(265) Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000 November 9;408(6809):239-47.

(266) Buijsse B, Feskens EJ, Moschandreas J et al. Oxidative stress, and iron and antioxidant status in elderly men: differences between the Mediterranean south (Crete) and northern Europe (Zutphen). Eur J Cardiovasc Prev Rehabil 2007 August; 14(4):495-500.

(267) Harrison-Findik DD. Gender-related variations in iron metabolism and liver diseases. World J Hepatol 2010 August 27;2(8):302-10.

(268) Rossi E, Olynyk JK, Jeffrey GP. Clinical penetrance of C282Y homozygous HFE hemochromatosis. Expert Rev Hematol 2008 December;1(2):205-16.

(269) Harrison-Findik DD, Schafer D, Klein E et al. Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. J Biol Chem 2006 August 11;281(32):22974-82.

(270) McNaughton SA, Mishra GD, Paul AA, Prynne CJ, Wadsworth ME. Supplement use is associated with health status and health-related behaviors in the 1946 British birth cohort. J Nutr 2005 July;135(7):1782-9.

(271) Zlotogora J. Penetrance and expressivity in the molecular age. Genet Med 2003 September;5(5):347-52.

(272) Asberg A, Hveem K, Kruger O, Bjerve KS. Persons with screening-detected haemochromatosis: as healthy as the general population? Scand J Gastroenterol 2002 June;37(6):719-24.

(273) Asberg A, Hveem K, Thorstensen K et al. Screening for hemochromatosis: high prevalence and low morbidity in an unselected population of 65,238 persons. Scand J Gastroenterol 2001 October;36(10):1108-15.

(274) Jackson HA, Carter K, Darke C et al. HFE mutations, iron deficiency and overload in 10,500 blood donors. Br J Haematol 2001 August;114(2):474-84.

(275) Beaumont C, Simon M, Fauchet R et al. Serum ferritin as a possible marker of the hemochromatosis allele. N Engl J Med 1979 July 26;301(4):169-74.

(276) Meyer T, Baynes R, Bothwell T et al. Phenotypic expression of the HLA linked iron-loading gene in males over the age of 40 years: a population study using serial serum ferritin estimations. J Intern Med 1990 June;227(6):397-406.

(277) Milman N, Clausen JO, Jordal R. Iron status in young Danish men and women: a population survey comprising 548 individuals. Ann Hematol 1995 April; 70(4):215-21.

(278) Bassett ML, Halliday JW, Ferris RA, Powell LW. Diagnosis of hemochromatosis in young subjects: predictive accuracy of biochemical screening tests. Gastroenterology 1984 September;87(3):628-33.

(279) Beutler E, Felitti V, Gelbart T, Ho N. The effect of HFE genotypes on measurements of iron overload in patients attending a health appraisal clinic. Ann Intern Med 2000 September 5;133(5):329-37.

(280) Crawford DH, Jazwinska EC, Cullen LM, Powell LW. Expression of HLA-linked hemochromatosis in subjects homozygous or heterozygous for the C282Y mutation. Gastroenterology 1998 May;114(5):1003-8.

(281) Bradley LA, Haddow JE, Palomaki GE. Population screening for haemochromatosis: a unifying analysis of published intervention trials. J Med Screen 1996;3(4):178-84.

(282) Gannon PO, Medelci S, Le PC et al. Impact of hemochromatosis gene (HFE) mutations on epithelial ovarian cancer risk and prognosis. Int J Cancer 2011 May 15; 128(10):2326-34.

(283) Osborne NJ, Gurrin LC, Allen KJ et al. HFE C282Y homozygotes are at increased risk of breast and colorectal cancer. Hepatology 2010 April;51(4):1311-8.

(284) Brissot P, Ball S, Rofail D, Cannon H, Jin VW. Hereditary hemochromatosis: patient experiences of the disease and phlebotomy treatment. Transfusion 2011 June; 51(6):1331-8.

(285) Swinkels DW, Aalbers N, Elving LD, Bleijenberg G, Swanink CM, van der Meer JW. Primary haemochromatosis: a missed cause of chronic fatigue syndrome? Neth J Med 2002 December;60(11):429-33.

