Thrombophilia and Genetics in Liver Disease

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Colofon

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Thrombophilia and Genetics in Liver Disease

Trombofilie en genetica in leverziekten

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Chapter 1

General introduction and outline of the thesis

The liver is the largest internal organ in the human body. In physiological circumstances, the liver receives 25-30% of the cardiac output. As a result, normal blood flow through the liver is approximately 1,600 ml per minute of which 80% is supplied by the portal vein and the remaining part by the hepatic artery. This dual blood supply is unique and enables the liver to play a crucial role in connecting the digestive system with the circulation. This is reflected by the fact that one of the main functions of the liver is to absorb, process, store and distribute metabolites. In addition, the liver is vital for detoxification and elimination of toxic substances. Finally, several proteins, including coagulation factors and albumin, are synthesized in the liver. Blood flows from the gastrointestinal organs to the liver through the superior mesenteric vein and the splenic vein, which unite in the portal vein. Within the liver parenchyma, terminal portal venules drain into sinusoids. Eventually, blood leaves the liver through one of three hepatic veins, which on their turn drain into the inferior vena cava (1, 2).

LIVER FIBROSIS

Damage to the liver can result in the development of liver fibrosis, this process is called liver fibrogenesis. Liver fibrogenesis is characterized by the deposition of extracellular matrix components within the liver parenchyma. It is the result of a complex interplay of chronic injury, environmental risk factors and genetic susceptibility. Possible causes of chronic liver injury include, but are not limited to, alcohol abuse, viral hepatitis, genetic disorders, immune disorders, toxic-metabolic insults, and obesity or presence of the metabolic syndrome. When these damaging factors persist, fibrogenesis proceeds and can ultimately result in liver cirrhosis, a condition in which the liver parenchyma is severely damaged. Cirrhosis on its turn can result in the development of hepatocellular carcinoma and liver failure, both life-threatening conditions (1, 2).

Advanced liver fibrosis and cirrhosis affects hundreds of millions of people and comprises a major cause of morbidity and mortality, with a worldwide annual mortality due to liver cirrhosis and primary liver cancer of approximately 1.7 million (3, 4). In the United States, chronic liver disease and cirrhosis are the eleventh leading cause of mortality (5). Nowadays, non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide. It is estimated that 30% of the current United States population is affected by NAFLD, which corresponds to 75 million to 100 million individuals. Nonalcoholic steatohepatitis (NASH), the progressive subtype of NAFLD, is present in 25-30% of this group (Figure 1.1). Presence of characteristics of the metabolic syndrome, i.e. central obesity, dyslipidemia, hypertension, and insulin resistance, is associated with an increased risk of NASH and more progressive disease. In patients with NASH, cardiovascular disease and malignancy are the most common causes of death (6). During their lifetime, 20% of patients with NASH will develop cirrhosis. As a result, NASH will become the most common indication for liver transplantation between 2020 and 2030 (7, 8).

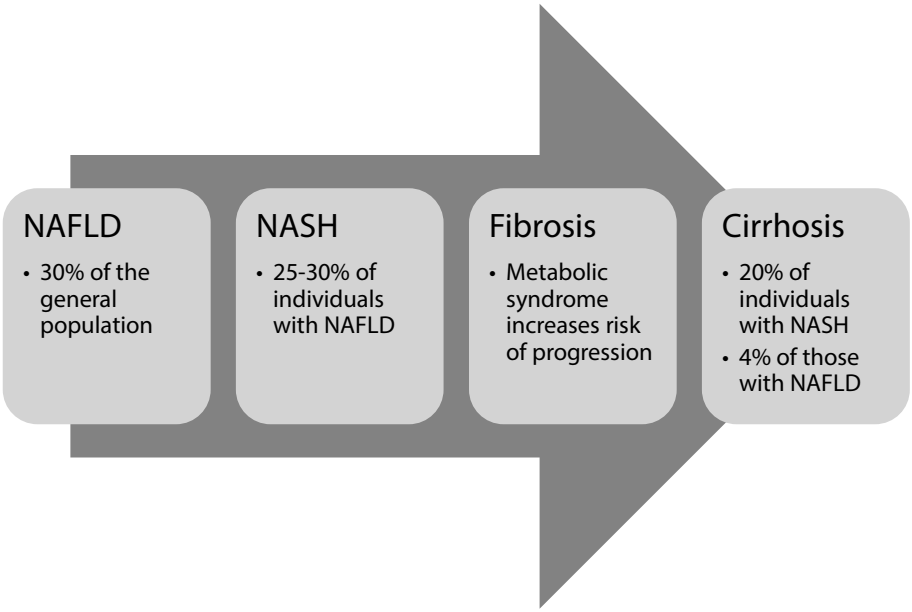

Figure 1.1 *Non-alcoholic fatty liver disease (NAFLD) is present in approximately 30% of the current Western population. Non-alcoholic steatohepatitis (NASH), the progressive subtype of NAFLD, is encountered in 25-30% of these individuals. NASH is characterized by steatosis associated with hepatic injury. Presence of characteristics of the metabolic syndrome, including insulin resistance, central obesity and dyslipidemia, is associated with a higher risk of NASH and more progressive disease. Ultimately, 20% of individuals with NASH will develop cirrhosis. In addition, approximately 4% of persons with NAFLD will progress towards cirrhosis.*

Traditionally, liver biopsy is considered the gold standard for the diagnosis of liver fibrosis, as it assesses the nature and severity of fibrosis, its distribution within the liver and other associated lesions. However, limitations of biopsy include sampling error and interobserver variation. In addition, because of its invasive nature, liver biopsy is associated with a risk of complications, including serious bleeding and even mortality (9-13). Therefore, performing a liver biopsy in healthy individuals is unethical. As a result, studying the prevalence of, and risk factors associated with liver fibrosis in the general population using liver biopsy is not feasible. Recently, several non-invasive alternatives for assessing presence and amount of liver fibrosis have been evaluated. These include serum biomarkers and imaging modalities measuring liver stiffness. Nowadays, the most widely used and best-validated method to non-invasively assess liver fibrosis is transient elastography (Fibroscan®, Echosens™, France) (14-16). Transient elastography is an effective, fast, easy to learn, reproducible and safe method to assess liver fibrosis and cirrhosis (16-19). Studies in patients with chronic liver disease have shown that transient elastography can also be used to predict liver decompensation, portal-hypertension related complications, and survival (20, 21).

Genetics in liver disease

Liver fibrosis is considered a multifactorial disease, in which the interaction between chronic liver injury, genetic susceptibility, and environmental risk factors results in fibrogenesis. Until 2000, genetic analysis focused on the identification of mutations underlying monogenic diseases, such as Wilson disease, in a single patient or small group of individuals. In that year, the *human genome project* was presented, paving the way for studies aiming to entangle the association between genetic information and complex human traits and diseases, such as liver fibrosis (22). The *International HapMap project* and *1000 genomes project* have provided an additional important contribution to understanding the role of genetics in many diseases and traits by characterizing the human genome (23, 24). These, and technical advancements have enabled studying common variants in the human genome. These common variants, also known as single nucleotide polymorphisms (SNPs), account for most of the genetic variability between individuals, together with microsatellites. SNPs can affect the development of disease by influencing the function, availability, or transcription of a protein (25). Genome-wide association studies (GWAS) aim at hypothesis-free testing of hundreds of thousands of SNPs throughout the genome to assess their role in complex disorders. This technique has been applied to various complex liver diseases, including viral hepatitis, gall stones, cholestatic liver diseases, alcoholic liver disease, and NAFLD (26-36). In addition, several SNPs have been identified that predispose to liver fibrosis in at-risk populations (27, 30, 31, 34, 37-39). However, the role of these SNPs in the general population remains to be elucidated. As a result, it is not known whether these genetic variants alter the effect of chronic liver injuries on liver fibrogenesis or whether they affect fibrosis development independently of other factors.

THROMBOPHILIA IN LIVER FIBROGENESIS

Increasing evidence suggests a role for hypercoagulability in the development and progression of liver fibrosis. Presence of prothrombotic risk factors, including the factor V Leiden mutation and prothrombin G2021A variant, has been shown to be associated with occurrence of more advanced fibrosis and/or cirrhosis in patients with chronic liver injury, while comparable liver injury in patients with haemophilia resulted in a lower rate of fibrogenesis (40-48). Until now, no studies have been performed assessing the role of prothrombotic risk factors in liver fibrogenesis in the general population.

The exact mechanism by which hypercoagulability influences liver fibrogenesis has not yet been fully elucidated. Two alternative hypotheses have been postulated, in which thrombin seems to be the key driver of fibrogenesis. The first theory comprises the formation of small occlusive thrombi following inflammation-induced thrombin production. The development of these thrombi will result in consequently tissue ischemia, parenchymal extinction and development of fibrosis and cirrhosis (49, 50). The second hypothesis states that thrombin causes direct activation of hepatic stellate

cells by binding the protease-activated receptor 1. As stellate cells are responsible for the production of extracellular matrix components, activation of these cells will cause fibrogenesis (2, 51-54).

In several animal and laboratory studies, administration of low molecular weight heparin or vitamin K antagonists had an antifibrotic effect (55-59). This beneficial effect of anticoagulant medication has also been demonstrated in humans. Administration of unfractionated or low molecular weight heparin in addition to regular treatment resulted in decreased collagen levels and proliferation in patients with chronic hepatitis B (60). Administration of low molecular weight heparin was also beneficial in a randomized controlled trial in patients with advanced cirrhosis. Enoxaparin administration for 48 weeks in a high prophylactic dose (4,000 IU/day) resulted in a significantly lower incidence of portal vein thrombosis (PVT) during treatment and during 2 years of follow-up. Even more notable was the observation that treatment with enoxaparin reduced the incidence of liver decompensation and improved survival without increasing the number and severity of bleeding complications (61).

VASCULAR LIVER DISEASES

Venous thrombosis is defined as venous occlusion caused by a blood clot. The incidence of venous thrombosis is age-dependent and ranges from 1 per 10,000 cases per year in young adults to 1 in 100 cases per year in the very old. In most cases, venous thrombosis develops in the deep veins of the lower extremities. However, in rare cases, thrombosis occurs in other veins, including the splanchnic veins (62). Splanchnic vein thrombosis (SVT) encompasses PVT, mesenteric vein thrombosis, as well as hepatic vein thrombosis, which is responsible for the vast majority of cases with Budd-Chiari syndrome (BCS).

Budd-Chiari syndrome

BCS is a rare variant of venous thrombosis, which is defined as occlusion of the hepatic venous outflow tract. This occlusion can occur in the outflow tract from the level of the small hepatic veins until the right atrium. Outflow obstruction caused by heart failure or sinusoidal obstruction syndrome is considered a different entity. Most often, BCS is caused by thrombosis of the hepatic veins, but other causes, including extravenous compression or invasion by a tumor or abscess are also described. Obstruction of the hepatic venous outflow tract can result in portal hypertension and ischemia of hepatocytes due to venous congestion. Clinical presentation of BCS is highly variable. Patients with BCS can be asymptomatic, have symptoms as abdominal pain, fever, ascites, hepatomegaly and jaundice, or can even present with fulminant liver failure (63, 64). With the current treatment algorithm, including a step up approach of anticoagulation,

recanalization, transjugular intrahepatic portosystemic shunt (TIPS) insertion and liver transplantation, survival in patients with BCS is 88% after 1 year and 72% after 5 years (65).

Portal vein thrombosis

PVT is characterized by obstruction of the portal vein or its branches. Although the prevalence of PVT was reported to be 1% in a large autopsy study, PVT is considered a rare subtype of venous thrombosis, like BCS (66). In clinical practice, PVT is classified as either acute or chronic depending on the amount of time between origin and diagnosis. In acute PVT, patients can be asymptomatic or present with various symptoms, including abdominal pain, nausea, fever and ileus. If acute PVT is not recognized and the thrombus persists, collaterals will form to restore blood flow from upstream of the thrombus to downstream of the thrombus. This network of collaterals is called portal cavernoma, which is a hallmark of chronic PVT. In addition to cavernoma development, compensatory dilation of other veins and the hepatic artery will occur. Despite these changes, portal hypertension is inevitable. Therefore, complications of portal hypertension, including gastrointestinal bleeding and splenomegaly, are the most important clinical features of chronic PVT (64, 67). In addition, gastrointestinal ischemia is considered a concerning complication of PVT. Ischemia can result in intestinal infarction, a condition that requires immediate surgical treatment. However, data on gastrointestinal ischemia in PVT is scarce. It has been suggested that extension of thrombus from the portal vein into the superior mesenteric vein and/or splenic vein is associated with an increased risk of ischemia and infarction (68). Available studies report a prevalence ranging between 2% and 32% in patients with acute PVT. In chronic PVT, little is known about the occurrence and impact of gastrointestinal ischemia (69-74).

Etiology of splanchnic vein thrombosis

Local risk factors are important in the etiology of PVT. This is reflected by the fact that PVT is most often seen in patients with liver cirrhosis or hepatobiliairy malignancies. In BCS, local risk factors are of far less importance in the Western world. Nonetheless, there is considerable overlap in the etiology of BCS and non-cirrhotic, non-malignant PVT. Systemic, prothrombotic risk factors are pivotal in the etiology of both disorders, as they account for approximately 75-85% of the etiology of SVT (74, 75). These prothrombotic risk factors can be divided into genetic and acquired risk factors. As a multifactorial etiology is present in 46% of patients with BCS and 48% of those with PVT, a complete hematological work-up, assessing both genetic and acquired risk factors, should be performed in all patients with SVT (74-76). Genetic risk factors of SVT include the factor V Leiden mutation, prothrombin G20210A gene variant, protein C, protein S and antithrombin deficiency, all known risk factors of common venous thrombosis. Re-

cently, several genetic risk factors of common venous thrombosis were identified that have not yet been tested in the etiology of SVT (77-80). Myeloproliferative neoplasms (MPNs) – i.e. polycythemia vera, essential thrombocytosis and primary myelofibrosis – are the most important acquired risk factor for SVT with a prevalence of 20-50% (74, 75, 81). Diagnosing MPN in patients with SVT is often challenging as portal hypertension, resulting from obstruction of the portal or hepatic vein, can mask the blood cell changes associated with MPN due to splenomegaly and hemodilution (82). The *JAK2V617F* mutation is an easily detectable and highly specific marker for presence of (occult) MPN, testing for this mutation is therefore recommended in all patients with SVT (64, 67, 83). However, the *JAK2V617F* mutation is not present in all patients with MPN. In some patients, an additional *MPL* mutation is identified (84, 85). Recently, new somatic mutations in *CALR* were detected in patients with MPN lacking *JAK2V617F* and *MPL* mutations (86, 87). The prevalence and role of these *CALR* mutations in patients with SVT remains to be elucidated.

AIMS AND OUTLINE OF THE THESIS

The aims of this thesis were:

1. to determine the prevalence of and risk factors associated with liver fibrosis in the general population
2. to explore the association between thrombophilia and liver fibrosis in the general population
3. to assess the role of new genetic risk factors in SVT and to study the occurrence and characteristics of gastrointestinal ischemia in portal vein thrombosis

To address these aims, the current thesis is divided in three parts. As previously described, relatively little is known about the occurrence of and factors associated with liver fibrosis in the general population, as previous studies focused mainly on fibrosis in risk groups, such as patients with alcohol abuse and/or chronic viral hepatitis. Therefore, the first part of this thesis addresses the prevalence of and risk factors associated with liver fibrosis in the general population. The studies described in this part are conducted within the Rotterdam Study, a large, ongoing, prospective population-based cohort study among subjects aged 45 years and over. **Chapter 2** describes the prevalence of liver fibrosis, assessed non-invasively using transient elastography, in participants of the Rotterdam Study. In addition, this chapter focuses on the risk factors associated with liver fibrosis in this population-based cohort. In **chapter 3**, we assess the association between common genetic variants associated with liver fibrosis in at-risk populations and liver fibrogenesis in the general population. The association between interferon gamma receptor 2 gene variants and liver fibrosis is discussed into more detail in this chapter.

Part II of this thesis focuses on the role of thrombophilia in liver fibrogenesis. In **chapter 4**, we aim to describe the role of anticoagulation in liver disease. In the first part of this chapter, the use of anticoagulation in the treatment of vascular liver diseases is addressed. The second part evaluates the potential role of anticoagulation in the prevention of liver fibrogenesis. In the next two chapters of this thesis, the role of thrombophilia in liver fibrogenesis in the general population is further explored. In **chapter 5,** we assess the role of the factor V Leiden mutation, prothrombin G20210A gene variant and blood group type non-O – well-known genetic prothrombotic risk factors – in liver fibrogenesis in participants of the Rotterdam Study. As von Willebrand factor (VWF) is a key player in hemostasis and VWF levels are known to be elevated and associated with long-term outcome in patients with cirrhosis, we assess the association between VWF levels and liver fibrosis in the general population in **chapter 6**.

The third part of this thesis aims to provide more insight in the etiology and ischemic complications of vascular liver diseases. Diagnosing MPNs, the most important acquired risk factor for SVT, in clinical practice is often challenging, especially in patients without the *JAK2V617F* mutation, as mentioned before. Therefore, we describe in **chapter 7** the role of somatic Calreticulin mutations, recently identified mutations found in patients with MPN lacking *JAK2V617F* and *MPL*, in patients with SVT. In **chapter 8**, we describe the role of genetic variations associated with common venous thrombosis in the etiology of SVT. Gastrointestinal ischemia is one of the most feared complications of PVT, but data on its prevalence and clinical presentation are scarce. Therefore, we explore the occurrence and characteristics of gastrointestinal ischemia in patients with PVT in **chapter 9**. Finally, in **chapter 10**, the main findings and conclusions of our studies will be summarized and discussed.

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Part I

Prevalence and risk factors of liver fibrosis in the general population

Chapter 2

Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: The Rotterdam Study

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ABSTRACT

Background and aims

Since little is known about the prevalence of and factors associated with liver fibrosis in the general population, we aimed to investigate this in a large, well-characterized cohort, by means of transient elastography (TE).

Methods

This study was part of the Rotterdam Study, a population-based study among individuals ≥45 years. All participants underwent abdominal ultrasound and TE. Liver stiffness measurement (LSM) ≥8.0 kPa was used as cut-off suggesting clinically relevant fibrosis.

