Osteoarthritis and Hemochromatosis

A genetic epidemiologic study

Behrooz Z. Alizadeh

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“This has all been wonderful
… I am on my way
… and the future gleams.”

To my lovely Leila
To sweaty Aylar
To my parents

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1

GENERAL INTRODUCTION

Osteoarthritis (OA) refers to a heterogeneous group of distinct diseases which express a common radiographic and clinical phenotype at diarthrodial joints.[1,2] It is the leading cause of pain and disability in the elderly with a prevalence of 75 percent in the population aged 70 years.[3-5] There is a substantial genetic component in the etiology of osteoarthritis.[6-9] It is expected that genetic heterogeneity, the extent of which is unknown yet, contributes to a spectrum of osteoarthritis related phenotypes. Linkage as well as candidate gene studies are being performed to identify the genes involved.[10-13] The overall aim of our research project is to identify genes that contribute to osteoarthritis. The aim of the present thesis was to understand the relation between hemochromatosis and osteoarthritis. As a part of this project, candidate osteoarthritis genes were analysed. These include the gene encoding the alpha domain of collagen type IX (COL9A1) and the HFE gene involved in hereditary hemochromatosis, a disorder that coincides with arthropathy. This thesis, therefore, brings together two related complex diseases, that share related phenotypes and possibly some of the underlying genetic components.

The COL9A1 gene encodes the alpha domain of type IX collagen fibrils. In mice, knockout or transgenic experiments have shown that the absence of a functional type IX collagen fibril leads to cartilage instability and early onset generalized osteoarthritis. In humans, a genomewide scan linked the COL9A1 gene, among other loci, to a severe form of hip osteoarthritis in women.[14] The second candidate gene studied for osteoarthritis is the HFE gene in which two common mutations explain type I hereditary hemochromatosis, a common disorder of iron metabolism that leads to pathology at multiple organs as well as the joints.[15,16] In fact, arthropathy is one of the most common features of hemochromatosis affecting up to 80 percent of patients.[16] The relation to arthropathy was studied first. These investigations have led us to study the implication of the HFE mutations on morbidity and mortality, addressing also the issue of penetrance.

Chapter 2.1 presents a review of the current knowledge on the genetic epidemiology of osteoarthritis. It uses osteoarthritis as an example to describe the current strategies in unraveling the genetic components of a complex disease. **Chapter 2.2** reviews the genetic epidemiology of hemochromatosis with special reference to the impact of a common mutation on public health and important considerations in population screening. **Chapter 3** summarizes the results of several association studies. **Chapter 3.1** presents the relationship between the COL9A1 gene and osteoarthritis and **Chapter 3.2** describes the results of an association study on the HFE C282Y and H63D mutations with arthralgia, chondrocalcinosis, and

osteoarthritis. **Chapter 3.3** presents the association between the H63D mutation, Heberden's nodes, with mortality and tests a hypothesis on the role of inflammation in hemochromatosis-associated arthropathy. **Chapter 3.4** describes the relation between HFE mutations, serum total bilirubin and mortality, and tests a hypothesis that high levels of serum bilirubin may explain, at least in part, the low penetrance of HFE mutations. **Chapter 4** addresses the heritability estimates for serum iron, ferritin and transferrin saturation. Finally, the findings and the future prospects are discussed.

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2

REVIEWS

2.1

GENETIC EPIDEMIOLOGY OF OSTEOARTHRITIS

Introduction

Osteoarthritis is a disorder of diarthrodial joints characterized clinically by pain and functional limitation, radiographically by osteophytes and joint space narrowing, and histopathologically by alterations in cartilage and sub-chondral bone integrity. Osteoarthritis has considerable impact on public health in terms of morbidity i.e. productivity, hospitalization and prolonged treatment, and it may predict a higher mortality in patients. From etiological prospect, osteoarthritis has been shown to be a family of disorders in which genetic factors play a central role. Other risk factors include age, gender, weight, biomechanical stress, and occupation. Since there is no treatment to prevent or ameliorate the underlying disease process, medical interventions are aimed primarily at relieving symptoms i.e. pain, preserving joint function and replacing the severely damaged joints. Currently efforts are focused on unraveling genetic factors that underlie the pathologic pathways leading to osteoarthritis. The genetic studies, as described here, may eventually reveal the underlying disease pathways that may provide new targets for intervention.

Definition and classification of phenotype

Osteoarthritis can be defined radiographically, clinically or etiologically. The main radiographic features used to define osteoarthritis include joint space narrowing, osteophyte formation, subchondral sclerosis, cysts and abnormality of bone contour. Most epidemiologic studies have used the scoring system described by Kellgren and Lawrence to characterize osteoarthritis in the studied population.[1] This system scores one of the five grades i.e. 0 to 4 for osteoarthritis at various joint sites (Table 1). Grading is performed by comparing various joint sites i.e. knee, hip, hand and spine with reproductions in a radiographic atlas. A cut-off score on the Kellgren and Lawrence scale to diagnose radiographic osteoarthritis is 2. In clinical practice, different criteria, based on the presence of joint pain and radiographic features are used for a clinical definition of osteoarthritis. The most widely used clinical criteria for the definition of osteoarthritis was developed by the American College of Rheumatology and are based on pain.[2-4] This contrasts with the use of radiographic changes, as many subjects do not report pain and the discrepancy depends on the affected joint sites.[5]

Table 1. Radiographic grading system for osteoarthritis.

Grade	Classification	Description
0	Normal	No feature of osteoarthritis
1	Doubtful	Minute osteophyte, doubtful significance
2	Minimal	Definite osteophyte, unimpaired joint space
3	Moderate	Moderate diminution of joint space
4	Severe	Joint space greatly impaired with sclerosis of subchondral bone

Adapted from the atlas of standard radiographs. Oxford: Blackwell Scientific.

Osteoarthritis affects one i.e. monoarticular or multiple joint sites. The pattern of joint involvement is influenced by age, gender, race, familial predisposition, previous joint injury, presence of metabolic risk factors such as weight and occupational history. When multiple joints are affected, there is a stronger association between hand and knee osteoarthritis in Caucasian populations.[5,6] Generalized osteoarthritis refers to a condition in which Heberden's nodes are found in combination with polyarticular disease.[7,8]

Etiologically, osteoarthritis can be defined as primary or secondary. Four main categories of disorders can cause secondary osteoarthritis i.e. metabolic disorders such as hemochromatosis and chondrocalcinosis, anatomic derangement such as epiphyseal dysplasia, major trauma or surgery and inflammatory arthropathy such as rheumatoid arthritis. The term inflammatory osteoarthritis is used to identify patients with obvious inflammation and multiple joints' involvement.[9] But, in most forms of osteoarthritis, the joints pass through phases in which the inflammation is less or more prominent. In inflammatory osteoarthritis, some patients develop erosions, an aggressive form of joint destruction,[10] which represent the end point of the spectrum of disease. Variability in the joint sites and number of sites involved, and in etiopathogenesis suggest that osteoarthritis may not represent a single disease entity. Osteoarthritis has been defined as a group of overlapping distinct diseases, which may have different etiologies but with similar morphologic, and clinical outcomes.[2,11] In this prospect the articular cartilage degeneration is the ultimate end of several underlying pathologic processes.[2,11] For genetic studies different osteoarthritis phenotype definitions are being used. Primary osteoarthritis is expected to be heterogeneous at the genetic level, meaning that different genetic variation predispose to different forms of the disease.[12,13]

Prevalence

The prevalence of osteoarthritis has been variously estimated in epidemiologic studies based on the inclusion criteria for osteoarthritis: pathological, radiographic or clinical. Pathological studies reported that the prevalence of cartilage erosion, subchondral reaction and osteophytes are present at the knees of 60 percent of men and 70 percent of women aged 70 years or older. Epidemiological surveys based on radiographic findings showed that the prevalence of osteoarthritis increases steadily from less than 2 percent in women younger than 45 years of age to 30 percent in those aged 45 to 65 years and to 68 percent in those older than 65 years of age.[14] The Dutch population-based Zoetermeer Study showed that more than 75 percent of women aged 60 to 70 years had osteoarthritis hand joints.[15] These findings were confirmed in another Dutch population-based study, the Rotterdam Study.[16] However, when clinical criteria are used, the prevalence of symptomatic osteoarthritis drops dramatically from 17 percent radiographic osteoarthritis to only 2 percent for knee osteoarthritis in women aged 65 years or younger.[17,18] This shows a large discrepancy between radiographic and clinical osteoarthritis. From this point of view, one may define osteoarthritis, in general, as a silent disease that allows the underlying causal process to progress without obvious clinical manifestation and thus remains undetected leading to severe irreversible consequences and associated morbidity.

Risk factors

There are two groups of factors predisposing to osteoarthritis, factors influencing a generalized susceptibility to osteoarthritis such as heredity, obesity, osteoporosis, hypermobility and systemic diseases, and factors resulting in a single joint pathology such as abnormal biomechanical loading, trauma, joint shape, occupation, and physical activity. Next, we will elaborate in detail on the role of hereditary factors in osteoarthritis.

Evidence for inheritance of osteoarthritis

In 1941 Stecher[19] introduced the possible role of heredity in susceptibility to nodular hand osteoarthritis. Later, twin-pair, segregation and population-based studies demonstrated a strong hereditary predisposition to generalized osteoarthritis.[12,16,19-23] Several studies showed that osteoarthritis clusters within families.[7,16,20,24,25] Two factors can explain intrafamilial clustering of osteoarthritis. First, close relatives inherit the same osteoarthritis predisposing DNA variants, and second, they share environmental factors. Twin-pair studies have been

used to determine the influence of genetic factors on osteoarthritis at specific joint sites. These studies estimated a heritability of 39 to 65 percent for osteoarthritis independent of known environmental factors.[26-30] Similarly, large population-based family studies confirmed the findings of the twin-pair studies with similar heritability estimates for osteoarthritis at hand, knee, and hip joints.[16,21,22,31,32] Several studies investigated the mechanism by which osteoarthritis segregated within families. A large segregation analysis of nuclear families suggested a recessive genetic model.[21] But, an autosomal dominant mode of inheritance has also been reported indicating that different types of mutations/genes influence the susceptibility for osteoarthritis.[33] Overall, twin-pair and segregation studies revealed a substantial genetic component often with a polygenic inheritance for osteoarthritis in hand,[31,34] knee[24] and hip[22,24,25,27] joints, which is influenced by environmental factors. At this point it is not clear whether genetic heterogeneity underlies the various phenotype definitions that are used to establish heritability.

Genome scans and osteoarthritis susceptibility genes

The fact that osteoarthritis is heritable raises the question which genes are causal. Investigators used two main research tools to identify genes involved in osteoarthritis: positional cloning and candidate gene association studies. The evidence for the presence of osteoarthritis susceptibility loci has emerged from linkage studies in families with rare Mendelian forms of generalized osteoarthritis. In 1994, the first candidate gene for osteoarthritis was suggested through the work of Ritvaniemi and colleagues[35] who reported the type II procollagen gene (COL2A1) is associated to spondyloepiphyseal dysplasia, a mild form of generalized osteoarthritis. These and other investigations provided evidence for the presence of a disease susceptibility locus for dominant forms of the disease on chromosome 2q (personal communication, Slagboom PE, 2004), 4q35,[36] and 16p.[37] Genomewide or directed genome screens were also performed for other and milder phenotypes. Linkage studies revealed osteoarthritis to be linked to loci on chromosomes 1p,[31] 2q,[38-41] 4q12-21,[42,43] 6p,[44] 6q,[31] 7q,[31] 9q,[31] 11q,[45-47] 13,[31] and 16p.[37,42] Association studies addressed a large numbers of candidate genes, in particular on chromosome 6p and 12q where linkage studies failed to identify osteoarthritis predisposing regions. Here we will summarize the overlap in linkage in the main osteoarthritis studies that support the relevance of some chromosomal loci and candidate genes for different definitions of osteoarthritis. The inclusion criteria and joint sites that were investigated in the main studies are shown in Table 2.

Two genomewide screens carried out within the Framingham Study suggested a linkage to 1p in 296 pedigrees with radiographic hand osteoarthritis.[31,34] These studies did not find a responsible gene for the observed linkage on this region.[31] One of the candidate genes located at this region is the Matrilin-1gene. However, a study of siblings with generalized osteoarthritis found no linkage to this gene.[13] Neither a relationship between the Matrilin-1 gene and severe hip osteoarthritis was found in the UK cohort of patients with total hip or knee replacement.[48] However, the Matrilin-1 gene has been associated to radiographic osteoarthritis at hip or knee in the population-based Rotterdam Study.[49] Overall, the two population-based studies, the Framingham Study and the Rotterdam Study, found linkage and association to chromosome 1p and the Martilin-1 gene in different osteoarthritis phenotypes: radiographic hand and knee[45] or hip osteoarthritis.[31,34]

Chromosome 2q is the most replicated region implicated in osteoarthritis in both linkage and association studies. Chromosome 2q12-13 is linked to distal interphalangeal osteoarthritis,[41] 2q31 to hip osteoarthritis,[40] and 2q23-35 to nodal osteoarthritis.[38] The interleukin-1 gene cluster, mapped on chromosome 2q12-13 has been associated to knee osteoarthritis in the UK cohort,[50] hip radiographic osteoarthritis in the Rotterdam Study,[51] and to severe erosive hand osteoarthritis.[52] Recently, chromosome 2q13-31 encompassing the Frizzled 2B gene that is involved in bone development, has been linked to female hip osteoarthritis,[23] and to generalized osteoarthritis in the Leiden osteoarthritis cohort (the GARP Study) and in the Rotterdam Study (in press). However, others found no linkage of 2q11.2-36.3 to nodal or knee,[53] or hand osteoarthritis.[54] These negative findings are supported by the Framingham Study.[31] Taken together, the findings suggest this region may harbor multiple osteoarthritis susceptibility genes.

Osteoarthritis was also linked to 2p. A two steps genomewide scan recently found a significant evidence of linkage of chromosome 2p to hand osteoarthritis in an Icelandic population[43] that was close to a peak reported earlier in the Framingham Study.[31] This region coincided with a gene encoding the non-collagenous cartilage extracellular matrix protein, Matrilin-3 with missense mutation that cosegregates with hand osteoarthritis in several families. This finding is in complement with linkage of the Matrilin-3 region i.e. 2p24-23 to multiple epiphyseal dysplasia (MED), a disease associated to osteoarthritis in a genomewide scan, as well as in candidate gene studies.[55] Two different missense mutations in the exon encoding the von Willebrand factor A domain of Matrilin-3 explained MED in two unrelated families.[55] Overall, there is a substantial repetition for osteoarthritis susceptibility being

Table 2. Characteristics of major ongoing osteoarthritis (OA) cohorts around the world.

Name	Ethnicity	Study design	Study population (Inclusion criteria)	Studied phenotypes	Studied joints/
The Framingham Heart Study [21,31,34,56,57]	American	Population-based multigenerational cohort; began 1948	>1300 pedigrees	Radiographic OA using K-L ≥2	Hand, & knee
The Rotterdam Study[49,51,58-64]	Dutch	Population-based cohort; began 1990-1993	12000 subjects aged 55 years or over living in Rotterdam	Clinical & radiographic OA using K-L ≥2	Hand, hip, knee & spine /
The UK OA Cohort[13,23,39,42,44,48,50,65,66]	British	Sibling-pairs	481 families (1054 subjects) with ≥2 affected sibs	Primary severe OA/ THR, or/and TKR	Hip & knee
The Iceland OA Cohort[43,67,68]	Icelandic	Sibling- and affected relative pairs; began 1992	2919 subjects from families with ≥2 affected members and 3 first degree relatives/unrelated controls	Clinical OA/ Patients having two HN or squaring of CMC1 or THR	Hand & hip
The Leiden OA Cohort (GARP)[5,69]	Dutch	Sibling-pairs; nuclear families; began 2000		Clinical & radiographic OA	Hand, hip, knee & spine
Finland Study[41]	Finnish	Twin-pairs	Unrelated patients/ affected twin pairs	Clinical & radiographic OA using K-L ≥2	Hand
British National Cohort Study[70]	British, Scottish,	Population-based cohort; began 1946	13687 subjects born between March the 3-9 1946	Clinical OA at least in 1 joint	Hand

Abbreviations: K-L: Kellgren/Lawrence OA scoring system; THR total hip replacement therapy; TKR Total knee replacement therapy; FOS Framingham OA scoring system; HN Heberden's nodes.

linked to 2q and 2p regions.

Although several studies reported a linkage to chromosome 4q, no responsible gene for observed linkage has yet been mapped. One study fine mapped the chromosome 4q region to a 4 cM interval using a high density of microsatellite markers in female hip osteoarthritis in the UK cohort.[42] Another study also reported linkage to an 11 cM interval on 4q35 for an autosomal dominant form of hip osteoarthritis.[36] With respect to hand osteoarthritis, two independent genomewide scans have suggested a linkage to chromosome 4q at marker D4S2980,[43] and 4q26-27.[41] No association analysis has been reported. Overall, 4q is likely to harbor a common osteoarthritis susceptibility locus for hip and hand osteoarthritis.

In the interplay of genes for susceptibility to osteoarthritis, chromosome 6 has an inarguable position, as it harbors at least two osteoarthritis susceptibility regions namely 6q12–23.1 and 6p21.3. Each of the two regions harbors at least two known osteoarthritis susceptibility loci (Figure 1). When considering the chromosome 6q region, a genomewide scan has found a suggestive linkage interval of 50 cM on 6q to a severe form of primary hip osteoarthritis in the UK cohort.[66] The investigators used several strategies i.e. expanding the cohort to higher number of families affected with severe osteoarthritis, genotyping the candidate chromosome 6 interval to a higher density, and stratification of the statistical analysis by gender, to refine the candidate region to a 11.4 cM female specific interval.[66] Evidence for the role of the COL9A1 gene that encodes the alpha 1 domain of type IX collagen polypeptide, a structural protein in cartilage matrix, emerged from different sources. Using a two stage linkage analysis of 11 candidate genes following a genome scan, the investigators found suggestive evidence for linkage of the COL9A1 8B2 marker to severe hip osteoarthritis in 132 concordantly affected female sibling-pairs.[71] This group concluded that the COL9A1 8B2 marker is in strong linkage disequilibrium with an osteoarthritis susceptibility mutation within or close to the COL9A1 gene. Moreover, others reported linkage of COL9A1 to multiple epiphyseal dysplasia.[72] The other 6q osteoarthritis susceptibility gene is the estrogen receptor gene mapped to 6q22.3-23.1. The estrogen receptor α gene encodes a protein that is involved in signal transduction pathway. It has been associated to radiographic severe osteoarthritis in a young Korean population.[73] This finding has been replicated in an independent Korean study of patients with knee osteoarthritis.[74] In the population-based Rotterdam Study, a polymorphism in this gene has been associated with knee osteoarthritis in particular with osteophytosis.[61]

When considering chromosome 6p21.3, this region has shown a weak evidence of

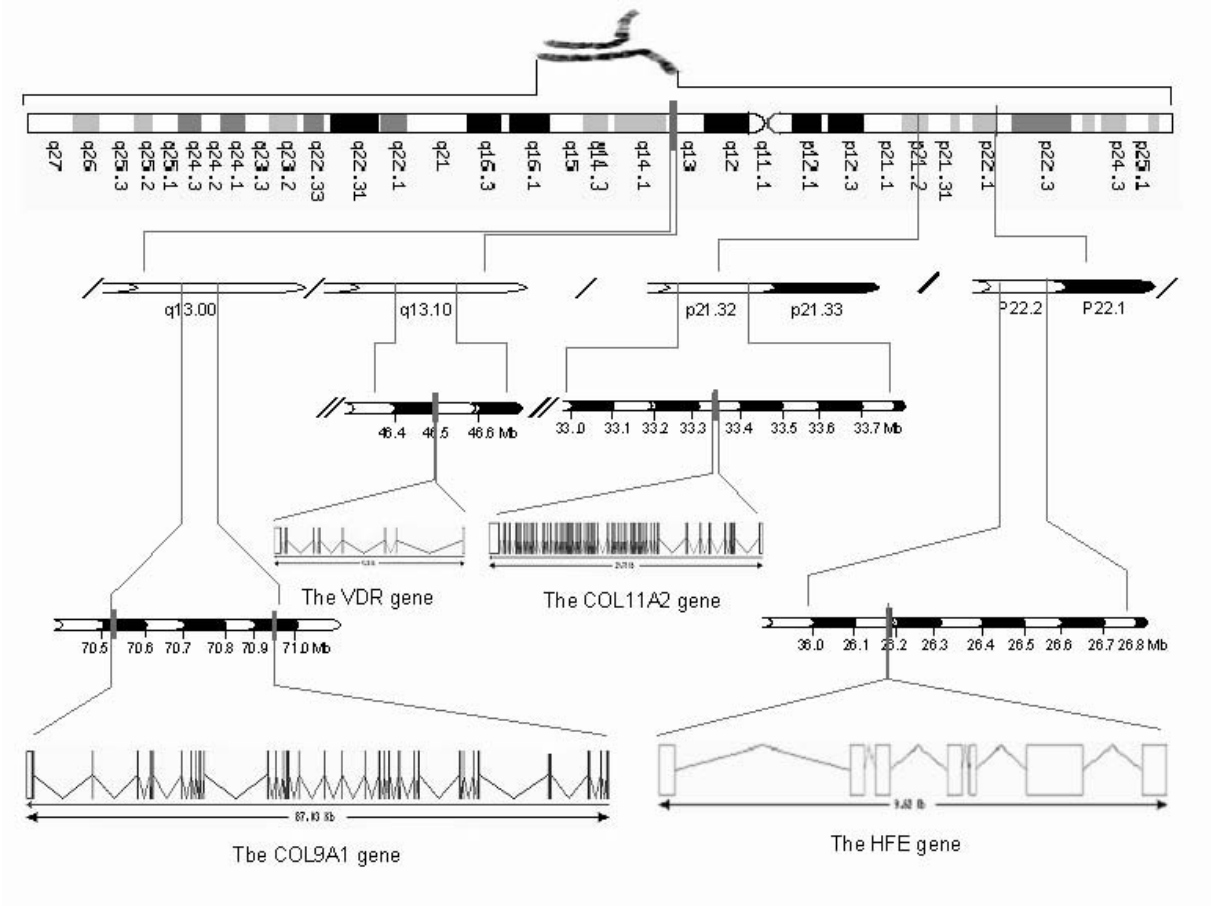

Figure 1.The chromosome 6 osteoarthritis susceptibility loci.

linkage to female hip osteoarthritis in the UK cohort.[71] It harbors at least two osteoarthritis susceptibility loci namely the COL11A2 mapped to 6p21.3 and the hereditary hemochromatosis gene (HFE) mapped to 6p22.2 (Figure 1). On chromosome 6p21.3, COL11A2 has been proposed as a potential hip osteoarthritis susceptibility locus in the UK cohort.[71] Further, this gene has been associated to autosomal dominant and recessive osteochondrodysplasias,[75] and implicated in cartilage formation.[76] However the role of this gene in osteoarthritis at hip as well as other joint locations remains to be confirmed. The other candidate locus mapped to 6p21.3 is the HFE gene, which encodes a protein involved in iron homeostasis. An abnormal or non-functional HFE protein leads to a form of iron overload known as type I hereditary hemochromatosis. The two common C282Y and H63D variants of this gene explain type I hereditary hemochromatosis.[77] Type 1 hemochromatosis is the most common autosomal recessive disorder in Caucasians with a prevalence rate of up to 1 in 200-400. Arthropathy including arthralgia, osteoarthritis and chondrocalcinosis, is the most common early clinical feature in patients with hereditary hemochromatosis,[78-81] and occurs in

45 percent of symptomatic cases at the time of diagnosis,[82] and up to 85 percent of patients during the course of the disease.[80] Clinically arthropathy presents in hemochromatosis with acute inflammation and associated bilateral destruction of metacarpophalangeal joints.[80,81] Hemochromatosis arthropathy includes mainly osteoarthritis like changes at hand, hip and knee joints that are more striking at hand metacarpophalangeal joints. Radiographic features includes, hook-like osteophytes, joint space narrowing, subchondral cyst formation and sclerosis.[80,81] These changes may resemble immune related arthropathy such as rheumatoid arthritis and may be accompanied by Heberden's nodes. Chondrocalcinosis is also often seen in patients with hemochromatosis, but is usually asymptomatic. Radiographic and histological characteristics consist of isolate deposition of calcium crystals in both fibrous and hyaline cartilage, i.e. calcium pyrophosphate dihydrate and calcium hydroxiapatite in cartilage at knee, hip, symphysis pubis and shoulder joints.[80,83] Typical arthritis features strongly suggest the diagnosis in the pre-cirrhotic phase when organ damage can still be prevented.[82] Although linkage studies suggested chromosome 6 as a candidate for osteoarthritis, the evidence for a role of the HFE gene in osteoarthritis is still weak, since it is not known whether either or both of the common HFE C282Y or H63D variants contribute to increased risk of arthropathy.