(286) Milewicz DM, Chen H, Park ES et al. Reduced penetrance and variable expressivity of familial thoracic aortic aneurysms/dissections. Am J Cardiol 1998 August 15; 82(4):474-9.

(287) Kotnis A, Sarin R, Mulherkar R. Genotype, phenotype and cancer: role of low penetrance genes and environment in tumour susceptibility. J Biosci 2005 February; 30(1):93-102.

(288) Barton JC, Lee PL, West C, Bottomley SS. Iron overload and prolonged ingestion of iron supplements: clinical features and mutation analysis of hemochromatosis-associated genes in four cases. Am J Hematol 2006 October;81(10):760-7.

(289) Wallace DF, Summerville L, Crampton EM, Frazer DM, Anderson GJ, Subramaniam VN. Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload. Hepatology 2009 December;50(6): 1992-2000.

(290) Olsson KS, Ritter B, Raha-Chowdhury R. HLA-A3-B14 and the origin of the haemochromatosis C282Y mutation: founder effects and recombination events during 12 generations in a Scandinavian family with major iron overload. Eur J Haematol 2010 February 1;84(2):145-53.

(291) Milet J, Dehais V, Bourgain C et al. Common variants in the BMP2, BMP4, and HJV genes of the hepcidin regulation pathway modulate HFE hemochromatosis penetrance. Am J Hum Genet 2007 October;81(4):799-807.

(292) Flaten TP, Aaseth J, Andersen O, Kontoghiorghes GJ. Iron mobilization using chelation and phlebotomy. J Trace Elem Med Biol 2012 June;26(2-3):127-30.

(293) Laine F, Reymann JM, Morel F et al. Effects of phlebotomy therapy on cytochrome P450 2e1 activity and oxidative stress markers in dysmetabolic iron overload syndrome: a randomized trial. Aliment Pharmacol Ther 2006 October 15;24(8): 1207-13.

(294) Houschyar KS, Ludtke R, Dobos GJ et al. Effects of phlebotomy-induced reduction of body iron stores on metabolic syndrome: results from a randomized clinical trial. BMC Med 2012;10:54.

(295) Rajapurkar MM, Hegde U, Bhattacharya A, Alam MG, Shah SV. Effect of deferiprone, an oral iron chelator, in diabetic and non-diabetic glomerular disease. Toxicol Mech Methods 2013 January;23(1):5-10.

(296) Zacharski LR, Shamayeva G, Chow BK. Effect of controlled reduction of body iron stores on clinical outcomes in peripheral arterial disease. Am Heart J 2011 November;162(5):949-57.

(297) Fernandez-Real JM, Lopez-Bermejo A, Ricart W. Iron stores, blood donation, and insulin sensitivity and secretion. Clin Chem 2005 July;51(7):1201-5.

(298) Zheng H, Cable R, Spencer B, Votto N, Katz SD. Iron stores and vascular function in voluntary blood donors. Arterioscler Thromb Vasc Biol 2005 August;25(8):1577-83.

(299) Edgren G, Nyren O, Melbye M. Cancer as a ferrotoxic disease: are we getting hard stainless evidence? J Natl Cancer Inst 2008 July 16;100(14):976-7.

(300) Zhang X, Ma J, Wu K, Chan AT, Fuchs CS, Giovannucci EL. Blood donation and colorectal cancer incidence and mortality in men. PLoS One 2012;7(6):e39319.

(301) Tuomainen TP, Loft S, Nyyssonen K, Punnonen K, Salonen JT, Poulsen HE. Body iron is a contributor to oxidative damage of DNA. Free Radic Res 2007 March; 41(3):324-8.

(302) Poulsen HE, Specht E, Broedbaek K et al. RNA modifications by oxidation: a novel disease mechanism? Free Radic Biol Med 2012 April 15;52(8):1353-61.

(303) Broedbaek K, Siersma V, Henriksen T et al. Urinary markers of nucleic acid oxidation and long-term mortality of newly diagnosed type 2 diabetic patients. Diabetes Care 2011 December;34(12):2594-6.