Results

Of 3041 participants (age 66.0±7.6 years) with reliable LSM, 169 (5.6%) participants had LSM ≥8.0 kPa. Age (OR 2.40, 95%CI 1.72-3.36,p<0.001), ALT (OR 1.24, 95%CI 1.12-1.38,p<0.001), smoking (OR 1.77, 95%CI 1.16-2.70, p=0.008), spleen size (OR 1.23, 95%CI 1.09-1.40,p=0.001), HBsAg or anti-HCV positivity (OR 5.38, 95%CI 1.60-18.0,p=0.006), and combined presence of diabetes mellitus (DM) and steatosis (OR 5.20, 95%CI 3.01-8.98, p<0.001 for combined presence) were associated with LSM ≥ 8.0 kPa in multivariable analyses. The adjusted predicted probability of LSM ≥8.0 kPa increased per age decade, with probabilities ranging from 1.4% (0.9-3.6) in participants aged 50-60 years to 9.9% (6.8-14.5) in participants >80 years. Participants with both DM and steatosis had the highest probabilities of LSM ≥8.0 kPa (overall probability 17.2% (12.5-23.4), this probability did not increase with age (p=0.8).

Conclusions

In this large population-based study of older adults, LSM ≥8.0 kPa, suggestive of clinically relevant fibrosis, was present in 5.6% and was strongly associated with steatosis and DM. In the context of an aging population and an increased prevalence of diabetes mellitus and obesity, this study illustrates that liver fibrosis may become a more prominent public health issue in the nearby future.

INTRODUCTION

Advanced liver fibrosis and cirrhosis affects hundreds of millions of people and comprises a major cause of morbidity and mortality, with a worldwide mortality rate due to liver cirrhosis and primary liver cancer of over 1.5 million per year (1, 2). In the United States, chronic liver disease and cirrhosis are the eleventh leading cause of mortality (3). In the past few decades, non-alcoholic fatty liver disease (NAFLD) has become one of the major causes of liver disease (4). In previous European population-based cohort studies, the reported prevalence of steatosis, assessed by abdominal ultrasound, ranged from 25-35% (5-8). In patients with diabetes mellitus (DM) the prevalence of NAFLD was shown to be as high as 40-70% (9, 10). Considering population ageing and the current epidemic in obesity and type II DM in developed countries, the burden of chronic liver diseases – in particular non-alcoholic steatohepatitis (NASH) – is projected to increase substantially in the next decades (11-13).

To date, limited studies have been performed focusing on the prevalence of, and risk factors for liver fibrosis in the general population, as data are mainly derived from autopsy studies or biopsy studies in selected populations. In theory, one way to investigate the prevalence of liver fibrosis and cirrhosis in the general population would be to perform a liver biopsy in a large population of healthy volunteers. However, an important disadvantage of liver biopsies is the invasive nature of the procedure, and healthy volunteers are rarely representative of the general population at risk. In recent years, non-invasive techniques have been developed to circumvent the need for liver biopsy. One of these techniques is transient elastography (TE), which is strongly associated with histological stages of liver fibrosis (14-19). Previous studies examining factors associated with elevated liver stiffness measurements (LSM) in community-based populations were performed in volunteers attending a free medical check-up and in a Chinese population-based cohort study (20, 21). Until now, these factors have not been studied in a large random sample of a general Caucasian population. Therefore, the aim of our study was to investigate the distribution of and factors associated with clinically relevant liver fibrosis, as measured by TE, in a large population-based cohort of Caucasians.

SUBJECTS AND METHODS

Study population

The Rotterdam Study is a large prospective population-based cohort study conducted among adults aged 45 years and above living in Ommoord, a district of Rotterdam, The Netherlands. The rationale and study design have been described previously (22). The medical ethics committee at the Erasmus University of Rotterdam approved the study, and written informed consent was obtained from all participants.

All consecutive participants (age range 51-98 years) that visited the research center between January 2011 and September 2013 were included in the current study. Each participant completed an extensive interview and clinical examination that included abdominal ultrasonography, TE, a fasting blood collection, and an anthropometric assessment.

Interview

The interview preceded the clinical examination and was designed to obtain data concerning demographics, medical history, comorbid conditions, current and past smoking behaviour, current alcohol consumption (drinks/week), and drug use. Detailed information on dispensed drug prescriptions was obtained from automated pharmacies, where nearly all participants are registered.

Biochemistry

Fasting blood samples were collected on the morning of ultrasound examination and LSM. Blood lipids, platelet count, glucose and alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), and total bilirubin were measured using automatic enzymatic procedures (Roche Diagnostics GmbH, Mannheim, DE). Insulin, hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (anti-HCV) were measured by an automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE). According to local cut-off criteria, the upper limit of normal (ULN) of ALT was defined as 40 U/L for men and 30 U/L for women.

Abdominal ultrasonography

Abdominal ultrasonography was performed by a certified and experienced technician on Hitachi HI VISION 900. Images were stored digitally and re-evaluated by a single hepatologist with more than ten years of experience in ultrasonography (JS). The diagnosis of steatosis was determined by the ultrasound technician according to the protocol by Hamaguchi et al (23). Severity of fatty liver was classified as 'no fatty liver' (score 0-1), 'mild fatty liver' (score 2-3), or 'moderate to severe fatty liver' (score 4-6). NAFLD was defined as steatosis in absence of any of the following possible secondary causes of steatosis: 1) excessive alcohol consumption (>14 drinks/week), 2) positive HBsAg or anti-HCV tests, and 3) use of pharmacological agents associated with steatosis (i.e. amiodarone, corticosteroids, methotrexate, and tamoxifen). In addition, spleen size, presence of collaterals, and Doppler examination of the hepatic veins, hepatic artery and portal vein were evaluated during ultrasound examination. Splenomegaly was defined as a spleen size >12.0 cm.

Transient elastography

LSM (Fibroscan®, EchoSens™, Paris, France) was performed by a single operator, who carried out more than 1000 examinations before start of the study. LSM was performed on the right lobe of the liver, through the intercostal spaces, with the participant lying flat on his/her back with the right arm laying in maximal abduction. Either M- or XL-probe was applied, according to instructions by the manufacturer. Participants with intracardiac devices and participants with physical disabilities, making TE impossible, were excluded from the study. Because of necessary maintenance of probes (for calibration), TE was not performed on four days during the study period. Failure was recorded when no LSM was obtained after at least 10 shots. Reliability of LSM was categorized according to the criteria by Boursier et al (24). LSM was considered 'poorly reliable' if interquartile range (IQR)/median LSM >0.30 with median LSM ≥7.1 kPa. These 'poorly reliable' LSM were excluded from our analyses. LSM ≥8.0 kPa and >13.0 kPa were taken as cut-offs suggesting clinically relevant liver fibrosis and cirrhosis, respectively. These cut-off levels were deliberately chosen, for they are known to yield high positive predictive values for presence of clinically relevant fibrosis and cirrhosis in previous studies (16, 21, 25).

Covariables

Anthropometric measurements were performed by well-trained research assistants. Body Mass Index (BMI) was calculated as weight (kg)/height (m^2). Waist circumference was measured in centimetres. The average of two blood pressure measurements, obtained at a single visit in upright position after a minimum of 5 minutes rest, was used for analysis. Metabolic syndrome was defined, according to Adult Treatment Panel III criteria (26), as the presence of at least 3 of the following 5 traits: 1) abdominal obesity, defined as a waist circumference in men >102 cm (40 inch) and in women >88 cm (35 inch), 2) serum triglycerides ≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides, 3) serum HDL cholesterol (HDL-C) <40 mg/dL (1.0 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL-C, 4) blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure, 5) fasting plasma glucose (FPG) ≥100 mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose. Hypertension was defined as blood pressure ≥140/90 mmHg or drug treatment for elevated blood pressure. DM was defined as FPG ≥126 mg/dL (7.0 mmol/L) or drug treatment for elevated blood glucose. The insulin resistance index was calculated using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR): fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5 (27).

Statistical analysis

Baseline data were done using descriptive statistics. Chi-square tests and Student's t-tests (means) or Wilcoxon rank sum tests (medians) were used to assess the significance of differences in distributions of categorical data and continuous data respectively. To examine associations between traits and LSM as continuous (log-transformed) dependent variable or LSM ≥8.0 kPa, we performed linear or logistic regression analyses, respectively. Since there is some discordancy regarding the cut-off level of LSM for presence of advanced liver fibrosis using XL-probe, we performed additional sensitivity analyses and we tested our model applying a one point lower cut-off for XL probe (15). In addition we tested our model applying a cut-off of 9.5kPa for advanced fibrosis, a cut-off that has been proposed in studies including subjects with viral hepatitis (25). Interaction terms between age, sex and other covariables were tested in linear and logistic multivariable regression models. A combined interaction term of DM and steatosis with age was constructed by dividing the total cohort into 4 groups: 1) participants without DM and steatosis, 2) participants with DM and without steatosis 3) participants with steatosis and without DM and 4) those with both DM and steatosis. We calculated predicted probabilities of having LSM ≥8.0 kPa by using all covariables of our multivariable logistic regression model, i.e. age, sex, spleen size, ALT, BMI, alcohol consumption/week, smoking behaviour, presence of HBsAg and/or anti-HCV, the presence of DM and/or steatosis, and the combined interaction term. Probabilities were expressed as median (IQR) percentage. Linear regression analyses was used to assess the significance of differences in predicted probabilities between subgroups of our cohort. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Study population

Of 3439 participants aged 51 or above who visited the research center between January 2011 and September 2013, a total of 3342 participants underwent TE (Figure 2.1). Due to LSM failure, an additional 162 participants (4.8%) were excluded. LSM was reliable in 3041 participants. Baseline characteristics of the study population are shown in Table 2.1. Fifty-five percent of 3041 participants were women, mean age of the participants was 66.0 ± 7.6 years and mean BMI was 27.3 ± 4.0 kg/m^2. Participants were predominantly of Caucasian ethnicity (95.1%). Median LSM was 4.7 kPa (3.8-5.8) and 1080 participants (35.5%) had presence of steatosis on abdominal ultrasound. LSM ≥8.0 kPa, suggesting presence of clinically relevant fibrosis, was detected in 169 participants (5.6%). Twenty-two participants had positive viral serology (7 were HBsAg positive and 16 were anti-HCV positive; one participant was both HBsAg and anti-HCV positive); only

one of these participants had ALT of 70U/L, which is one time the upper limit of normal. Normal liver enzymes were present in 2216 (76.0%) of all participants. Of these 2216 participants, 81 (3.7%) had LSM ≥8.0 kPa.

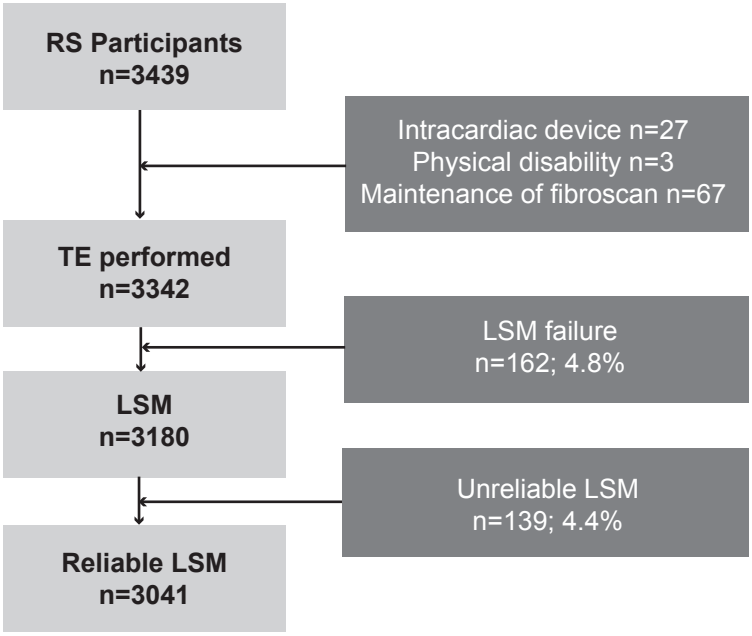

Figure 2.1 *Flow chart of the study. In total, 3041 participants had a reliable liver stiffness measurement.*

Factors associated with liver stiffness

The distribution of LSM values in our cohort of 3041 participants is illustrated in Figure 2.2. Results of linear univariable analyses are illustrated in Supplementary Table 2.1. In linear multivariable regression analysis, log-transformed LSM was associated with higher age, male sex, presence of diabetes mellitus, larger spleen size, higher ALT, presence of positive viral serology for hepatitis B and/or C and presence of steatosis (Table 2.2). Greater HOMA-IR, indicative of more insulin resistance, was associated with higher LSM when we replaced presence of diabetes mellitus with HOMA-IR in the model (β=0.003, 95%CI 0.001-0.004, p<0.001). Replacing BMI with waist circumference in the model did not alter the association with LSM (β-0.001, 95%CI -0.001-<0.001, p=0.08). No interaction between age, sex and other covariables was observed for the association with LSM.

Table 2.1 *Baseline characteristics*

Characteristic	Total	<8.0 kPa	≥8.0 kPa	p-value*
	n=3041	*n=2872*	*n=169 (5.6%)*	
Age (years)	66.0 ± 7.6	65.8 ± 7.5	68.7 ± 8.9	<0.001
Female	55.0	56.1	37.9	<0.001
Caucasian	95.1	95.0	96.9	0.3
BMI (kg/m^2)	27.3 ± 4.0	27.2 ± 3.9	28.9 ± 5.2	<0.001
Normal; BMI < 25	28.5	28.9	22.2	
Overweight; 25 ≤ BMI < 30	49.2	49.7	40.1	
Obese; BMI ≥ 30	22.3	21.4	37.7	
Waist circumference (cm)	93.7 ± 12.2	93.3 ± 12.0	99.7 ± 14.2	<0.001
Alcohol consumption (drinks/week)	3.8 (0.4-8.3)	3.8 (0.4-8.3)	3.8 (0.4-8.8)	0.4
Presence of HBsAg and/or anti-HCV	0.8	0.7	2.5	0.03
Smoking				0.002
Never	35.1	35.9	22.2	
Former	51.8	51.2	61.1	
Current	13.1	12.9	16.7	
Hypertension¥	62.4	61.5	76.7	<0.001
Diabetes Mellitus†	11.1	9.8	33.7	<0.001
Metabolic syndrome	44.0	42.9	61.9	<0.001
Waist circumference >88cm (♀) or >102 cm (♂)	44.6	43.9	56.9	0.001
Triglycerides >150 mg/dL or drug treatment for elevated triglycerides	37.2	36.8	44.3	0.05
HDL-C <40 mg/dL(♂) or <50 mg/dL(♀) or drug treatment for low HDL-C	33.5	33.0	42.5	0.01
BP ≥130/85 mmHg or drug treatment for elevated BP¥	77.7	77.2	86.0	0.01
FPG >100 mg/dL or drug treatment for elevated blood glucose	44.7	43.2	69.9	<0.001
Ultrasound characteristics				
Steatosis	35.5	34.1	59.2	<0.001
Spleen size (cm)‡	9.8 ± 1.3	9.8 ± 1.3	10.4 ± 1.5	<0.001
Portal vein flow velocity (cm/s)§	22.0 ± 4.7	22.0 ± 4.7	22.2 ± 5.4	0.6
Laboratory data				
ALT (U/L)	18 (14-24)	18 (14-24)	22 (16-30)	<0.001
AST (U/L)	24 (21-28)	24 (21-28)	27 (23-36)	<0.001
Bilirubin (umol/L)	8 (6-11)	8 (6-11)	9 (6-12)	0.07
ALP (U/L)	67 (56-78)	67 (56-78)	70 (56-87)	0.08
GGT (U/L)	24 (17-35)	24 (17-34)	38 (25-67)	<0.001
HOMA-IR	2.6 (1.7-3.9)	2.5 (1.7-3.8)	3.9 (2.5-6.3)	<0.001
Platelets (*10^9/L)	263 (224-306)	263 (226-307)	242 (205-288)	<0.001
Transient elastography				
LSM (kPa)	4.7 (3.8-5.8)	4.6 (3.8-5.6)	9.1 (8.6-10.4)	<0.001
IQR/M	0.19 (0.12-0.27)	0.19 (0.12-0.27)	0.17 (0.12-0.24)	0.02

Table 2.1 *Continued*
Data are represented as mean ± standard deviation, median (25th-75th percentile) or percentage.
** Based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test.*
¥ BP measurement and data on drug treatment for elevated BP was available for 2611participants.
† Data on presence of diabetes mellitus was obtained in 3001 participants
‡ Spleen size measurement was available for 2587 participants
§ Portal blood flow velocity was available for 2887 participants.
Abbreviations: kPa, kilopascal; BMI, Body Mass Index; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatise; GGT, gamma glutamyl transferase; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; LSM, liver stiffness measurement; IQR/M, interquartile range/ median LSM.

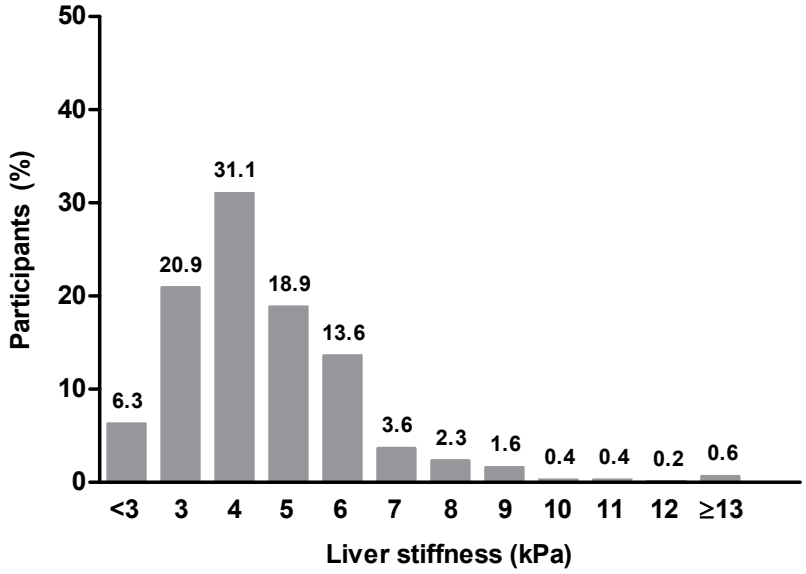

Figure 2.2 *Distribution of reliable liver stiffness measurements in 3041 participants.*

Using linear regression analysis in 1565 participants without apparent liver disease, excluding participants with positive HBsAg, anti-HCV, steatosis, excessive alcohol intake, and ALT above the upper limit of normal, only higher age (β=0.029 per 10 years, 95%CI 0.020-0.037, p<0.001) and male sex (β=0.066, 95%CI 0.049-0.082, p<0.001) remained associated with log LSM. In these participants, the 5th and 95th percentile values of LSM were 2.8 kPa and 7.3 kPa respectively. Age (β=0.021 per 10 years, 95%CI 0.013-0.030, p<0.001) and male sex (β=0.067, 95%CI 0.051-0.082, p<0.001) remained associated with higher log LSM after excluding an additional 51 cases with LSM ≥8.0 kPa.