The Framingham linkage analysis in 267 families with radiographic hand osteoarthritis, suggested a locus for osteoarthritis on chromosome 7q.[31,34] This finding was replicated by a genome wide scan study of Finnish patients with distal interphalangeal osteoarthritis.[41] Although this region encompasses several candidate genes for osteoarthritis, such as the COL1A2 gene, no responsible gene for the observed linkage has so far been identified.

Chromosome 11q has been linked to osteoarthritis in females at marker D11S901 in the UK cohort.[46] This region has been narrowed into two distinct linkage intervals of 11.9 cM and of 6.5 cM.[47] However, a recent study of 295 Russian nuclear families failed to find an association and linkage of 11q12-13 to hand osteoarthritis.[45] Overall, it remains unclear whether the observed linkage in the UK cohort is a true finding.

Several strong osteoarthritis genes have been mapped on chromosome 12. Chromosome 12q12-13.1 encompasses three closely located strong candidate genes i.e. the COL2A1 gene, the vitamin D receptor gene (VDR), and the insulin like growth factor 1 gene. The COL2A1 gene encodes α1 chain of type II collagen. This gene has been associated to osteoarthritis at multiple joints.[65,84] These findings have been replicated within the Rotterdam

study, where COL2A1 has been significantly associated to generalized,[62] and knee osteoarthritis.[58,85] However, others found no relation between this gene and osteoarthritis at hip or hand joints.[56,86,87] The other potential chromosome 12 osteoarthritis susceptibility locus is the VDR gene. This gene has been associated to knee osteoarthritis in the UK cohort,[88] and the Rotterdam Study.[58,85] Several studies, on the other hand, failed to associate the VDR gene to osteoarthritis in knee, hip, or spinal joints in particular in women.[86,89,90] The third chromosome 12 osteoarthritis susceptibility gene is the IGF-I gene that has been associated with radiographic osteoarthritis at multiple joint sites in the Rotterdam Study.[59] Further analysis of osteoarthritis patients within the Rotterdam Study suggested an interaction between the COL2A1 and IGF-1 on susceptibility to radiographic osteoarthritis in persons aged less than 65 years.[60]

Chromosome 16 shows two regions of weak linkage to osteoarthritis, the first on 16p and the second on 16q in the UK cohort.[42] A significant association was found between the interleukin 4 receptor mapped on chromosome 16p12.3-12.1 and female hip osteoarthritis in the UK cohort.[91]

There are several other chromosomal regions that have been linked or associated to osteoarthritis, but those linkages remain to be replicated. Chromosome 3p was linked to hand osteoarthritis at D3S1566.[43] Within the Framingham Study, chromosome 15 and chromosome 20 have been linked to osteoarthritis in the first carpometacarpal joint.[34] There are other candidate genes, which have been described for osteoarthritis in small studies and are not mentioned in this review as they remain to be confirmed.

Discussion

We briefly reviewed the findings in genetic epidemiology of osteoarthritis. Hereafter few methodological points are further discussed.

Model free or non-parametric linkage analysis looks for allele or chromosomal regions that are shared by affected individuals.[92-94] Many osteoarthritis linked genomic regions clearly do not coincide and for many of them the gene has not yet been identified. The studies may differ in population structure,[95] in underlying pathogenic pathways, in phenotype definitions i.e. radiographic and symptomatic osteoarthritis, in liability classes i.e. early onset versus late onset osteoarthritis, gender, pre-menopausal versus post-menopausal women with osteoarthritis, in osteoarthritis endo-phenotypes i.e. osteophytosis, nodal osteoarthritis, or cartilage loss, the extent of osteoarthritis i.e. generalized or a local form of osteoarthritis,

studied joints sites i.e. hand, hip, knee or spine, in interacting environmental factors, and in sample sizes.[96-99]

Among the regions that have been linked or associated to osteoarthritis, regions on chromosome 6 are of special interest. There are several functional, experimental and knock-out studies indicating a role for chromosome 6q COL9A1 gene in osteoarthritis. Nevertheless, to date, only the UK cohort found linkage to COL9A1 in affected sibling pairs with severe osteoarthritis. No other linkage studies have replicated this finding. The question still to be answered is whether COL9A1 is associated to osteoarthritis at hip as well as at other joint sites in population-based samples. Another chromosome 6p candidate gene for osteoarthritis is the HFE gene. Although, osteoarthritis is the main feature of hemochromatosis-associated arthropathy, little is known about the relation between the HFE mutations and osteoarthritis. Several studies associated the C282Y mutation to osteoarthritis and chondrocalcinosis, however, the generalizability of these studies has been questioned.[100] Overall, the findings from experimental and linkage studies on the relationship between the COL9A1 gene and the strong association between hemochromatosis and arthropathy, warrants more detailed studies on the relationship between the COL9A1 and HFE genes and osteoarthritis.

In summary, the term osteoarthritis refers to a group of etiologically and phenotypically heterogeneous disorders that mainly affects the joints. Twin studies and segregation analysis revealed a substantial heritability for osteoarthritis. Multiple genes may contribute to the development of osteoarthritis. From a pathologic perspective, the step forward is to identify these genes and determine how they function. From the public health perspective, the question is whether measuring a set of identified osteoarthritis genes can predict in future the susceptibility to osteoarthritis in an individual. Genetic studies aimed to identify new genes implicated in osteoarthritis may help to distinguish homogeneous groups of osteoarthritis or identify new pathways to underlying susceptibility to osteoarthritis.

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2.2

GENETIC EPIDEMIOLOGY OF HEMOCHROMATOSIS

Introduction

Hemochromatosis includes several disorders of iron metabolism characterized by pathological accumulation of iron in tissues.[1] Although there is no consensus on the definition of hemochromatosis, the disease is usually categorized into primary and secondary forms.[2] Primary hemochromatosis is referred to as hereditary hemochromatosis. It is an inherited disorder resulting from an inborn error of iron metabolism that leads to progressive iron loading of the parenchymal cells in the liver, pancreas, and heart.[1] Secondary hemochromatosis referred to as acquired hemochromatosis, is an iron overload disorder that occurs as a result of chronic disorders of erythropoiesis such as thalassemia or sideroblastic anemia.[2]

Hereditary hemochromatosis is one of the most common genetic disorders in populations of northern European descent with a prevalence of 0.2 to 0.5 percent.[2-6] Hemochromatosis can lead to multiple diseases like cirrhosis, hepatocellular carcinoma, cardiomyopathy, diabetes mellitus, amenorrhoea, impotence, arthritis, pituitary hypogonadism, and skin hyper pigmentation.[1,7,8] Early symptoms and complaints include joint pain, abdominal pain, weakness and fatigue.[8] Expression of the disease is modified by several factors, in particular dietary iron intake, blood loss associated with menstruation and pregnancy, and blood donation. The disease is 5 to 10 times more frequent in men than women and the age of onset is delayed in women.[9] Hemochromatosis does not usually express before 20 years of age, although with genetic screening and periodic health examinations, asymptomatic subjects with iron overload can be identified in adulthood.

For long, the diagnosis of hemochromatosis was based on the presence of excess iron in a liver biopsy in combination with serum iron, serum transferrin, and total iron binding capacity (TIBC).[10,11] In 1996, Feder and colleagues reported that two mutations in the HFE gene, the C282Y and the H63D are associated with hereditary hemochromatosis. The C282Y mutation is found in about 85 percent of patients with hereditary hemochromatosis. Since then, diagnostic procedures have shifted to biochemical and genetic tests.[12] Biochemical tests including serum iron, ferritin, and transferrin saturation level are now widely used in combination with genetic tests.[13,14] Hemochromatosis has been regarded as a model disease for large-scale genetic screening.[15,16] The aim of this chapter is to critically review the potential of genetic testing in hemochromatosis. Before we turn to preventive screening we will start with a brief review of the genetic epidemiology of hemochromatosis.

Genetic epidemiology of hemochromatosis

In 1935, Sheldon suggested that hemochromatosis is an inborn error of iron metabolism.[17] Studies of familial aggregation have extended from the 1970's up to the 1990's. Hemochromatosis is indeed found more commonly in relatives of patients.[18-23] Studies of the transmission of the disease in families suggest that hemochromatosis segregates usually as an autosomal recessive trait.[24-26] Genetic and phenotypic heterogeneity are well-recognised features in hemochromatosis and it is becoming more and more evident that several genes or environmental factors may lead to the disease.[17] Depending on the localisation of the genetic defect and the clinical phenotype, several types of hemochromatosis are distinguished.

Type 1 hemochromatosis

Type 1 hereditary hemochromatosis (*HFE*1 or simply *HFE*) is by far the most common form of hemochromatosis.[1,2,13,27,28] The culprit gene, termed *HFE,* is located on human chromosome 6p21.3 and has two major mutations, c.845G→A (C282Y) and c.187C→G (H63D).[12] Since its identification, over 37 other allelic variants of the *HFE* gene have been described.[29] The localization of the *HFE* protein in the crypt cells of the duodenum, the site of dietary iron absorption and its association with the transferrin receptor in those cells are consistent with a role in regulating iron absorption.[30-32] In *HFE* associated forms of hemochromatosis, the progression of iron overload is usually slow and affected individuals do not often present with clinical signs or symptoms until the fifth or sixth decade of life.[33] Type 1 hemochromatosis explains for a large part the prevalence of hemochromatosis (0.2 to 0.5 percent) found in northern Europeans.[3,5,28,33,34] *HFE* segregates in families as an autosomal recessive trait,[23,25] and about 80 percent of clinically diagnosed probands of hemochromatosis patients are homozygous for the C282Y mutation in the *HFE* gene.[1,12,35]

Type 2 hemochromatosis

Type 2 hemochromatosis (HFE2), also called juvenile hemochromatosis, differs distinctly from type 1 hemochromatosis.[36,37] This is a rare recessive form with a more severe disease phenotype that affects both sexes equally in the second decade of life.[13] There is rapid iron loading and early onset of cardiac symptoms, endocrine dysfunction (hypogonadotrophic hypogonadism) and premature death.[38,39] Kelly and colleagues (1998) reported a mean onset

of 22 years in patients from 3 pedigrees.[40] It has been recently suggested that more than one gene may underlie the phenotype of juvenile hemochromatosis.

Linkage to a locus on chromosome 1q has been found in patients with juvenile hemochromatosis. Recently, the putative gene encoding a protein designated hemojuvelin has been cloned that cause the main form of juvenile hemochromatosis.[41] A deleterious G320V mutation in the hemojuvelin gene[41] modulate hepcidin expression, a key protein implicated in iron metabolism.[41-43] Others also confirmed that mutations in hemojuvelin cause juvenile hemochromatosis.[44] A second rare form of juvenile hemochromatosis, with clinical expression identical to the 1q-linked form, is due to mutations in the HAMP gene leading to inactivation of hepcidin.[45-47] Hepcidin is a hepatic antimicrobial-like peptide the deficiency of which leads to iron overload.

Type 3 hemochromatosis

Type 3 hemochromatosis (HFE3) is phenotypically similar to HFE1.[13] The disease has been associated to the transferrin receptor 2 (TFR2) gene on human chromosome 7q22.[48-50] The TFR2 gene is homologous to the transferrin receptor (TFRC) gene and is able to bind transferrin with lower affinity than TFRC. The TFR2 function is still unclear. TFR2 is spliced in two alternative forms, Alfa and Beta. The Alfa form is strongly expressed in the liver. The Beta form, coded from a start site in exon 4 of the Alpha has a low and ubiquitous expression.[50] TFR2 mutations are very rare mutations.

Type 4 hemochromatosis

Contrary to the previously described forms of hemochromatosis, type 4 hemochromatosis or *HFE*4 segregates as an autosomal dominant trait.[51-53] The clinical phenotype of patients in this case is quite similar to that of patients with HFE1 hemochromatosis but differs in that the disease is less severe and the pattern of iron loading is distinct.[52-56] Iron accumulates predominantly in Kuppfer cells and other macrophages. Type 4 hemochromatosis (HFE4) is associated with various mutations (N144H, A77D, V162 del) in the SLC11A3 gene encoding the metal transporter called ferroportin (FPN1) alias, iron regulated transporter (IREG1) or metal transporter protein (MTP1) on human chromosome 2q.[52-56] The exact mechanism by which mutations in the SLC11A3 gene causes autosomal dominant iron overload is still not known. Gain of function and loss of function of the protein have both been suggested,[52,53] but

it is becoming more apparent that interactions between the SLC11A3 protein and other proteins involved in iron metabolism occur and can lead to iron accumulation.[57] A form of autosomal dominant iron overload clinically distinct from type 4 hemochromatosis and which is due to a single point mutation (A49U) in the iron responsive element of the H ferritin mRNA has been reported in a single Japanese family.[58]

Other types of hemochromatosis

Other forms of hereditary iron overload include neonatal hemochromatosis, hyperferritinemia cataract syndrome, aceruloplasminemia, congenital atransferrinemia, and African iron overload. African iron overload is common in sub-Saharan Africa and is a distinct type of iron storage disorder.[59] It is believed to result from increased dietary iron derived from traditional home-brewed beer. The etiology of neonatal hemochromatosis and hereditary hyperferritinemia cataract syndrome is not well understood. Aceruloplasminemia, and congenital atransferrinemia are due to the absence of ceruloplasmin and transferrin respectively and are secondary forms of iron overload. The pattern of iron deposition in patients suffering from these diseases is clearly different from that of classical hemochromatosis. Each of these disorders is rare.

Occurrence of mutations involved in hemochromatosis

HFE is the most widely studied gene that is involved in hemochromatosis. In the general Caucasian population, the carrier frequency of the C282Y mutation is estimated to be 10 percent, and for the H63D mutation, 22 percent.[28] In Caucasians, the most common form of hemochromatosis is due to homozygosity for the C282Y mutation or compound heterozygosity for the C282Y and H63D mutations in the HFE gene.[12] The proportion of hemochromatosis due to HFE mutations varies in different parts of the world. Figure 1 summarizes the published frequencies of carriers of HFE mutations in different populations (adapted from Hanson and colleagues 2001).[34] Most C282Y and H63D carriers are found in the United States of America and Europe. About 65 percent of the population of these two continents are homozygous for the wild type or normal allele compared to 85 percent in India, and about 95 percent in Africa, the Middle East, and Asia.

Up until now all other mutations involved in hemochromatosis are found to be rare, the contribution of HFE2 gene to the occurrence of hemochromatosis is thought to be limited

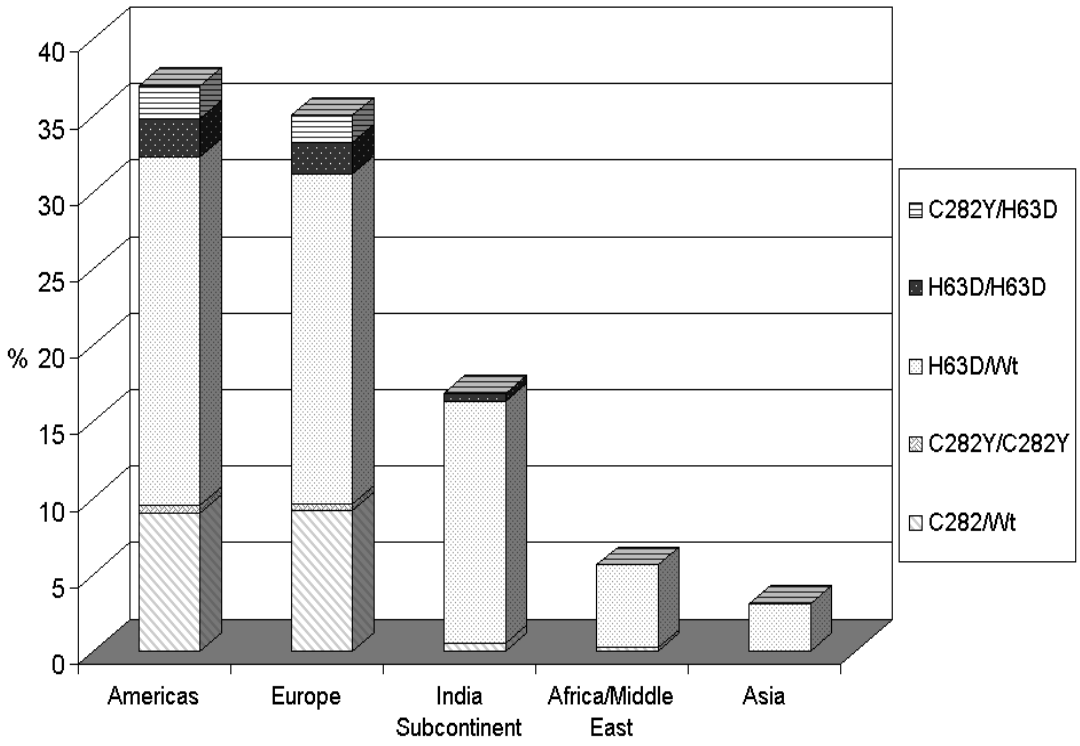

Figure 1. Frequencies of HFE C282Y and H63D mutations in different populations.

to a few families.[40,60] TFR2 mutations are rare but may occur frequently in the Italian population.[61] Although several mutations have been reported for the SCL11A3 gene, these mutations are thought to be rare in the general population.

Is genetic testing worthwhile?

In recent years there has been increasing interest in screening populations for hemochromatosis.[5,32,62,63] Hemochromatosis is an excellent example of a disease that meets the World Health Organization recommendations and the US preventive services task force criteria for a screening program. The disorder is common, it has a prolonged asymptomatic and early symptomatic phase, and if untreated can result in serious morbidity and premature death. Simple and effective screening tests for iron overload are available and there is a reliable confirmatory test. The treatment is safe and acceptable and in some countries the blood collected from venesection treatment is utilized by the blood transfusion services. It is still a matter of debate whether we should screen for hemochromatosis and if yes whether the test should be based on biochemical levels of serum iron parameters or based on the presence of common mutations in the HFE gene.[15,63-66] On the other hand, screening using DNA analysis is simple to carry out and has the additional advantage of detecting subjects with

delayed or incomplete penetrance, allowing diagnosis at an early age and treatment to prevent clinically significant iron overload.[67] However, not all subjects with iron overload carry the C282Y mutation.[27] This mutation is mainly found in Caucasians. This limits the application of this screen test to other ethnic groups. On the other hand, phenotypic measures such as biochemical iron levels are early indicators of disease but they have a low specificity and are less valuable for screening strategy. Phenotypic expression of hemochromatosis is very much influenced by age, diet, blood loss and menstruation, pregnancy and gene-gene interaction.

Another important parameter in evaluating a screening program is the cost-effectiveness of the latter. This is assessed by comparing the total diagnostic costs to the extra costs arising from managing the disease. Studies have shown that population screening for hemochromatosis is cost effective.[68,69] However screening for hemochromatosis like many other diseases has several disadvantages, among others ethical concerns, psychological troubles, over-medicalisation, and if screening is based on the genotype, many subjects with iron overload due to other reasons will be missed. Little is known of the psychological impact and ethical implications of a screening program for hemochromatosis. There is still lack of information on the natural history of the disease and the age-related penetrance of the disease is still unknown.

In deciding whether or not to screen, important quantitative parameters that should help in the decision are the positive predictive value (PPV), the sensitivity and the specificity of the test used. The PPV, the probability that a person with a positive test result will develop the disease is approximately equal to the penetrance of the disease and is a function of the frequency of the susceptibility-conferring genotype, the relative risk of the disease and the risk of disease in a given population.[70] It can be calculated as follows: PPV=[R (D) *100] / [G (R-1) +1], where R is the relative risk, D the incidence of the disease and G the frequency of the susceptibility conferring genotype.[71] Our study in the elderly population has shown that for all HFE mutations, the PPV was 10 percent in men and 5 percent in women.

The sensitivity (the probability that the test correctly classifies people with preclinical disease as positive) was 70 percent for men and 52 percent for women and the specificity (the probability that the test classifies as negative those who will not have the disease) was 62 percent for men and 64 percent for women.[72] A more or less important quantitative parameter is the population attributable risk (PAR). This is the proportion of cases of a disease that can be attributed to the susceptibility-conferring genotype. It can be calculated as follows; PAR= [G (R-1) *100]/(G (R-1) +1, where G is the frequency of a susceptibility conferring genotype

and R is the relative risk.[71] Only in the case of polymorphisms that have frequencies in the range of 10 to 30 percent and that increase susceptibility to disease will the PAR be appreciable. Single, highly penetrant gene mutations cause only a small proportion of the disease.[73] Our results in a population-based setting suggest that many sub-clinical cases of hemochromatosis will be missed when screening is based on HFE genotypes. These findings in the general elderly population suggest that the value of screening for high iron based on HFE genotypes is limited in that only a small percentage of subjects with elevated levels of iron will be detected. However, the aim of a population-based screening is to identify at an early stage individuals at risk of developing serious iron overload, to prevent organ damage. Although not all patients may be found, the implications for those who are found are high despite the controversy of the role of HFE in disease.

One reason why genetic screening for complex diseases is not advocated is that the risk for disease does not only depend on the gene but also on other factors like the environment, nutrition and genetic modifiers. Penetrance depends on at least six factors: (1) the importance of the function of the protein encoded by the gene, (2) the functional importance of the mutation, (3) the interaction with the environment, (4) onset of somatic mutations, (5) interaction with other genes, and (6) existence of alternative pathways that can substitute for the loss of function.

Another point of concern is the definition of the phenotype. There is no consensus on the definition of hemochromatosis and also no agreement on the clinical features of the disease among clinicians and experts. This situation has led to several approaches in estimating the penetrance of HFE mutations. While some authors consider clinical hemochromatosis as the end point, others have used combinations of signs and symptoms of hemochromatosis as end point to estimate the penetrance while other investigators have used phenotypic measures such as serum iron indices. These diverging outcomes have obviously led to diversity in quantification and estimates of penetrance. Four stages of the disorder are generally recognized; the genetic predisposition but without any abnormality, iron overload without any symptom, iron overload with early symptoms (lethargy, arthralgia), and iron overload with organ damage (cirrhosis especially).[1] Some authors[74] have argued that the excess of iron may not translate to associated diseases of hereditary hemochromatosis such as diabetes, but other disorders such as atherosclerosis, cancer. This hypothesis is supported by our own data suggesting that HFE is involved in artherosclerosis, particularly in smokers.[75] We have studied the association between the HFE mutations, carotid artherosclerosis, and

stroke. We observed that in the presence of additional risk factors (smoking and hypertension), there is increased risk of carotid artherosclerosis and stroke in carriers of HFE mutations.[75] HFE mutations only showed an overall weak association with stroke (odds ratio (OR) 1.3; 95% confidence interval (CI), 0.8 to 2.2). But patients with hypertension who were also carriers of the HFE mutations showed a significantly increased risk of stroke (OR 3.0; 95% CI, 1.9 to 4.6). Also HFE carriers who were also smokers had an increased risk of stroke (OR 2.6; 95% CI, 1.4 to 5.0). We conclude that HFE mutations modify the risk of stroke in subjects who already carry traditional risk factors.

Concerning the relationship with diabetes, we conducted a meta-analysis of the association between HFE mutations and diabetes and did not find any indication of an increased risk of diabetes in carriers of the HFE mutations (Figures 2 and 3).[76] Also in a population-based sample of elderly, we observed that 11 percent of patients with type 2 diabetes and 10.6 percent of controls were carriers of the C282Y mutation (OR 1.0, 95% CI, 0.6 to 1.7). For the H63D mutation, 25.7 percent of type 2 diabetes patients and 28.5 percent of control subjects were carriers (OR 0.8, 95% CI, 0.6 to 1.1).[70] Are the studies biased towards the null due to survival bias? This is difficult to believe but not impossible.