Table 2.2 *Factors associated with (log-transformed) LSM in linear multivariable regression analysis (n=3041)*

Variable	β (95%CI)	p-value
Age, per 10 years	0.029 (0.022-0.036)	<0.001
Sex, male	0.055 (0.042-0.067)	<0.001
Diabetes Mellitus	0.041 (0.023-0.059)	<0.001
Spleen size, cm	0.012 (0.007-0.016)	<0.001
ALT, per 10 U/L	0.014 (0.009-0.018)	<0.001
BMI, kg/m^2	-0.001 (-0.002-0.001)	0.28
Steatosis	0.024 (0.012-0.037)	<0.001
Alcohol consumption, drinks/week	<0.001 (-0.001-0.001)	0.50
Current or former smoking	0.002 (-0.009-0.013)	0.74
HBsAg or anti-HCV positive	0.090 (0.025-0.156)	0.007

Abbreviations: LSM, liver stiffness measurement; ALT, alanine aminotransferase; BMI, Body Mass Index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus

Factors associated with and predicted probability of clinically relevant fibrosis

Of 3041 participants, 169 (5.6%) had LSM ≥8.0 kPa, suggesting clinically relevant fibrosis, and 19 (0.6%) had LSM >13.0 kPa, suggesting presence of advanced fibrosis or cirrhosis. General characteristics of participants according to a LSM cut-off of 8.0 kPa are shown in Table 2.1. One participant with LSM ≥8.0 kPa had positive serum anti-HCV and 3 participants had positive serum HBsAg. Results of logistic univariable analyses are illustrated in Supplementary Table 2.2. Factors associated with LSM ≥8.0 kPa in multivariable logistic regression analysis are shown in Table 2.3. Severity of steatosis was not associated with elevated LSM, applying different cut-offs (p=0.31 for ≥ 8.0 kPa, and p=0.79 for ≥ 9.5 kPa). Thirteen of 19 (68%) participants with LSM >13.0 kPa were female, mean age of this group was 70.0 ± 11.8 years and mean BMI 28.7 ± 6.4 kg/m^2. Two of these participants drank more than 14 alcoholic beverages per week, 11 (57.9%) participants had steatosis on ultrasonography, and 7 (38.9%) had diabetes mellitus. Splenomegaly was present in 5 of 19 participants with LSM >13.0 kPa. None of the participants with LSM >13.0 kPa had positive viral serology for hepatitis B or C.

We performed several sensitivity analyses to determine the robustness of our findings. Consistency was shown, when a one point lower cut-off for elevated LSM was applied for use of the XL probe or when 9.5kPa was used as a cut-off for clinically relevant liver fibrosis. When participants with ALT >2x the ULN were excluded from the analyses, as severe inflammation of the liver may have influenced the positive predictive value of presence of liver fibrosis, the prevalence of LSM ≥8.0 kPa was 5.2% and the same factors, with sex instead of spleen size, remained associated with elevated log-transformed LSM.

We calculated predicted probabilities of having LSM ≥8.0 kPa, taken into account all factors associated with LSM ≥8.0 kPa in our multivariable model. These predicted probabilities increased incrementally with age (Figure 2.3). The adjusted probability of having LSM ≥8.0 kPa was 1.4% (0.9-3.6) for participants of 50-60 years of age, 3.4% (2.1-6.0) for those aged 60-70 years, 5.5% (3.6-8.5) for participants aged 70-80 years, and 9.9% (6.8-14.5) for participants aged >80 years.

Since the association between age and LSM ≥8.0 kPa was significantly influenced by the presence of DM and/or steatosis (p for combined interaction term=0.04), we also calculated predicted probabilities of having LSM ≥8.0 kPa per age decade for participants with and without DM and/or steatosis (Figure 2.4). These probabilities increased per age decade for participants without DM or steatosis (n=1814) (1.1% (0.8-1.6) in participants aged 50-60 years versus 10.5% (6.6-14.6) in participants >80 years, $p=4.0*10^{-162}$). Probabilities also increased with increasing age for participants with steatosis, but without DM (n=853) (5.1% (3.6-7.0) versus 8.8 (5.0-11.0), p=0.001). For participants with only DM (n=118), probabilities did not increase with age (p=0.76). Participants with both DM and steatosis had the highest probabilities of having LSM ≥8.0 kPa for all age decades. For these 216 participants the overall probability of LSM ≥8.0 kPa was 17.2% (12.5-23.4). These probabilities did not increase with age (14.3 (12.1-18.4) versus 17.0 (12.3-24.1) versus 20.1 (13.5-23.9) versus 16.3 (12.5-23.8) respectively per increasing age decade, overall p=0.76).

Table 2.3 *Factors associated with LSM ≥8.0 kPa (n=169) in logistic multivariable regression analysis*

Variable	OR (95%CI)	p-value
Age for no DM, no steatosis, per 10 years	2.40 (1.72-3.36)	<0.001
Sex, male	1.35 (0.91-2.02)	0.14
Spleen size, cm	1.23 (1.09-1.40)	0.001
ALT, per 10 U/L	1.24 (1.12-1.38)	<0.001
BMI, kg/m^2	1.00 (0.95-1.06)	0.97
Alcohol consumption, drinks/week	1.00 (0.98-1.03)	0.76
Current or former smoking	1.77 (1.16-2.70)	0.008
HBsAg or anti-HCV positive	5.38 (1.60-18.0)	0.006
Interaction		
For age 66.0 years*		
No DM, no steatosis	1 (ref)	ref
Steatosis, no DM	1.99 (1.28-3.10)	0.002
DM, no steatosis	3.22 (1.39-7.42)	0.006
DM and steatosis	5.20 (3.01-8.98)	<0.001

** 66.0 years was the mean age in the total population*
Abbreviations: LSM, liver stiffness measurement; kPa, kilopascal; DM, diabetes mellitus; ALT, alanine aminotransferase; BMI, Body Mass Index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ref, reference

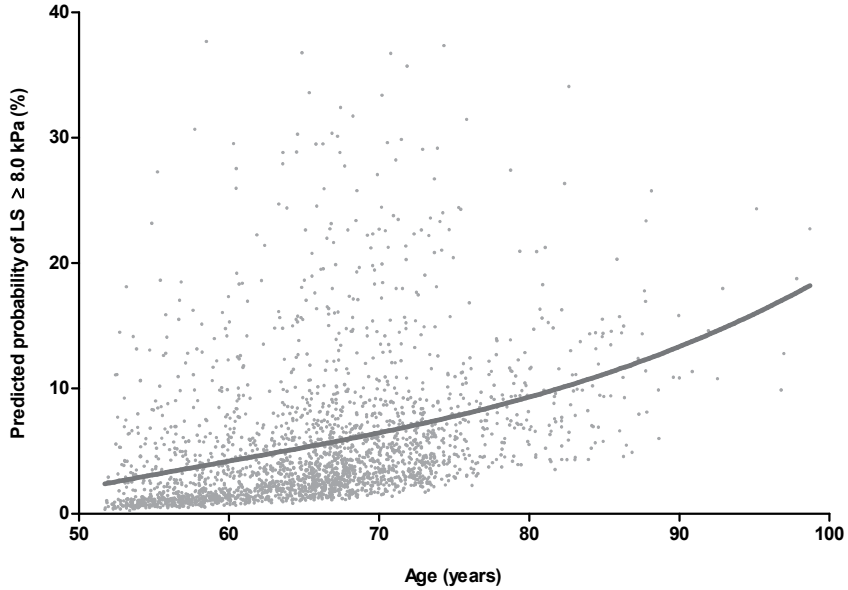

Figure 2.3 *Predicted probabilities (%) of having LSM ≥ 8.0 kPa increase with age. Probabilities are adjusted for age, sex, BMI, current or former smoking, presence of diabetes mellitus and/or steatosis, ALT, presence of HBsAg and/or anti-HCV, weekly alcohol consumption, spleen size, and the interaction between age and presence of DM and/or steatosis.*

We also observed a second interaction in our analyses: the association between current or former smoking and LSM ≥8.0 kPa was significantly influenced by sex (p for interaction term=0.024). Of 1902 previously or currently smoking participants, males had a median predicted probability of having LSM ≥8.0 kPa of 6.2% (3.6-10.2) compared to a probability of 3.7% (2.1-6.8) in female smokers (p<0.001). These probabilities were 2.8% (1.6-5.0) and 2.4% (1.3-3.8) respectively for males and females who had never smoked (p<0.001).

Liver stiffness in participants with NAFLD

A sub analysis was performed to investigate factors associated with LSM in participants with NAFLD. Of 3041 participants, 532 participants were excluded due to presence of secondary causes associated with steatosis (excessive alcohol consumption (n=449), positive anti-HCV (n=16), positive HBsAg (n=7), pharmacological agents associated with steatosis (n=69)). Eight hundred twenty-two (32.8%) participants had NAFLD. Mean age of participants with NAFLD was 66.0 ± 7.2 years, mean BMI was 29.8 ± 4.0 kg/m^2, and median LSM was 4.9 kPa (4.0-6.1). Sixty-nine (8.4%) participants of this subgroup had LSM ≥8.0 kPa. In multivariable linear regression analysis, higher age (β=0.027 per 10 years,

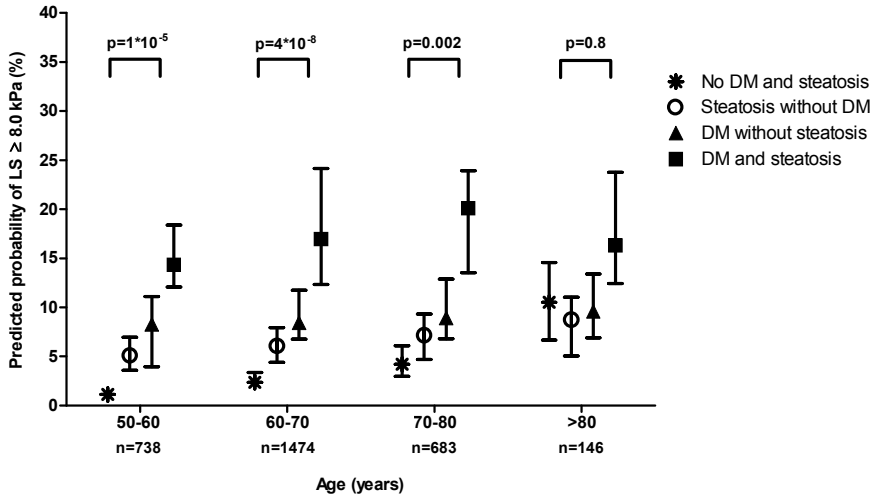

Figure 2.4 *Predicted probabilities (%) of having LSM ≥ 8.0 kPa per age decade for participants with and without diabetes mellitus and/or steatosis. Probabilities are adjusted for age, sex, BMI, current or former smoking, presence of diabetes mellitus and/or steatosis, ALT, presence of HBsAg and/or anti-HCV, weekly alcohol consumption, spleen size, and the interaction between age and presence of DM and/or steatosis.*

95%CI 0.012-0.042, p<0.001), presence of diabetes mellitus (β=0.038, 95%CI 0.010-0.065, p=0.007), male sex (β=0.044, 95%CI 0.019-0.068, p=0.001), higher ALT (β=0.023 per 10 U/L, 95%CI 0.014-0.032, p<0.001), and larger spleen size (β=0.014, 95%CI 0.006-0.023, p=0.001) were associated with higher log LSM. Replacing presence of diabetes mellitus by HOMA-IR did not alter results. Presence of diabetes mellitus (OR 2.96, 95%CI 1.60-5.47, p=0.001), higher ALT (OR 1.43 per 10 U/L, 95% CI 1.19-1.72, p<0.001), and increasing weekly alcohol consumption (OR 1.09, 95%CI 1.01-1.18, p=0.023) were associated with having LSM ≥8.0 kPa in participants with NAFLD in multivariable logistic regression analysis.

DISCUSSION

To date, this is the largest study to investigate factors associated with and distribution of liver fibrosis, assessed by TE, in a general Caucasian population of older individuals. In this cross-sectional study the prevalence of LSM ≥8.0 kPa, suggestive of clinically relevant liver fibrosis, was 5.6%. Higher age, presence of DM and/or steatosis, higher ALT, larger spleen size, current or former smoking, and positive viral serology for hepatitis B and/or C were independently associated with LSM ≥8.0 kPa.

Only few community-based studies have assessed liver fibrosis in adults by means of TE. In a study by Roulot *et al*, in which reliable LSM was obtained in 1190 subjects older than 45 years attending a free medical check-up, prevalence of LSM ≥8.0 kPa was 7.5% and risk factors of LSM ≥8.0 kPa were rather consistent with our findings and included age, elevated liver enzymes and factors associated with the metabolic syndrome (21). We were able to include ultrasonographic findings in our analyses, and found that both steatosis and spleen size were significantly associated with greater liver stiffness. Although the prevalence of LSM ≥8.0 kPa in our cohorts is nearly comparable, our study population may have included a less selected population, but had a higher mean age, greater prevalence of metabolic syndrome and included more women than the population described by Roulot *et al*. A cross-sectional community-based study by Wong *et al*. assessed the prevalence of NAFLD and LSM ≥9.6 kPa in Hong Kong Chinese subjects by using MRI and TE (20). LSM ≥9.6 kPa was present in 2.0% of the total cohort of 759 subjects without a hepatitis B or C infection and in 3.7% of subjects with NAFLD. These percentages were comparable with our results (data not shown), even though the prevalence of steatosis and metabolic syndrome and the mean age of our cohort were higher.

In the present study, higher age was associated with continuous LSM or elevated LSM in both linear and logistic regression analysis, respectively. An association of age with LSM has been corroborated in some previous studies, but not in others (21, 28-32). Indeed, the incidence of liver diseases increases with advancing age, which may in part explain a higher prevalence of LSM ≥8.0 kPa at higher age (33). However, we demonstrated that higher age remained associated with higher LSM after exclusion of subjects with positive viral serology for hepatitis B and/or C, steatosis, ALT above the upper limit of normal and LSM ≥8.0 kPa. Elastic properties of the normal liver may change as a result of ageing for several reasons. Firstly, age-related changes in histological architecture of the liver may cause increased liver stiffness. With ageing there is a decline in hepatic blood flow, hepatic volume, and number and volume of individual hepatocytes (34, 35). Hepatocytes in aged livers show increased polyploidy (36). Secondly, livers of older individuals may be stiffer as a result of accumulation of collagen. It is hypothesized that older individuals have reduced collagenolytic activity (37). In addition, cellular senescence, caused by telomere dysfunction, and increased mitochondrial damage and oxidative stress, may increase susceptibility of the older liver to liver damage and may reduce the capacity of the liver to regenerate (33).

A compelling observation in our study was that the association of age with LSM ≥8.0 kPa was positively influenced by presence of steatosis and/or DM. The predicted probabilities of LSM ≥8.0 kPa increased with age for participants without DM. For participants with DM on the other hand, the probability of having clinically relevant fibrosis was already as high as 8.2-14.0% for participants aged 50-60 years, depending on the concurrent presence of steatosis, and these probabilities did not increase with age. These findings underline the significant role of these – potentially modifiable – risk factors in liver fibrosis and stress the importance of early targeting insulin resistance

and/or DM. We observed that the presence of steatosis and/or DM did not influence the probability of LSM ≥8.0 kPa in octo- and nonagenarians, which can be the result of a survival effect, but it may also suggest that both steatosis and insulin resistance or DM play a less important role in liver fibrosis at very old age. However, this remains speculative as some selection bias may exist. In addition, this study was cross-sectional by design and we were therefore unable to determine the role of duration of presence of DM or steatosis on probability of high LSM in the very old.

Furthermore, a strong association between increased liver stiffness and presence of DM and/or greater insulin resistance was not only observed in the study population as a whole, but also in a subgroup of subjects with ultrasonographically defined NAFLD. The prevalence of LSM ≥8.0 kPa in participants with NAFLD was as high as 8.4%. These findings suggest that NAFLD may be an important determinant of clinically relevant fibrosis in a population that has a very low prevalence of viral hepatitis. Since NAFLD will become more prevalent considering population ageing and the current increasing prevalence of DM and obesity, and because the presence of NASH is associated with an increased overall, cardiovascular, and liver related mortality, NAFLD will become a more urgent public health issue in the next few decades (38-41).

In contrary to the observations by Roulot et al, we found that previous or current smoking was significantly associated with elevated liver stiffness (21). Smoking may cause liver injury through various mechanisms, including (in)direct toxic effects, immunological effects through pro-inflammatory cytokine release, and oncogenic effects through free radical damage (42). The harmful effects of smoking on the liver have been investigated in some experimental studies with rats and in smaller clinical studies including patients with various liver diseases, however, epidemiological evidence of an independent association is limited (43-50). Although a synergism between alcohol consumption and smoking is well-known, smoking was shown to increase the risk of liver cirrhosis independent of alcohol consumption in a previous population-based cohort study, consistent with our study (45).

The strengths of this study encompass the large number of participants that were included and extensive data that were available for characterization of metabolic and environmental traits. In addition, abdominal ultrasound was performed in all participants. Our study also has some limitations. For diagnosing liver fibrosis, biopsy remains the gold standard. However, performing a biopsy in all participants of this population-based cohort would have been unethical. Therefore, we used TE to obtain LSM for all participants. Although limited research is available addressing the positive predictive value to rule in advanced fibrosis and cirrhosis in subjects of the general population, TE is considered to be a reproducible and effective non-invasive alternative to assess liver fibrosis in patients with chronic liver diseases (25, 51). In addition, TE has also been shown to be able to predict portal hypertension-related complications, decompensation and survival in patients with chronic liver disease (52, 53). There is some debate regarding which criteria to apply to determine reliability of TE. Applying the

other reliability criteria – success rate ≥ 60%, ≥ 10 valid measurements, and IQR/median LSM ≤0.3 – to our cohort resulted in comparable results. Unfortunately, we did not have access to histology to corroborate our results. However, we expect the cut-off of 8.0 kPa for clinically relevant fibrosis to be feasible, since Roulot et al could determine a cause of liver disease in all subjects with LSM ≥8.0 kPa in whom a biopsy was performed (21). XL-probe may have a lower cut-off value for advanced liver fibrosis and different cut-offs have been applied for advanced fibrosis in patients with chronic liver diseases depending on etiology (15, 25). Consistency of results was found when different cut-offs were used in our study. Steatosis was diagnosed by means of ultrasonography, which may identify a degree of steatosis greater than 30%. Nevertheless, abdominal ultrasonography has an acceptable sensitivity of 80-100% for detecting steatosis, and its accuracy for diagnosis of steatosis meets other imaging modalities (54-56). Finally, there are controversial data about the influence of liver steatosis on LSM values in chronic hepatitis C patients (57-60). In patients with NAFLD most studies have shown no or an inverse relationship between degree of steatosis and LSM (16, 19, 61). Petta et al. demonstrated an association between severity of steatosis and LSM in NAFLD patients without significant fibrosis at biopsy. In this study, a cut-off of 7.9 kPa – similar to the cut-off used in the present study – was identified as the best LSM cut-off for discriminating significant fibrosis in presence of severe steatosis (≥66%) or severe bright liver echo pattern (62). We demonstrated no association between severity of steatosis and elevated liver stiffness in participants with steatosis. However, we cannot completely rule out the possibility that steatosis on itself may have affected LSM, independent of fibrosis stage, due to the lack of biopsies in this general population.

In summary, prevalence of LSM higher than 8.0kPa, suggestive of clinically relevant liver fibrosis, was 5.6% in this older adult population-based cohort. Factors associated with clinically relevant fibrosis were higher age, presence of DM and/or steatosis, higher ALT, greater spleen size, current or former smoking, and positive viral serology for hepatitis B and/or C. Presence of DM, especially in concurrent presence of steatosis, resulted in increased probabilities of having clinically relevant fibrosis. In the context of an ageing population and increasing prevalence of obesity, DM and NAFLD, this study illustrates that liver fibrosis and its complications may become a more prominent public health issue in the nearby future.

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Supplementary table 2.1 *Factors associated with (log-transformed) LSM in linear univariable regression analyses (n=3041)*

Variable	β (95%CI)	p-value
Age, per 10 years	0.028 (0.022-0.035)	<0.001
Sex, male	0.075 (0.065-0.085)	<0.001
Diabetes Mellitus	0.083 (0.067-0.099)	<0.001
Spleen size, cm	0.024 (0.020-0.028)	<0.001
ALT, per 10 U/L	0.019 (0.015-0.023)	<0.001
BMI, kg/m^2	0.004 (0.003-0.005)	<0.001
Hypertension	0.033 (0.021-0.044)	<0.001
Steatosis	0.046 (0.035-0.056)	<0.001
Waist circumference, cm	0.002 (0.002-0.003)	<0.001
Alcohol consumption, drinks/week	0.001 (<0.001-0.002)	0.004
Current or former smoking	0.012 (0.001-0.023)	0.030
Platelets (*10^9/L)	<0.001 (<0.001-<0.001)	<0.001
Bilirubin, umol/L	0.001 (<0.001-0.001)	0.037
HBsAg or anti-HCV positive	0.089 (0.029-0.149)	0.004

Abbreviations: LSM, liver stiffness measurement; ALT, alanine aminotransferase; BMI, Body Mass Index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus

Supplementary table 2.2 *Factors associated with LSM ≥8.0 kPa (n=169) in logistic univariable regression analyses*

Variable	OR (95%CI)	p-value
Age, per 10 years	1.57 (1.30-1.90)	<0.001
Sex, male	2.09 (1.52-2.88)	<0.001
Diabetes Mellitus	4.68 (3.32-6.61)	<0.001
Spleen size, cm	1.39 (1.25-1.55)	<0.001
ALT, per 10 U/L	1.28 (1.17-1.40)	<0.001
BMI, kg/m^2	1.10 (1.06-1.14)	<0.001
Hypertension	2.06 (1.40-3.03)	<0.001
Steatosis	2.80 (2.04-3.84)	<0.001
Waist circumference, cm	1.04 (1.03-1.05)	<0.001
Alcohol consumption, drinks/week	1.01 (0.99-1.03)	0.19
Current or former smoking	1.96 (1.34-2.86)	0.001
Platelets (*10^9/L)	0.995 (0.99-1.00)	<0.001
Bilirubin, umol/L	1.02 (1.00-1.03)	0.047
HBsAg or anti-HCV positive	3.85 (1.29-11.5)	0.016

Abbreviations: LSM, liver stiffness measurement; ALT, alanine aminotransferase; BMI, Body Mass Index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus

Chapter 3

Interferon gamma receptor 2 gene variants are associated with liver fibrosis in the general population: The Rotterdam Study

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ABSTRACT

Background and aims

Increasing evidence suggests that genetic factors play a role in the development of liver fibrosis. The association between several single nucleotide polymorphisms (SNPs) and liver fibrosis is well-established in patients with viral hepatitis or non-alcoholic fatty liver disease. The aim of this study was to investigate the association between these candidate SNPs and liver fibrosis in the general population, using liver stiffness (LS) as a proxy.

Methods

This research is part of the Rotterdam study, a large population-based cohort of subjects aged ≥ 55 years. In all patients, LS was measured using transient elastography. We investigated SNPs in 13 loci known to be associated with fibrosis and/or cirrhosis in at-risk populations.

Results

In 1059 participants, two linked SNPs in the interferon gamma receptor 2 (*IFNGR2*) gene were independently associated with liver fibrosis in multivariable analysis (p=0.04 for *rs9976971* and p=0.01 for *rs2284553*). A third variant in the *IFNGR2* gene, *rs9808753*, showed a trend towards a significant association with fibrosis (p=0.08). The association between these three *IFNGR2* SNPs and fibrosis was significantly modified by body mass index (BMI). For participants with the *IFNGR2* AA genotypes, LS was higher as BMI increased. Subgroup analysis in participants with steatosis also showed an association between the *IFNGR2* SNPs and fibrosis.

Conclusions

In this large population-based cohort, *IFNGR2* gene variants were independently associated with liver fibrosis. These results suggest that the *IFNGR2* SNPs do not only play a role in liver fibrogenesis in at-risk populations, but in the general population as well.

INTRODUCTION

Liver fibrosis is known to develop from many types of chronic liver injuries. Persistence of hepatic injuries leads to progression of liver fibrosis into cirrhosis and hepatocellular carcinoma, conditions associated with high morbidity and mortality.(1, 2) In addition to chronic liver injuries, other factors are known to influence the development of liver fibrosis; among these are genetic variations.(3)

Single nucleotide polymorphisms (SNPs) are variations in an individual's DNA that consist of a single nucleotide which differs from the one in the majority of the population. Together with microsatellites, SNPs account for most of the genetic variability between individuals.(4) As of July 2013, more than 62 million SNPs have been described in humans.(5) SNPs can affect the function, availability or transcription of a protein and can therefore also affect the development of disease.(4)

Genome wide association studies have identified several SNPs associated with liver fibrosis in at-risk populations.(6, 7) In addition, SNPs in or near the interleukin 28B (*IL28B*) gene and patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene were shown to be associated with liver fibrosis in patients with chronic hepatitis C and non-alcoholic fatty liver disease respectively in genetic association studies.(8, 9) Furthermore, SNPs in the interferon gamma receptor 2 (*IFNGR2*) gene, known to have antifibrogenic activity, have also been studied in order to determine their association with liver fibrogenesis in at-risk populations. SNPs in this gene were found to be associated with progression of liver fibrosis in patients with chronic hepatitis C.(10)

Until now, all studies on the role of genetic variants in liver fibrogenesis have focused on associations in at-risk populations, such as individuals with chronic viral hepatitis or fatty liver disease. As far as we are aware, no studies have been performed to investigate whether these associations between genetic variants and liver fibrosis are also present in the general population. As a result, we do not know whether the genetic variants discovered in at-risk populations alter the effect of chronic liver injuries on liver fibrogenesis or whether they affect fibrosis development independently of other factors. Therefore, the aim of our study was to determine the association between candidate genetic variants identified in at-risk populations and liver fibrosis, assessed using transient elastography (TE), in a large population-based cohort.

MATERIALS AND METHODS

Study population and design

This study was part of the Rotterdam study, a large ongoing prospective population-based cohort study in the Netherlands, which started in 1990.(11) All inhabitants of Ommoord, a suburb in the city of Rotterdam, aged 55 years and over were asked to

participate in this study. As of 2000, a new cohort was added to the initial study population. This second cohort consisted of participants who had turned 55 years of age since the start of the study or who had moved into the study district. Participants visit the designated research center every 3-4 years. Each assessment cycle consists of a home interview and a range of physical examinations at the research center. For more details, the reader is referred to previous publications.(11) As of 2009, each examination cycle includes an abdominal ultrasound and TE measurement.

The Rotterdam study has been approved by the Medical Ethics Committee of the Erasmus University Medical Center and written informed consent has been obtained from all participants.(11)

Assessment of liver fibrosis and steatosis

Liver fibrosis was assessed non-invasively by measuring liver stiffness (LS) using TE (Fibroscan®, Echosens™). LS measurements were considered reliable if 10 valid measurements were obtained with a success rate of at least 60% and an interquartile range of less than 30% of the median LS value. All LS measurements were performed by a single experienced ultrasonographer using either an M or XL-probe according to the manufacturer's recommendations. TE is contraindicated in patients with an intracardiac device and was therefore not performed in these participants. A liver stiffness cut-off value of 8.0 kPa was used as suggestive of clinically relevant fibrosis.(12, 13)

Abdominal ultrasound (Hitachi HI VISION 900) was used to assess the presence of steatosis, to measure spleen size and to examine the hepatic parenchyma. Images were re-evaluated by an expert hepatologist with vast experience in ultrasound. The presence of steatosis was always reassessed by the expert hepatologist.

Biochemistry and assessment of covariables

During the assessment cycle, fasting venous blood samples were collected and stored at -80˚C. Alanine aminotransferase (ALT) and glucose levels were determined using automated procedures (Roche Diagnostics GmbH, Mannheim, Germany). Levels of hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (HCV) antibodies were measured to determine the presence of viral hepatitis. Insulin, HBsAg and anti-HCV antibodies were measured using immunoassays (Roche Diagnostics GmbH, Mannheim, Germany). The extent of insulin resistance was determined using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) defined as fasting glucose (mmol/L) x fasting insulin (mU/L) ÷ 22.5.(14)

During the home interview, extensive data was obtained on demographics, medical history, comorbidity, alcohol consumption, smoking behavior, and drug use. Excessive alcohol consumption was defined as an intake of more than 14 units of alcohol per week. Trained research nurses performed anthropometric measurements at

the research center, from which body mass index (BMI) was calculated as weight (kg) ÷ height squared $(m)^2$.

Genotyping

DNA was isolated from whole blood samples and extracted according to standard automated procedures.(15) Genotyping in the Rotterdam study cohort was performed in batches in the Erasmus University Medical Center, Rotterdam, The Netherlands using the Infinium II HumanHap 550K Genotyping Bead-Chip® version 3 (Illumina Inc., San Diego, CA, USA). Sample-specific quality control included filters for low call rate, heterozygosity and sex mismatch. SNP-specific quality control measures comprised filters for call rate, minor allele frequency and Hardy-Weinberg equilibrium (HWE). The Markov Chain Haplotyping package (16) was used for imputation with the cohort of the 1000 Genomes Project (17) as reference population. A detailed description of these methods has been published elsewhere.(18-21)

We analyzed SNPs that have been associated with liver fibrosis in large at-risk populations (Table 3.1).(6-10) Additionally, all SNPs of the cirrhosis risk score were analyzed for a possible association with LS in our cohort. This previously published cirrhosis risk score was constructed in a group of Caucasian patients with chronic hepatitis C to estimate the risk of development of cirrhosis.(22) One of the imputed SNPs, *rs2290351*, is in full linkage disequilibrium with a genotyped SNP, *rs4932145*. Therefore, we have chosen to use this genotyped SNP as proxy for *rs2290351* in our analyses.

Statistical analysis

Baseline characteristics are expressed as numbers with proportions for categorical variables and as median with interquartile range or mean with standard deviation for continuous variables. Genotypes were coded as 0,1 or 2 based on the number of effect alleles. The fractional allele count (imputed dosage of the effect allele) was used in case of imputation. All SNPs were analyzed in an additive genetic model. LS measurements were not normally distributed and therefore logarithmically transformed prior to the analyses. We performed linear regression analysis to examine associations between SNPs and LS measurements, adjusting for age, sex, presence of steatosis, type of elastography probe used, spleen size, ALT, BMI, alcoholic intake, presence of viral hepatitis and HOMA-IR. Effect modification was tested for age, sex, BMI, alcoholic intake and steatosis by adding interaction terms to the regression models. Logistic regression analysis was used to assess the association between the *PNPLA3* SNP and presence of steatosis.

This was a candidate gene study, investigating SNPs with a well-established association with fibrosis in at-risk populations. All observed univariable associations were tested for independency by adjusting for possible confounders in multivariable models. Therefore, a p-value of <0.05 was considered statistically significant. Statistical

Table 3.1 *Single nucleotide polymorphisms associated with fibrosis in at-risk populations.*

SNP id	Chromosome (forward strand)	Gene	Minor allele	MAF
rs738409	22:44324727	*PNPLA3*	G	0.22
rs12979860	19:39738787	*IL28B*	T	0.32
rs12980275	19:39731783	*IL28B*	G	0.31
rs8099917	19:39743165	*IL28B*	G	0.17
rs9976971	21:34768097	*IFNGR2*	A	0.42
rs2284553	21:34776695	*IFNGR2*	A	0.39
rs9808753	21:34787312	*IFNGR2*	G	0.16
rs17882748	21:34775721	*IFNGR2*	C	0.48
rs62522600	8:103841709	*AZIN1*	A	0.05
rs4986791	9:120475602	*TLR4*	T	0.06
rs886277	11:2439767	*TRPM5*	C	0.37
rs2290351	15:90374781	*AP3S2*	A	0.25
rs4290029	1:224400640	*None*	C	0.14
rs17740066	3:121100283	*STXBP5L*	A	0.10
rs2878771	12:50352393	*AQP2*	C	0.19
rs2229738	11:68562328	*CPT1A*	T	0.09
rs1140409	17:62496670	*DDX5*	C	0.09
rs343062	7:35549066	*None*	T	0.36

Abbreviations: SNP, Single nucleotide polymorphisms; PNPLA3, patatin-like phospholipase domain-containing protein 3; IL28B, interleukin 28B; IFNGR2, interferon gamma receptor 2; AZIN1, antizyme inhibitor 1; TLR4, toll-like receptor 4; TRPM5, transient receptor potential cation channel, subfamily M, member 5; AP3S2, adaptor-related protein complex 3, sigma 2 subunit; STXBP5L, syntaxin binding protein-5-like; AQP2, aquaporin 2; CPT1A, carnitine palmitoyltransferase 1A; DDX5, DEAD (Asp-Glu-Ala-Asp) box helicase 5, MAF, Minor allele frequency in individuals from European descent (EUR) in the 1000 Genomes project (http://browser.1000genomes.org).

analyses were performed using SPSS 21 (SPSS Inc., Chicago, IL, USA). The STREGA recommendations were applied for reporting this study.(23)

Genetic risk scores were constructed using the SNP with the strongest association of every locus, i.e. adding only one SNP per gene to the risk score. The SNPs included in this score are listed in supplementary Table 3.1. For every participant, the weighted sum of the total number of fibrosis risk alleles was calculated. Weighting was based on effect estimates obtained from previous studies.(6, 7, 10, 22, 24, 25) This method has been described in more detail elsewhere.(26) For every participant, the genetic risk score was subsequently calculated by dividing the sum of weighted fibrosis risk alleles by the total number of SNPs included in the score. Two genetic risk scores were calculated. The first consisted of all SNPs, except those included in the cirrhosis risk score. In the second genetic risk score, all SNPs were included. Next to these genetic risk scores we also calculated the cirrhosis risk score for every participant using the method as described by Huang et al.(22)

RESULTS

Study population

Of the 1324 participants in which reliable LS measurements were available, genetic data was obtained for 1059 participants, comprising the study population. The baseline characteristics of this study population are presented in Table 3.2. Mean age was 74.2 ± 5.6 years and 50.2% of participants were male. Almost all participants were of Caucasian origin (99.6%). Median LS was 5.1 kPa (interquartile range 4.1-6.3). In 101 participants (9.5%) a liver stiffness ≥ 8.0 kPa was present. Two participants appeared infected with the hepatitis B virus (0.2%) and 9 participants with the hepatitis C virus (0.9%). Steatosis was diagnosed in 338 participants (31.9%).

Table 3.2 *Baseline characteristics of the study population.*

Characteristic	n=1059
Age (years)	74.2 ± 5.6
Male sex	532 (50.2%)
Caucasian *	999 (99.6%)
BMI (kg/m2)	26.7 ± 3.6
BMI (kg/m2) categories	
Normal weight (<25)	361 (34.1%)
Overweight (25-30)	521 (49.2%)
Obese (≥30)	177 (16.7%)
HOMA-IR	2.5 (1.7-3.6)
Alcoholic intake >14 IU/week †	121 (11.6%)
Chronic viral hepatitis ‡	
B	2 (0.2%)
C	9 (0.9%)
Spleen size (cm)	9.6 ± 1.3
ALT (u/mL)	18.0 (14.0-23.0)
Steatosis	338 (31.9%)
Fibroscan probe	
M	531 (50.1%)
XL	528 (49.9%)
LSM (kPa)	5.1 (4.1-6.3)

Values are mean ± standard deviation, number (proportion) or median (interquartile range)
* *Data on ethnicity was missing for 56 participants.*
† *Data on alcoholic intake was missing for 12 participants.*
‡ *Data on presence of viral hepatitis was missing for 14 participants.*
Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; ALT, alanine aminotransferase; LSM, liver stiffness measurement; kPa, kilopascal.