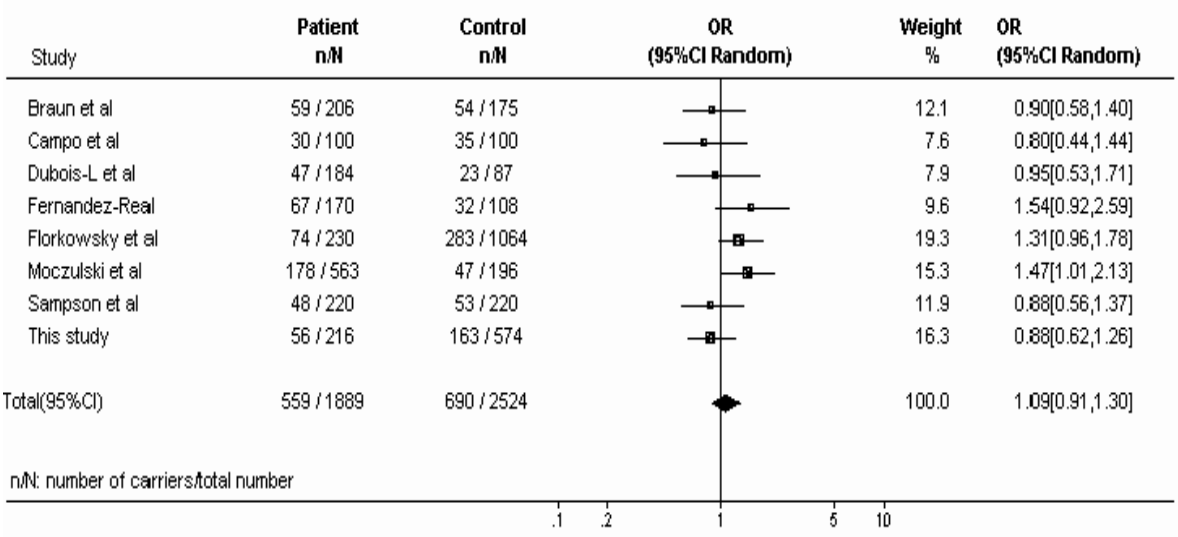

Figure 2. Meta-analysis of the frequency of the C282Y mutation in patients with type 2 diabetes.

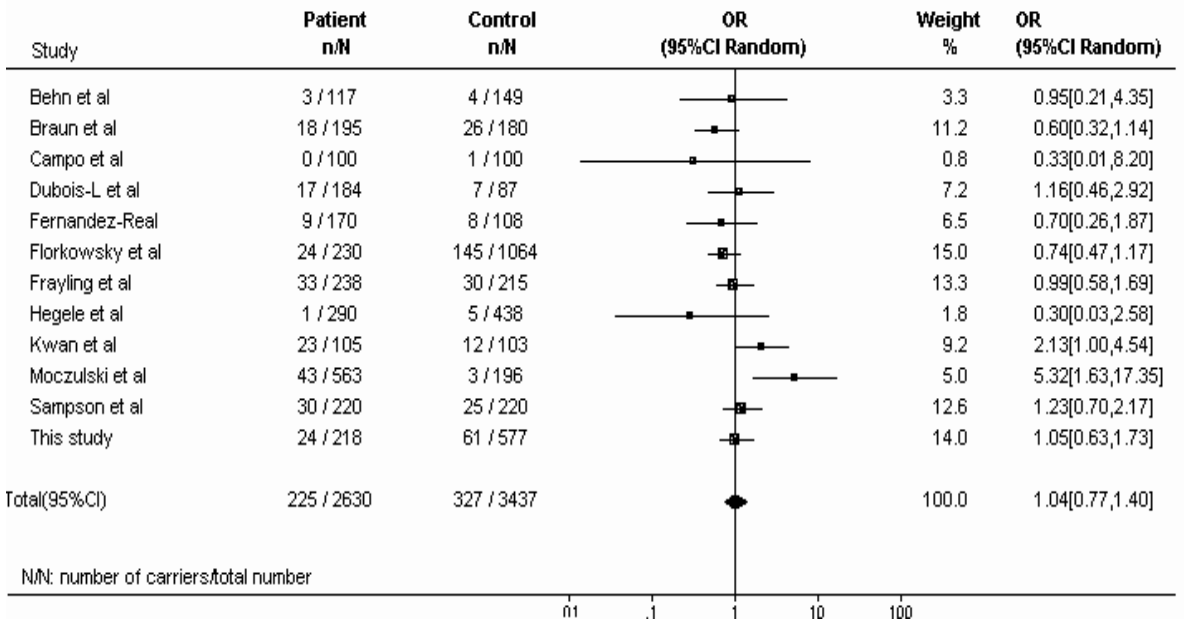

Figure 3. Meta-analysis of the frequency of the H63D mutation in patients with type 2 diabetes.

Conclusion

Hemochromatosis is a common genetic disease in Western populations. The potential for genetic screening for mutation carriers is *a priori* high. Although findings have been negative in the largest study to date, the possible biases in this study, addressed elsewhere,[77] leave the question to be answered whether screening for the HFE mutations is worthwhile. Our findings in the general elderly population suggest that the value for high iron based on HFE genotypes is limited, and only a small percentage of subjects with elevated levels of iron will be detected. However, the aim of a population-based screening is to identify at an early stage individuals at risk of developing serious iron overload so that treatment can be started to prevent organ damage. Not all patients may be found; the implications for those who are found are high. Thus, screening is helpful to identify high-risk groups.

In assessing the feasibility of screening for hemochromatosis, attention should not be directed only to the disease genotype or phenotype but also to the human being as end beneficiary. The translation of genetic and epidemiological advances in the field of hemochromatosis calls for studies on the cost-effectiveness, cost-benefit and cost-utility of screening for hemochromatosis to be carried out. From this point of view, all information critical for the assessment and implementation of population screening for hemochromatosis are still lacking and need the input and cooperation of scientists in several fields of research. Although many consider hemochromatosis as a good example of a disease that meet the

criteria for genetic screening, some key information is still necessary before genetic screening can be assessed.

The differential risk of disease seen with different genotypes and the evidence of incomplete penetrance for the genotype conferring the highest risk make genetic screening for hemochromatosis less worthy.

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3

ASSOCIATION STUDIES

3.1

THE COL9A1 GENE AND OSTEOARTHRITIS

Abstract

Collagen IX proteoglycan is an important protein in collagen networks and has been implicated in hip osteoarthritis. We studied two COL9A1 markers (509-8B2 and 509-12B1) in relation to radiographic osteoarthritis in the Rotterdam Study, a population-based study of 7983 subjects aged 55 years or over. We used two different designs. First a sibling-pairs study of 83 probands with radiographic osteoarthritis at multiple joints, and their 221 siblings yielding 445 sibling pairs who participated in the study. Second, an association study in a series of 71 patients with hip radiographic osteoarthritis and 269 controls. All subjects were characterized for the two COL9A1 509-8B2 and 509-12B1 markers. The mean test was used to assess the proportion of alleles shared in concordantly affected and unaffected sibling pairs. The chi-squared test was used to compare the allele distributions in cases and controls. We found that affected sibling-pairs with radiographic osteoarthritis at hip joints shared significantly ($p<0.05$) more often alleles identical by descent (IBD) at the 8B2 (mean 0.66± standard error 0.07) and 12B1 (0.65±0.08) markers than expected. No excess sharing was observed for radiographic osteoarthritis at other joint sites. When comparing the allele frequency of 8B2 and 12B1 in cases and controls, the frequency of 8B2 alleles in cases differed significantly ($p<0.01$) from that of controls. Our data suggest that susceptibility for hip osteoarthritis is conferred within or close to the COL9A1 gene in linkage disequilibrium with the COL9A1 509-8B2 marker.

Osteoarthritis is a common complex disorder worldwide[1] and is the leading cause of disability and pain in the elderly.[2] Family-based and candidate gene studies demonstrated a clear genetic component for primary osteoarthritis.[3,4] One of the main pathological characteristics of osteoarthritis is the degradation of hyaline cartilage. The collagen fibril networks is one of the essential components that maintains the integrity of hyaline cartilage and prevents its degradation.[5] Among collagen fibrils, type IX collagen links the collagen type II-containing fibrils to the rest of the cartilage matrix, and thus plays a role in the cartilage integrity.[6] Type IX collagen is composed of three genetically distinct alpha (α) polypeptide chains i.e. α1(IX), α2(IX), and α3(IX), encoded by COL9A1, COL9A2, and COL9A3, respectively.[6]

Deficiency of α1(IX) polypeptide has been shown to lead to functional abnormality in collagen IX fibrils, and thus to instability of hyaline cartilage.[7] This observation suggests that mutations in the COL9A1 gene that leads to a non-functional α1(IX) polypeptide may be implicated in osteoarthritis. There are some evidences to support this view. Transgenic mice that express a non-functional protein as well as knock-out mice indeed develop osteoarthritis[8] suggesting COL9A1 as a candidate gene for osteoarthritis in human. There is some evidence that this gene is involved in hip osteoarthritis.[9,10] Affected sibling-pairs studies found linkage of COL9A1 to a severe form of hip osteoarthritis in women.[9,10] However, association studies failed to show any relationship.[9] Another question that remains to be answered is whether COL9A1 is involved in other joints than the hip.

In the present study, we investigated two polymorphisms in the COL9A1 gene (509-8B2 and 12B1) in relation to radiographic osteoarthritis at different joint sites in two independent studies, a sibling-pairs study including 445 pairs with hip, knee, and hand radiographic osteoarthritis or spinal disk degeneration, and an association study of 71 persons with radiographic osteoarthritis at hip joints and 269 controls.

Methods

Study population The present study was conducted in the framework of the Rotterdam Study, a population-based cohort study of chronic diseases in 7983 subjects aged 55 years or over.[11] The medical ethics committee of the Erasmus Medical Center has approved the study. Written informed consent was obtained from all participants. Baseline examination took place between 1990 and 1993 by means of a structured interview using standardized questionnaires.

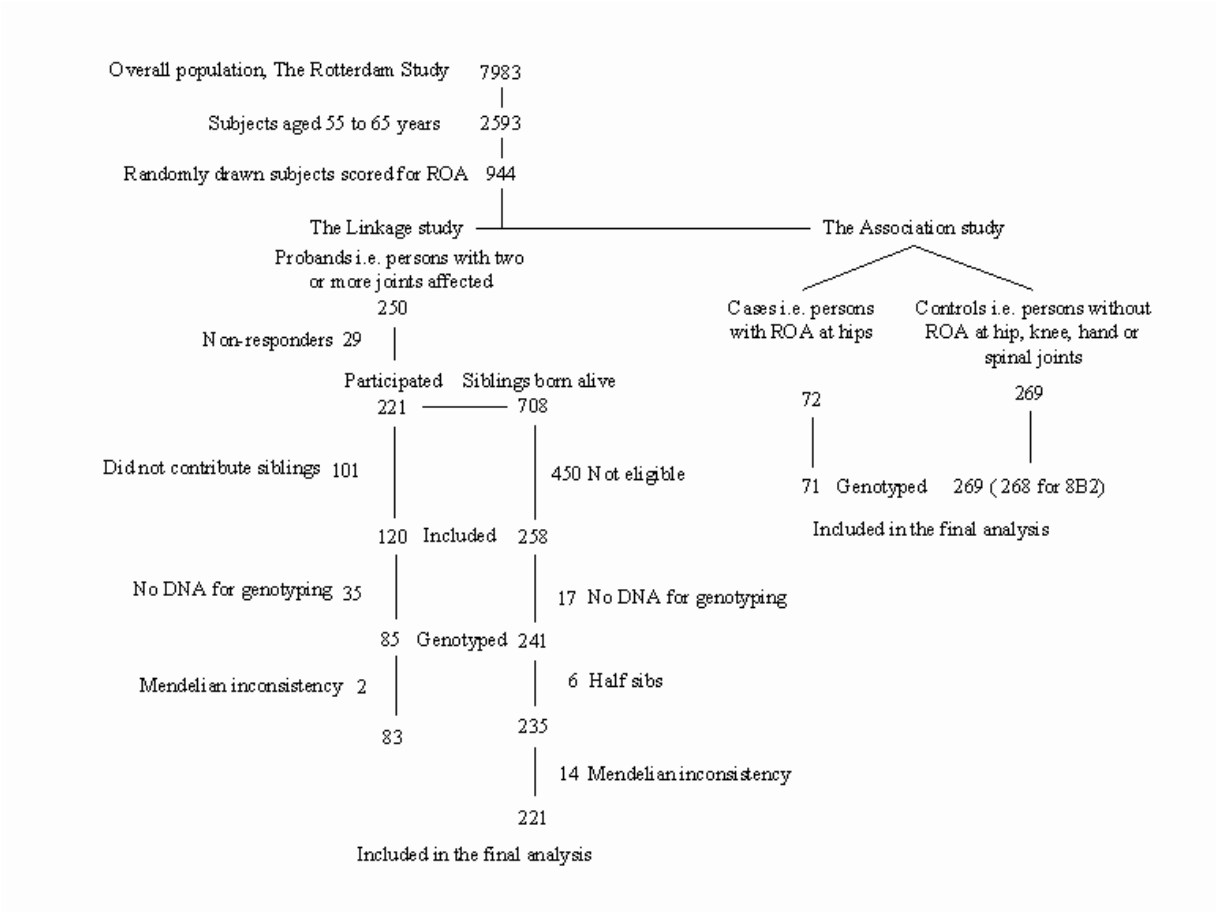

Figure 1. Flow-chart outlining the participation of probands, and siblings in the linkage study and cases and controls in the association study.

Figure 1 presents a flow-chart of the participation of the study population. From the total cohort of subjects aged 55 to 65 years (n=2593), a random cohort including 944 non-institutionalized persons was drawn and scored for radiographic osteoarthritis at hip, knee, and hand joints and for disk degeneration of the spine.

Radiographic examination Radiographs of hip, knee, and hand joints of participants of the Rotterdam Study, and the siblings were scored for the presence of radiographic osteoarthritis. Radiographs of the spine were evaluated for the presence of disk degeneration as proposed by Kellgren and Lawrence.[12] The diagnosis of radiographic osteoarthritis was considered for any joint with a Kellgren score two or higher. Two independent readers scored all radiographs. After each set of about 150 radiographs, the scores of the two readers were evaluated. Whenever the scores were two or more points different, or was two for one reader but one for

the other, a consensus was agreed upon. All radiographs were scored before genotyping and this was performed blind to clinical data.

Linkage study- For linkage analysis, probands were derived from the random cohort. Persons who had radiographic osteoarthritis at two or more joint sites of the four joint groups i.e. hips, knees, hands, or the spine were selected as probands. In case individuals had hand radiographic osteoarthritis and disk degeneration of the spine, which was the most common combination observed, additionally they had to have Heberden's nodes to be included as probands. This criterion was applied to maximize the probability of a genetic form of radiographic osteoarthritis. Two hundred and twenty-one (response rate 88 percent) probands were willing to contribute to the study, yielding 708 siblings born alive (Figure 1). Four hundred and fifty siblings of 101 probands were not eligible for the study due to siblings death, refusal, emigration, disease, and non-response. In total, 258 siblings and 120 probands derived from 120 pedigrees were included in the study. The siblings were examined at the research center using the same protocol and methods as those used to examine the participants in the random cohort.

Association study- Within the random cohort, 72 persons with radiographic osteoarthritis at hip joints were genotyped. The 269 persons who did not have radiographic osteoarthritis at hip, knee, or hand joints were selected as controls (Figure 1).

Genotyping for COL9A1 509-8B2 and 12B1 markers Participants were genotyped for *COL9A1* 509-8B2 and 12B1 short tandem repeat polymorphism (STRP) according to the protocol of Warman and colleagues.[13] Genotyping was successful for 85 probands and 241 siblings in the sibling pair study, and in the association study, for 71 cases, and all controls except for 8B2 in 1 control subject (Figure 1).

Data analysis

Linkage study- Familial relation between siblings was confirmed using the genealogical data. There were six half sibs who were excluded from the analysis (Figure 1). Mendelian inconsistency in pedigrees was checked using MARKERINFO module. Given the siblings genotypes in nuclear families, this module reconstructs siblings' genotypic sets and thereafter the parental genotypes. Pedigrees with Mendelian inconsistency are identified whenever one or two alleles of the studied markers in any sibling do not match with the family genotypic sets. Two probands and 14 full siblings, who belonged to 4 pedigrees with Mendelian

inconsistencies in 1 or both of the two markers, were excluded from the analysis (Figure 1). The remaining 83 probands and 221 siblings of 100 pedigrees yielded a total number of 445 sibling-pairs. Sibling pairs were classified according to affection status as concordantly affected i.e. both siblings had radiographic osteoarthritis, concordantly unaffected i.e. both siblings had no radiographic osteoarthritis and discordant siblings i.e. one sib was affected with another sib unaffected at the studied joint site. We used the mean test, which is a powerful test for additive inheritance to compare the average proportion of allele shared IBD with the expected value of 0.5.[14] On average, sibling pairs share half of the alleles at a given locus IBD. Concordantly affected sibling-pairs should share alleles IBD more than 50 percent at COL9A1 if this locus is linked to radiographic osteoarthritis. The analysis was adjusted for age and gender, the two major determinants of osteoarthritis. Sibling-pairs data was analyzed using S.A.G.E. version 4.4.

Association study- Allele and genotype frequencies for the 8B2 and 12B1 markers were estimated by counting alleles and estimating sample proportion. Allele and genotype proportions were tested for Hardy Weinberg equilibrium. The chi-squared test was used to compare allele frequencies between cases and controls.

Results

Table 1 shows the characteristics of the study population. The mean age of siblings was significantly ($p<0.001$) higher and body mass index was lower ($p<0.05$) than that of probands. In the final analysis each pedigree contributed on average 4.5 (range 1 to 36) sibling pairs to the linkage study. Among probands, 33 percent had radiographic osteoarthritis at hip, 78 percent at knee, 78 percent at hand joints, and 64 percent had spinal disk degeneration. Among the siblings, 7 percent had radiographic osteoarthritis at hip, 19 percent at knee, 75 percent at hand joints, and 79 percent had spinal disk degeneration. In the association study, there was no significant difference in gender, body mass index, or bone mineral density between cases with hip radiographic osteoarthritis and controls. Cases were slightly (1 year) older than controls ($p=0.05$). Allele and genotype proportions were in Hardy Weinberg equilibrium.

Table 2 shows the results of the linkage analysis in affected and unaffected sibling pairs. Affected sibling pairs (n=11) with radiographic osteoarthritis at hip joints had a significant ($p<0.05$) excess in IBD allele sharing in the COL9A1 8B2 (mean 0.66± standard error 0.07) and 12B1 (0.65±0.08) markers.

Table 1. Characteristics of the study population.†

	Linkage study		Association study	
	Probands	Siblings	Cases	Controls
Number	83	221	71	269
Age (years)	60.90±2.71*	65.80±8.02	60.76±2.43**	59.71±2.84
Women (%)	69.22	50.25	41.66	49.07
Body mass index (kg/m^2)	27.36±4.23	26.71±4.01	26.45±3.46	25.65±3.26
Bone mineral density (cg/cm^2)	0.91±4.23*	0.86±0.14	0.90±0.13	0.87±0.13

Frequency of families by the number of sibling pairs

	Number of families	Number of contributing sibling-pairs
1 sibling-pair	54	54
3 sibling-pairs	20	60
6 sibling-pairs	12	72
10 sibling-pairs	4	40
11 sibling-pairs	1	11
15 sibling-pairs	3	45
21 sibling-pairs	3	63
28 sibling pairs	1	28
36 sibling-pairs	2	72

†Mean± standard deviations are presented. *$p<0.05$ compared to siblings; **$p<0.05$ compared to controls.

Table 2. Mean proportion (± standard errors) of COL9A1 509-8B2 and 12B1 alleles shared identical by descent (IBD) over the presence of radiographic osteoarthritis.

COL9A1 marker	Sibling-pairs phenotype†	Joint site with radiographic osteoarthritis							
		Hip		Knee		Hand		Spine	
		n		n		n		n	
509-8B2	Concordantly affected	11	0.66±0.07*	41	0.49±0.05	251	0.51±0.02	243	0.52±0.02
	Concordantly unaffected	327	0.50±0.02	205	0.51±0.02	26	0.52±0.06	39	0.54±0.06
509-12B1	Concordantly affected	11	0.65±0.08*	30	0.50±0.05	251	0.50±0.02	243	0.50±0.02
	Concordantly unaffected	327	0.49±0.02	212	0.49±0.02	26	0.52±0.07	39	0.50±0.06

*$p<0.05$ indicating a significant increase in mean proportion of alleles shared IBD from the expected value of 0.5. †The data on discordant pairs was not presented.

The 11 sibling pairs with hip radiographic osteoarthritis belonged to 9 families consisted of a total number of 19 siblings (1 family contributed 3 affected sibling-pairs). Among the sibling pairs with radiographic osteoarthritis at hip joints, 3 pairs were homozygous for COL9A1 8B2 allele 5/ allele 6 genotype i.e. both siblings had the 5/6 genotype, 2 pairs for 5/5 and 1 pair for 4/6. The remaining sibling-pairs were heterozygous for 8B2 i.e. two sibling-pairs had a 5/5 and 5/6 genotype set, one 5/2 and 5/6, one 5/4 and 9/4, one 5/6 and 9/6. When considering the 12B1 marker, 2 sibling-pairs were homozygous for 4/6 genotype, 1 pair for 4/8 and 1 pair for 4/4 genotype. The rest of sibling-pairs were heterozygous for 12B1 i.e. two pairs had 4/4 and 4/8 genotype sets, 2 pairs had 4/6 and 5/6, 1 pair had 4/8 and 8/8, 1 pair had 4/4 and 4/6, and 1 pair had 3/6 and 3/5 genotype set. No significant differences for the other joints were observed. The number of allele shared in affected and unaffected sibling-pairs were similar suggesting there is no evidence for a role of COL9A1 in radiographic osteoarthritis at other joints. The frequency of 8B2 or 12B1 alleles was not significantly different between negative controls and the total population. Table 3 shows the frequency of 8B2 and 12B1 alleles by the

Table 3. Frequency of COL9A1 509-8B2 and 12B1 alleles by radiographic osteoarthritis (ROA) at hip joints.

COL9A1 marker	Hip ROA	Alleles							P value
		5	6	7	8	9	Others*		
509-8B2	Present	50 (0.35)	27 (0.19)	14 (0.10)	10 (0.07)	20 (0.14)	21 (0.15)		0.01
	Absent	231 (0.43)	140 (0.26)	29 (0.05)	22 (0.04)	62 (0.12)	52 (0.10)		
		2	4	5	6	7	8	Others*	
509-12B1	Present	14 (0.10)	50 (0.35)	27 (0.19)	15 (0.11)	5 (0.03)	23 (0.16)	8 (0.06)	0.10
	Absent	96 (0.18)	172 (0.32)	67 (0.12)	44 (0.08)	33 (0.06)	92 (0.17)	34 (0.06)	

Figures are numbers (frequencies). *Alleles with a frequency of less than 0.05 are summed in the category others.

presence of radiographic osteoarthritis at hip joints. The frequency of 8B2 alleles differed significantly ($p \leq 0.01$) between subjects with compared to those without radiographic osteoarthritis at hip joints. The frequency of 12B1 alleles was not significantly different between subjects with and without radiographic osteoarthritis at hip joints.

Discussion

In this population-based study, we found that affected sibling pairs with radiographic osteoarthritis at hip joints shared significantly higher number of alleles IBD at 2 markers in COL9A1 (8B2 and 12B1 STRPs). Further, in the association study, we found that 8B2 marker was significantly associated to radiographic osteoarthritis at hip joints.

The positive linkage of the COL9A1 locus in our sibling-pairs confirmed earlier findings of linkage in female sibling-pairs with hip osteoarthritis,[9,10] although we could not stratify for gender as the numbers were too low for a meaningful statistical analysis. Despite the fact that the number of sibling-pairs was small in our study, the excess of sharing was statistically significant. Also in our association study, we found a significant relation between the COL9A1 8B2 marker and radiographic osteoarthritis at hip joints. The relevance of our finding is not completely clear since the significance was marginal, various alleles together

contribute to the association and no association was found to the nearby 12B1 marker. One previous association study on the relation between COL9A1 8B2 and 12B1 markers and radiographic osteoarthritis has been reported. No association of 8B2 or 12B1 with severe hip osteoarthritis was found in a study of 146 women selected from families with osteoarthritis.[9]

There are two important points of consideration when interpreting the difference between our findings and those of Loughlin and colleagues (2000).[9] First, in contrast to a linkage study the relation in an association study can be easily missed since the marker used in the two studies is not very powerful for association analysis due to a large number of rare alleles. The genetic information content of a marker depends on heterozygosity index, a function of marker allele frequencies, as well as on the location of the marker on genome map, and the functional effect of the marker variants. In the present study, the polymorphic nature of the studied markers resulted in multiple strata of cases and controls thus demolishing the power of the association study. Second, although we hypothesize that the COL9A1 locus contributes to osteoarthritis susceptibility, the 8B2 marker is not likely causally related to radiographic osteoarthritis. 8B2 is located in COL9A1 intron 4 that resides in 17.7 kilobase (kb) downstream of the start of a haplotype block of 65 kb within COL9A1. This haplotype block is encompassed by intron 1 (-501) to intron 34 (+32).[9] Thus 8B2 may be in strong linkage disequilibrium with other COL9A1 mutations. 12B1 is located 14.3 kb upstream of exon 1 and resides outside the COL9A1 haplotype block. Further, COL9A1 mapped to a region where other FACIT-like collagen e.g. COL19A1[15] have been also mapped. Although the association of marker 8B2 with hip osteoarthritis might be explained by linkage disequilibrium with adjacent loci which suggests an osteoarthritis susceptibility locus may map near to COL9A1 locus, several experimental studies support the role of COL9A1 locus in osteoarthritis.[7,8] Those studies[7,8] showed that COL9A1 gene knockout mice developed early-onset osteoarthritis.

Taken together with earlier findings, our data suggest that osteoarthritis susceptibility may map within or near to the COL9A1 gene, with 509-8B2 simply being a marker for this. In our sibling-pairs data, there was no evidence for a role of COL9A1 in other forms of osteoarthritis. Further studies are necessary to identify the underlying mutation in COL9A1 or within a nearby osteoarthritis susceptibility locus.

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3.2

THE HFE GENE AND ARTHROPATHY

Abstract

Arthropathy is one of the most common manifestations in patients with hereditary hemochromatosis. The HFE C282Y and H63D mutations are the most common causes of hereditary hemochromatosis. We investigated the relation between the HFE C282Y and H63D mutations with arthralgia and joint pathology in the population-based Rotterdam Study. From a cohort of 7983 people aged 55 years or over, 2095 randomly drawn subjects were genotyped for C282Y and H63D mutations. We compared the frequency of arthralgia, and the presence of chondrocalcinosis, osteophytes, joint space narrowing and osteoarthritis at radiographs of hand, hip and knee joints, and Heberden's nodes in carriers of HFE mutations to that in non-carriers. Overall, there was a significantly higher frequency of arthralgia (odds ratio 1.6; 95 percent confidence interval 1.0 to 2.6), oligoarthralgia (2.3; 1.2 to 4.4) and Heberden's nodes (2.0; 1.1 to 3.8) in those homozygous for H63D compared to non-carriers. In persons aged 65 years or younger, H63D homozygotes had significantly more often polyarthralgia (3.1; 1.3 to 7.4), chondrocalcinosis at hip or knee joints (4.7; 1.2 to 18.5), increased number of hand joints with osteophytes (mean 6.1± standard deviation 1.0 versus 4.4±0.3), joint space narrowing (2.8±0.5 versus 1.0±0.1), radiographic osteoarthritis (4.4±0.7 versus 2.0±0.2), and Heberden's nodes (3.1; 1.3 to 12.8). We found no relation of arthralgia or joint pathology to C282Y, but compound heterozygotes had a significantly higher frequency of arthralgia (2.9; 1.0 to 9.3), chondrocalcinosis at hip (6.5; 1.8 to 22.3), and increased number of osteophytes at knee (6.9±1.2 versus 2.4±0.1) joints at late age (65 years or over). The HFE H63D mutation may explain at least in part the prevalence of arthralgia at multiple joints sites, chondrocalcinosis, and hand osteoarthritis in the general population.

In type I hereditary hemochromatosis, arthralgia affects up to 85 percent of patients,[1-6] seriously influencing quality of life.[7] Hand and knee are the joints most often affected.[8-10] Most of our knowledge on the relationship between hemochromatosis and arthropathy is developed studying patients or families with the hereditary form of the disease. In patients with hemochromatosis, arthropathy may originate from a progressive degenerative arthritis initially presenting at hand joints,[9, 10] but can also originate from an inflammatory mediated condition like chondrocalcinosis.[9, 10, 12] Occasionally, arthropathy in hemochromatosis may resemble rheumatoid arthritis,[8, 9] accompanied by Heberden's nodes.[13] Main radiographic findings in hemochromatosis arthropathy are calcium crystal depositions, osteophytes and joint space narrowing.[9, 10]

The C282Y and H63D mutations in the HFE gene are the most common mutations involved in hereditary hemochromatosis.[5, 14-16] Eleven percent of Caucasians are carriers of C282Y and 23 percent of the total population worldwide are carriers of H63D.[15] The risk of hemochromatosis is increased for those homozygous for C282Y (4383 folds) or compound heterozygotes i.e. carriers of both H63D and C282Y (32 folds).[15] Also, H63D homozygotes are estimated to have a 6 fold increased risk of hemochromatosis,[15] although iron levels are modestly increased.[15, 17, 18]

Findings on the relation between HFE mutations and arthropathy are neither consistent nor conclusive. Some studies found no relation between C282Y and self-reported arthropathy,[19] inflamed joints,[20] chondrocalcinosis,[21, 22] or subchondral arthritis.[21] Other studies reported a significant association between C282Y and chondrocalcinosis,[23] or late onset hand osteoarthritis.[22] Few studies addressed the role of H63D.[21, 22] The generalisability of these studies has been a matter of concern.[24] We have studied the HFE C282Y and H63D mutations in the population-based Rotterdam Study.[18, 25] The mutations were studied in relation to arthralgia as well as joint pathology assessed at radiographs including chondrocalcinosis at hip or knee joints, presence of osteophytes, joint space narrowing, radiographic osteoarthritis at hand, hip or knee joints, and Heberden's nodes. Further, we investigated the relation between HFE, joint pain and overall mortality.

Methods

Population This study was carried out in the framework of the Rotterdam Study, a population-based cohort study of major chronic diseases. The medical ethics committee of

Erasmus Medical Center has approved the study and informed consent was obtained from all participants. The design and objectives of the study have been described elsewhere.[26] In brief, the study population consists of 7983 inhabitants aged 55 years or over living in the district of Ommoord in Rotterdam. Baseline examinations took place between 1990 and 1993 by means of a structured interview using a standardized questionnaire. From the total cohort, 2095 subjects randomly drawn were genotyped for the HFE C282Y and H63D mutations. In the Rotterdam Study, participants were followed up to 11.3 years. During the follow up period, information on the vital status of all participants was obtained at regular intervals from municipal health authorities in Rotterdam. The data on hospital admissions and a corresponding diagnosis of hemochromatosis were retrieved from interviewing participants, and medical records of the participants' general practitioners.

Main outcome measures At baseline examination, participants were asked whether they had any pain or other complaints in or around their joints. If yes, the research physicians questioned participants about the site and duration of joint complaints. The study physicians asked participants whether they had a medical diagnosis of orthopedic, traumatic, rheumatologic, or other diseases and whether they used any kind of pain medication or were treated with physiotherapy because of their joint complaints. Further at the research center, study physicians examined the hand of participants for the presence of Heberden's nodes, a common local form of osteoarthritis at distal interphalangeal joint with inflammatory episodes associated with generalized osteoarthritis.[29, 30] Within the randomly selected cohort (n=2095), clinical data were available on the presence of arthralgia for 2047 and on the presence of Heberden's nodes for 1833 of subjects.

The baseline anteroposterior radiographs of hip and knee joints of a random subset of the population were scored for the presence of chondrocalcinosis by two independent observers who were blinded to all information on participants as explained elsewhere.[27] Presence of osteophytes and space narrowing in anteroposterior radiographs of hands were assessed in the distal and proximal interphalangeal joints, the interphalangeal joint of thumb, the metacarpophalangeal joints, the first carpometacarpal joints, and the trapezoscaphoideal joints. Radiographic osteoarthritis at hand, hip and knee joints were graded as proposed by Kellgren and Lawrence.[28] The diagnosis of radiographic osteoarthritis was considered for any joint with a Kellgren score two or higher. Within the randomly selected cohort, the data on presence of chondrocalcinosis at hip or knee joints were available for 1132 persons, on the

presence of osteophytes, joint space narrowing and radiographic osteoarthritis at hand joints for 1274, at knee joints for 1112 and at hip joints for 1352 persons. Finally, for H63D or C282Y homozygotes (n=65), all radiographs at baseline and follow up were re-examined for the presence of osteophyte, joint space narrowing, sclerosis, cyst formation, calcification, and chondrocalcinosis in subchondral bone at hand, hip and knee joints and at spinal joints for disk degeneration, spondylophytes, and calcification by a rheumatologist who was blinded to clinical data.

Blood samples were collected on the day of baseline examination by venepuncture. Mutations analysis was performed as described elsewhere.[14]

Data analysis The extent of arthralgia was classified into 4 groups. The first group consisted of those without arthralgia (the reference group), the second group of those with pain at one joint site, the third group of those with pain at two joint sites (oligoarthralgia), and the fourth group of those with pain at three or more joint sites (polyarthralgia). Presence of osteophytes at hand joints was transformed to a quantitative trait by summing up the number of joints with osteophytes. The same procedure was applied for the presence of joint space narrowing and radiographic osteoarthritis. The HFE C282Y genotypes were modeled by assigning a value of 0, 1 or 2 for carriers of no (non-carriers), one (C282Y heterozygotes), or two (C282Y homozygotes) copies of the C282Y mutation, respectively. The same procedure was carried out for H63D. Genotype proportions were tested for Hardy Weinberg equilibrium. Independent *t* statistics, ANOVA and χ^2 tests were used for comparisons of means and frequencies. We fitted statistical models using logistic regression analysis to test the association of C282Y or H63D and the risk of arthralgia overall and at different joint sites, chondrocalcinosis at hip or knee joints, or Heberden's nodes in the right and/or left hand, and radiographic osteoarthritis at hip or knee joints. The magnitude of the association was expressed as odds ratio (OR) with 95 percent confidence interval (95% CI). Univariate regression analysis was used to estimate mean with the standard errors for the number of hand joints with osteophytes, joint space narrowing, or radiographic osteoarthritis by the HFE genotypes. For the study of mortality, we used Cox proportional regression analysis. All analyses were adjusted for age and gender. As a relation of C282Y heterozygosity to hand osteoarthritis was found in patients aged 65 years or over,[22] and since differences may exist in the etiopathogenesis of early and late onset arthralgia or arthropathy,[34] we stratified our

analysis by age using a cut-off point of 65 years. A two sided p<.05 was considered as statistically significant.

Results

Baseline characteristics The baseline characteristics of the participants are presented in Table 1. Persons with arthralgia were more often women and users of pain medications (p<0.001). Genotype frequencies and baseline characteristics did not differ between persons aged 65 years or younger and those aged 65 years or over, and between persons who had data on genotype, clinical and radiographic findings compared to others (data not shown). In persons with arthralgia, the number of joints with pain for each subject ranged from 1 to 10 (median=2). Allele and genotype proportions were in Hardy Weinberg equilibrium overall and in persons without arthralgia. The baseline characteristics did not differ across the HFE genotypes, except that H63D homozygotes aged 65 years or younger were significantly (p<0.02) more often user of pain medications and/or physiotherapy than non-carriers (data not shown).

Table 1. Participants' characteristics by age and presence of arthralgia†

	Age ≤ 65 years			Age > 65 years		
Characteristics	With arthralgia (n=473)	No arthralgia (n=493)	p-value*	With arthralgia (n=526)	No arthralgia (n=555)	p-value*
Age (years)	60.3±0.1	60.3±0.1	0.2	71.2±0.2	70.8±0.2	0.7
Women (%)	58.9	41.1	<0.001	63.7	42.7	<0.001
Body mass index (kg/m^2)‡	26.1±0.2	26.3±0.2	0.1	25.9±0.2	26.3±0.2	0.4
User of painkiller or physiotherapy (%)	69.8	30.2	<0.001	68.7	31.3	<0.001
Frequency of HFE mutations (%)						
C282Y	6.2	6.3	0.9	5.5	6.0	0.3
H63D	16.7	15.7	0.3	14.4	15.5	0.4

†Plus-minus values are means± standard errors. ‡Body mass index was calculated as weight in kilograms divided by the square of height in meters.*p value for comparison of subjects with and without arthralgia.

HFE mutations and arthralgia Overall, H63D homozygotes had significantly a higher frequency of polyarthralgia (OR 1.6; 95% CI 1.0 to 2.6; p<0.05) and oligoarthralgia (2.3; 1.2 to 4.4; p<0.01) compared to non-carriers. The frequency of arthralgia was not increased in C282Y or H63D heterozygotes compared to non-carriers. Table 2 presents the analysis stratified by age. H63D homozygotes aged 65 years or younger had a significantly higher frequency of arthralgia (3.1; 1.3 to 7.4; p<0.01) compared to non-carriers. Figure 1A shows that H63D homozygotes had a significantly increased risk of arthralgia at hands (4.0; 1.4 to 11.7; p<0.001), hips (3.2; 1.0 to 10.8; p<0.05) and knees (3.5; 1.2 to 10.1; p<0.05). In those aged 65 years or over, the frequency of arthralgia did not differ by *HFE* genotypes (Table 2 and Figure 1B).

Table 2. The frequency of arthralgia at any joint site by HFE genotypes

		Age ≤ 65 years			Age > 65 years		
HFE genotypes		n	Percent	OR (95% CI)†	n	Percent	OR (95% CI)†
C282Y	Non carriers	847	49.1	1.0 (Reference)	959	49.0	1.0 (Reference)
	Heterozygotes	116	47.4	0.9 (0.6-1.4)	119	47.5	0.9 (0.6-1.3)
	Homozygotes	3	66.7	1.5 (0.1-17.2)	3	33.3	0.6 (0.1-6.7)
H63D	Non carriers	679	49.2	1.0 (Reference)	791	49.6	1.0 (Reference)
	Heterozygotes	261	46.0	0.9 (0.6-1.2)	257	45.5	0.9 (0.6-1.1)
	Homozygotes	26	73.1	3.1 (1.3-7.4)*	33	51.5	1.0 (0.5-2.1)

Abbreviation: OR, Odds ratios compare the prevalence of arthralgia among subjects heterozygous or homozygous for the C282Y or H63D mutations to that of non-carriers, calculated using logistic regression analysis while adjusting for age and gender; CI, Confidence interval. *p<0.01 for comparison with non-carriers.

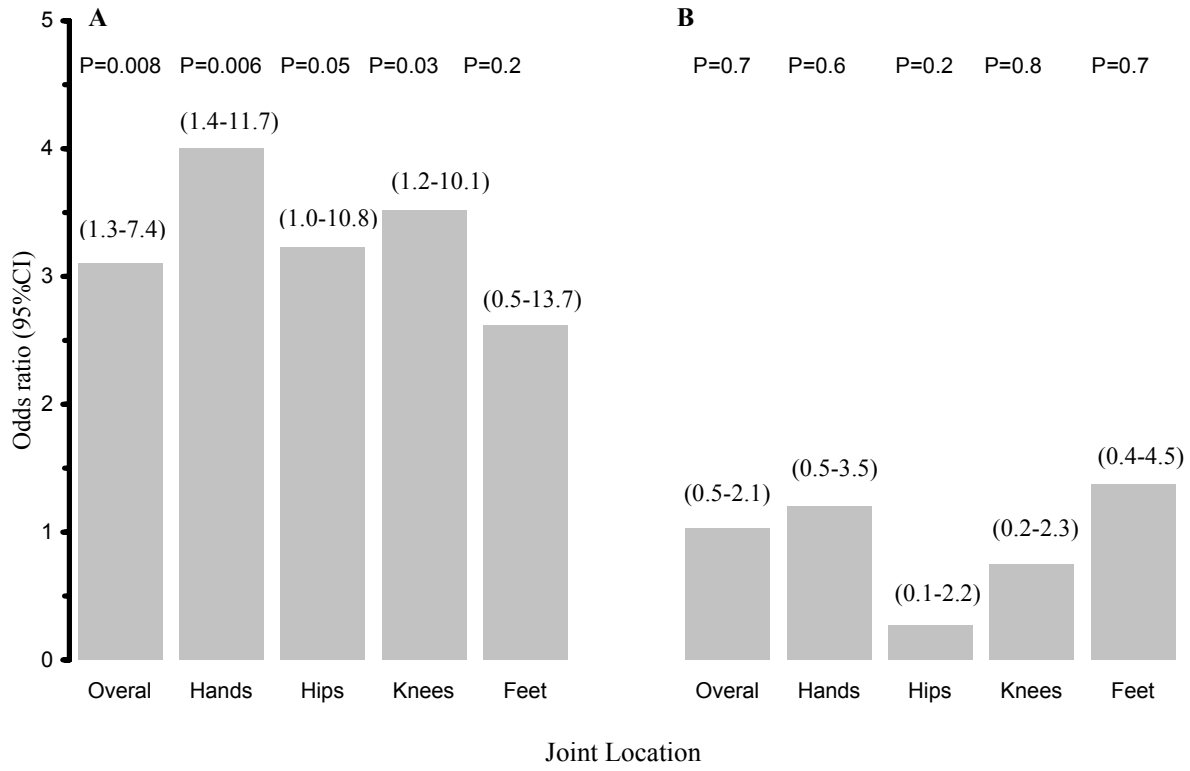


Figure 1. Odds ratio for arthralgia in H63D homozygotes aged (A) ≤ 65 and (B) > 65 years. Figures within the brackets indicate 95 percent confidence intervals.

HFE mutations and chondrocalcinosis Overall, there was no significant difference in the frequency of chondrocalcinosis at hip or knee joints by HFE genotypes. When stratifying by age (Table 3), H63D homozygotes aged 65 years or younger had a significantly higher frequency of chondrocalcinosis compared to non-carriers (4.7; 1.2 to 18.5; p<0.02).

HFE mutations and radiographic osteoarthritis Overall, the number of joints with osteophytes at hands increased significantly with the numbers of H63D mutation (p for trend<0.01). Among persons aged 65 years or younger, the number of joints with osteophyte was increased in H63D heterozygotes (mean 5.2± standard error 0.4; p<0.03) or homozygotes (6.1±1.0; p=0.08) compared to non-carriers (4.4±0.3; p for trend<0.03; Table 4). In H63D homozygotes compared to non-carriers, the number of hand joints with space narrowing (2.8±0.5 versus 1.0±0.1), or with radiographic osteoarthritis (4.4±0.7 versus 2.0±0.2) were significantly increased. Again, no relation to HFE genotypes was found in persons aged 65 years or over. We found no significant difference in number of osteophytes, presence of joint space narrowing or radiographic osteoarthritis across HFE genotypes at either hip or knee joints (data not shown).

Table 3. The frequency of chondrocalcinosis at hip or knee joints by HFE genotypes.

		Age ≤ 65 years			Age > 65 years		
HFE genotypes		n	Percent	OR (95% CI)*	n	Percent	OR (95% CI)*
C282Y	Non carriers	469	4.5	1.0 (Reference)	516	7.4	1.0 (Reference)
	Heterozygotes	74	2.7	0.7 (0.1-2.9)	60	8.3	1.2 (0.1-3.3)
	Homozygotes	2	0.0	-	2	0.0	-
H63D	Non carriers	372	4.0	1.0 (Reference)	434	6.9	1.0 (Reference)
	Heterozygotes	146	4.1	1.1 (0.4-2.8)	147	10.2	1.8 (0.9-3.5)
	Homozygotes	14	21.4	4.7 (1.2-18.5)*	19	0.0	-

Abbreviation: OR, Odds ratios compare the prevalence of arthralgia among subjects heterozygous or homozygous for the C282Y or H63D mutations to that of non-carriers, calculated using logistic regression analysis while adjusting for age and gender; CI, Confidence interval. *p<0.02 for comparison to non-carriers.

Table 4. Number of hand joints with osteophytes, joint space narrowing or radiographic osteoarthritis (ROA) by HFE genotypes.†

		Osteophytes				Joint space narrowing		ROA‡	
		Age ≤ 65 years		Age > 65 years		Age ≤ 65 years	Age > 65 years	Age ≤ 65 years	Age > 65 years
HFE genotypes		n		n					
C282Y	Non carriers	590	5.0±0.3	534	6.3±0.4	1.4±0.1	1.6±0.2	2.4±0.5	3.3±0.4
	Heterozygotes	78	5.5±0.6	71	5.8±0.7	1.9±0.3	1.2±0.4	2.3±0.4	2.6±0.6
	Homozygotes	3	5.3±2.5	3	2.3±4.9	0.2±1.5	2.3±3.0	-	3.6±4.2
H63D	Non carriers	466	4.4±0.3	446	4.7±1.6	1.0±0.1	2.0±1.0	2.0±0.2	3.5±1.4
	Heterozygotes	184	5.2±0.4*	142	4.7±1.7	1.2±0.2	1.6±1.0	2.4±0.3	3.2±1.4
	Homozygotes	18	6.1±1.0	18	4.9±2.0	2.8±0.5**	1.5±1.2	4.4±0.7**	2.8±1.7

†Figures are mean± standard error, calculated using univariate linear regression analysis while adjusting for age and gender. ‡ROA was diagnosed for any joint with a Kellgren score 2 or higher. *p<0.03, **p<0.01 for comparison with non-carriers.

HFE mutations and Heberden's nodes Overall, 21.5 percent of H63D homozygotes (n=51) compared to 16.9 percent of non-carriers (n=1316) had Heberden's nodes (OR 2.1; 95% CI 1.1 to 3.9; p<0.02). Again, H63D homozygotes aged 65 years or younger had a significantly (p<0.01) higher frequency of Heberden's nodes (3.1; 1.3 to 12.8; Table 5). The frequency of Heberden's nodes by HFE genotypes did not differ in H63D or C282Y heterozygotes, or in those aged 65 years or over.

Compound heterozygotes and outcomes Compound heterozygotes aged 65 years or younger were associated with none of the outcomes under the study. Compound heterozygotes aged 65 years or over had a significantly higher frequency of polyarthralgia (2.9; 1.0 to 9.3; p<0.05), increased number of osteophytes at knee joints in the overall analysis (4.9±0.6 versus 2.2±0.1; p<0.01) and in those aged 65 years or over (6.9±1.2, n=5 versus 2.4±0.1, n=374; p<0.01). At hands, the number of joints with osteophytes, space narrowing or radiographic osteoarthritis and the frequency of Heberden's nodes did not significantly differ between compound heterozygotes aged 65 years or over and non-carriers.

Table 5. The frequency of Heberden's nodes by HFE genotypes.*

		Age ≤ 65 years			Age > 65 years		
HFE genotypes		n	Percent	OR (95% CI)	n	Percent	OR (95% CI)
C282Y	Non carriers	701	19.7	1.0 (Reference)	835	19.2	1.0 (Reference)
	Heterozygotes	107	15.0	0.9 (0.5-1.7)	110	11.8	0.6 (0.3-1.0)
	Homozygotes	2	50.0	4.0 (0.2-65.3)	2	0.0	-
H63D	Non carriers	637	16.0	1.0 (Reference)	726	17.9	1.0 (Reference)
	Heterozygotes	246	16.3	1.0 (0.7-1.6)	240	19.6	1.1 (0.7-1.6)
	Homozygotes	23	34.8	3.1 (1.3-12.8)*	28	25.0	1.4 (0.6-3.5)

Abbreviation: OR, Odds ratios compare the prevalence of arthralgia among subjects heterozygous or homozygous for the C282Y or H63D mutations to that of non-carriers, calculated using logistic regression analysis while adjusting for age and gender; CI, Confidence interval. *p=0.02 for comparison with non-carriers.

HFE mutations, arthralgia and mortality To explore why we found a strong relation of H63D homozygosity to arthralgia and arthropathy before age 65 years but not later in life, we studied the mortality in H63D homozygotes. In persons aged 65 years or younger, H63D homozygotes with arthralgia at any joint had a 4 (95% CI 1.4 to 11.7; $p<0.01$) fold increased risk of mortality compared to non-carriers without arthralgia during the follow up period.

C282Y or H63D homozygotes and clinical arthropathy When the radiographs of H63D homozygotes (n=59) or C282Y (n=6) were re-examined by a rheumatologist specifically for the presence of pathology related to hereditary hemochromatosis, most subjects had two or more joints affected with multiple pathologies such as osteophytes, sclerosis, joint space narrowing and calcification (Figure 2). The clinical findings with regard to the features that did not discuss earlier are summarized in Table 6. Only in three persons (4.6 percent), the radiographic findings were recognized as compatible with hereditary hemochromatosis. Of C282Y homozygotes, three persons aged less than 65 years had osteoarthritis at hands and among them one underwent total hip replacement. Among the others, one had mild generalized osteoarthritis, another one had articular calcification, and the last had a moderate spondylophytosis.