Table 3.3 *Univariable regression models with logarithmically transformed liver stiffness as outcome (n=1059). Betas represent the increase in logarithmically transformed liver stiffness per fibrosis risk allele.*

SNP id	Fibrosis risk allele/frequency	Beta (SE)	p-value
rs738409	G/0.24	-0.006 (0.008)	0.44
rs12979860	C/0.68	-0.009 (0.007)	0.21
rs12980275	A/0.70	-0.005 (0.007)	0.47
rs8099917	T/0.80	-0.011 (0.008)	0.17
rs9976971	**A/0.43**	**0.016 (0.007)**	**0.018**
rs2284553	**A/0.40**	**0.017 (0.007)**	**0.011**
rs9808753	A/0.86	0.012 (0.009)	0.21
rs17882748	C/0.45	-0.007 (0.007)	0.33
rs62522600	G/0.93	-0.008 (0.013)	0.55
rs4986791	C/0.95	-0.016 (0.015)	0.26
rs886277	C/0.41	-0.001 (0.007)	0.94
rs4932145	A/0.23	-0.009 (0.008)	0.26
rs4290029	G/0.85	0.003 (0.009)	0.75
rs2878771	G/0.84	0.003 (0.010)	0.78
rs2229738	C/0.97	0.011 (0.018)	0.53
rs1140409	C/0.06	0.004 (0.014)	0.77
rs343062	T/0.40	0.003 (0.006)	0.69

Genotyping

Two SNPs in the *IFNGR2* gene, *rs9976971* and *rs2284553*, were in strong linkage disequilibrium (R^2=0.80, D'=0.96). Quality of imputation was high, with R^2-values ranging from 0.94 to 1.0 (Supplementary Table 3.2). All SNPs were in HWE, except for *rs17740066* (*DDX5*, X^2=16.36, p<0.001), which was therefore excluded from all analyses. Frequencies and call rates of the different SNPs in our cohort are shown in supplementary Table 3.3.

Associations between SNPs and liver fibrosis

Associations between liver fibrosis and the different SNPs are listed in Table 3.3. Two linked SNPs in the *IFNGR2* gene, *rs9976971* and *rs2284553*, were associated with increased fibrosis in our cohort (β=0.016, p=0.018 for *rs9976971* and β=0.017, p=0.011 for *rs2284553*) (Figure 3.1).

The association between *IFNGR2* SNPs *rs9976971* and *rs2284553* and fibrosis remained significant in a multivariable model (β=0.013, p=0.044 for *rs9976971*; β=0.017, p=0.010 for *rs2284553*) (see Table 3.4). Other factors independently associated with liver fibrosis in these models were age, sex, HOMA-IR, spleen size and ALT. Moreover, *rs9808753* (*IFNGR2* SNP 3) showed a trend towards a significant association with fibrosis in multivariable analysis (β=0.016, p=0.081).

There was no association between fibrosis and SNPs near *IL28B* (p-values >0.17) or *PNPLA3* (p=0.19 in adjusted analysis).

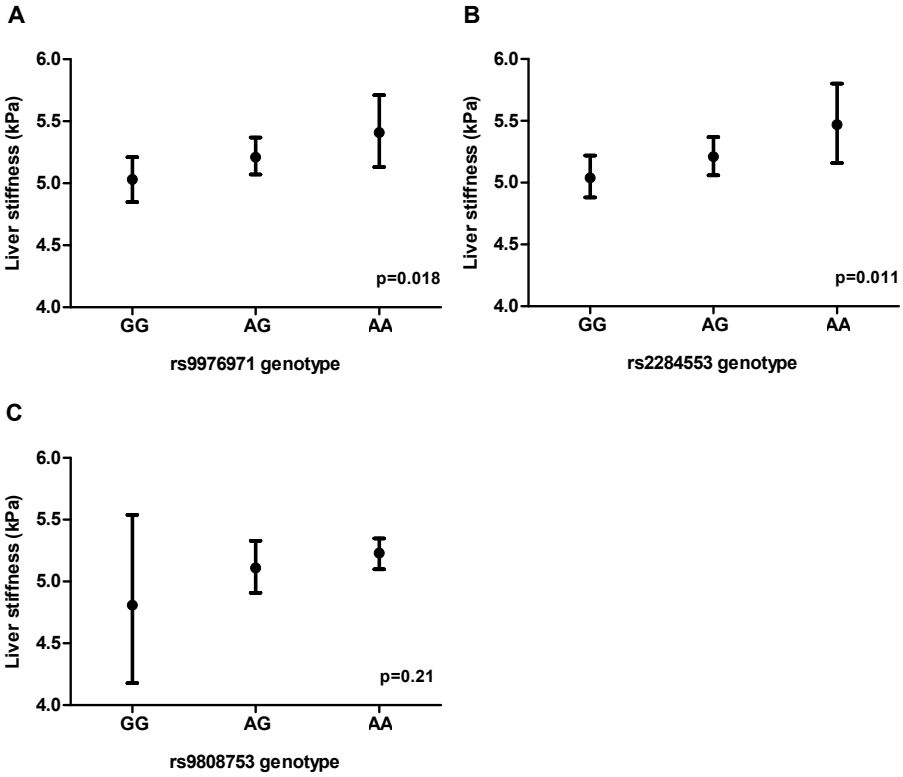

Figure 3.1 *Association between single nucleotide polymorphisms (SNP) in the interferon gamma receptor 2 gene and liver stiffness measurements. Panel (A) represents rs9976971, panel (B) rs2284553 and panel (C) depicts rs9808753. For all three SNPs, liver stiffness increases with the addition of an A allele. Error bars represent the geometric means of liver stiffness values for each genotype per SNP and their 95% confidence interval.*

Subgroup analyses

We performed a sensitivity analysis by excluding all participants infected with hepatitis B or C (n=11) as well as those who had an alcohol intake of 14 or more international units per week (n=121). In the remaining subgroup of 928 participants (one participant had both viral hepatitis and a high alcohol intake), the association between the *IFNGR2* SNPs and liver fibrosis was comparable with that observed in the total cohort (β=0.019, p=0.008 for *rs9976971* and β=0.022, p=0.002 for *rs2284553* respectively in univariable analysis). In adjusted analysis, we also observed a significant association between *rs9976971*, *rs2284553* and liver fibrosis (β=0.015, p=0.03 and β=0.020, p=0.004 respectively). Results for the other SNPs were comparable to those obtained in the total cohort.

The association between the *IFNGR2* SNPs and fibrosis was also present in a subgroup of participants with steatosis. In this group of 338 participants, *rs9976971*, *rs2284553* and *rs9808753* were all significantly associated with liver fibrosis (p=0.046,

0.044 and 0.022 respectively). In a multivariable model, only *rs9808753* remained independently associated with fibrosis in this subgroup (β=0.045, p=0.010).

Modification of the association between IFNGR2 SNPs and liver fibrosis

BMI significantly influenced the association between the *IFNGR2* SNP *rs2284553* and fibrosis, with higher LS measurements found in participants with an increasing BMI (p-value for the interaction term was 0.036). Similar results were obtained for the interaction term between BMI and *IFNGR2* SNPs *rs9976971* and *rs9808753* (p=0.050 for *rs9976971* and p=0.044 for *rs9808753*). Associations between the *IFNGR2* SNPs, BMI and liver fibrosis are shown in Figure 3.2. We explored this interaction further by dividing BMI into 3 groups: normal weight (BMI 0-25), overweight (BMI 25-30) and obese (BMI≥30). Interaction terms of BMI with all three *IFNGR2* SNPs and fibrosis as dependent variable were significant in ordinal regression analyses (p=0.037 for *rs2284553*, p=0.037 for *rs9976971* and p=0.023 for *rs9808753*). In obese participants, the association between *rs9976971* or *rs2284553* and fibrosis was stronger than that observed in the total cohort (β=0.055, p=0.002 for *rs9976971* and β=0.056, p=0.002 for *rs2284553*).The same applied to overweight participants for the association between SNP *rs9808753* and fibrosis (β=0.034, p=0.01).

The association between *rs9808753* and liver fibrosis was also significantly modified by the presence of steatosis. The association between the A allele of *rs9808753* and fibrosis was stronger in participants with steatosis than in those without steatosis (p-value for the interaction term was 0.003). There were no interactions between the *IFNGR2* SNPs and age, sex or alcohol intake.

Table 3.4 *Multivariable analyses in 2 separate models for the association between single nucleotide polymorphisms in the interferon gamma receptor 2 gene and liver fibrosis (n=1059).*

rs9976971			**rs2284553**		
Variable	**Beta**	**p-value**	**Variable**	**Beta**	**p-value**
Sex, male	0.063	<0.001	Sex, male	0.063	<0.001
Age, years	0.007	<0.001	Age, years	0.007	<0.001
HOMA-IR	0.004	0.004	HOMA-IR	0.004	0.005
FS-probe, M	0.011	0.27	FS-probe, M	0.011	0.27
Steatosis	0.023	0.050	Steatosis	0.023	0.050
Spleen size, cm	0.012	0.002	Spleen size, cm	0.012	0.002
ALT, u/mL	0.003	<0.001	ALT, u/mL	0.003	<0.001
Body mass index	<0.001	0.88	Body mass index	<0.001	0.88
Alcohol intake, IU per week	-0.001	0.37	Alcohol intake, IU per week	-0.001	0.34
Viral hepatitis B or C	0.041	0.39	Viral hepatitis B or C	0.040	0.40
rs9976971	**0.013**	**0.044**	**rs2284553**	**0.017**	**0.010**

Abbreviations: HOMA-IR, homeostasis model assessment of insulin resistance; FS, Fibroscan®; ALT, alanine aminotransferase.

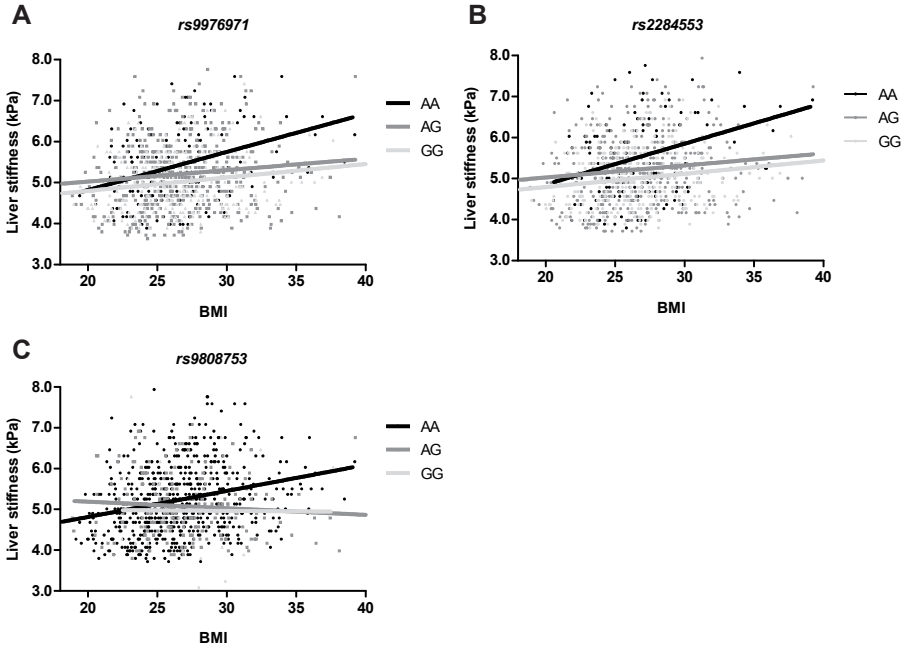

Figure 3.2 *Predicted liver stiffness measurements with body mass index (BMI) for all genotypes of the interferon gamma receptor 2 single nucleotide polymorphisms (SNPs). Panel (A) represents rs9976971, panel (B) rs2284553 and panel (C) depicts rs9808753. Predicted liver stiffness increases for genotypes AA with increasing BMI for all SNPs. Models are adjusted for age, sex, presence of steatosis, type of Fibroscan probe used, alanine aminotransferase, homeostasis model assessment of insulin resistance, spleen size, units of alcoholic intake per week and presence of viral hepatitis B or C.*

Genetic risk scores

The first composed genetic risk score, consisting of a group of SNPs representing all loci except for those included in the previously reported cirrhosis risk score (see Supplementary Table 3.1), was not associated with liver fibrosis in a univariable model (β=0.038, p=0.14). However, we found it to be independently associated with fibrosis in a multivariable model (β=0.051, p=0.047). No association between the second genetic risk score, including SNPs representing all loci, and liver fibrosis was observed (β=0.051 and p=0.18 in a multivariable model).

The cirrhosis risk score was not associated with liver fibrosis in our cohort (p=0.26). When we compared participants with LS values suggestive of significant fibrosis or cirrhosis (LS≥9.5 kPa) with participants with LS values suggestive of no or minimal fibrosis (LS<7.1 kPa) only, again no relationship between the cirrhosis risk score and liver fibrosis was observed (p=0.66).

PNPLA3 in steatosis

We observed an association between the *PNPLA3* SNP and steatosis in our cohort (OR 1.58, 95%CI 1.27-1.97, p<0.001 for addition of an 148M allele). *PNPLA3* was independently associated with steatosis after adjustment for age, sex, BMI, HOMA-IR, serum triglyceride levels and high density lipoprotein cholesterol (HDL-C) levels (additive OR 1.87, 95%CI 1.43-2.45, p<0.001).

DISCUSSION

This study shows that SNPs in the *IFNGR2* gene are independently associated with liver fibrosis, as assessed by TE, in a population-based cohort of elderly participants. After exclusion of participants who had viral hepatitis and those who had an excessive alcohol intake – both well-known causes of liver fibrosis – the association between these SNPs and liver fibrosis was still present. The association between the *IFNGR2* SNPs and fibrosis was also confirmed in a subgroup of participants with steatosis.

The SNPs investigated in this study have all been reported in previous studies. In such studies, an association with liver fibrosis in at-risk populations was detected for all of the loci included in our analyses.(6-10, 22) Since we discovered that SNPs in the *IFNGR2* gene are also associated with liver fibrosis in the general population, this implies that these SNPs affect fibrosis development independently of chronic liver injuries, which is a novel finding.

The gene variants that were associated with liver fibrosis in our cohort were situated in the *IFNGR2* gene. Interferon gamma, the type II interferon, has been shown to influence liver fibrogenesis. The precise mechanism of this effect has not been fully elucidated but interferon gamma has been shown not only to inhibit collagen synthesis in hepatic stellate cells but also to induce progression of the stage of liver fibrosis in patients with chronic hepatitis C.(27, 28) Of the two known interferon gamma receptors, type 1 and 2, *IFNGR2* seems to be the most important for interferon gamma to exert its effect.(29) In a recent study, an association between variants in the *IFNGR2* gene and liver fibrosis was described for the first time in a large cohort of patients with chronic hepatitis C.(10) In this study, two highly linked SNPs, *rs9976971* and *rs2284553*, were significantly associated with liver fibrosis. Since we also found these two linked SNPs to be independently associated with liver fibrosis in our cohort of healthy participants, as well as in a subgroup of participants with steatosis, interferon gamma seems to promote fibrogenesis independently of any interaction with viral replication. Studies to further elucidate the biological mechanism by which the *IFNGR2* SNPs influence liver fibrogenesis are warranted. Once the mechanism by which *IFNGR2* exert its effect on fibrogenesis is known, treatment options targeting this pathway could be developed.

An interesting finding of our study was that the effect of the *IFNGR2* gene variants on liver fibrosis was significantly altered by BMI: in obese participants, LS was consider-

ably higher for the AA genotypes of SNP *rs2284553*, *rs9976971* and *rs9808753* than for the other genotypes. A possible explanation is related to the fact that obesity is characterized by a state of chronic inflammation in which adipose tissue is infiltrated by macrophages and starts producing cytokines.(30, 31) It is certainly possible that this pro-inflammatory state reinforced the effect of interferon gamma on liver fibrogenesis in those obese individuals in our cohort who had the fibrosis-prone genotypes of the *IFNGR2* SNPs.

None of the SNPs in other fibrosis loci were associated with liver fibrosis in our cohort. A possible explanation for this finding is that these loci have an effect in at-risk populations only. To further clarify this, validation of our results in other population-based cohort studies is required.

A strength of our study is that it was conducted in a large, well-described, homogeneous cohort of elderly, Caucasian citizens living in a suburb of Rotterdam. This suburb was chosen because it is a representative subset of the Dutch population and because it has an extensive network of health care providers and pharmacists.

In this study, liver fibrosis was assessed using TE. Liver biopsy remains the gold standard for detecting liver fibrosis. However, this study was conducted in the general population and performing a biopsy in these healthy participants would have been unethical. TE was therefore used as a non-invasive alternative for assessing the degree of liver fibrosis. TE has several advantages. It is non-invasive, assesses an area approximately 100 times greater than a liver biopsy specimen, has good reproducibility, and is as effective as other non-invasive tests that assess liver fibrosis.(32-34) TE is therefore an ideal method for population-based studies. In two recent studies, TE was also able to predict 5-year survival and was as effective as the hepatic venous pressure gradient in predicting portal hypertension-related complications and decompensation in patients with chronic liver disease.(35, 36) The fact that we used LS as a continuous variable in our analyses – rather than categorizing LS measurements into Metavir fibrosis stages - enabled us to assess trends in the effects of gene variants on liver fibrosis.

To assess the combined effect of the fibrosis-associated loci, we created genetic risk scores based on those SNPs that had the highest associations with fibrosis. Weighting of these scores was based on effect estimates obtained in previous studies. In these studies, effect estimates of the fibrosis risk associated with specific alleles were determined only in at-risk populations, such as individuals with viral hepatitis or non-alcoholic liver disease. Since these populations differ substantially from the individuals in our cohort, these effect estimates are suboptimal for application in our study. However, since no effect estimates are currently available from cohorts similar to ours, we were therefore forced to use the effect estimates determined in at-risk populations.

The association between IFNGR2 SNPs and liver fibrosis was previously reported in patients with chronic hepatitis C.(10) Nonetheless, it is desirable to validate our findings in a cohort of healthy participants. However, to the best of our knowledge, no such cohort is yet available. Therefore, studies in population-based cohorts assessing both genetics and liver fibrosis are warranted.

In conclusion, this is the first study that shows an association between SNPs and liver fibrosis in the general population. Our findings suggest that gene variants in the *IFNGR2* gene influence liver fibrogenesis, irrespective of other causal factors of liver fibrosis. This association was even stronger in obese individuals. These results could have implications for the development and tailoring of interferon-gamma based therapy for the treatment of liver fibrosis. Influencing the interferon-gamma pathway could be a treatment option in liver fibrosis, regardless of its etiology.