Table 6. Radiographic findings in subjects homozygous for the *HFE* C282Y or H63D mutations.*

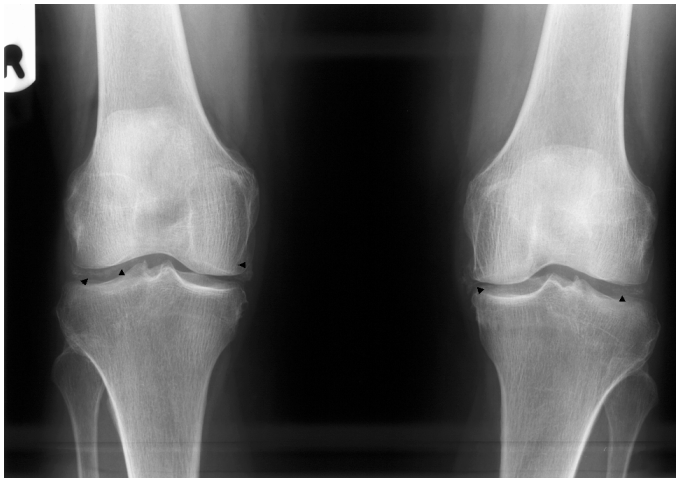
	Age ≤ 65 years				Age > 65 years			
Radiographic findings	Hips	Knees	Hands	Spine	Hips	Knees	Hands	Spine
C282Y homozygotes (n=6)		(n=3)				(n=3)		
Spondylophytes	-	-	-	100.0	-	-	-	100.0
Articular or periarticular calcifications	33.3	33.3	0.0	0.0	0.0	33.3	0.0	0.0
Subchondral bony sclerosis	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Subchondral bony cysts	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
H63D homozygotes (n=59)		(n=25)				(n=34)		
Spondylophytes	-	-	-	96.0	-	-	-	50.0
Articular or periarticular calcifications	20.0	16.0	20.8	16.0	0.0	6.0	37.0	13.3
Subchondral bony sclerosis	16.0	4.0	20.8	12.0	17.9	0.0	29.6	6.6
Subchondral bony cysts	8.0	0.0	4.0	0.0	0.0	3.0	14.3	0.0

*Figures are percentages.

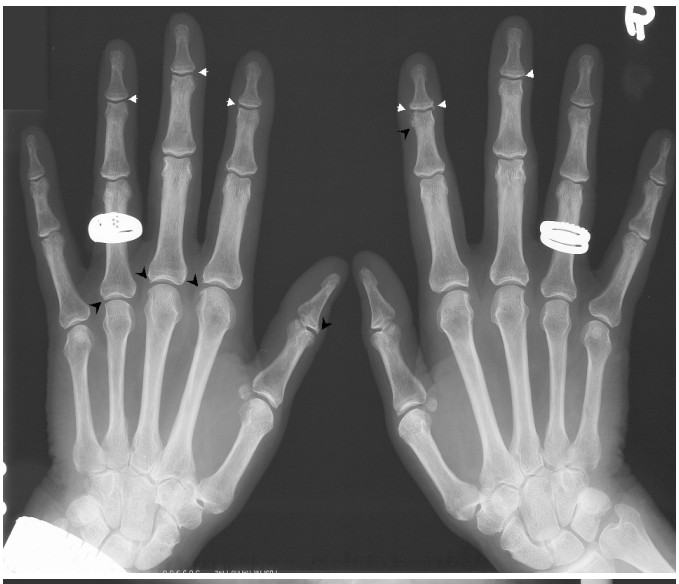
HFE mutations and clinical hemochromatosis None of C282Y or H63D homozygotes, or compound heterozygotes had received a diagnosis of clinical hemochromatosis from their general practitioner or any other physician at the baseline or during the follow up.

Figure 2. Arthropathy in H63D homozygotes.
2a shows a knee with chondrocalcinosis, arrows show a marked deposition of calcium crystal in synovial cartilage. 2b shows osteoarthritis at hands accompanied by clear calcium crystal deposition (➔) in cartilage of bone and synovium in distal interphalangeal, osteophytes (➤) in metacarpophalangeal joints. 2c shows a severe osteoarthritis in both hips with large osteophytes (➤), severe joint space narrowing particularly in right joint (➔), large subchondral cysts (▷) in femoral neck and left trocanter, stigma of therapeutic osteotomy for osteoarthritis (➔).

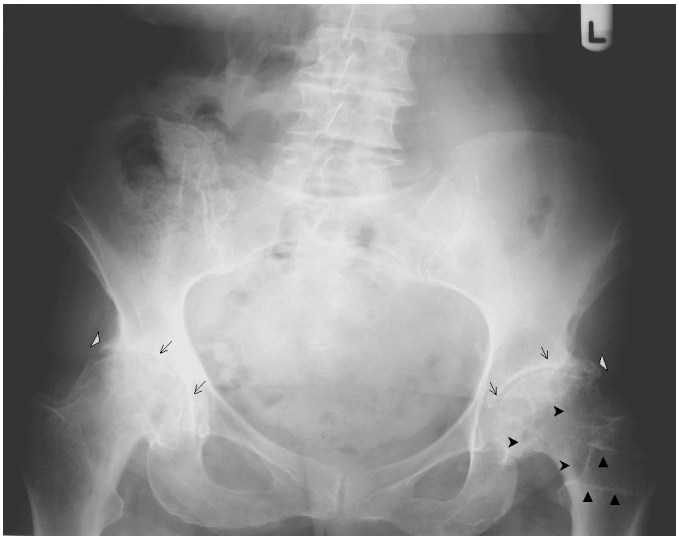
2a



2b



2c



Discussion

Main findings This study evaluated the relation between HFE and arthropathy in the general population. Overall, we found that H63D homozygotes had more often arthralgia. In persons aged 65 years or younger, H63D homozygosity was consistently associated to arthralgia at multiple joint sites, chondrocalcinosis, radiographic osteoarthritis at hands, and Heberden's nodes. H63D homozygotes used more often pain medication. We found that H63D homozygotes with arthralgia had a higher mortality. We found no association to C282Y homozygotes or heterozygotes. In persons aged 65 years or over, compound heterozygosity was associated to arthralgia, chondrocalcinosis at hip and osteophytes at knee joints.

Advantages and limitations of the current study A point of concern for population-based studies of genetic factors is the probability of bias due to population admixture.[35, 36] The Rotterdam Study consists of an ethnically homogenous population. Typing of multiple genetic markers has not revealed any evidence for the presence of population admixture.[37] Another source of bias may be observer related misclassification. All radiographs were scored blinded to other clinical data and genotyping. Therefore, the occurrence of spurious associations due to population admixture or a selective misclassification is unlikely. The major strength of our study is its population-based design. Most studies on the HFE gene mutations have been on clinical based samples. Another strength of our study was the use of several related clinical (subjective) and radiographic (objective) outcomes.

C282Y, H63D and arthropathy We observed that H63D homozygotes had a consistent increased risk of early onset arthralgia and arthropathy at multiple joint sites. In line with this finding, H63D homozygotes used more often pain medication in our study population. We found no relation to arthropathy in C282Y homozygotes or heterozygotes for C282Y. The effect of C282Y on iron metabolism is much stronger than that of H63D,[18, 38] and thus the risk for hemochromatosis is the highest.[15] Therefore, one may expect a stronger association to arthropathy in C282Y carriers. There are a number of explanations why we failed to find this trend. One may speculate that the numbers of C282Y homozygotes were too few to draw a definite conclusion in our study. However, this finding is not unique to our population. Others also found no relation to arthralgia or joint pathology in carriers of C282Y.[19-21] One of these studies composes over 40000 persons who were screened for *HFE* and showed no relation of

arthralgia to C282Y homozygosity (n=128) or compound heterozygosity (n=616).[19] Together, these findings suggest that C282Y is not a determinant of arthralgia in the general population. One study reported a small relation of C282Y to chondrocalcinosis,[23] and another study reported a relation between C282Y heterozygosity and late onset hand osteoarthritis.[19] For C282Y heterozygosity we found an effect on arthralgia and arthropathy only in compound heterozygotes for C282Y and H63D after age 65 years. These findings suggest that C282Y heterozygosity may have a late effect, whereas H63D homozygosity showed an early effect in our study.

From a pathological prospect, the question is whether the levels of iron determine the relationship between H63D and early onset arthropathy. In fact, there is clinical support for the view that the iron overload may not be the main determinant of arthropathy as arthropathy shows a poor response to phlebotomy,[2] neither did arthropathy show a relation to iron concentration in the liver,[3] nor to levels of serum iron or ferritin in our population (Data not shown). Moreover, arthropathy can occur with moderate iron overload,[1] and is uncommon in other forms of iron storage diseases,[39] suggesting the arthropathy may not be explained directly by iron overload.[2,3,8] Further research will be needed to determine the precise mechanism by which H63D may affect the risk of arthralgia and arthropathy. The report on the relation between H63D and rheumatoid arthritis,[40] the consistent relation of H63D to arthralgia at multiple joint sites, to Heberden's nodes, which represents an inflammatory component in pathogenesis of osteoarthritis,[29,41] to chondrocalcinosis, an inflammatory mediated condition,[12] and to early onset hand osteoarthritis suggest an alternative mechanism i.e. the involvement of an inflammatory component in H63D associated arthropathy. Understanding the underlying pathologic process may provide new targets for intervention in arthropathy associated to hemochromatosis.

Clinical implications In the present study, H63D homozygosity was associated to arthralgia at multiple joint sites and arthropathy. Earlier, we have shown that C282Y and H63D homozygotes had higher levels of serum iron and ferritin in the same study population.[18] However, these persons did not have diabetes mellitus,[42] a disease associated to hemochromatosis. But those HFE homozygotes who smoked or had hypertension, had a higher risk for atherosclerosis or stroke.[25] C282Y or H63D homozygotes or compound heterozygotes had no other complaint to the treating physicians recognized as hemochromatosis; and thus did not have a clinical diagnosis of hemochromatosis. This

suggests that carriers of H63D may initially presents with arthropathy perhaps together with excess iron but without other associated diseases of hereditary hemochromatosis like diabetes mellitus, or liver pathology. As a result at the early stages and in the absence of typical clinical features, the disease may remain undiagnosed or be misdiagnosed and thus untreated. Untreated, disease may progress to irreversible complications like liver diseases,[5] or may lead to cerebro- cardiovascular events like stroke,[25] leading to early death. In this respect, the significant higher mortality in a subgroup of H63D homozygotes with arthralgia aged 65 years or younger is of concern. Further, the early mortality may explain why the association of H63D homozygosity to arthralgia or arthropathy is stronger early in life and weak in those older than 65 years. Further studies are necessary to translate our findings into clinical and public health practice.

Conclusions Taken together, our findings suggest that H63D may explain at least in part the early onset arthropathy in the general population. Although this remains to be confirmed by others, our observation suggests that testing for HFE mutations in patients with arthralgia aged less than 65 years may be clinically relevant.

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3.3

THE HFE H63D MUTATION, INFLAMMATION AND MORTALITY

Abstract

The H63D mutation in the hemochromatosis gene (HFE) has been associated to pain and osteoarthritis at hand joints, and to mortality in the general population. We investigated the relation between H63D mutation, Heberden's nodes, and their joint effect on overall and cause-specific mortality. Within the total population of the Rotterdam Study, a population-based cohort study of 7983 persons aged 55 years or over, 2332 randomly drawn subjects have been genotyped for the H63D mutation. Participants were followed up to 13.6 years. Cox proportional regression analysis was used to estimate the risk of mortality (Hazard ratio; HR) and all analyses were adjusted for age and gender. Overall, no relation was found between mortality and the HFE H63D genotypes. The presence of Heberden's nodes was significantly related to a modest increase in mortality (HR 1.3; 95% CI 1.0 to 1.6, $p \leq 0.05$). Persons homozygous for the H63D mutation with Heberden's nodes had a substantial increase in mortality risk compared to subjects homozygous for the wild type allele without Heberden's nodes (HR 2.7; 95% CI 1.2 to 5.7, $p \leq 0.01$). This was explained by an increase in mortality risk due to stroke (HR 4.0; 1.2 to 12.9, $p \leq 0.05$). Persons homozygous for H63D with Heberden's nodes are characterized by increased levels of C-reactive protein (CRP) in serum ($p < 0.001$). Increased levels of serum CRP were not found in those with Heberden's nodes who were not homozygous for the H63D mutation. The increased inflammatory state in carriers may explain in part the increased mortality due to stroke. Our study suggests that inflammation may explain the increased risk of mortality in H63D homozygotes with Heberden's nodes.

The common HFE H63D mutation has been associated to hand osteoarthritis, a common complaint in hemochromatosis patients.[1-3] In a previous study,[4] we found that H63D homozygotes with arthralgia are at an increased risk of early mortality. Further we found that Heberden's nodes are more prevalent in persons homozygous for this mutation.[4] We hypothesized that H63D homozygosity may be associated with a high state of inflammation based on evidence that the prevalence of Heberden's nodes, an inflammatory associated condition, was higher in HFE H63D homozygotes in our previous study.[4] Consequently, patients homozygous for the H63D mutation with Heberden's nodes are expected to be at increased risk of mortality due to increased inflammation. Within a population-based follow up study of 7983 persons aged 55 years or over, we tested whether the H63D homozygosity and Heberden's nodes lead to increased mortality due to increased levels of inflammation. We examined the relation between the H63D mutation, Heberden's nodes to the levels of serum CRP in a population-based study, the Rotterdam Study.

Methods

Population The present study was carried out within the framework of the population-based Rotterdam Study, a cohort study of major chronic diseases in the elderly. The medical ethics committee of the Erasmus Medical Center has approved the study, and informed consent was obtained from all the participants. The design and objectives of the study have been described elsewhere.[5] In brief, 7983 (response rate 78 percent) inhabitants of the district of Ommoord in Rotterdam aged 55 years or over participated in the study. Baseline examinations took place between 1990 and 1993 by means of structured interview using a standardized questionnaire. Participants were followed up to 13.6 years. From the total population, 2332 randomly drawn subjects were genotyped for the HFE C282Y and H63D mutations.

Assessment of Heberden's nodes During the visit to the research center, trained study physicians examined the hand of the participants for the presence of Heberden's nodes. Within the random cohort (n=2332), clinical data on the presence or absence of Heberden's nodes were available for 2005 subjects.

Assessment of mortality Information on the vital status and cause of death of all participants was obtained at regular intervals from municipal health authorities in Rotterdam. Causes of

death were coded according to the ICD-10 system.[6] For the cause specific study, we focused on the three major causes of death i.e. cancer defined as code C00 to D48, coronary heart disease as code I20 to I25.9, I70, I70.9, and cerebrovascular disease as code I60 to I69.4. Mortality data was available for all subjects within the random cohort. For 1664 persons the data on H63D genotypes, Heberden's nodes and mortality was available.

Measurement of serum CRP Blood samples were collected on the day of baseline examinations by venepuncture. Serum CRP (mg/dL) was quantified by nephelometric method using the Beckman Coulter High Sensitivity C-Reactive Protein reagent on the fully automated IMMAGE® Immunohistochemistry System. Within the random cohort, measurement of the levels of serum CRP was successful for 1940 subjects.

HFE genotyping Genomic DNA was extracted from a frozen buffy coat using the salting out protocol as described elsewhere.[7] Mutation analysis was performed as described previously[8] and was successful for both mutations in 2122 subjects. Subjects with the C282Y mutation (n=253) were excluded from the present study. The remaining 1869 subjects were homozygous for the wild type allele or carriers of the H63D mutation. For 1559 subjects H63D genotyping, amount of Heberden's nodes and measurement of CRP levels were available. Allele and genotype frequencies were in Hardy Weinberg equilibrium.

Data analysis Presence of Heberden's nodes at the distal interphalangeal joint at both hands was considered as a dichotomous variable. The H63D mutation was coded as 0 (wild type homozygotes i.e. H63D non-carrier), 1 (H63D heterozygous), or 2 (H63D homozygous). To study the joint effect of the H63D mutation and Heberden's nodes on mortality as well as the levels of serum CRP, we stratified the random cohort into four categories. The first category consisted of subjects homozygous for the wild type allele who did not have Heberden's nodes (the reference group), the second category of H63D homozygotes without Heberden's nodes, the third category of wild type homozygotes with Heberden's nodes, and the last category of H63D homozygotes with Heberden's nodes. Independent *t,* ANOVA and chi-square tests were used to compare means and frequencies. Cox proportional regression analysis was used to estimate the risk (Hazard ratio; HR) of mortality in carriers of H63D compared to subjects homozygous for the wild type allele. All analyses were adjusted for gender and age (years) at

the baseline examination. A two tailed p-value <0.05 was considered as statistically significant.

Results

Table 1 shows the baseline characteristics of the study population. The mean (±S.E.) age of the random cohort at the baseline examination was 66.5 (±0.1) years and participants were more often women (54.0 percent). Overall 19.6 percent of subjects had Heberden's nodes.

Heberden's nodes were significantly (p=0.001) more often present among women (24.6 percent) than men (13.9 percent). The overall population risk of mortality was 27.1 percent during the follow-up period. Table 2 presents the mortality by H63D genotypes and Heberden's nodes. H63D by itself was not associated to increased risk of mortality. The mortality in persons with Heberden's nodes was modestly but significantly (p<0.05) increased (Table 2).

Figure 1 shows the joint effect of the H63D mutation, and Heberden's nodes on mortality. The risk of mortality of H63D was only significantly (p<0.01) increased for persons who were homozygous and had Heberden's nodes compared to wild type

Table 1. Baseline characteristics of the study population.

Characteristics	Overall	H63D genotypes		
		Wt/Wt homozygotes	H63D/Wt heterozygotes	H63D/H63D homozygotes
Number of participants	2122	1314	496	59
Age (years)	66.5±0.1	66.2±0.2	66.0±0.3	66.8±0.9
Women (%)	54.0	53.6	49.2	50.8
Heberden's nodes (%)	19.6	19.1	20.1	29.4
Body mass index (kg/m^2)	26.1±0.1	26.2±0.1	26.1±0.2	25.8±0.1

Plus minus figures represent mean (±S.E.).

Table 2. Mortality by (a) HFE H63D mutation or (b) Heberden's nodes.

	n	Percent of Death	HR (95% CI)
a. The H63D genotype			
Wt/Wt homozygotes	1314	28.4	1.0 (Reference)
H63D/Wt heterozygotes	496	25.6	0.9 (0.8-1.1)
H63D homozygotes	59	33.9	1.1 (0.7-1.8)
b. Heberden's nodes			
Absent	1611	22.8	1.0 (Reference)
Present	394	26.9	1.3 (1.0-1.6)*

HR, hazard ratios were adjusted for age and gender. *p<0.05.

homozygotes without Heberden's nodes (HR 2.7; 1.2 to 5.7). The mortality in this subgroup was also significantly increased compared to the other subgroups. H63D homozygotes with Heberden's nodes died significantly ($p<0.05$) more often of stroke compared to wild type homozygotes without Heberden's nodes (4.0; 1.2 to 12.9). No other association was found to other causes of death i.e. cancer, or coronary heart disease (data not shown).

Figure 2 shows the relation between H63D genotypes, Heberden's nodes and their joint effects on CRP levels at the baseline examination. Levels of serum CRP were increased in persons homozygous for H63D mutation (mean±S.E. 6.1±0.9, n=46) compared to persons homozygous for the wild type allele (2.7±0.2, n=1208). However, this difference was not statistically significant (Figure 2, Graph A). When the levels of serum CRP were analyzed by Heberden's nodes, persons with Heberden's nodes showed a modest non-significant increase in the levels of serum CRP (3.5±0.3, n=365) compared to those without this condition (2.7±0.1; n=1496; Figure 2, Graph B). H63D homozygotes with Heberden's nodes had increased serum levels of CRP (15.5±1.6; n=13) compared to those homozygous for the wild type allele who did not have Heberden's nodes (2.8±0.2; n=1220; Figure 2, Graph C).

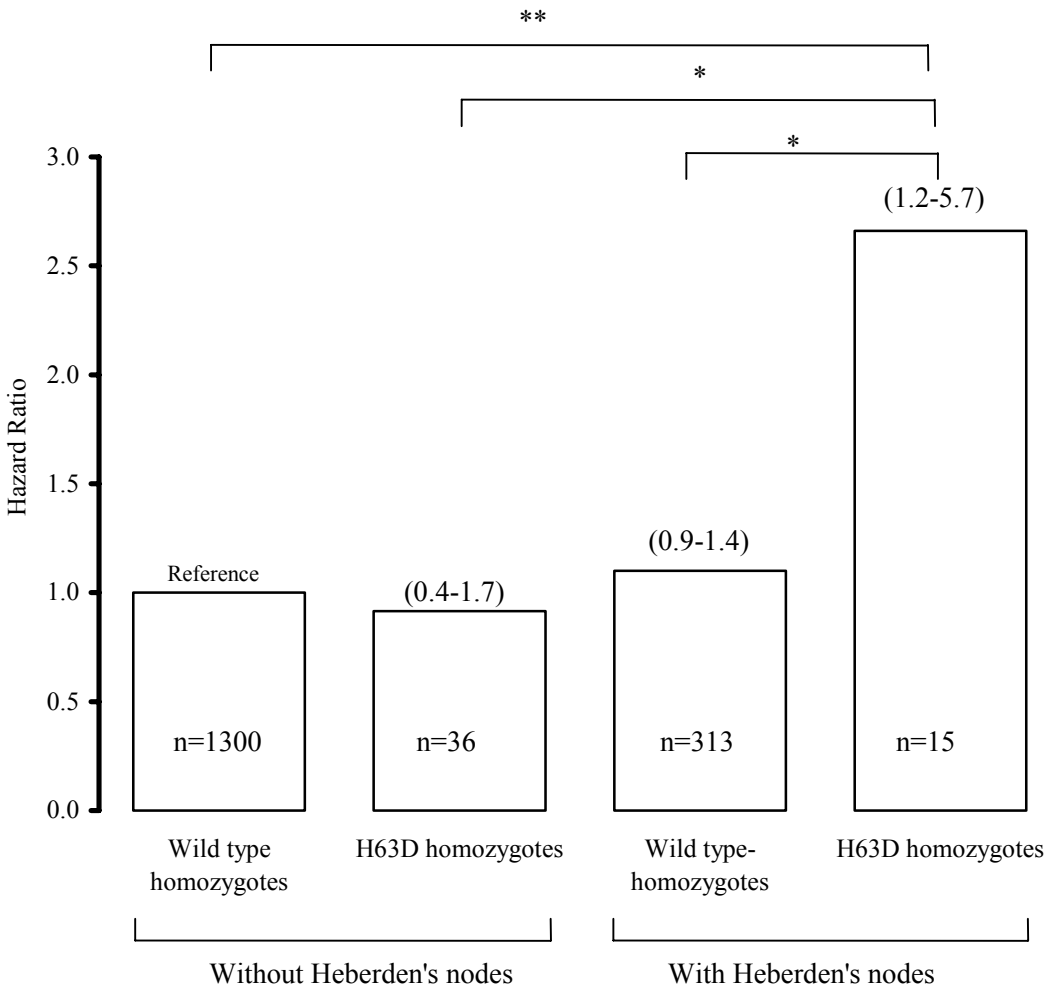

Figure 1. Mortality by possible combinations of homozygosity for the HFE H63D mutation and Heberden's nodes. Figure within the brackets present 95 percent confidence interval of the corresponding hazard ratio. Significance: *p<0.03; **p<0.01.

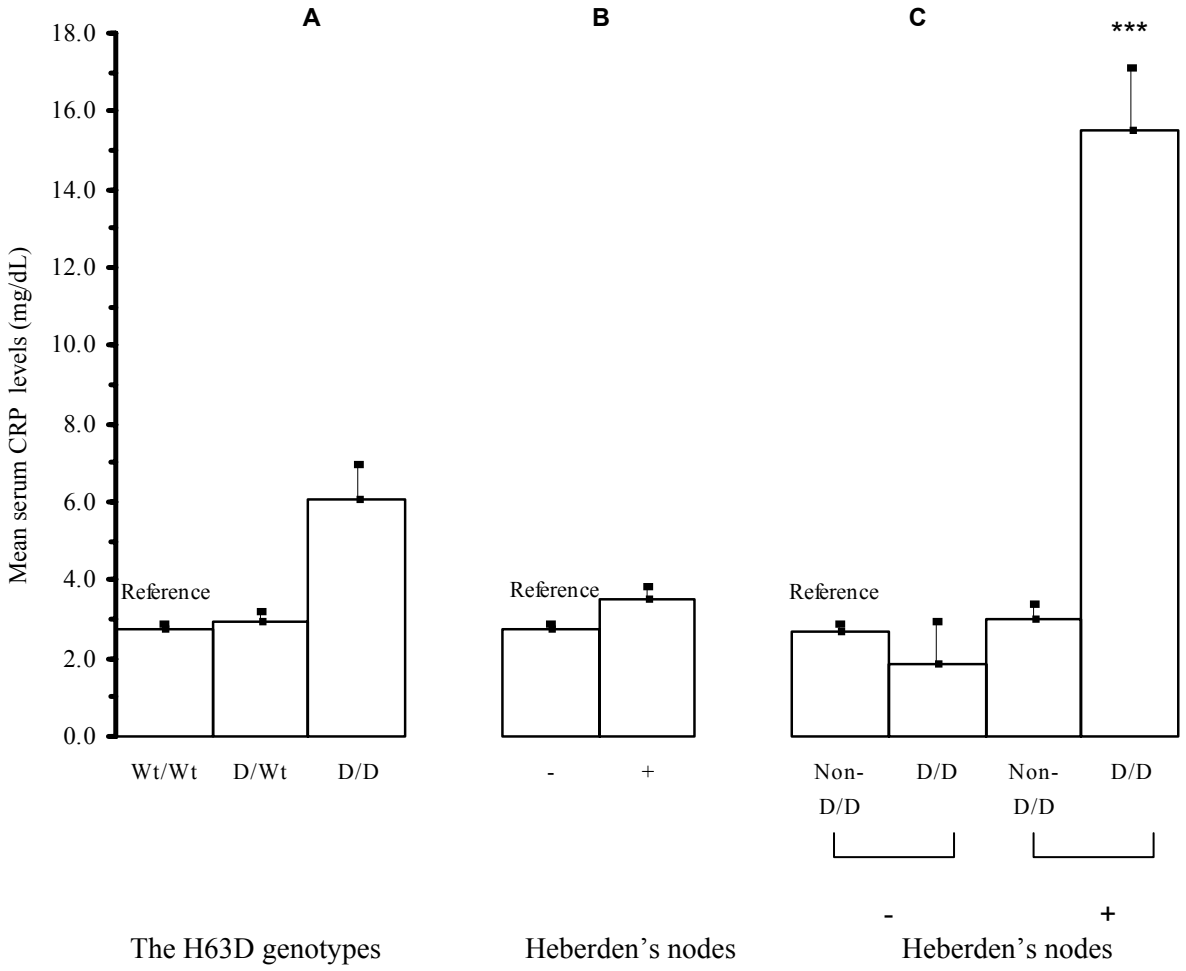

Figure 2. Levels of serum C-reactive protein (CRP) by (A) H63D genotypes, (B) Heberden's nodes and (C) combination between H63D and Heberden's nodes. Wt/Wt represents persons homozygous for the wild type allele; Non-DD represents persons non-homozygous for H63D, D/Wt represents persons heterozygous for H63D, and D/D represents persons homozygous for H63D. Error bars represent the standard error of means. ***Significance compared to the reference group: $p<0.001$.

Discussion

Overall, the H63D mutation was not associated to mortality in our population-based study. We found that subjects with Heberden's nodes had a slight but significantly higher risk of mortality. Persons homozygous for the H63D mutation with Heberden's nodes were at a significant increased risk of mortality mostly due to cerebrovascular events and had increased levels of serum CRP at the baseline examination compared to subjects homozygous for the wild type allele without Heberden's nodes.

This is the first study that addressed the role of HFE and inflammation in relation to mortality. In our population, the H63D mutation was not associated to mortality. This finding is consistent with several other studies.[9-12] In line with this finding, others also found no decrease in prevalence of the H63D mutation in elderly people.[9,10] Overall, our findings together with those of others,[9-12] suggest that the H63D mutation by itself is not associated to mortality.

In the present study, we observed that Heberden's nodes were associated to mortality due to cerebrovascular events. This finding may echo other studies[13,14] which found a relation between osteoarthritis at distal interphalangeal joints[13] or generalized osteoarthritis[14] and mortality due to cardiovascular events, and adverse risk profile for coronary heart disease.[15] To our knowledge, no previous study investigated the relationship between Heberden's nodes and mortality. As H63D was associated with Heberden's nodes in our study population,[4] we tested whether Heberden's nodes modify the relation between H63D and mortality. We found a significant increased risk of early mortality due to cerebrovascular event i.e. stroke in H63D homozygotes with Heberden's nodes. Heberden's nodes has been known as an inflammatory associated condition, we tested a hypothesis that the high inflammatory status in H63D homozygotes compared to non-carriers may explain the relation between H63D, Heberden's nodes and their positive interaction with an early mortality due to stroke. We observed that H63D homozygotes as well as Heberden's nodes had an increase in levels of serum CRP. But, H63D homozygous with Heberden's nodes had a significant increase in levels of serum CRP.

In summary, our epidemiological findings suggest that H63D is not independently associated to early mortality. Our findings suggest that subjects homozygous for the HFE H63D mutation who also have Heberden's nodes before age 65 years are at increased risk of early mortality due to cerebrovascular events. H63D homozygosity has a joint effect with Heberden's nodes and coincides with higher inflammatory status that may explain increased

mortality due to cerebrovascular events. Our findings may have a potential preventive value in clinical practice but remains to be confirmed by others.

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3.4

THE HFE GENE, BILIRUBIN AND MORTALITY

Abstract

Serum bilirubin is an important antioxidant that is found at increased levels in hereditary hemochromatosis patients. We hypothesized that increased levels of serum bilirubin may play a protective role against oxidative stress induced by iron overload in carriers of mutations in the hereditary hemochromatosis gene (HFE*)*. We studied the relation between serum total bilirubin, serum iron levels, HFE C282Y and H63D mutations, and mortality. The study was conducted in 2332 randomly selected subjects from the Rotterdam Study, a population-based follow up study of people aged 55 years or over. Serum bilirubin levels were significantly correlated with serum ferritin (Pearson's correlation coefficient (*r)*=0.2, p<0.05), iron (*r* =0.4, p<0.001) and transferrin saturation (*r*=0.4, p<0.001). Carriers of the HFE mutations had higher levels of bilirubin compared to wild type homozygotes. The relation was the strongest in H63D heterozygotes or homozygotes and C282Y heterozygotes. High levels of serum bilirubin were associated with a 2.8 (95% CI 0.9 to 8.8) fold reduction in mortality in H63D homozygotes and a 2.2 (1.0 to 4.7) fold reduction in mortality in C282Y heterozygotes. Taken together, our data suggest that the high levels of the antioxidant bilirubin may counteract the adverse effect of oxidative stress induced by iron overload. This may explain in part the reduced penetrance of the HFE mutations.

Hereditary hemochromatosis is one of the most common genetic disorders in Caucasians with a prevalence rate up to 1 in 200 to 400.[1,2] The disease is characterized by iron overload in multiple organs.[3] In over 80 percent of patients, the disease is explained by mutations in the HFE gene.[4] The predominant mutation in patients is a single base transition, c.845G→A (C282Y), leading to substitution of a cysteine residue by tyrosine at position 282 of the HFE protein.[4] The second common mutation is the c.187C→G (H63D) transversion leading to a substitution of histidine by aspartic acid at position 63 of the HFE protein.[4]

While for long the penetrance was thought to be high in C282Y homozygous and compound heterozygous, recent studies suggested a low penetrance of clinical disease based on hemochromatosis pathology.[5] Also the common H63D polymorphism is associated with only a mild increase in risk of clinical hemochromatosis.[6-8] This raises the question whether there are physiological mechanisms in the body that counteract the adverse effects of excess iron in carriers. Edwards and colleagues[9] reported hyperbilirubinemia in 31 percent of patients with hereditary hemochromatosis. These patients did not have signs of hemolysis, or liver pathology, one of the most common and lethal disorder in patients with hereditary hemochromatosis.[10,11] One of the most important pathways through which the HFE mutations may lead to chronic disorders is thought to be oxidative stress that is induced by iron overload.[12,13] Bilirubin, in any sub fractions i.e. conjugated, unconjugated or bound to serum albumin, is a strong endogenous antioxidant.[13-17] We hypothesized that high levels of bilirubin may counteract the high oxidative stress due to excess iron in HFE carriers and may thus contribute to the reduced penetrance of HFE mutations. To test this hypothesis, we addressed two main research questions in asymptomatic carriers derived from a population-based study, the Rotterdam Study. First, we studied the relation between serum iron indices, the HFE H63D and C282Y genotypes and serum bilirubin. Second, we evaluated the relation between levels of serum bilirubin and mortality in carriers of HFE C282Y and H63D mutations.

Methods

From the Rotterdam Study (n=7893), 2332 subjects were randomly selected and genotyped for the HFE C282Y and H63D mutations. The design of the Rotterdam Study has been described elsewhere.[18] In brief, this study is a population-based follow up study of inhabitants of the district of Ommoord in Rotterdam aged 55 years or over. The aim of the study is to investigate the determinants of chronic and disabling disorders in the elderly. Full subjects' recruitment, data acquisition and baseline examinations took place between 1990

and 1993 by means of a structured interview and a physical examination by research physicians. The medical ethics committee of Erasmus Medical Center has approved the study and written informed consents and permission to retrieve information from medical records were obtained from all participants. Participants were followed for 13.6 years. Information on the vital status of all participants was obtained at regular intervals from municipal health authorities in Rotterdam. The data on hospital admissions and corresponding diagnosis of hemochromatosis or other liver diseases were retrieved from medical records of participants' general practitioner and hospitals' registry databases. From the total cohort genotyped, serum iron, ferritin and transferrin saturation were determined in a total of 342 persons. We included all subjects with rare genotypes i.e. C282Y homozygotes (n=8) and compound heterozygotes (n=51). Further, based on power calculations (α=0.05 and β=0.8) for the other genotypes about 70 subjects were selected, i.e. those without any mutation (the wild type homozygotes, Wt/Wt, n=74), the H63D heterozygotes (Wt/H63D, n=73), the C282Y heterozygotes (Wt/C282Y, n=71), and the H63D homozygotes (H63D/H63D, n=61).

For 108 men and 124 women serum levels for both iron indices and bilirubin were available. For a total of 1394 participants (men 627, women 767) data on vital status, serum bilirubin and HFE genotypes were complete.

At the baseline examination at the research center blood samples were collected by venepuncture in the morning. Serum and plasma was separated immediately, and kept frozen at –80°C until the laboratory analysis. Genomic DNA was extracted from buffy coat using the salting out protocol as described elsewhere.[19] The HFE C282Y and H63D mutations analysis was performed as described previously.[4] Serum total bilirubin (µmol/l) was measured according to the protocol of Bartels and Bohmer [1971]. Serum ferritin (µg/l), iron(µmol/l) and transferrin (µmol/l) were measured as described elsewhere.[20] All measurements were done in the same laboratory by the same experienced technicians.

Serum ferritin levels were not normally distributed therefore they were transformed to a logarithmic scale to achieve normality. One-way analysis of variance or t-test was used to compare means and the χ^2 test was used to compare frequencies between groups. The correlation between serum iron indices and serum total bilirubin was estimated using Pearson's correlation coefficients. Median of serum bilirubin was used as the cut-off point to categorize the participants into two subgroups of those with high (above median) and those with low (below median) serum total bilirubin levels. Cox proportional regression analysis

was used to compare the cumulative survival rates in HFE carriers with high to low serum total bilirubin levels. As gender determines the penetrance of HFE genotypes all analyses were stratified by gender. Continuous variables are reported as mean±the standard error, unless otherwise specified.

Results

Table 1 summarizes the characteristics of the participants including the HFE genotypes frequencies. When comparing the 1394 subjects in whom bilirubin was assessed to those without data on serum bilirubin (n=938), we found no significant difference in the characteristics listed in Table 1. Mean age in men (66.3±0.3 years) did not differ from that in women (66.2±0.2 years). Five percent of the 1394 subject had a history of liver disease and none of the participants had received a diagnosis of hereditary hemochromatosis from their general practitioner or any other physician at the baseline or during the follow up. Serum bilirubin levels, iron indices, alanine aminotransferase, and hemoglobin differed significantly between men and women. Overall, HFE genotype or allele proportions were similar for men and women and were in Hardy-Weinberg equilibrium.

The relationship between levels of serum iron indices and serum bilirubin is summarized in Table 2. In both men and women, serum iron levels and transferrin saturation were significantly correlated with serum bilirubin levels. A significant correlation between serum ferritin and serum bilirubin was observed only in women. This can be explained by the fact that serum ferritin had the largest standard deviation.

Figure 1 shows the relation of HFE mutations to serum bilirubin. In the overall analysis and in the analysis of men, those heterozygous or homozygous for the H63D mutation and those heterozygous for the C282Y mutation had significantly increased levels of serum bilirubin compared to those homozygous for the wild type allele. In women homozygous for the H63D mutation, levels of serum bilirubin were significantly increased compared to those homozygous for the wild type allele.

Table 1. Characteristics of participants and HFE genotype frequencies.

	Overall n=1394	Men n=627	Women n=767
Age (years)	66.3±0.2	66.3±0.3	66.2±0.2
Total bilirubin (µmol/l)*†	9.1±0.1	10.1±0.2	8.3±0.1
Alanine aminotransferase (iu/l)*	19.2±0.3	20.4±0.5	18.2±0.4
Aspartate aminotranferase (iu/l)	20.7±0.2	21.2±0.3	20.3±0.3
Hemoglobin*	8.9±0.1	9.3±0.1	8.6±0.6
Ln serum ferritin (µg/l)†	4.9±0.1	5.0±0.1	4.8±0.1
Serum iron (µmol/l)*	18.0±0.4	19.3±0.6	17.0±0.5
Serum transferrin saturation* (%)	30.9±0.7	33.0±1.2	28.9±0.9
History of liver disease	5.4 %	5.1 %	5.6 %
HFE genotype-frequencies			
Wt/Wt	61.4 %	58.7 %	63.6 %
Wt/H63D	23.9 %	26.0 %	22.2 %
Wt/C282Y	9.6 %	10.7 %	8.7 %
H63D/H63D	2.9 %	3.2 %	2.6 %
C282Y/H63D	1.9 %	1.1 %	2.5 %
C282Y/C282Y‡	0.4 %	0.3 %	0.4 %

The figures are presented as means± the standard errors or as percentage. †Ln. natural logarihtm transformation. ‡Only 4 women and 2 men were homozygous for the C282Y mutation. The numbers were too small for meaningful statistical analysis. Comparison between men and women: *p<0.05.

Table 2. Partial Pearson's correlation coefficients between serum iron indices and total bilirubin.[#]

	Overall	Men	Women
Ferritin (µg/l)	0.2 *	0.0	0.2 **
Iron (ng/l)	0.4 **	0.4 **	0.4 **
Transferrin saturation (%)	0.4 **	0.4 *	0.4 **

[#] Correlations were adjusted for age and gender. Significance: *p<0.01; **p<0.001.

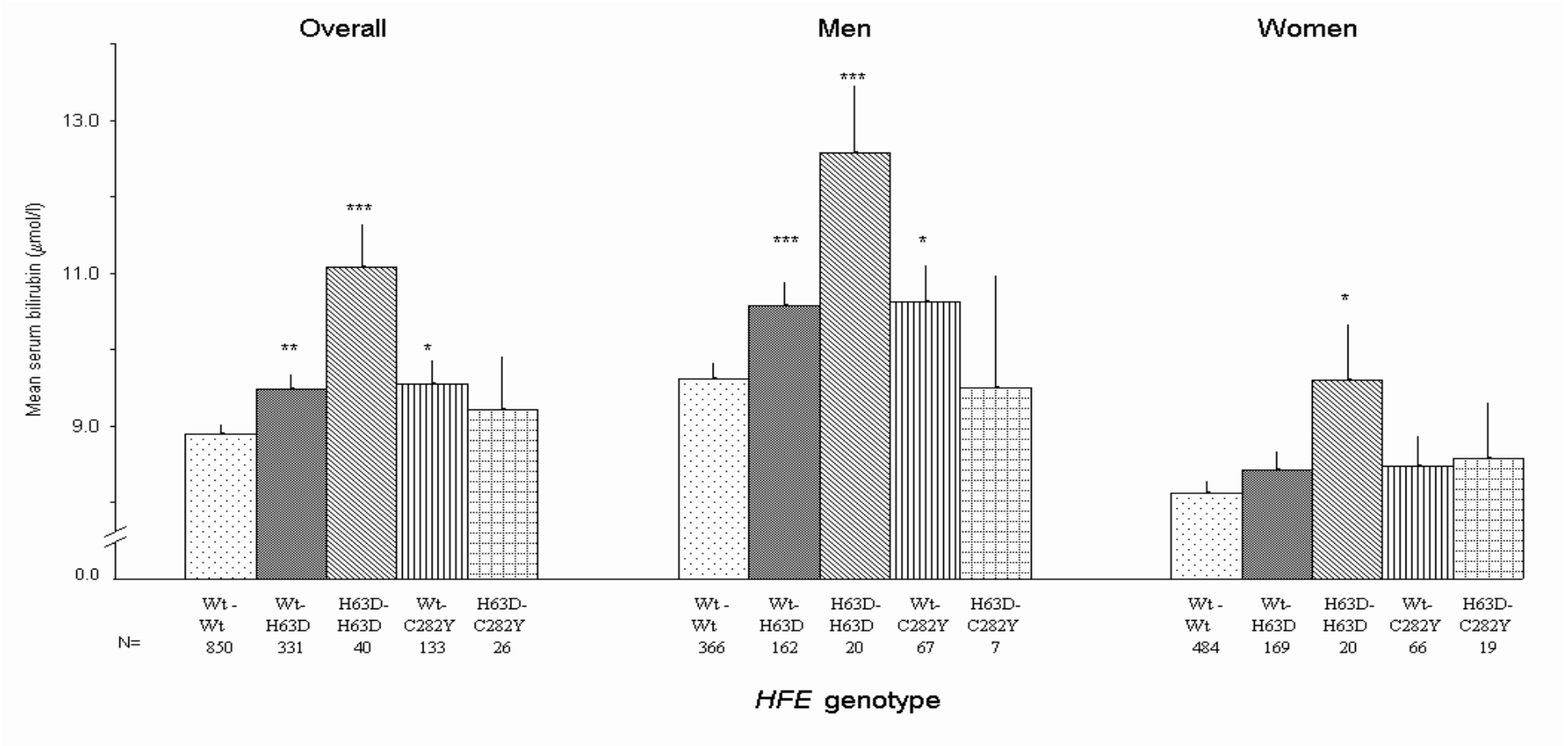

Figure 1. Mean serum total bilirubin levels by HFE genotypes overall, in men and in women. Error bars show standard error for the mean. Significance is given as *p<0.05, ** p<0.01, and *** p<0.001. N. Numbers of subjects. Serum bilirubin levels are not given for C282Y homozygotes since only 2 men and 4 women carried this genotype.

Figure 2 shows the total mortality for subjects by serum bilirubin levels and HFE genotypes. In those homozygous for the wild type allele or heterozygous for the H63D mutation, high bilirubin levels were not associated to mortality. In those homozygous for the H63D mutation, high bilirubin levels were associated with a 2.8 (95% CI 0.9 to 8.8) fold reduction in mortality overall, a 2.1 (0.4 to 12.5) fold reduction in men and a 3.3 (0.7 to 16.7) fold reduction in mortality in women. In those heterozygous for the C282Y mutation we observed a 2.2 (1.0 to 4.7) fold reduction in mortality overall, a 2.1 (0.7 to 7.1) fold in men, and a 1.6 (0.6 to 5.0) fold in women with high bilirubin compared to those with low bilirubin. Overall there was no significant difference in mortality among the HFE genotypes regardless of levels of bilirubin.

Discussion

Our population-based study showed that levels of serum total bilirubin were significantly related to serum iron indices and HFE genotypes in both men and women. High serum bilirubin levels were associated with a substantial reduction in mortality in those homozygous for H63D or heterozygous for the C282Y mutation.

A limitation of the present study was the lack of information on the conjugated fraction of the serum total bilirubin, and on the causes of mortality. The other limitation was the number of persons homozygous for C282Y was too low for a meaningful statistical comparison. The advantage of our study was its population-based design.

We found that the HFE mutations are associated with two counteracting metabolites. On the one hand, we and others[7,21,22] have found that H63D heterozygotes or homozygotes and C282Y heterozygotes or homozygotes had higher levels of serum iron, a major oxidant. On the other hand, in the present study, we found that H63D heterozygosity or homozygosity and C282Y heterozygosity were associated with increased levels of an efficient antioxidant, serum bilirubin. This counteracting effect may explain the observed non-penetrance of the HFE mutations with regard to chronic disorders that are linked to oxidative stress.

Serum bilirubin was significantly correlated to serum iron indices. The fact that H63D homozygotes had an elevated serum bilirubin level is striking. This genotype is reported to be associated with a mild increase in serum iron loading.[6,8] However, in our population sample, we have reported that this genotype was associated with a very high serum iron level.[20]

C282Y homozygosity was significantly associated to high iron levels, but there were not

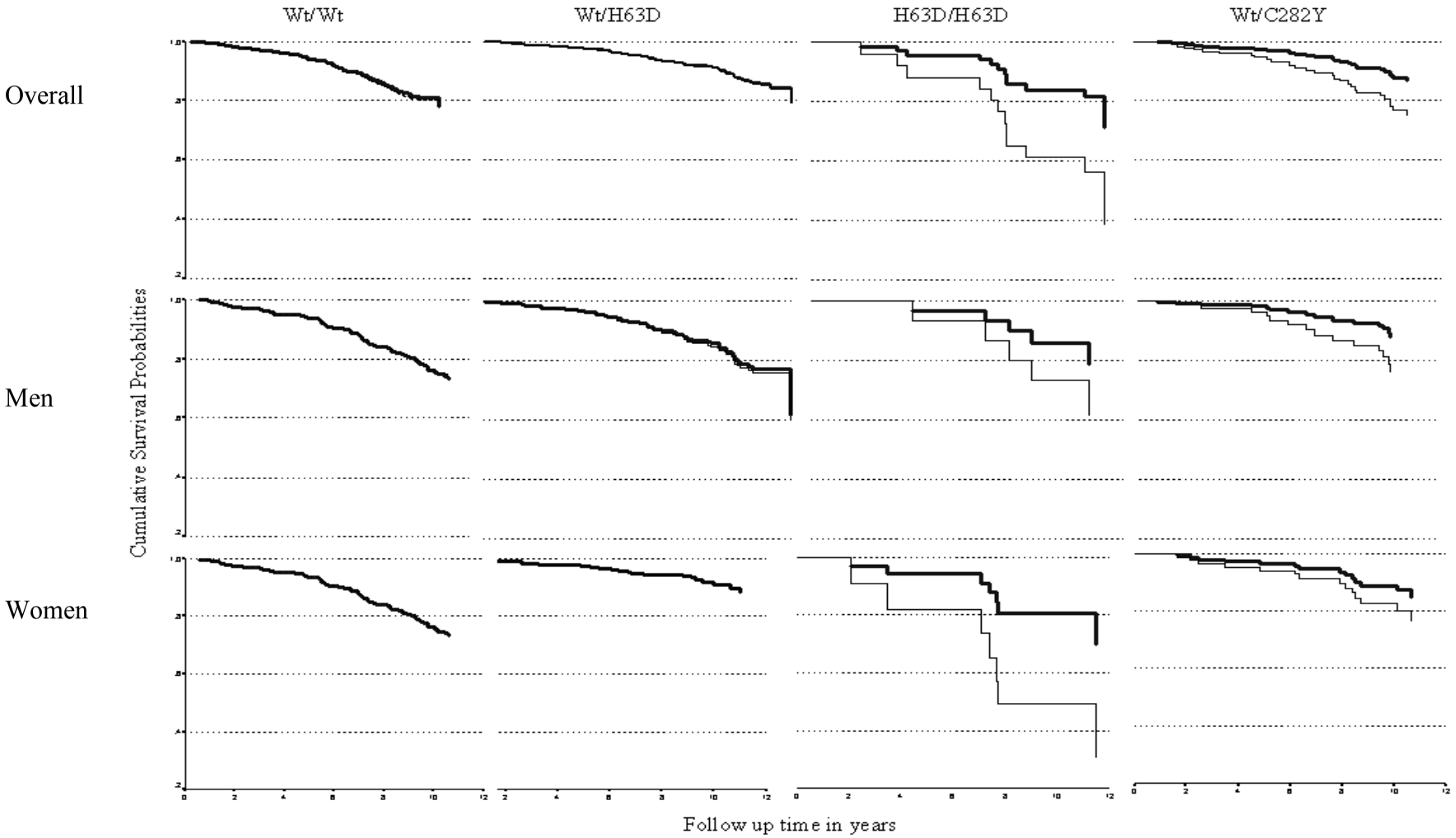

Figure 2. Cumulative survival probability by levels of serum total bilirubin and HFE genotypes in overall subjects, in men and in women. The thick line and the thin line represent subjects with high and low bilirubin levels respectively. For C282Y homozygotes and the compound heterozygotes the numbers were too low for meaning full statistical analysis.

enough subjects to study. In our study population compound heterozygotes had no increase in levels of iron and had no increase in levels of serum bilirubin in this study. Altogether, this points to the fact that the higher the levels of serum iron, the higher the levels of serum bilirubin will be. The mechanism through which bilirubin may be increased in iron overload conditions remains to be elucidated. Other factors such as liver diseases and hemoglobin can lead to a high bilirubin. But in our study population, these factors did not account for the observed associations. One probable mechanism to explain at least part of the variation of serum bilirubin by HFE genotypes is the heme-oxygenase pathway. This pathway is an inducible anti-oxidant and anti-inflammatory enzymatic complex that catalyses the degradation of heme to biliverdin, ferrous iron and carbon monoxide. The induction of the heme-oxygenase pathway by oxidant species or iron enhances the production of serum bilirubin,[14,23-25] and is known as a part of an antioxidant mechanism.[26,27]

We further showed that the high bilirubin levels were associated with a reduction in mortality in H63D homozygotes or C282Y heterozygotes. This may be due to the fact that the deleterious effects of oxidative stress due to excess iron induced by HFE mutations, is compensated by an increase in the levels of bilirubin, an antioxidant with well known cardio and neuroprotective effects.[28-30] In the same line of our findings, Temme and colleagues[31] reported a lower overall, and in particular a lower cancer mortality rate in men with high serum bilirubin levels. Taken together, our data suggests that the high levels of bilirubin may counteract the adverse effects of oxidative stress induced by iron overload.