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Supplementary table 3.1 *Single nucleotide polymorphisms (SNP) included in the genetic risk scores. The top SNP of each locus was included in the genetic risk scores.*

First genetic risk score	Second genetic risk score
rs738409	*rs738409*
rs12980275	*rs12980275*
rs2284553	*rs2284553*
rs2229738	*rs2229738*
rs343062	*rs343062*
rs1140409	*rs1140409*
	rs62522600
	rs4986791
	rs886277
	rs4932145
	rs4290029
	rs2878771

Supplementary table 3.2 *Quality of imputation of the single nucleotide polymorphisms.*

SNP	Imputation quality (R^2) RS-I	Imputation quality (R^2) RS-II
rs738409	*0.993*	*0.994*
rs12979860	*0.977*	*0.981*
rs17882748	*0.939*	*0.939*
rs62522600	*0.996*	*0.996*
rs4290029	*0.986*	*0.984*
rs2878771	*0.974*	*0.973*
rs2229738	*0.950*	*0.950*
rs343062	*0.995*	*0.996*

Supplementary table 3.3 *Frequencies of genotypes and call rates for all genotyped single nucleotide polymorphisms in the study cohort (n=1059).*

SNP	Genotypes	Frequency, n(%)	Call rate (%)
rs12980275	GG	103 (9.9%)	97.8
	GA	425 (41.0%)	
	AA	508 (49.0%)	
rs8099917	GG	40 (3.9%)	97.4
	GT	339 (32.9%)	
	TT	652 (63.2%)	
rs9976971	GG	325 (31.4%)	97.8
	GA	522 (50.4%)	
	AA	189 (18.2%)	
rs2284553	GG	373 (36.0%)	97.9
	GA	502 (48.4%)	
	AA	162 (15.6%)	
rs9808753	GG	19 (1.8%)	97.9
	GA	250 (24.1%)	
	AA	768 (74.1%)	
rs4986791	TT	3 (0.3%)	97.9
	TC	102 (9.8%)	
	CC	932 (89.9%)	
rs886277	TT	345 (33.3%)	97.7
	TC	524 (50.6%)	
	CC	166 (16.0%)	
rs4932145	GG	615 (59.3%)	97.9
	GA	362 (34.9%)	
	AA	60 (5.8%)	
*rs17740066**	GG	862 (83.3%)	97.7
	GA	153 (14.8%)	
	AA	20 (1.9%)	
rs1140409	AA	913 (88.9%)	97.0
	AC	112 (10.9%)	
	CC	2 (0.2%)	

** rs17740066 was out of Hardy-Weinberg equilibrium and therefore excluded from all analyses*

Part II

Thrombophilia in liver fibrogenesis

Chapter 4

4

Role of anticoagulant therapy in liver disease

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ABSTRACT

Anticoagulant therapy is a cornerstone in the treatment of different liver diseases. In Budd-Chiari syndrome (BCS), survival rates have increased considerably after the introduction of a treatment strategy in which anticoagulation is treatment of first choice. In all patients diagnosed with acute portal vein thrombosis (PVT) anticoagulant therapy for at least 3 months is indicated. Anticoagulation should also be considered in patients with chronic PVT and a concurrent prothrombotic risk factor. Current evidence suggests that patients with PVT in cirrhosis will benefit from treatment with anticoagulation as well. In severe chronic liver disease the levels of both pro- and anticoagulant factors are decreased, resetting the coagulant balance in an individual patient and making it more prone to deviate to a hypo- or hypercoagulable state. An increased activity of the coagulation cascade is not solely a feature of chronic liver disease; it influences the development of liver fibrosis as well. Several studies in animals and humans have shown that anticoagulation could prevent or improve fibrogenesis and even disease progression in cirrhosis. Anticoagulation is therefore a promising anti-fibrotic treatment modality.

INTRODUCTION

Haemostasis depends on the complex interaction of several variables including coagulation factors, tissue factor and platelets. The liver produces most of the pro- and anticoagulant factors and is therefore essential in coagulation.

For decades, chronic liver disease has been considered an acquired bleeding disorder caused by decreased levels of procoagulant factors. Prolonged activated partial thromboplastin time (APTT) and prothrombin time/international normalized ratio (PT/INR) in these patients supported this hypothesis. However, a growing body of evidence suggests this describes only part of the actual situation.

Already in 1981 was observed that bleeding after liver biopsy was poorly correlated with the PT (1). Abnormal coagulation tests were also not able to predict bleeding risk after invasive procedures (1, 2). In patients with chronic liver disease the administration of factor VIIa improves the PT, but fails to control variceal bleeding or bleeding during liver transplantation (3, 4). Moreover, patients with chronic liver disease are at an increased risk of developing venous thromboembolism and portal vein thrombosis, proving that, despite the increased coagulation times, the suggested state of auto-anticoagulation in these patients is a myth (5, 6). Patients with cirrhosis have elevated levels of factor VIII and von Willebrand factor and decreased levels of protein C and antithrombin, resetting the balance between pro- and anticoagulant factors to a new, more fragile, equilibrium (7, 8). This delicate balance can be disturbed more easily, resulting in hypo- or hypercoagulation. The conventional coagulation tests, PT/INR and APTT, do not accurately reflect the coagulation status in patients with cirrhosis. These tests determine whether a patient is deficient in procoagulant factors, but reveal nothing about the status of the anticoagulant factors or the increase in procoagulant factors (9). As a result, these tests can not be used to reliable predict the bleeding risk in these patients.

The coagulant imbalance in chronic liver disease can be detected with a thrombin-generation assay in the presence/absence of thrombomodulin, an activator of protein C (7). Since this laboratory test is not easy to perform, an alternative test using snake venom has been developed (10). In cirrhosis these tests show that the reduction of procoagulant factors is compensated for by a concomitant decrease in anticoagulant factors resulting in normal to even increased thrombin generation in these patients, provided there is an adequate amount of thrombocytes (11). In advancing stages of cirrhosis the resistance to trombomodulin increases, resulting in an increasing hypercoagulable state (12, 13).

Disturbances in the coagulation cascade are also observed in vascular liver diseases, providing the rationale for anticoagulant therapy in these disorders. This review will focus on the various applications of anticoagulant therapy in liver disease.

THE ROLE OF ANTICOAGULATION IN THE TREATMENT OF VASCULAR LIVER DISEASES

Vascular liver diseases encompass a range of mostly rare disorders. In some of them anticoagulant therapy is indicated. In this review the role of anticoagulant therapy in BCS, PVT, idiopathic noncirrhotic portal hypertension (INCPH) and sinusoidal obstruction syndrome (SOS) will be discussed.

Budd-Chiari syndrome

BCS is a rare disorder caused by an obstruction of the hepatic venous outflow tract. The location of the obstruction can be situated from the level of the small hepatic veins to the terminal portion of the inferior vena cava (14). In the majority of patients with BCS an underlying prothrombotic risk factor is present. A large European multicentre cohort study showed that 84% of the patients had at least 1 thrombotic risk factor; 46% of the total cohort had even more than 1 risk factor for thrombosis (15). It is recommended to test for risk factors of thrombosis, including myeloproliferative disorders, in all patients diagnosed with BCS (16).

The current recommendations for management of BCS are based on cohort studies and expert opinions. No prospective randomized trials have been performed in patients with BCS due to the rarity of this disorder. BCS is however one of the most life threatening forms of thrombosis and anticoagulation should therefore be recommended to all BCS patients and initiated immediately to prevent progression of thrombosis and, if possible, to achieve recanalization (14, 16, 17).

Table 4.1 *Survival rates in patients with Budd-Chiari syndrome after anticoagulant treatment*

References	Patients included	Treatment strategy	Time of diagnosis	Survival rates				
				1 year	*2 years*	*3 years*	*5 years*	*10 years*
Zeitoun et al. (18)	120	AC and/or shunt	Before 1985	62			50	47
			After 1985	88			75	63
Langlet et al. (19)	69	AC and/or shunt		91			82	74
Darwish Murad et al. (20)	237	AC and/or shunt/ TIPS/ LTx		82			69	62
Plessier et al. (21)	51	AC and RC/TIPS/ LTx if necessary		96		89	89	
Darwish Murad et al. (15)	163	AC and RC/shunt/ TIPS/ LTx if necessary		87	82			

Note: Only studies with an inclusion of 50 or more patients are described here
AC, anticoagulant treatment; shunt, surgical shunt; TIPS, transjugular intrahepatic portosystemic shunt; LTx, liver transplantation; RC, recanalization

After the introduction of a more thorough screening for prothrombotic risk factors and an earlier initiation of anticoagulant therapy in 1985, an improvement in survival was observed (see Table 4.1) (18). A survival rate of 82% at 5 years was observed in BCS patients treated with anticoagulation. Surgical portosystemic shunting did not significantly influence this survival rate (19). BCS patients with a good prognosis have an improved survival on anticoagulation therapy (relative risk 0.14), in contrary to patients with an intermediate (relative risk 0.88) or poor prognosis (relative risk 1.3) (20). Treatment according to a regimen of step-wise therapeutic procedures with increasing invasiveness has improved survival rates. An overall 5-year survival rate of 89% was reported for a treatment regimen starting with low molecular weight heparin (LMWH) followed by vitamin K antagonists (VKA) targeting at an INR of 2 to 3. Additional steps in the management of BCS were percutaneous recanalization of the hepatic veins or inferior vena cava; transjugular intrahepatic portosystemic shunt (TIPS) insertion and liver transplantation if all previous treatments were unsuccessful (21). An international prospective multicentre study demonstrated an overall survival of 87% at 1 year with this step-up treatment regimen (15).

In patients with BCS special features must be taken into account. The incidence of heparin-induced thrombocytopenia in BCS patients with a myeloproliferative disorder is high, with a reported incidence up to 14%, so monitoring platelet count during heparin treatment of these patients is advised (22-24). The most important complication of anticoagulant therapy is obviously the occurrence of bleeding events. The incidence of major bleeding episodes was reported to be 22.8 per 100 patient years (95% confidence interval (CI) 14.3-31.3), which is higher in comparison with the bleeding incidence in anticoagulated patients with a venous thromboembolism. Esophageal varices and invasive therapeutic procedures were the most important determinants of major bleeding occurrence (25).

Treatment with thrombolysis has only been studied in small samples. Due to major bleeding complications and limited recanalization rates thrombolysis by itself should be used with caution in patients with BCS. Local thrombolysis is sometimes used together with stenting of short segment stenosis in the hepatic veins (26, 27).

Portal vein thrombosis

PVT is defined as the development of a thrombus in the portal vein and/or its branches. In a large autopsy study the estimated population prevalence of PVT was 1%. In most of the cases PVT was associated with cirrhosis or malignancy (28). PVT can be classified as acute or chronic, according to the duration of symptoms (29). In more than half of the patients diagnosed with PVT one or more general prothrombotic conditions are present. In addition, local factors, such as cirrhosis and malignancy, contribute to the development of PVT (30). Screening for these disorders and risk factors is therefore recom-

mended in patients with PVT (16). The management of patients with PVT depends on the stage at which the thrombosis is detected and whether cirrhosis is present (14, 16).

Non-cirrhotic non-malignant portal vein thrombosis

Acute portal vein thrombosis

In acute PVT therapy aiming at recanalization of the obstructed veins and prevention of further development of the thrombus is indicated to prevent portal hypertension and intestinal infarction due to thrombosis of the mesenteric veins (14, 16). An international prospective study in 102 patients with acute PVT showed recanalization of the portal vein in 39% of patients treated with anticoagulant therapy. The inability to achieve recanalization was independently associated with the presence of ascites and an occluded splenic vein. The occurrence of bleeding, further development of the thrombus and death was rare (30). International consensus guidelines advise to start LMWH without delay in patients diagnosed with acute PVT. If the patient is stable, therapy can be switched to oral anticoagulation with VKA targeting at an INR between 2 and 3 (16, 17). The optimal duration of anticoagulant treatment has not yet been established. An international expert panel has recommended to anticoagulate all patients with acute PVT for at least 3 months. If persistent prothrombotic risk factors can not be corrected otherwise, anticoagulation should be continued lifelong to prevent recurrent thrombotic events (14).

Management of acute PVT with thrombolysis has only been studied in small cohorts. The results of these studies vary considerably, with recanalization rates ranging from 15-60%. Major bleeding is a frequently observed complication of thrombolysis, with incidences up to 60% (27, 31, 32). Thrombolysis should therefore only be used with great caution.

Chronic portal vein thrombosis

Despite the development of collaterals, complications of portal hypertension will occur in case of a persisting thrombus in the portal venous system (33, 34).

Patients with chronic PVT may well have prothrombotic risk factors, making them prone for recurrent thrombotic events (33, 34). An autopsy study showed that patients with PVT had an increased risk of other venous thromboembolisms (odds ratio 2.9, 95% CI 2.2-3.7), suggesting the presence of a hypercoagulable state in patients with idiopathic PVT (35). Therefore, anticoagulant therapy has been suggested as treatment option in patients with chronic PVT to prevent further development of portal hypertension and/or recurrent thrombosis. On the other hand anticoagulation may increase the risk of bleeding form gastro-esophageal varices, which is one of the most feared complications of PVT. Screening for varices in all patients with chronic PVT must be performed and it is recommended to treat patients with PVT-induced varices endoscopically according to the guidelines for cirrhotic patients (14, 16).

No controlled prospective trials assessing the role of anticoagulant therapy in patients with chronic PVT are available. Only few studies describe anticoagulation as treatment modality in these patients. Current evidence shows a reduced recurrence rate of thrombotic events in the portal venous system and an improved survival in patients treated with anticoagulant therapy, including those with previous variceal bleeding. Reported one- and five-year survival rates are 82% and 78% respectively (36). Bleeding rates were not increased in anticoagulated patients (34). Given the scarcity of evidence, international guidelines advise to consider long-term anticoagulant therapy only in patients with chronic PVT with a prothrombotic risk factor. Prophylaxis of bleeding should be initiated before starting anticoagulant therapy in patients with gastrointestinal varices (14, 16, 17).

Portal vein thrombosis in cirrhosis

PVT is frequently observed in patients with end stage liver disease (28). The incidence of PVT increases with the severity of cirrhosis and reduction of portal flow (37). In patients with liver cirrhosis a prevalence ranging from 8% up to 14% at the time of liver transplantation has been reported (6, 38, 39). Furthermore, in cirrhotic patients with PVT higher prevalences of prothrombotic factors are observed in comparison with cirrhotic patients without PVT (6, 40). Complete and incomplete recanalization can be achieved with anticoagulant therapy in a significant amount of cases (42-86%) without the occurrence of significant bleeding events (38, 41, 42). In a recently published study, early initiation of anticoagulant therapy was the only parameter independently associated with recanalization. After discontinuation of anticoagulant therapy a recurrent thrombotic event was observed in 39% of patients with initial complete recanalization (42). In a prospective case-control study 33 cirrhotic patients with PVT were treated with LMWH. In 6 patients a TIPS was additionally inserted. Complete recanalization was observed in 36% of treated patients; in total 63% of patients showed some form of recanalization in comparison with 1/21 among controls. Thrombus extension occurred significantly more often in patients not treated with anticoagulation ($p<0.001$). One major bleed was observed during anticoagulant therapy. The rate of variceal bleedings was lower in the treatment group (43).

Special caution should be taken in patients on the waiting list for liver transplantation. In patients with a complete PVT at the time of liver transplantation, survival is significantly decreased. Screening for PVT and anticoagulant treatment of patients listed for liver transplantation may be beneficial, given the significantly higher recanalization rates in anticoagulated patients in comparison with listed patients who were untreated, making the transplantation procedure technically more feasible (38).

Current guidelines advise to decide on a case-by-case basis which patients should be treated with anticoagulation. Given the recently emerged evidence, we believe that anticoagulant therapy could have a more prominent role in the treatment of PVT in patients with liver cirrhosis. However, in patients with severe decompensated cirrhosis caution should be taken in administering anticoagulant therapy.

Idiopathic noncirrhotic portal hypertension

INCPH is characterised by the presence of portal hypertension in the absence of liver cirrhosis and known causes of noncirrhotic portal hypertension (44). The etiology of INCPH is diverse and differs between ethnic groups, but thrombotic events in the microcirculation of the liver may well play an important role (45). Particularly in Western patients thrombophilia is considered an important risk factor in the etiology of this disease. PVT is a frequently encountered entity in patients with INCPH, especially in those with a concomitant human immunodeficiency virus (HIV) infection (44, 46, 47). Based on the current knowledge it is advised to treat INCPH with anticoagulant therapy in case of prothrombotic disorders and/or development of PVT (44, 46, 48-50). Further studies are nonetheless required to determine the effect of anticoagulant therapy on mortality in patients with INCPH.

Sinusoidal obstruction syndrome

SOS (previously known as veno-occlusive disease) is a disorder most commonly caused by toxicity from chemotherapeutic regimens used in the work-up for stem cell transplantation. This syndrome is characterized by sinusoidal non-thrombotic circulatory obstruction (16). A meta-analysis of 12 studies showed that primary prophylaxis with heparin was associated with a non-significant trend towards a decreased risk of SOS in patients undergoing hematopoietic stem cell transplantation (pooled RR 0.9; 95% CI 0.62-1.29). Methodological flaws and heterogeneity between studies made it nonetheless impossible for the authors to draw solid conclusions from the pooled analysis (51). Defibrotide, an anticoagulant with a relatively low risk of bleeding, was evaluated as treatment modality in patients with SOS in four studies (52-55). The day 100 survival of patients treated with defibrotide ranged from 32-65%. No bleeding episodes were observed in these studies (52-55). Future studies in this area are required to determine the role of anticoagulation as prophylaxis and treatment modality in patients with SOS (16).

ANTICOAGULANT THERAPY TO PREVENT FIBROGENESIS AND DISEASE PROGRESSION

Hypercoagulability is not only a feature of chronic liver disease, it also seems to play an important role in the development of fibrosis. In an autopsy study, the extent of microthrombi in branches of the hepatic and portal vein correlated with fibrosis and cirrhosis progression (56). In the current literature, two hypotheses are described explaining how coagulation can influence liver fibrogenesis (see Figure 4.1). The first involves microthrombi caused by inflammation resulting in tissue ischemia with parenchymal extinction and fibrous septa as a consequence. The other hypothesis states that thrombin, produced upon activation of the coagulation cascade due to inflammation, directly

activates hepatic stellate cells (HSC) by binding the thrombin receptor (protease activated receptor (PAR)) resulting in fibrogenesis (57). Evidence for this second, not organ specific, hypothesis is reported in several studies. In rodents, PAR1-antagonism or PAR-1 knockout inhibited HSC activation and reduced fibrogenesis (58, 59). In patients with hepatitis C virus (HCV) infection PAR-1 gene polymorphisms influenced the progression of liver fibrosis (60).

The association between coagulation and fibrogenesis has been supported by multiple clinical studies. In patients with HCV and haemophilia liver fibrosis progressed slower in comparison with HCV infected patients without haemophilia (61, 62). The risk of fast development of fibrosis was significantly increased in HCV patients who are carriers of the factor V Leiden mutation or prothrombin G20210A mutation (63, 64). The presence of other procoagulant factors has also been associated with more advanced fibrosis and/or cirrhosis (65-68).