We propose that high bilirubin levels induced by HFE mutations may have a protective effect, preventing at least in part the damage induced by iron overload. This may explain in part the reduced penetrance of the HFE mutations. Further experimental and epidemiological studies are needed to confirm our hypothesis and its clinical implications.

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4

FAMILY-BASED STUDY

4.1

HERITABILITY OF SERUM IRON INDICES

Abstract

Iron plays a crucial role in the pathogenesis of complex disorders such as atherosclererosis, neurodegenerative diseases, and cancer. Both iron deficiency and iron overload are common public health problems. From a genetic perspective, iron metabolism is a complex trait, in which both genetic and environmental factors are involved. The purpose of the present study was to estimate the magnitude of genetic influences on serum levels of iron indices including iron, ferritin and transferrin saturation in relatives from a recent genetic isolate in the Netherlands. Estimation of how much of the variation in the levels of iron and ferritin could be explained by additive genetic factors was done using the variance component method implemented in Sequential Oligo-genic Linkage Analysis Routines (SOLAR). This study included 90 nuclear families with a total of 988 subjects. The proportion of the residual phenotypic variance due to additive genetic effects i.e. heritability estimates were approximately 0.20 (S.E. 0.06, $p<0.0001$) for iron, 0.28 (S.E. 0.08, $p<0.001$) for transferrin saturation, and 0.24 (S.E. 0.08, $p<0.0001$) for ferritin while adjusting for gender and age. Further adjustment for serum albumin levels, a significant co-variable of serum iron levels, the heritability estimates changed to 0.17 (S.E. 0.07, $p<0.0001$) for iron, 0.26 (S.E. 0.08, $p<0.0001$) for ferritin, and 0.24 for transferrin saturation (S.E. 0.07, $p<0.001$). A modest proportion of the variance of iron, transferrin saturation, and ferritin can be explained by heredity, independent of gender, age and environmental effects. Our results demonstrate the influence of both genetic and environmental factors on iron levels.

Iron is a crucial component of biochemical reactions.[1, 2] High and low levels of body iron are associated with common human diseases.[1-5] To maintain iron levels within the normal limits and thus prevent pathologic consequences of iron excess or deficiency, iron haemostasis evolved as a complex and tightly coordinated process in which numerous genes and environmental factors are involved.[1, 3, 6] The role of genetics on iron haemostasis is supported by investigations that proved iron overload as a heritable disease,[7] and identified several genes involved in iron metabolism.[8-12] Arthropathy is one of the most common complaint in hemochromatosis patients.[1-3] This raised the question whether the genetic factors involved in iron haemostasis, are also involved in osteoarthritis.

Overall, the aim of the present study is to unravel the genetic determinants of iron metabolism. Few studies investigated whether levels of serum iron indices are heritable.[15, 16] One twin study[15] found no significant heritability for the levels of serum iron. Others have estimated, respectively in men and women, 23 and 31 percent heritability for serum iron, 47 percent for serum ferritin, an iron associated protein, and 21 and 47 percent for transferrin saturation.[16] The point of concern is that in these studies,[15, 16] heritability is overestimated as monozygotic twins share more environmental factors than dizygotic twin pairs to which they are compared. This may confound the heritability estimations. One approach to overcome this problem is the use of an extended pedigree, which also includes second and third degree relatives who do not share a common environment.

Within a recent genetic isolate in the Netherlands, we investigated the magnitude of genetic and environmental influences on levels of serum iron and ferritin in 988 individuals related to each other in one extended pedigree. Next, we assessed the phenotypic, genotypic and environmental correlation between the studied serum iron indices.

Methods

Population This study was carried out within a family-based study of 2500 inhabitants of a genetically isolated community in the Southwest region of the Netherlands, the Erasmus Rucphen Families (ERF) study. The aim of the ERF study is to unravel the genetic determinants of several common complex disorders. The target population was founded in the middle of the 18th century by about 150 people and was characterized with minimal inward migration (less than 5 percent) and considerable population growth. Since 1848, the

population expanded to 20000 inhabitants scattered over 8 adjacent villages. Genealogical data on this population is currently available including over 63000 individuals. The medical ethics committee of the Erasmus Medical Center Rotterdam has approved the study. Written informed consent was obtained from all participants.

Participants' selection For the purpose of the ERF study, twenty couples, who had at least 6 children from 1880 to 1900, were identified with the help of genealogical record of the church and municipality. Each of these couples could be traced back to one or more of the 10 couples who lived in middle of the 16th century in this community. All third, fourth and fifth generational descendent of these couples and their spouses were invited to participate in the study.

Data collection and measurements Phenotypic data collection, and baseline examination have been performed since June 2002 by means of a structured questionnaire. Participants were invited for a series of clinical examinations at the research center. In the present study, we will focus on the first 988 participants for whom complete phenotypic data have been collected.

At the start of clinical examination, fasting blood samples were drawn by venepuncture, which was done between 7:00 and 10:00 o'clock. Serum samples were obtained from the whole blood after clotting. Plasma samples were obtained from whole blood collected in disodium EDTA. Serum iron (μmol/l) was measured by means of using the Ferrozine method, an immuno(chemi)-luminescence assay, using Roche/Hitachi 747 - 400 Kit (Roche). Serum ferritin levels (ng/ml) were measured by a two-site chemiluminescencent immunometric assay using the Immulite 2000 (Diagnostics Products Corporation). Transferrin saturation (%) was calculated as serum iron levels divided by serum total iron binding capacity. Plasma albumin was measured according to standardized protocol. For 988 persons levels of serum iron, and for 957 persons levels of serum ferritin, and for 988 persons levels of serum transferrin saturation were successfully measured. For 953 subjects both measurements of serum iron levels and ferritin, for 988 subjects both measurements of serum iron levels and transferrin saturation, for 953 subjects both measurements of serum transferrin saturation and ferritin were available. Height and weight were measured with participants dressed in light under clothing and body mass index was calculated as weight divided by height square.

Data analysis Inbreeding coefficients, the probability that the two alleles at any locus in an individual are inherited from a common ancestor i.e. identical by descent, were calculated using PEDIG software (http://dga.jouy.inra.fr/sgqa/diffusions/pedig/pedigE.htm). Prior to data analyses, levels of serum iron and ferritin were regressed for the baseline variables including age, gender, levels of serum albumin, weight and body mass index using stepwise multivariate linear regression analysis. To correct for the amount of genetic materials shared between relatives, inbreeding coefficient was also included in the model. Age, gender, and serum albumin showed a significant association to serum iron indices and were included as covariables in the heritability estimation. From the regression model, we explored standardized residuals. As these residuals were skewed, we derived natural logarithmic for serum iron and ferritin.

Heritability estimation- A standard maximum likelihood variance decomposition techniques was used to partition the phenotypic covariance of the trait among the relatives into variance due to additive genetic factors, and variance due to dominance (non additive allelic effects) and environmental i.e. random individual-specific components.[17-19] This approach is implemented in Sequential Oligo-genic Linkage Analysis Routines (SOLAR) software. SOLAR calculates heritability, in the narrow sense, as the ratio of the variance explained by additive effects of multiple genes to the total phenotypic variance of the trait.[17-19] The significance of the heritability estimate was obtained by comparing a model in which additive heritability was estimated with the one that this parameter fixed to zero. The two times difference between natural logarithm likelihood values of the two models distribute as a chi-squared distribution with one degree of freedom.[17] Heritability was first estimated while the model was regressed for age, gender, and inbreeding coefficients (model I), and then analyses were repeated including serum levels of albumin. The significance between these two models was tested using the likelihood-based chi-square statistics.

Bivariate correlation analysis- The phenotypic correlation between the levels of serum iron and ferritin, iron and transferrin saturation, and serum transferrin saturation and ferritin were estimated using Pearson's correlation coefficients (r). To examine the underlying determinants of the phenotypic correlation, series of bivariate analyses between serum iron and ferritin, between serum iron and transferrin saturation, and between serum ferritin and transferrin saturation were performed to estimate the additive genetic and environmental correlation.[20] Whether the environmental correlation differs significantly from zero, SOLAR compares the likelihood of a model in which this correlation was fixed to zero with a model in

which environmental correlation was estimated using a likelihood based chi-square test with one degree of freedom. The same procedure was performed for genetic correlation. Further, we tested whether the genetic correlation between serum iron indices was significantly different from the value of 1. This test exploits the pleiotropic genetic effects.[21] Pleiotropy describes the phenomena that one or a set of related genes with additive effects, explains more than one trait.

Results

Overall 988 subjects were included in the analysis consisting of 907 first-degree relative pairs, 659 second degree relatives pairs, and 2370 third degree relative pairs. Table 1 presents the baseline characteristics of the study population. Mean (±S.E.) age was 54.46 (±0.47) years. The inbreeding coefficient was 0.007 (range $0.58*10^{-7}$ to 0.04) in 685 subjects; for the remaining subjects (n=368) no inbreeding was detected. Within the total population, 143 persons (13.58 percent) had a transferrin saturation of higher than 45 percent. Among subjects with serum ferritin available (n=958), 10.35 percent had a serum ferritin higher than 300 µg/L. In total, 34 (3.22 percent) persons had both transferrin saturation higher than 45 percent and a serum ferritin level higher than 300 µg/l. These subjects had a significantly higher inbreeding coefficient (mean natural logarithm transformed value 4.79±0.51) compared to the remaining cohort (3.45±0.09).

Table 2 presents the components of phenotypic variance of serum iron levels. The heritability estimate was 0.20±0.04 (S.E.) while the model was adjusted only for age and gender (model I). The hypothesis of no polygenic effects was rejected ($p<0.0001$). Adjusting for serum levels of albumin (model II) further reduced the heritability estimate to 0.17±0.07 ($p<0.001$). With regard to ferritin (Table 3), the heritability estimate was 0.24±0.08 in the model adjusted for age and gender. The hypothesis of no additive polygenic effects was rejected ($p<0.0001$). In model adjusted for age, gender, and albumin levels, the heritability estimate increased to 0.26±0.08. This was statistically significant ($p<0.001$). With regard to the levels of serum transferrin saturation (Table 4), the heritability estimate was 0.28±0.07 in the model when adjusting for age and gender. The hypothesis of no polygenic effects was rejected ($p<0.0001$).

Table 1. Characteristics of the study population.

n	988
Men (%)	40.20
Age (years)	54.46±0.47
Body mass index (kg/m^2)	31.47±1.25
Serum albumin (g/dl)	40.30±0.13
Serum iron (µmol/l)	19.85±0.22
Median of serum ferritin (ng/ml)	107 (2.84 - 4732.00)
Serum transferrin saturation (%)	33.12±0.41
Inbreeding coefficients	0.007 (0.58*10^{-7} - 0.04)
Number and type of relative pairs	
Parent-offspring	371
Siblings	563
Half siblings	43
Avuncular	875
Grandparents-grandchild	35
Half avuncular	55
First cousins	2262
Half first cousins	53

Plus-minus figures represent mean±S.E.

Table 2. Heritability estimates of serum iron.

Model	n	Additive polygenic effect (Heritability)	Random environmental factors	Proportion of variance explained by covariates	2 log likelihood polygenic model	χ^2 test
I	988	0.20±0.04**	0.80±0.06	0.04	551.98	-
II	905	0.17±0.07*	0.83±0.07	0.03	535.55	32.85**

Figure presents mean proportion± S.E.

Model I. Ln serum iron = {2.98 - 0.01*(age-53.48) - 0.13*female}.

Model II. Ln serum iron = {3.00 - 0.001*(age-52.10) - 0.12*female - 8.76e-05*(serum levels of albumin - 111.49)}.

p-value: *<0.001; **<0.0001.

Table 3. Heritability estimates for the levels of serum ferritin.

Model	n	Additive polygenic effect (Heritability)	Random environmental factors	Proportion of variance explained by covariates	2 log likelihood polygenic model	χ^2 test
I	957	0.22±0.08**	0.76±0.08	0.25	312.95	-
II	874	0.26±0.08*	0.74±0.08	0.27	274.22	97.46**

Figure presents mean proportion± S.E.

Model I. Ln serum ferritin = {5.15 + 0.01*(age - 53.42) - 0.91*female}.

Model II. Ln serum ferritin = {5.13 - 0.01*(age - 51.97) - 0.93*female - 3.52e-05*(serum levels of albumin - 113.94)}.

p-values: *<0.001; **<0.0001.

Table 4. Heritability estimates of levels of serum transferring saturation.

Model	n	Additive polygenic effect (Heritability)	Random environmental factors	Proportion of variance explained by covariates	2 log likelihood polygenic model	χ^2 test
I	988	0.28±0.06***	0.72±0.06	0.04	468.84	-
II	905	0.24±0.07***	0.74±0.08	0.04	448.90	39.87***

Figure presents mean proportion ±S.E.

Model I. Ln serum transferring saturation = {3.49 - 0.001(age-53.48) - 0.16*female)}.

Model II. Ln serum transferring saturation = {3.50 - 3.44*(age-5.10) - 0.15*female - 1.93e-05*(serum levels of albumin - 111.49)}.

p values: ***<0.0001.

Further adjustment for serum levels of albumin reduced the heritability estimate to 0.24±0.07 (p<0.001).

We found significant positive phenotypic correlations between serum iron and ferritin (n=953, r =0.21; p<0.001), serum iron and transferrin saturation (n=988, r=0.90, p<0.001), and between serum ferritin and transferrin saturation (n=957, r=0.37, p<0.001). There was a

substantial genetic correlation between serum iron and ferritin (0.42±0.25), or transferrin saturation (0.72±0.07), and between serum ferritin and transferrin saturation (0.75±0.15). These estimates differed significantly ($p<0.001$) from the value of 1.

Discussion

Within a large family-based sample from a recent genetic isolate, we investigated the influences of genetic, and environmental factors on the variation in the levels of serum iron indices. We found that a significant ($p<0.001$) proportion of the levels of serum iron (0.17±0.07), ferritin (0.26±0.08), and transferrin saturation (0.24±0.07) was explained by the additive polygenic effects. We observed significant phenotypic correlations between serum iron, ferritin, and transferrin saturation, which were explained by significant underlying shared genotypic and environmental factors.

The major advantage of our study was the use of extended families within a genetic isolate. The present sample includes also spouses, second and third degree relatives who do not live in the same households. This design reduces the confounding of genetic influences by shared household, and environmental effects, which is problematic when samples include only nuclear families that may inflate the heritability estimates.

Our study demonstrated a significant heritability for the levels of serum iron, an essential element. In our study population, the heritability estimate was 0.17 for iron and 0.24 for transferrin saturation when the models were adjusted for age, sex and levels of serum albumin. Earlier, Whitefield and colleagues (2000),[16] reported 20 to 30 percent of the variance of the serum iron levels and 33 percent and 47 percent of the variance in transferrin saturation could be explained by additive polygenic effects. For serum ferritin, a protein, we found a heritability estimate of 0.24 that was increased to 0.26 when correcting for the levels of serum albumin. Comparing to the study of Whitefield and colleagues (2000),[16] who estimate a heritability of 47 percent for serum ferritin levels in both men and women, the heritability estimates of serum ferritin levels is lower in the present study. There are several explanations for this. First, the finding from the study of Whitefield and colleagues (2000),[16] is overestimated due to a higher shared environmental factors in monozygotic twin compare to dizygotic twins. Our data suggest a modest heritability to serum iron indices while we analyzed relatives who share less environmental factors. Second, we did not correct for the effect of other proteins, which induce serum ferritin levels. This may lead to an increase in random environmental residuals and thus a decrease in the residual heritability estimate.

However, the heritability estimates variations between our and the study of Whitefield and colleagues (2000)[16] is not unique for serum iron indices. Variations in heritability estimates across different populations have been previously observed for other complex traits as well.[22, 23] These variations may be regarded as random or explained by differences in designs, data analysis techniques, population genetic make up, or environmental components. Together, our findings indicate that a modest proportion of body iron contents is explained by additive polygenic effects and thus is modestly heritable.

We found a significant phenotypic correlation between serum iron and ferritin levels. This correlation, were significantly modulated by shared environmental factors, as we found a significant environmental correlation between the studied traits. Also, bivariate analysis showed a genetic correlation between serum iron, ferritin and transferrin saturation levels that were significantly different from the value of 1. This finding may indicate a degree of pleiotropy for serum iron, ferritin and transferrin saturation. This may be due to the fact that levels of serum iron exert the strongest regulation on ferritin and transferrin production.[24, 25] Thus, the gene pool involved in regulation of iron levels, may also regulate ferritin as well as transferrin metabolism.

We confirmed that a modest proportion of the body iron content could be explained by heredity, independent of age, gender and environmental effects.

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5

GENERAL DISCUSSION

5.1. Main findings and their relevance

Introduction

In medicine the concern is to cure, and ultimately, is to prevent osteoarthritis pathology in susceptible persons and to slow the joint degeneration, in an affected individual. One way is to reduce environmental factors such as biomechanical pressure, or other risk factors among them obesity, physical activity or repetitive trauma,[1-3] and the other way is to identify susceptible subjects, and identify individualized effective interventions. The latter approach requires the detailed knowledge of the underlying molecular process leading to joint destruction, to which understanding the genetic components of osteoarthritis plays a crucial role.

The overall aim of our research project is to identify genes that contribute to osteoarthritis. As a part of this project, candidate genes were analysed in relation to osteoarthritis in subpopulations of the Rotterdam Study. We investigated the relationship between two candidate genes i.e. the gene encoding the alpha domain of collagen type IX (COL9A1) and the hereditary hemochromatosis (HFE) gene, with osteoarthritis. Here, we will discuss our main findings and their relevance, followed by future prospects in the field of research in osteoarthritis and hemochromatosis.

5.1.1. Osteoarthritis definitions: Renew classifications

The first part of **Chapter 2** describes the definitions and classifications of osteoarthritis in brief. For long, epidemiological studies use the Atlas of Standard Radiographs of Arthritis developed in 1963.[4] It is clear that there is no clear relation between radiographic abnormalities and clinical sign and symptoms of osteoarthritis,[5] and thus, the definition of osteoarthritis needs to develop. The ACR criteria, renewed the definitions of clinical osteoarthritis for knee,[5] hand[6] and hip[7] joints for clinicians. A different classification method for osteoarthritis, taking into account the knowledge of the underlying molecular basis of osteoarthritis in a subset of patients will eventually lead to a more homogenous classification of the disease. As yet, however, genetic studies on osteoarthritis apply for too many definitions to decide which phenotype classes rely on different genetic etiology.

5.1.2. Genetics of osteoarthritis: many studies, few replications

The second part of **Chapter 2** focuses on the genetic epidemiology of osteoarthritis, summarizing, first, the evidences on the heritability of osteoarthritis. Heritability estimates of osteoarthritis range from 27 up to 65 percent. Heritability has been estimated as 56 percent for hand osteoarthritis,[8] 58 to 65 percent for hip,[9,10] and 44 percent for knee,[11] joints. The heritability estimates among the studies may not be completely comparable due to differences in design i.e. population-based versus twin studies, definition of phenotypes i.e. radiographic, clinical or pathological, and applied statistical approaches to analyze the data and influences of potential confounding factors such as age, gender and body mass index. Indeed one may argue true differences among populations may exist due to differences in genetic background, and biomechanical stress in these populations. Overall, the fact that osteoarthritis is a heritable condition is beyond any doubt.

The finding that osteoarthritis is a heritable condition raises the question of where osteoarthritis susceptibility genes are located across the genome. As reviewed in **Chapter 2.1**, multiple genomic regions have been linked to osteoarthritis on almost all chromosomes i.e. chromosomes 1, 2, 3, 4, 7, 9,11, 13, 15, 16, 19, 20 and X.[13-31] As it is clear, while some of the identified loci implicated in osteoarthritis appear to be involved in several joint sites, others may express a site-specific phenotype.

One of the major concerns in genetic studies that have been conducted in the field of osteoarthritis is the reproducibility. There is hardly any replication among the studies, with the exception of chromosome 2. No replication may of course be due to both false positive and false negative findings. In the field of osteoarthritis, this may be reasonably expected as repeated sub-cohort analyses were performed on the same participants of the main cohorts to a great extent. The lack of replication may be explained by phenotypic diversity due to clinical-based classification of patients or it may partly represent, genetic diversity of osteoarthritis. Lack of replication may also arise from differences between the design of studies including a priory mapping strategies i.e. map-based or sequence-based designs,[32,33] choice of type and number of SNPs or markers in terms of variant frequencies and effect size,[33] utilization of appropriate technologies for genotyping.[32] Differences in applied statistical methods and inferences at the level of statistical significance,[32] and levels of multiple comparisons,[32] are other points to be considered when dealing with lack of replication in linkage studies.

The other issue to be considered when comparing the results of linkage or association studies is the population structure. Lack of replication may originate from differences in the degree to which population differ in terms of genetic susceptibility and linkage disequilibrium structure,[33] the extend and structure of the pedigree and population,[33] and population stratification.[34] Another point that needs to be addressed is at what distance do we reject the hypothesis that two location estimates in a genomic region represent the same gene? It is suggested that even with relatively large numbers of multiplex families, chance variation in the location estimate is substantial and may be a function of magnitude of the estimated LOD score.[35]

5.1.3. The COL9A1 gene and hip osteoarthritis: A replication study

Chapter 3.1 describes our investigations on the relationship between the two 12B1 and 8B2 COL9A1 markers and radiographic osteoarthritis at hand, hip, knee and spinal joints in the general population. We used two different designs; in a sibling-pairs study, we found that concordant affected sibling-pairs with radiographic osteoarthritis at the hip shared significantly more often alleles at the maker 8B2 in the COL9A1 gene than expected. No linkage of COL9A1 509-12B1 or 509-8B2 to radiographic osteoarthritis at other joints was found. To confirm and extend the findings to an out-bred population, we found the frequency of 8B2 alleles were significantly different between persons with radiographic osteoarthritis and controls within the population-based Rotterdam Study.

In our study, several issues are of important consideration. At the design step of a candidate gene approach, a key point to success is the selection of candidates. There is evidence supporting a role for the COL9A1 gene, mapped to 6q12-13, in osteoarthritis. The evidence can be summarized as (a) the role of COL9A1 polypeptide, as a structural protein, in the stability of joint cartilage, (b) functional studies that showed that synthesis of alpha1(IX) polypeptides to be essential for the assembly of heterotrimeric collagen IX molecules,[36] (c) transgenic mice that express a non-functional protein as well as knock-out mice that develop generalized,[37] or early onset knee,[38] osteoarthritis, and in human, (d) the COL9A1 mutation that was identified as one of the causes of multiple epiphyseal dystrophy, a phenotype associated with osteoarthritis,[39] (e) the COL9A1 509-8B2 marker that has been linked to hip osteoarthritis in women in an affected sibling-pairs study of female patients with severe form of osteoarthritis in the UK cohort.[40] The other issue to be considered when interpreting our findings is the characteristics of the candidate markers genotyped. First, the 12B1 and 8B2

COL9A1 markers are short tandem repeat polymorphism leading to 10 and 12 different variants of the COL9A1 gene, respectively. In contrast to a linkage study, the relation in an association study, can be easily missed since the repeat markers used have a large number of rare alleles. In the present study, the polymorphic nature of the studied markers resulted in multiple strata of cases and controls, thus, demolishing the power of the association study. The other point is that although, we hypothesize that the COL9A1 locus contributes to osteoarthritis susceptibility, the 8B2 marker is not likely causally related to radiographic osteoarthritis. Thus, 8B2 may be in linkage disequilibrium with an osteoarthritis susceptibility mutation within or close to the COL9A1 locus. The association was not specific for a single allele. This could be explained if a casual mutation resides on different haplotypes in linkage disequilibrium with 8B2 alleles. The last point to be mentioned is that in our sibling-pairs data, there was no evidence for a role of COL9A1 in other forms of osteoarthritis. Further studies are necessary to identify the underlying mutation in COL9A1 or within a nearby osteoarthritis susceptibility locus.