The important role of coagulation in the development of liver fibrosis, irrespective of etiologic pathology, suggests that prevention of fibrogenesis and possibly even improvement of already developed fibrosis could be achieved by inhibiting co-

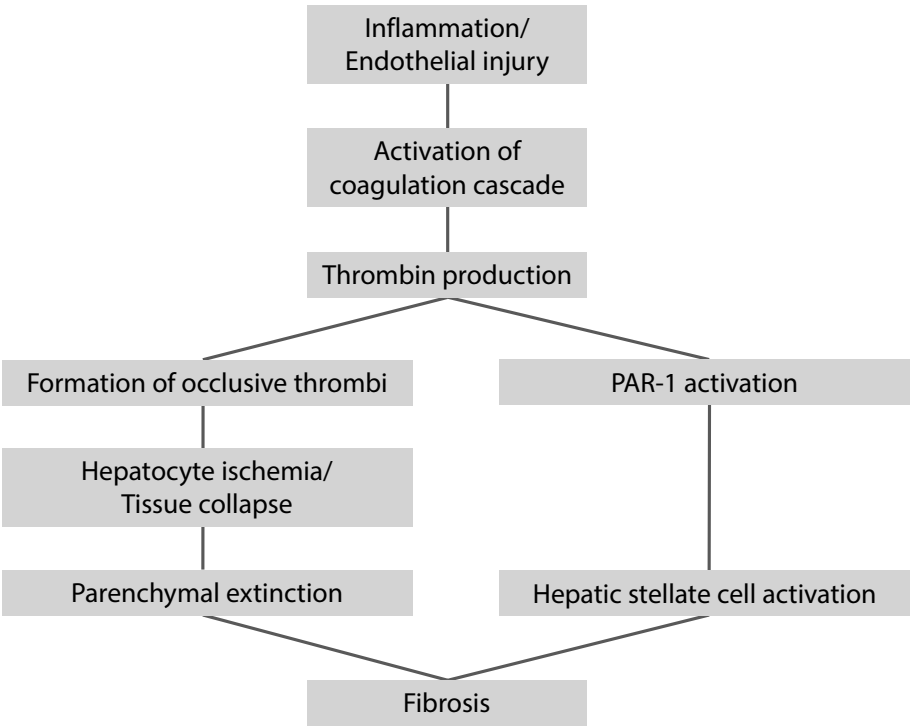

Figure 4.1 *Schematic overview of two hypotheses describing the role of the coagulation cascade in hepatic fibrogenesis*

Table 4.2 *Anticoagulant medication improves fibrogenesis in animals and humans*

Drug name	Class of drugs	References
Animal models		
SSR182289	Thrombin antagonists	Duplantier (72)
Enoxaparin	LWMH	Abdel-Salam (69); Assy (71)
Nadroparin	LWMH	Abdel-Salam (69)
Dalteparin	LMWH	Abe (70)
Warfarin	VKA	Anstee (73)
Humans		
Unfractionated heparin	Heparins	Shi (74)
Parnaparin	LMWH	Shi (74)
Enoxaparin	LWMH	Villa (75)

LMWH, low molecular weight heparin; VKA, vitamin K antagonists

agulation. This may be important, especially given the lack of anti-fibrotic therapy in current clinical practice. In several animal and laboratory studies the effect of different kinds of coagulation inhibiting medication on fibrogenesis was studied (see Table 4.2). Reduction of liver fibrosis, prevention of fibrogenesis and improvement of hepatic regeneration was seen after administration of LMWH in rodents (69-71). An antifibrotic effect was also achieved by thrombin antagonism and Warfarin, a VKA (72, 73).

In humans, treatment with unfractionated heparin or LMWH for 3 weeks in addition to regular treatment in patients with chronic hepatitis B decreased collagen levels and proliferation significantly (74).

Recently, a landmark study has been published. In this trial 70 patients with cirrhosis were randomized between treatment with Enoxaparin in a prophylactic dose for 48 weeks and no treatment. In the treated group a lower incidence of PVT was observed during treatment and during 2-years of follow-up ($p=0.025$ and $p=0.001$ respectively). Liver decompensation occurred significantly less frequent in the treated group ($p<0.001$). After discontinuation of Enoxaparin the incidence of decompensation was comparable between the two groups. The probability of survival was higher in the treated group ($p=0.02$). Enoxaparin was well tolerated; only one patient had to discontinue treatment due to thrombocytopenia. The bleeding incidence did not differ between the two groups. The authors explain these results by suggesting that Enoxaparin might be able to improve portal hypertension induced intestinal microthrombosis, reducing bacterial translocation and liver decompensation as a consequence (75). Larger, double-blind, multicentre trials with concurrent evaluation of portal hypertension are required to confirm these findings.

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Chapter 5

Prothrombotic genetic risk factors are associated with an increased risk of liver fibrosis in the general population: The Rotterdam Study

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ABSTRACT

Background and aims

The coagulation system is known to be involved in fibrogenesis in patients with liver disease. We investigated whether common genetic prothrombotic risk factors are associated with an increased risk of fibrosis in the general population.

Methods

This investigation was part of the Rotterdam Study, an ongoing, population-based cohort study. Liver stiffness (LS) was measured using transient elastography (Fibroscan®) and associated with single nucleotide polymorphisms determining blood group type and presence of the Factor V Leiden (FVL) mutation or Prothrombin G20210A gene variant.

Results

Reliable LS measurements and genetic data were obtained from 1055 Caucasian participants. LS ≥8.0 kPa, suggestive of clinically relevant fibrosis, was observed in 101 subjects (9.6%). Presence of FVL or prothrombin G20210A was independently associated with an increased risk of LS ≥8.0 kPa (OR 2.09, 95%CI 1.07-4.07, p=0.03). Combination of blood group type non-O and the FVL mutation or prothrombin G20210A variant resulted in an even higher risk of LS ≥8.0 kPa (OR 3.36, 95%CI 1.50-7.56, p=0.003). Presence of the FVL mutation or prothrombin G20210A variant in participants with blood group non-O was associated with a predicted probability of 14.3% (7.7-23.8) of LS ≥8.0 kPa.

Conclusions

Participants carrying the FVL mutation or prothrombin G20210A variant have an increased risk of clinically relevant liver fibrosis, which is even higher in blood group type non-O carriers. The fact that genetic prothrombotic risk factors are associated with an increased risk of liver fibrosis suggests that coagulation plays an important role in fibrogenesis in the general population.

INTRODUCTION

Liver fibrosis develops as the result of a complex interplay between chronic liver injury, genetic susceptibility and environmental risk factors. Hypercoagulability is considered to be one of the factors influencing liver fibrosis development and progression in liver disease [1]. The exact mechanism of this influence has not yet been fully elucidated, but thrombin seems to play a key role. Thrombin could cause liver fibrosis by directly activating hepatic stellate cells by binding the protease-activated receptor 1 [2-5]. An alternative hypothesis describes that thrombin production causes the formation of occlusive thrombi which eventually results in tissue ischemia, parenchymal extinction and ultimately liver fibrosis and cirrhosis [6, 7].

The Factor V Leiden (FVL) mutation, prothrombin G20210A gene variant and ABO blood group type non-O are well-known genetic prothrombotic risk factors. Presence of these risk factors is associated with a two- to five-fold increased risk of venous thrombosis [8-11]. In addition, the combined presence of FVL or prothrombin G20210A with blood group type non-O is known to result in an additionally increased risk of venous thrombosis. In previous studies, patients with both FVL and blood group type non-O were reported to have a 4- to 23-fold increased risk of venous thrombosis compared to patients with blood group O and without FVL [12-16]. Combined presence of prothrombin G20210A and blood group type non-O was associated with a nine-fold increased risk of venous thrombosis compared to controls and a two-fold increased risk compared to patients with prothrombin G20210A and blood group type O [15, 17].

Several studies have reported an association between presence of FVL, prothrombin G20210A, blood group type non-O and liver fibrogenesis in liver disease. Two studies in patients with chronic hepatitis C showed that the rate of fibrosis progression and the risk of cirrhosis was increased in patients carrying the FVL mutation [18, 19]. In addition, presence of FVL caused a significant acceleration of liver fibrogenesis when exposing mice to chronic liver injury [20]. An increased rate of liver fibrosis development was also observed in patients with hepatitis C carrying the prothrombin G20210A gene variant [21]. Finally, increased severity of liver fibrosis was observed in chronic hepatitis C patients with blood group type non-O, the most common prothrombotic genetic risk factor [12, 22]. It is unknown whether these prothrombotic risk factors also play a role in liver fibrogenesis in the general population. Therefore, the aim of the current study was to investigate whether presence of the FVL mutation, prothrombin G20210A gene variant and/or blood group type non-O is associated with an increased risk of liver fibrosis, assessed by using liver stiffness (LS) as proxy, in a population-based study.

MATERIALS AND METHODS

Study population and design

This study was part of the Rotterdam Study, a large ongoing prospective population-based cohort study in the Netherlands. The rationale and study design of the Rotterdam Study have been described elsewhere [23]. At the start of the Rotterdam Study in 1990, all inhabitants of Ommoord, a suburb in the city of Rotterdam, aged 55 years and over were asked to participate in this study. In 2000, a new cohort was added to the initial study population. This second cohort consisted of participants who had moved to Ommoord or who had turned 55 years of age since the start of the study. Participants are evaluated at the designated research center every 3-4 years. Each assessment cycle consists of an extensive home interview and a range of physical examinations, including fasting blood collection, at the research center. As of 2009, each examination cycle additionally includes an abdominal ultrasound and transient elastography (TE) measurement. Abdominal ultrasound and TE were performed after obtaining fasting blood samples at the research center. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC University Medical Center and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and permission to obtain information from their treating physicians.

Liver stiffness measurement and abdominal ultrasonography

Liver fibrosis was assessed non-invasively by measuring LS using TE (Fibroscan®, Echosens™). A single, experienced ultrasonographer measured LS. Measurements were performed on the right lobe of the liver, through the intercostal spaces, with the participant lying flat on his/her back with the right arm laying in maximal abduction and in between breathing movements. The failure rate of TE in our study was 4.3%. LS measurements were considered reliable if 10 valid measurements were obtained with a success rate of at least 60% and an interquartile range of less than 30% of the median LS. Based on these criteria, 21.6% of LS measurements was considered unreliable, which is comparable to other studies [24, 25]. Either an M or XL-probe was used to obtain LS measurements, according to the manufacturer's recommendations. XL-probe was used to measure LS in participants with a skin capsule distance larger than 2.5 cm, as assessed by abdominal ultrasound. In the remaining patients LS was measured using the M-probe. Participants with an intracardiac device were excluded from the current study, since TE is contraindicated in these participants. Consistent with others, an LS cut-off value of 8.0 kPa was used to identify clinically relevant liver fibrosis [26, 27]. In a subgroup analysis, LS ≥9.5 kPa was used as cut-off to determine presence of severe fibrosis or cirrhosis [28].

Abdominal ultrasonography (Hitachi HI VISION 900) was used to assess presence of steatosis, to measure spleen size, and to examine the hepatic parenchyma amongst others. Images were stored digitally and re-evaluated by an expert hepatologist with large experience in abdominal ultrasound. The diagnosis of steatosis was determined by the ultrasound technician according to the protocol by Hamaguchi et al [29]. Presence of steatosis was reassessed by the expert hepatologist in all cases.

Evaluation of the Factor V Leiden mutation, prothrombin G20210A gene variant and ABO blood group type

Presence of FVL and prothrombin G20210A was determined by assessing the genotype present at *rs6025* (G→T) and *rs1799963* (G→A) respectively. Participants were classified as having either ABO blood group type O or ABO blood group type non-O based on the genotype present at *rs505922*, used as proxy for *rs687289* (R^2=1.0, D'=1.0) (presence of genotype GG at *rs687289* corresponds with presence of blood group type O). To determine presence of these single nucleotide polymorphisms (SNPs), DNA was isolated from whole blood samples and extracted according to standard automated procedures [30]. Genotyping in the Rotterdam study cohort was performed in batches in the Erasmus MC University Medical Center, Rotterdam, The Netherlands using the Infinium II HumanHap 550K Genotyping Bead-Chip® version 3 (Illumina Inc., San Diego, CA, USA). Sample-specific quality control included filters for low call rate, heterozygosity and sex mismatch. SNP-specific quality control measures comprised filters for call rate, minor allele frequency and Hardy-Weinberg equilibrium. The Markov Chain Haplotyping package [31] was applied for imputation using the cohort of the 1000 Genomes Project as reference population [32]. A detailed description of these methods has been published elsewhere [33-36].

Interview, anthropometry and biochemistry

During the home interview, extensive data was obtained on demographics, medical history, comorbidity, alcohol consumption, smoking behavior, and drug use. Excessive alcohol consumption was defined as an intake of 14 or more international units (IU) of alcohol per week for women. For men, an intake of >21 IU per week was considered to be excessive. History of venous thromboembolism (VTE) was determined by using medical charts. VTE was defined as presence of pulmonary embolism or deep venous thrombosis. Trained research nurses performed anthropometric measurements at the research center, from which body mass index (BMI) was calculated as weight (kg) divided by height squared $(m)^2$.

During the assessment cycle, fasting venous blood samples were collected and stored at -80˚C. Alanine aminotransferase (ALT) and glucose levels were determined using automated procedures (Roche Diagnostics GmbH, Mannheim, Germany).

Diabetes mellitus (DM) was defined as fasting plasma glucose ≥7.0 mmol/L or drug treatment for elevated blood glucose. To determine presence of viral hepatitis, hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (HCV) antibodies were measured using immunoassays (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

Baseline characteristics are expressed as numbers with proportions for categorical variables and as median with interquartile range or mean with standard deviation for continuous variables. Significance of differences in baseline characteristics was assessed by using Chi-squared tests (counts), Student t-tests (means) or Mann-Whitney U tests (medians) respectively. Logistic regression analysis was used to examine associations between presence of FVL, prothrombin G20210A and/or blood group type and LS ≥8.0 kPa. In multivariable logistic regression analysis, we adjusted for age, sex, and logarithmically transformed ALT. In a second multivariable model was adjusted for age, sex, ALT, and steatosis. We adjusted for additional possible confounders in a multivariable model including age, sex, ALT, steatosis, presence of diabetes mellitus, current or former smoking, and alcohol intake. Finally, we assessed the effect of type of Fibroscan probe used by adding this variable to the initial multivariable regression model.

Predicted probabilities of having LS ≥8.0 kPa were calculated for four subgroups based on the combination of blood group type O or non-O and presence or absence of FVL or prothrombin G20210A. These probabilities were calculated using logistic regression analysis adjusting for age, sex, and ALT. Probabilities were expressed as median percentage with their interquartile range. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 21 (IBM SPSS Statistics for windows, Version 21.0, Armonk, NY, USA: IBM corp).

RESULTS

Characteristics of the study population

Reliable LS measurements were available in 1324 participants. Of these participants, 269 were excluded, because of lack of informed consent for determining genetic variants or non-Caucasian ethnicity (n=43). As a result, the total study cohort described in this manuscript consisted of 1055 participants. Baseline characteristics of this cohort are described in Table 5.1. Mean age of these 1055 participants was 74.2 ± 5.6 years and 528 (50.0%) of them were male. Six participants (0.6%) had a history of VTE, i.e. five had a history of pulmonary embolism and one had experienced a deep venous thrombosis. These thrombotic events all occurred between 1996 and 2010. Steatosis was detected at abdominal ultrasonography in 337 participants (31.9%) and 56 participants (5.4%)

Table 5.1 *Baseline characteristics of the study population*

Characteristic	Total cohort (n=1055)	LS <8.0 kPa (n=954)	LS ≥8.0 kPa (n=101)	p-value‡
Age (years)	74.2 ± 5.6	73.9 ± 5.3	76.6 ± 7.2	<0.001
Sex, male	528 (50.0%)	464 (48.6%)	64 (63.4%)	0.005
BMI (kg/m^2)	26.7 ± 3.6	26.6 ± 3.4	27.7 ± 4.4	0.003
Diabetes mellitus	117 (11.3%)	94 (10.1%)	23 (23.5%)	<0.001
Current or former smoking	678 (64.8%)	602 (63.7%)	76 (75.2%)	0.02
Excessive alcohol intake*	56 (5.4%)	53 (5.6%)	3 (3.0%)	0.3
History of venous thromboembolism	6 (0.6%)	6 (0.6%)	0 (0%)	1.0
Viral hepatitis	11 (1.1%)	10 (1.1%)	1 (1.0%)	1.0
Positive HBsAg	2 (0.2%)	1 (0.1%)	1 (1.0%)	
Presence of anti-HCV	9 (0.9%)	9 (1.0%)	0 (0%)	
Steatosis	337 (31.9%)	288 (30.2%)	49 (48.5%)	<0.001
Spleen size (cm)†	9.6 ± 1.3	9.6 ± 1.3	10.2 ± 1.6	<0.001
ALT (U/L)	18 (14-23)	18 (14-22)	20 (16-30)	<0.001
Fibroscan probe, M	529 (50.1%)	474 (49.7%)	55 (54.5%)	0.4

Values are represented as count (proportion), mean ± standard deviation, or median (interquartile range)
* *Excessive alcohol intake was defined as intake >14 IU per week for women and >21 IU per week for men.*
† *Spleen size measurements were missing in 135 participants.*
‡ *p-value for the comparison of participants with LS <8.0 kPa versus those with LS ≥8.0 kPa*
Abbreviations: BMI, body mass index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ALT, alanine aminotransferase; LS, liver stiffness.

had an excessive alcohol intake. Median LS was 5.1 kPa (4.1-6.3). One hundred and one participants (9.6%) had LS ≥8.0 kPa, suggesting presence of clinically relevant liver fibrosis. These participants were significantly older, more frequently male, had a higher BMI and had more often DM as compared to participants with LS <8.0 kPa (Table 5.1). Besides, they were also more frequently current or former smokers, had more often steatosis, a larger spleen size and higher ALT than participants with LS <8.0 kPa.

Association of the Factor V Leiden mutation, prothrombin G20210A gene variant, blood group type and LS ≥8.0 kPa

There was no linkage disequilibrium between the SNPs determining the FVL mutation (*rs6025*), prothrombin G20210A (*rs1799963*) and ABO blood group type (*rs687289*). Quality of imputation of *rs6025* and *rs1799963* was high (R^2>0.99). The call rate for *rs505922*, used as proxy for *rs687289*, was 97.8%. All SNPs were in Hardy-Weinberg Equilibrium.