5.1.4. The HFE gene and arthropathy

Chapter 3.2 describes the relationship between the C282Y and H63D mutations in the HFE gene and arthropathy. In a random cohort drawn from the population-based Rotterdam Study, overall, we found that subjects homozygous for H63D compared to non-carriers had significantly more often arthralgia, oligoarthralgia, and Heberden's nodes. When the data was stratified by age, in persons aged 65 years or younger, H63D homozygotes had significantly more often polyarthralgia, chondrocalcinosis at hip or knee joints, increased number of hand joints with radiographic osteoarthritis, and Heberden's nodes. We found no relation of arthralgia or joint pathology to C282Y. We conclude that the H63D mutation may explain at least in part the prevalence of arthralgia, chondrocalcinosis, and hand osteoarthritis in the general population.

When discussing our findings, several points need to be addressed. The first point is why we did not find any relation to the C282Y mutation. This is important as C282Y is the main mutation causing hemochromatosis and has been associated with the highest levels of serum iron levels in patients with hemochromatosis,[41] and in the general population.[42,43] Lack of a significant association between osteoarthritis and C282Y may be due to the low number of subjects homozygous for this mutation due to other mortality. This may not be true, as a large population-based study,[44,45] also did not find a significant difference in the prevalence of

pain between C282Y homozygosity and controls. Two studies reported a small but significant association between C282Y and chondrocalcinosis,[46] and hand osteoarthritis at age more than 65 years.[47] The earlier finding were only based on two individuals, and the frequency of chondrocalcinosis in the control group was unknown, and the base population for cases (UK) and controls (Australia) was different.[48] In the latter study, also, the prevalence of radiographic hand osteoarthritis is unknown in the control group. Further, the authors did not discuss the lower frequency of H63D homozygotes in elder subjects with osteoarthritis which preclude any assessment on their potential effect on osteoarthritis.[48] The lower frequency of H63D homozygotes can be a result of selective survival at young age. Overall, based on our findings and given the low prevalence of C282Y homozygosity, we conclude that C282Y is not an important factor for osteoarthritis in the general population.

Our findings support the H63D mutation as one of the candidate mutations implicated in osteoarthritis in the general population. We found a strong and consistent association to H63D homozygotes not only in arthralgia, a subjective outcome, but also, in the underlying pathology including chondrocalcinosis, radiographic osteoarthritis at hands, and Heberden's nodes. Based on our findings, H63D homozygosity may explain 4 percent of the occurrence of pain, 13 percent of chondrocalcinosis, and 6 percent of hand nodal osteoarthritis in the general population aged 65 years or younger. Previous studies did not investigate this mutation in details.[48] Both C282Y and H63D are associated to significant iron overload in the Rotterdam study. In brief, C282Y homozygotes and heterozygotes, compared to non-carriers, had significantly higher levels of serum iron ($p<0.001$), ferritin ($p<0.01$), and transferrin saturation ($p<0.001$). Similarly we found that H63D homozygotes, compared to non-carriers, had significantly higher levels of serum iron ($p<0.001$), ferritin ($p<0.03$) and transferrin saturation ($p<0.001$). As discussed in Chapter 3.2, however, there is strong evidence suggesting that iron overload alone may not explain hemochromatosis-associated arthropathy. In fact, in our population and in those of others there was a poor correlation between serum iron indices and arthropathy in hemochromatosis[49-51] suggesting the involvement of an alternative mechanism i.e. an inflammatory components, in H63D associated arthropathy.

5.1.5. The HFE gene, osteoarthritis and mortality: A new role for inflammation

The investigation of why we did find a strong and consistent association to H63D homozygosity in persons aged 65 years and younger and why we did find no relation in persons aged 65 years or over, led us to another striking finding (**Chapter 3.3**). Subjects

homozygous for the H63D mutation with pain had a significantly earlier mortality than non-carriers without pain in persons aged 65 years or younger (**Chapter 3.2**). We hypothesized that an underlying inflammatory pathway may explain the relation between H63D homozygosity, joint pain, and mortality. In particular, Heberden's nodes, a known hereditary condition,[52] has an inflammatory component. We tested this hypothesis in a population-based Rotterdam Study where the participants have been followed up to 14 years. We found that subjects homozygous for H63D and Heberden's nodes died earlier most likely due to stroke than wild-type homozygotes without Heberden's nodes (**Chapter 3.3**). This observation led us to test the relation between H63D homozygosity and levels of serum C-reactive protein (CRP). We found that H63D homozygotes with Heberden's nodes had significantly higher levels of serum CRP compared to wild type homozygotes with or without Heberden's nodes. Our epidemiological investigations suggest that H63D homozygosity has a joint effect with Heberden's nodes coincides with a higher inflammatory status, and to an increased mortality due to vascular pathology.

Some points are of consideration. First, our findings remain to be confirmed by others. Second in depth experiments are required to unravel the detailed mechanism by which H63D lead to a higher inflammatory status. The finding of such studies will prove or reject our hypothesis.

5.1.6. The HFE gene, and longevity: Bilirubin opposes inflammation

The other point of interest in our study population was the course of the C282Y mutation. In our cohort, subjects heterozygous or homozygous for the C282Y mutation had a significant iron overload.[53] These subjects did not have a clinical diagnosis of hemochromatosis, neither did diabetes mellitus,[54] arthropathy (**Chapter 3.2**), or liver pathology. These observations are in line with previous reports.[44,55,56] Further, similar to the findings of others,[57] survival analyses revealed none of C282Y homozygotes died during a follow-up of 15 years and indeed C282Y carriers did not show a shorter life span in this elderly cohort. The low penetrance of C282Y mutation, while the carriers have a higher iron status, encourages investigators, as well as us, to hypothesize the presence of modifiers, which counteract the adverse effects of iron overload. As discussed in **Chapter 3.4** serum bilirubin, a strong antioxidant, was found at increased levels in patients with hereditary hemochromatosis.[58] We hypothesized that the increased serum bilirubin levels may play a protective role against oxidative stress induced by iron overload in carriers of mutations in HFE.[59,60]

We found that serum bilirubin levels were significantly correlated with serum ferritin iron and transferrin saturation, and carriers of C282Y and H63D had a significantly higher levels of serum bilirubin. Further, high serum bilirubin was associated with a 2.8 fold reduction in mortality in H63D homozygotes and two folds reduction in mortality in C282Y heterozygotes. We suggested that the high levels of bilirubin may counteract the adverse effects of oxidative stress induced by iron overload, which may explain in part the reduced penetrance of the HFE mutations. Hemeoxygenase pathway, a strong anti-inflammatory and antioxidant mechanism in organism,[59,60] may explain the observed associations, although this remains to be tested in epidemiological and experimental studies.

5.1.7. Heritability of serum iron indices in a Dutch isolate; First step to identify genes in iron metabolism

The observed low penetrance or the genotype phenotype correlation in hemochromatosis raised the question to what extend the HFE mutations can explain the variation in the levels of iron in the general population. We as well as others have found that only 5 percent of body iron levels can be explained by the HFE mutations.[53,61] The remaining proportion is explained by other genetic and environmental factors, or gene-environmental interactions that still remains to be identified. As a first step towards identifying genes involved in iron metabolism, we investigated the heritability of serum iron indices, including iron, ferritin, and transferrin saturation (**Chapter 4**). In a Dutch isolate, we found a heritability estimate of 0.17 for iron, 0.24 for serum transferrin saturation, and 0.26 for ferritin. We conclude that a modest proportion of the variance of iron and ferritin can be explained by heredity, independent of sex, age and environmental effects. Our results demonstrate the influence of both genetic and environmental factors on iron levels. The next question remains to answer is to what extent the heritability estimates can be explained by known genes in the studied genetic isolate.

5.2. Future Perspectives

There are several challenges in the head of both hemochromatosis and osteoarthritis. **Chapter 2.1** discusses classification of osteoarthritis. In osteoarthritis, like other complex disorders, clinical definition of disease obscures multiple mechanistically distinct subtypes. New genes revealed previously unsuspected biochemical pathways that could explain the pathogenesis. This will help to a predictive diagnosis and introduce an appropriate individualized therapy.

Future research will show to how many sub-phenotypes do really exist; and to what extent the subgroups of osteoarthritis differ in causal pathway, risk of developing disabilities, prognosis and response to treatment.[62,63]

It is clear that few of the genes found to be associated with osteoarthritis mapped to the known linkage regions (**Chapter 2.1**). This indicates that most of the causal mutations responsible for the found genomic intervals remain unknown. The first challenge in the future will be to identify those yet unknown genes. This can be addressed by a careful sub-phenotyping (different sub-phenotypes should not lumped together), analyzing the genes involved in the same pathway or in the same regulatory network, careful evaluation or interpretation of the findings from the association or linkage studies, development of internationally collaborative consortium, which share the databases and genomic information.[62-68]

The other challenge in front of osteoarthritis and hemochromatosis is uncovering the pathways involved in the disease pathogenesis. It is clear within the large well-defined cohorts such as the Rotterdam Study, the Framingham Study, and the UK Sibling-pairs cohort, multiple genes or genomic regions were associated or linked to osteoarthritis. In hemochromatosis also multiple genes are involved in the pathogenesis. And still for both diseases many genes will yet come.

Elucidating the relationship between genotype and phenotype is one of the most challenging and important tasks of the future research in osteoarthritis as well as hemochromatosis. The question that also needs to be answered is how genes interact with each other and environmental factors. The large national epidemiological population-based follow up studies with well characterized participating individuals for their diseases, biomarkers and genetic variations are necessary to demonstrate multiple effects of a single genotype, the detailed relationship between genetic markers and clinical phenotypes, the course of the disease over time, and the final outcome of gene-gene, and gene-environmental interactions.

The next challenge, for the area of osteoarthritis, is to translate the genomic information to clinical practice. In spite of recent advances in osteoarthritis, current treatment in osteoarthritis is palliative, focusing on analgesics and surgical interventions and the genetic counseling plays no more than nothing in the disease prediction and prevention. Development of the genome variations involved in osteoarthritis or hemochromatosis, raises the question whether screening based on such a genomic portrait can be used to predict or to prevent the

disease, and to identify drug targets and predict therapeutic response. This is a major challenge for many complex disorders in the coming decade.[62,65,69]

For hemochromatosis, this prospect is far more advanced than osteoarthritis, as the causal mutations have already been identified, molecular-based disease sub-phenotyping is possible, the effective therapeutic treatment is available, genotype-phenotype correlation has been widely investigated and early diagnosis and intervention before organ damage improves prognosis. Our data (**Chapter 3.2**) suggest that one of the indications for genetic testing is hand osteoarthritis which has already been included as one of the criteria for hemochromatosis.[64] Hemochromatosis is one of the diseases that fulfill the WHO guidelines for screening.[64] Still the challenge forward is to characterize the at risk population for genetic screening and prevention effectiveness of population-screening.[64,70] Although simple and effective biochemical tests for iron overload are available, genetic testing may be a cost effective alternative. In 1996, Feder and colleagues showed that bout 85 percent of hemochromatosis patients are carriers for the common C282Y and H63D mutation.[41] From this, one may predict that screening for the C282Y mutation should ascertain most patients reliably. This encouraged investigators and public health experts to initiate genetic screening programs in young population,[71] blood donors,[72] or children of hemochromatosis homozygotes.[44,73,74] However, these initiatives were soon hampered by the findings of a poor correlation between the HFE C282Y genotypes and clinical hemochromatosis.[75,76] If genetic screening is not informative for hereditary hemochromatosis, there remains little hope for the usefulness of genetic screening for other disorders. Perhaps the most important lesson to be learned is that predictions from selected families with hereditary forms of diseases such as hemochromatosis and other diseases cannot be translated to the general population without thorough research in large population samples. Although not impossible, it will be a tall order to study major genes such as *HFE* in the general population with sufficient statistical power.

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6

SUMMARY

SUMMARY

Chapter 2.1 provides a review on the genetic epidemiology of osteoarthritis. Twin and family studies showed that heritability estimate varies between 27 to 60 percent, depending on the inclusion criteria for ascertainment of subject i.e. clinical, radiographic or pathologic phenotype and the affected joint locations. Using positional cloning multiple genomic region have been linked to osteoarthritis. These regions barely overlap. Few regions have been replicated in different studies. Candidate gene studies have associated multiple genes, most of them do not map to the known linkage regions, to osteoarthritis. This leaves most of the genes responsible for linked regions unidentified.

Chapter 2.2 reviews the genetic epidemiological aspects of hereditary hemochromatosis. Multiple genes have been identified for different clinically distinct phenotypes of hemochromatosis. Type I hemochromatosis, is the most common form of the disease, which is explained by mutations in the HFE gene. The discovery of the common C282Y (carriers rate 13 percent and associated to high iron levels in Caucasians) and H63D (carrier rate 23 percent worldwide and associated to a modest increase in iron levels) mutations in the HFE gene provides a potential mutation testing to prevent an adult-onset disease phenotype.

Chapter 3.1 presents the results of our linkage and association study on the relationship between the COL9A1 gene and osteoarthritis at hand, knee, hip and spinal joints. Within the Rotterdam Study, a population-based study of 7983 subjects aged 55 years or over, we used two different designs. We found that affected sibling pairs with hip radiographic osteoarthritis shared significantly more often alleles IBD at the 8B2 and 12B1 markers than expected. No excess sharing was observed for radiographic osteoarthritis at other joint sites. When comparing the allele frequency of 8B2 and 12B1 in cases and controls, the frequency of 8B2 alleles in cases differed significantly from those of controls. Our data suggests that susceptibility for hip osteoarthritis is conferred within or close to the COL9A1 gene in linkage disequilibrium with the COL9A1 509-8B2 marker.

Chapter 3.2 discusses our findings on the relationship between the HFE gene and osteoarthritis. We investigated the relation between the HFE C282Y and H63D mutations with arthralgia and joint pathology in the population-based Rotterdam Study. Overall, there

was a significantly higher frequency of arthralgia, oligoarthralgia and Heberden's nodes in those homozygous for H63D compared to non-carriers. In persons aged 65 years or younger, H63D homozygotes had significantly more often polyarthralgia, chondrocalcinosis at hip or knee joints, increased number of hand joints with osteophytes space narrowing, radiographic osteoarthritis, and Heberden's nodes. We found no relation of arthralgia or joint pathology to C282Y, but compound heterozygotes had a significantly higher frequency of arthralgia, chondrocalcinosis at hip, and increased number of osteophytes at knee joints at late age (65 years or over). We conclude that the H63D mutation may explain at least in part the prevalence of arthralgia, chondrocalcinosis, and hand osteoarthritis in the general population.

Chapter 3.3 reports our findings on the relationship between the H63D mutation, Heberden's nodes and mortality. Our study on the relation between the H63D mutation, Heberden's nodes, an inflammatory related local form of osteoarthritis, and their joint effect on overall and cause-specific mortality. Within the Rotterdam Study, we found no relation to HFE H63D genotypes in mortality. Presence of Heberden's nodes was significantly related to a modest increase in mortality. Persons homozygous for the H63D mutation with Heberden's nodes had a substantial increase in risk of mortality compared to subjects homozygous for the wild type allele without Heberden's nodes. This increase in mortality was explained by an increase risk of mortality due to stroke. Persons homozygous for H63D with Heberden's nodes are characterized by increased levels of C-reactive protein (CRP) in serum ($p<0.001$). Increased levels of serum CRP were not found in those with Heberden's nodes who were not homozygous for the H63D mutation. The increased inflammatory state in carriers may explain in part the increased mortality due to stroke. Our study suggests that inflammation may explain the increased risk of mortality of H63D homozygotes with Heberden's nodes.

Chapter 3.4 explains our findings on the relationship between HFE mutations and serum bilirubin. Within the Rotterdam Study, overall, serum bilirubin levels were significantly correlated with serum iron ($p<0.001$), transferrin saturation ($p<0.001$) and serum ferritin ($p=0.03$). Carriers of the HFE mutations had higher level of serum bilirubin compared to the wild type homozygotes in particular H63D homozygotes and C282Y heterozygotes. The high serum bilirubin was associated to a 2.8 fold reduction in mortality in H63D homozygotes and a 2.2 fold reduction in mortality in C282Y heterozygotes. Taken together, our data suggests

that the high levels of bilirubin may counteract the adverse effects of oxidative stress induced by iron overload, which may explain in part the reduced penetrance of the HFE mutations.

Chapter 4.1 describes the results of our study to estimate the magnitude of genetic influences on iron and ferritin levels in relatives from a recent genetic isolate in the Netherlands. The participants analyzed in this study included 90 nuclear families with a total of 988 subjects. The proportion of the residual phenotypic variance due to additive genetic effects i.e. heritability estimates were approximately 0.17 ($p<0.0001$) for iron, 0.24 for transferrin saturation ($p<0.001$) and 0.26 ($p<0.0001$) for ferritin, while adjusting for sex, age and levels of serum albumin. A substantial proportion of the variance of iron, transferrin saturation, and ferritin can be explained by heredity, independent of sex, age, and environmental effects. Our results demonstrate the influence of both genetic and environmental factors on iron levels. Identification of genes influencing iron and ferritin levels using a QTL approach is feasible.

Chapter 5 provides a general discussion of the studies presented in this thesis in context of current knowledge and ongoing research in the field of genetic epidemiology of osteoarthritis and hemochromatosis.

SAMENVATTING

Hoofdstuk 2.1 bevat een overzicht van de genetische epidemiologie van osteoarthritis. Tweeling studies en familiestudies toonden dat de bijdrage van de erfelijkheid varieert tussen 27 en 60 procent, afhankelijk van de inclusie criteria op grond van het klinisch, radiologisch of pathologisch fenotype en de ligging van de aangedane gewrichten. Met positional cloning technieken zijn meerdere gebieden op het genoom gelinkt met osteoarthritis. Deze gebieden vertonen nauwelijks overlap. Weinig gebieden zijn gerepliceerd in te verschillende studies. De kandidaatgen studies hebben associaties van osteoarthritis met meerdere genen laten zien. De ligging van de meeste genen correspondeert echter niet met de bekende chromosomale gebieden die koppeling vertonen. De meeste genen in gebieden die koppeling tonen zijn dan ook nog niet geïdentificeerd.

Hoofdstuk 2.2 geeft een overzicht van de genetisch epidemiologische aspecten van erfelijke hemochromatose. Meerdere genen zijn geïdentificeerd voor verschillende klinisch te onderscheiden kenmerken (fenotypen) van hemochromatose. Primaire hemochromatose, de meest voorkomende vorm van de ziekte, wordt verklaard door aanwezigheid van mutaties in het HFE-gen. De ontdekking van de algemene C282Y-mutatie (dragerschap frequentie bedraagt 13 procent en er in een associatie met hoge serum ijzer spiegels bij personen van Noord-Europese afkomst) en de H63D mutatie (wereldwijde dragerschap frequentie 23 procent met matig verhoogde ijzer spiegels) in het HFE-gen, maakt potentieel testen op mutaties mogelijk om de op volwassen leeftijd optredende vorm van de ziekte te voorkomen.

Hoofdstuk 3.1 toont de resultaten van onze koppeling- en associatie studie over het verband tussen het COL9A1 gen en osteoarthritis van de hand, knie, heup en wervelkolom. Binnen de ERGO-studie, een populatie studie bij 7983 personen van 55 jaar of ouder, gebruikten wij twee verschillende ontwerpen. Wij vonden dat aangedane sibling paren (broer-broer, zus-zus of broer-zus paren) met radiologisch gediagnosticeerde osteoarthritis van de heup significant vaker dan verwacht allelen deelden (Identical By Descent) op de DNA markers 8B2 en 12B1. Er werd geen bovenmatig delen van beide allelen voor een marker waargenomen bij radiologisch gediagnosticeerde osteoarthritis van andere gewrichten. Een vergelijking van de allel frequenties van 8B2 en 12B1 in patiënten en controles toonde een significant verschil in de frequentie van allelen 8B2 tussen patiënten en controles. Onze gegevens suggereren dat de

genetische gevoeligheid voor osteoarthritis van de heup binnen of dicht bij het gen COL9A1 linkage disequilibrium is met de marker COL9A1 509-8B2.

In **hoofdstuk 3.2** bespreken wij onze bevindingen over het verband tussen het gen HFE en osteoarthritis. Wij onderzochten de relatie van HFE C282Y en H63D mutaties met artralgie en gewricht pathologie in de ERGO-studie. Als totaal werd een significant hogere frequentie van artralgie, oligoartralgie en Heberden nodules gevonden bij homozygoten voor H63D dan bij niet-dragers. In personen van 65 jaar of jonger hadden H63D homozygoten significant vaker polyartralgie, chondrocalcinosis bij/van heup of knie gewrichten, een verhoogd aantal handgewrichten met osteophyten, radiologisch gediagnosticeerde osteoarthritis, en Heberden nodules. Wij vonden geen relatie van artralgie of gewricht pathologie met C282Y, maar samenstelde heterozygoten hadden een significant hogere frequentie van artralgie, chondrocalcinosis van de heup, en verhoogd aantal osteophyten bij knie gewrichten op latere leeftijd (65 jaar of ouder). Wij concluderen dat de H63D mutatie op zijn minst voor een deel de prevalentie van artralgie, chondrocalcinosis en osteoarthritis van de hand in de algemene bevolking kan verklaren.

Hoofdstuk 3.3 vermeldt onze bevindingen over het verband tussen de H63D mutatie, de nodules van Heberden en morbiditeit. Onze studie onderzoekt de relatie tussen de H63D mutatie, de nodules van Heberden, een inflammatoire verwante lokale vorm van osteoartritis, en hun gezamenlijk effect op algemene en oorzaak-specifieke mortaliteit. Binnen de ERGO-studie vonden wij geen relatie met HFE H63D genotypen en mortaliteit. De aanwezigheid van de nodules van Heberden was significant geassocieerd met een bescheiden verhoging van mortaliteit. Voor de H63D mutatie homozygote individuen met de nodules van Heberden hadden een aanzienlijke toename van het risico op mortaliteit in vergelijking met voor het wild-type homozygote personen zonder nodules van Heberden. Deze toename van mortaliteit werd verklaard door een verhoogd risico op mortaliteit ten gevolge van een beroerte. Voor de H63D mutatie homozygote individuen met de nodules van Heberden worden gekenmerkt door verhoogde serum spiegels c-reactieve proteïne (CRP; $p<0,001$). Verhoogde CRP serum spiegels werden niet gevonden in individuen met de nodules van Heberden die niet homozygoot waren voor de H63D mutatie. De verhoogde staat van ontsteking in dragers van deze mutatie kan voor een deel de verhoogde mortaliteit ten gevolge van een beroerte

verklaren. Onze studie doet vermoeden dat ontsteking het verhoogde risico op mortaliteit van H63D homozygoten met de nodules van Heberden kan verklaren.

Hoofdstuk 3.4 biedt een verklaring voor onze bevindingen over de relatie tussen HFE mutaties en serum bilirubine. Binnen de ERGO-studie als totaal waren serum bilirubine spiegels significant gecorreleerd met serum- ferritine ($p=0,03$), ijzer ($p<0,001$), en transferrine-saturatie ($p<0,001$). Dragers van de HFE mutaties bezaten hogere serum bilirubine spiegels dan the wild-type homozygoten in het bijzonder H63D homozygoten en C282Y heterozygoten. Hoog serum bilirubine was geassocieerd met een 2,8-voudige reductie in mortaliteit in H63D homozygoten en met een 2,2-voudige reductie in C282Y heterozygoten. Samenvattend suggereren onze gegevens dat de hoge bilirubine spiegels de nadelige effecten kunnen tegenwerken van door ijzer overbelasting geïnduceerde oxidatieve stress, wat voor een deel de gereduceerde penetrantie van de HFE mutaties kan verklaren.

Hoofdstuk 4.1 beschrijft de resultaten van onze studie die tot doel heeft de grootte in te schatten van genetische invloeden op ijzer en ferritine spiegels in verwanten uit een recent genetisch isolaat in Nederland. Tot de deelnemers aan deze studie behoorden 90 kernfamilies van in totaal 998 personen. Het deel van de residuele fenotypische variantie ten gevolge van additief genetische invloeden d.i. bijdragen van de erfelijkheid waren circa 0,26 voor ferritine ($p<0,0001$), 0,17 voor ijzer ($p<0,0001$) en 0,24 voor transferrine-saturatie ($p<0,001$) onder aanpassing voor geslacht, leeftijd en serum albumine spiegels. Een aanzienlijk deel van de variatie van ijzer, transferrine-saturatie en ferritine kan worden verklaard door erfelijkheid, onafhankelijk van geslacht, leeftijd en omgevingsinvloeden. Onze resultaten tonen de invloed van zowel genetische als omgevingsfactoren op ijzer spiegels aan. Identificatie van genen die de ijzer en ferritine spiegels beïnvloeden met een QTL aanpak is haalbaar.

Hoofdstuk 5 geeft een algemene discussie van de studies die in dit proefschrift worden gepresenteerd in samenhang met hedendaagse kennis en lopend onderzoek op het gebied van de genetische epidemiologie van osteoarthritis en hemochromatose.

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