A heterozygous FVL mutation was present in 49 participants (4.6%) and 20 participants (1.9%) had a heterozygous prothrombin G20210A gene variant (Table 5.2), which is in accordance with the general Dutch population. No homozygotes for FVL or prothrombin G20210A were present. One participant had both variants. Blood group type non-O was present in 568 participants (55.0%) of our cohort. FVL or prothrombin

Table 5.2 *Association of the Factor V Leiden mutation, prothrombin G20210A gene variant, ABO blood group type and presence of LS ≥8.0 kPa*

	Total cohort (n=1055)	LS <8.0 kPa (n=954)	LS ≥8.0 kPa (n=101)	OR (95%CI)*	p-value*
FVL mutation†	49 (4.6%)	41 (4.3%)	8 (7.9%)	2.00 (0.90-4.46)	0.09
Prothrombin G20210A gene variant‡	20 (1.9%)	16 (1.7%)	4 (4.0%)	2.04 (0.66-6.28)	0.2
FVL mutation or Prothrombin G20210A gene variant◊	68 (6.4%)	56 (5.9%)	12 (11.9%)	2.09 (1.07-4.07)	0.03
Blood group type non-O¥	568 (55.0%)	513 (54.9%)	55 (55.6%)	0.92 (0.60-1.40)	0.7

Prevalences are presented as count (percentage).

* *Multivariable logistic regression analyses testing the association between the FVL mutation, prothrombin G20210A gene variant, ABO blood group type and LS ≥8.0 kPa. All models are adjusted for age, sex and ALT (U/L)*

† *Determined by the genotype present at rs6025. Allele frequencies: G=97.7%, T=2.3%. Genotype distribution total cohort: GG=95.4%, GT=4.6%, TT=0%*

‡ *Determined by the genotype present at rs1799963. Allele frequencies: G=99.0%, A=1.0%. Genotype distribution total cohort: GG=98.1%, GA=1.9%, AA=0%*

◊ *Either an FVL mutation or Prothrombin G20210A gene variant was found in 67 participants. One additional participant had both an FVL mutation or Prothrombin G20210A gene variant*

¥ *Determined by the genotype present at rs687289. Allele frequencies: G=67.3%, A=32.8%. Genotype distribution total cohort: GG=45.2%, GA=44.3%, AA=10.6%*

Abbrevations: FVL, Factor V Leiden; LS, liver stiffness; OR, odds ratio; ALT, alanine aminotransferase.

G20210A was present in 11.9% of participants with LS ≥8.0 kPa versus 5.9% in those with LS <8.0 kPa (univariable OR 2.12, 95%CI 1.12-4.01, p=0.02). Prevalence of blood group type non-O did not differ between participants with LS ≥8.0 kPa versus those without LS ≥8.0 kPa (55.6% versus 54.9% respectively, univariable OR 1.02, 95%CI 0.68-1.54, p=0.9). None of the six participants with a history of VTE had the FVL mutation or prothrombin G20210A gene variant. Three of them (50%) had blood group type non-O. All participants with a history of VTE had LS <8.0 kPa.

In multivariable regression analysis, adjusting for age, sex, and ALT, presence of FVL or prothrombin G20210A was independently associated with an increased risk of having LS ≥8.0 kPa (OR 2.09, 95%CI 1.07-4.07, p=0.03). In a second multivariable model, additionally adjusting for steatosis, comparable results were obtained for the association between FVL or prothrombin G20210A and LS ≥8.0 kPa (OR 2.08, 95%CI 1.06-4.07, p=0.03). Additionally adjusting for presence of diabetes mellitus, current or former smoking, and alcohol intake (OR 2.12, 95%CI 1.08-4.18, p=0.03 for FVL or prothrombin G20210A) or type of Fibroscan probe (OR 2.11, 95%CI 1.08-4.11, p=0.03 for FVL or prothrombin G20210A) did not change results.

To test the robustness of our findings, we performed an analysis in a subgroup of our cohort consisting of all participants without signs of steatosis, with absence of HBsAg and anti-HCV and an alcohol intake ≤14 IU per week for women and ≤21 IU per

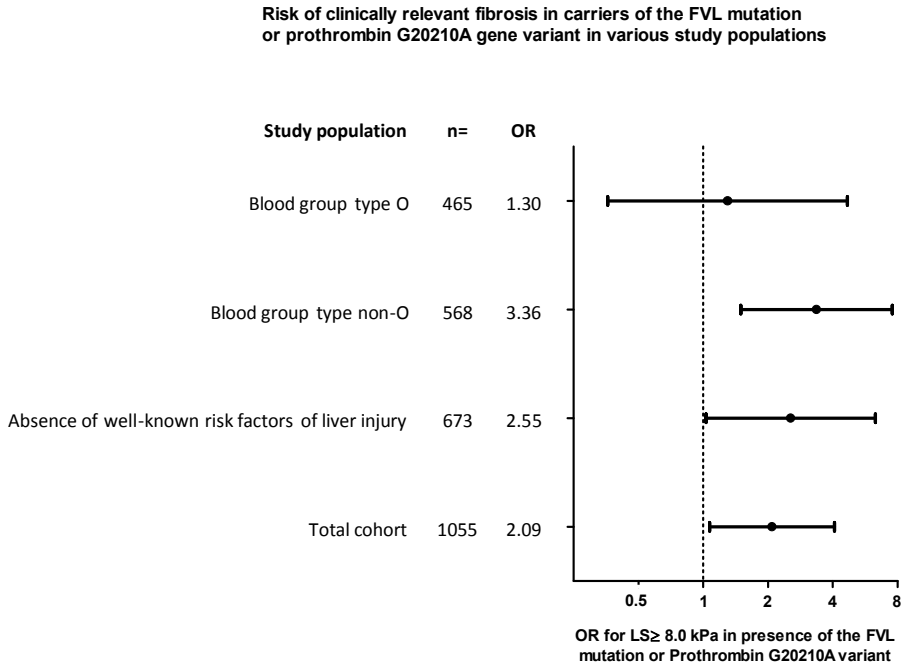

Figure 5.1 *Association between presence of the FVL mutation or prothrombin G20210A gene variant and LS ≥8.0 kPa in the total cohort and several subgroups, i.e. participants without well-known risk factors of liver injury (without steatosis, with absence of HBsAg and anti-HCV and an alcohol intake ≤14 IU per week for women and ≤21 IU per week for men), participants with blood group type non-O and participants with blood group type O. All logistic regression models are adjusted for age, sex and ALT (U/L).*
Abbreviations: FVL, Factor V Leiden; LS, liver stiffness; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; IU, international units; ALT, alanine aminotransferase

week for men, respectively. In this subgroup of 673 participants, presence of FVL or prothrombin G20210A was again independently associated with having LS ≥8.0 kPa in a multivariable model, adjusted for age, sex and ALT (OR 2.55, 95%CI 1.03-6.31, p=0.04) (Figure 5.1).

LS ≥9.5 kPa, suggestive of presence of severe fibrosis or cirrhosis, was present in 49 participants (4.6%). Seven of these participants (14.3%) had the FVL mutation or prothrombin G20210A gene variant compared to 61 (6.1%) participants with LS <9.5 kPa (OR 2.56, 95%CI 1.12-5.85, p=0.03 in univariable analysis and OR 2.33, 95%CI 0.98-5.51, p=0.06 in multivariable analysis). Six out of seven participants (86%) with FVL or prothrombin G20210A and LS ≥9.5 kPa had blood group type non-O. When performing a sensitivity analysis using a LS cut-off of 6.0 kPa, we did not find an association with presence of FVL or prothrombin G20210A (p=0.8).

Combined effect of blood group type and the Factor V Leiden mutation or prothrombin G20210A gene variant on the risk of having LS ≥8.0 kPa

We subsequently divided our cohort into two groups based on blood group type. Presence of both blood group type non-O and FVL or prothrombin G20210A was associated with an increased risk of having LS ≥8.0 kPa, adjusted for age, sex and ALT (OR 3.36, 95%CI 1.50-7.56, p=0.003) (Figure 5.1). Participants with blood group type O and presence of FVL or prothrombin G20210A on the other hand, did not have an increased risk of having LS ≥8.0 kPa (OR 1.30, 95%CI 0.36-4.68, p=0.7).

Predicted probabilities of having LS ≥8.0 kPa were calculated for four subgroups based on the combination of blood group type and presence or absence of FVL or prothrombin G20210A. These probabilities, adjusted for age, sex, and ALT, were comparable for all participants with blood group type O, irrespective of whether FVL or prothrombin G20210A was present (4.6% (3.1-7.9) with versus 4.9% (2.9-8.0) without FVL or prothrombin G20210A, p=0.8). For participants with blood group type non-O on the other hand, the median predicted probability of having LS ≥8.0 kPa was significantly higher for those participants with concurrent presence of FVL or prothrombin G20210A (14.3% (7.7-23.8)) than for participants with blood group type non-O without FVL or prothrombin G20210A (4.0% (2.4-6.6), p=0.008).

DISCUSSION

In this large population-based study, presence of the FVL mutation or prothrombin G20210A gene variant was associated with a twofold increased risk of having clinically relevant liver fibrosis, as indicated by presence of LS ≥8.0 kPa. Furthermore, the combined presence of FVL or prothrombin G20210A and blood group type non-O resulted in an even higher risk of having liver fibrosis. As a result, participants with both blood group type non-O and either FVL or prothrombin G20210A had a predicted probability of 14.3% of having clinically relevant liver fibrosis.

An increased risk of liver fibrosis was also observed in the subgroup of participants without well-known risk factors for liver injury. This finding indicates the relevance of FVL, prothrombin G20210A and blood group type non-O – common genetic prothrombotic risk factors – as risk factors of liver fibrosis, regardless of well-known liver-related causes of fibrosis.

Our results confirm the observations of previous studies showing that the presence of FVL, prothrombin G20210A or blood group type non-O was associated with an increased rate of development or progression of liver fibrosis in patients with chronic hepatitis C [18, 19, 21, 22]. Although the combined presence of FVL or prothrombin G20210A and blood group type non-O resulted in a strongly increased risk of fibrosis, presence of blood group type non-O solely was not associated with fibrosis in our cohort. This might be attributed to the fact that the extent of hypercoagulability associat-

ed with presence of blood group type non-O is lower compared to that associated with FVL or prothrombin G20210A, which may result in less impact on liver fibrogenesis.

The major strength of the current study is that data was derived from a large, well-described, prospective, ongoing, population-based cohort. The observed prevalences of FVL, prothrombin G20210A and blood group type non-O in our cohort were comparable to those of the general Caucasian population (2-8% for FVL and prothrombin G20210A [37-40] and 55% for blood group type non-O [12, 41] respectively). We did not demonstrate a significant association between liver fibrosis and either FVL or prothrombin G20210A alone, despite the fact that we were able to include more than 1,000 participants, as these mutations are not encountered frequently enough to provide sufficient power. Nonetheless, the observed risks of having liver fibrosis were comparable for either presence of FVL or prothrombin G20210A alone as well as for the combined analyses of presence of FVL or prothrombin G20210A, indicating that each of both mutations increases the risk of liver fibrosis in the general population.

The prevalence and impact on the risk of venous thrombosis differs for FVL or prothrombin G20210A on one hand and blood group type on the other hand. The first type of risk factors has a relatively low prevalence with a more profound effect, while the latter is widely encountered with an associated less prominent effect on venous thrombosis risk. Therefore, after addressing the separate effects of these types of risk factors, we also showed that combined presence of FVL or prothrombin G20210A in participants with blood group type non-O was associated with an additionally increased risk of liver fibrosis, as was previously demonstrated for the association between the combination of these genetic risk factors and venous thrombosis [15]. This additionally increased risk is in line with results from previous studies and cannot be explained by the effects of these prothrombotic risk factors separately [12-17]. A possible explanation for this phenomenon could be that FVL and presence of blood group type non-O both cause a decrease in sensitivity for activated protein C (APC), resulting in an additionally increased thrombosis risk if both factors are present [42]. The decreased sensitivity for APC in both conditions can be explained by 1) increased levels of factor VIII, which reduces APC sensitivity and is observed in participants with blood group type non-O [43] and 2) APC resistance, which is a hallmark feature of the FVL mutation [8]. Hence, this combined effect on APC sensitivity might cause an additionally increased risk of thrombosis [13]. In addition, factor V is required for inactivation of factor VIII by APC [44]. In patients with FVL, abnormal factor V will result in less inactivation of factor VIII. Combined with the abovementioned increased factor VIII levels associated with blood group type non-O, APC sensitivity is further reduced [13]. The additional increase in hypercoagulability could explain the enhanced risk of fibrosis in participants with both FVL and blood group type non-O.

The mechanism by which the increased thrombosis risk associated with the combined occurrence of the prothrombin G20210A gene variant and blood group type non-O could occur is to be explored. Only few studies have reported on the association

between combined presence of prothrombin G20210A, blood group type non-O and an increased risk of thrombosis, with varying results [14, 15, 17, 45]. Our study adds to the understanding that the coagulation system is involved in the pathogenesis and acceleration of liver fibrogenesis. Based on our findings, screening for presence of FVL, prothrombin G20210A and blood group type might be advocated in persons with LS measurements suggestive of fibrosis without a clear etiologic explanation for these findings. If recognized in time, individuals with thrombophilic risk factors as cause of fibrosis might benefit from anticoagulant therapy in the prevention of progression of fibrosis towards cirrhosis.

A limitation of this study is that we used transient elastography, as opposed to liver biopsy, to assess presence of liver fibrosis in this study. However, given the invasive nature of liver biopsies – the gold standard to detect liver fibrosis – it would be unethical to perform this in healthy participants. Measuring liver stiffness with TE is an effective, non-invasive, reproducible and currently well-established method to assess liver fibrosis [28, 46, 47]. Several studies have previously described associations between genetic variants and various liver diseases, using transient elastography as phenotype [48-56]. We used a cut-off of 8.0 kPa for clinically relevant liver fibrosis, as proposed and validated in previous community-based studies [26, 27]. Roulot et al. could detect a cause of liver disease in all subjects with LS ≥8.0 kPa and, among those in whom liver biopsy was performed, almost all showed liver fibrosis or cirrhosis. Therefore, we believe this cut-off to be suitable for detecting presence of liver fibrosis in healthy participants [26]. In addition, a cut-off of 9.5 kPa, suggestive of severe fibrosis or cirrhosis, was used in a sensitivity analysis [57]. We did not find an association with presence of FVL or Prothrombin G20210A and LS ≥6.0 kPa. However, this sensitivity analysis has to be interpreted with caution since TE is less accurate in quantitating intermediate fibrosis [47]. For this reason, applying a LS cut-off below 8.0 kPa could result in heterogeneous subgroups, as an increasing number of participants would be misclassified as having fibrosis.

In conclusion, our findings suggest that presence of the FVL mutation or prothrombin G20210A gene variant is associated with a twofold increased risk of liver fibrosis, as assessed by measuring LS, in the general population. Participants with combined presence of FVL or prothrombin G20210A and blood group type non-O were most at risk for fibrosis. Our results suggest that these genetic risk factors may be of clinical relevance for the development of liver fibrosis in the general population.

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Chapter 6

Is von Willebrand factor level a predictive marker of liver fibrosis in the general population? The Rotterdam Study

Manuscript submitted

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ABSTRACT

Background and aims

Liver fibrosis and its complications are a major health problem. In cirrhosis, von Willebrand factor (VWF) level is strongly elevated and predictive of clinical outcomes. The aim of the current study was to investigate whether VWF levels predict liver fibrosis, as measured by liver stiffness (LS), in a population-based cohort and in a subgroup of participants with steatosis.

Methods

Participants were recruited from the Rotterdam study, a large ongoing population-based cohort. LS was measured using transient elastography and hepatic steatosis using abdominal ultrasound on average 10.7±0.4 years after determining VWF levels.

Results

In 1,228 participants (age 74.0±5.6, 49.9% male), median VWF level at baseline was 1.11 (IQR 0.87-1.43) and median LS was 5.1 (4.1-6.3) kPa. VWF levels were positively associated with LS ($p=4.7*10^{-5}$). This association persisted after adjustment for factors known to influence LS, i.e. age, male sex, presence of diabetes mellitus, steatosis, spleen size, ALT, and current or former smoking (p=0.008). VWF levels were also independently associated with presence of LS ≥9.5 kPa, suggesting severe fibrosis (p=0.04). In a subgroup with steatosis (n=395), VWF was again independently associated with LS (p=0.04). If VWF levels were subdivided into quartiles, LS was more severe for similar VWF levels in participants with steatosis compared to those without steatosis.

Conclusions

In this population-based cohort and in a subgroup of participants with steatosis, VWF levels at baseline were independently associated with liver fibrosis after 10 years. Our data suggests that VWF might be used as marker of preclinical liver fibrosis.

INTRODUCTION

Liver fibrosis is a major health problem as it can ultimately result in the development of cirrhosis and liver cancer, which are conditions associated with a worldwide mortality of 1.7 million deaths per year (1, 2). Unlike cirrhosis, liver fibrosis can remain undetected for long. Indeed, we have previously shown that in a population-based cohort, 5.6% had unknowingly clinically significant liver fibrosis. We identified several factors which were associated with liver fibrosis in the general population, such as higher age, presence of diabetes mellitus and/or steatosis (3). However, an increasing body of evidence suggests that hypercoagulability should also be considered an important factor in liver fibrogenesis (4-13).

One of the key players in hemostasis is von Willebrand factor (VWF). VWF is produced by endothelial cells and is involved in platelet adhesion and thrombus formation at sites of vascular injury. It binds coagulation factor VIII (FVIII), thereby preventing it from clearance (14, 15). High plasma levels of VWF are a known risk factor for arterial and venous thromboembolism (16-18). VWF levels in plasma are determined mainly by genetic factors, with a reported heritability of 53-75%, of which the *ABO* gene is the most important determinant (19-21). In addition, VWF levels are also determined by non-genetic factors, including age and inflammation.

In patients with liver cirrhosis, VWF levels are strongly elevated (22). Previous studies showed that VWF levels correlate with the hepatic venous pressure gradient and can independently predict clinical outcome, including mortality, in cirrhotic patients (23, 24). However, the cause of these elevated VWF levels is not exactly known. In cirrhosis, endothelial dysfunction contributes to an increase in the hepatic vascular tone, resulting in portal hypertension (25, 26). Since VWF is produced by endothelial cells, the elevation of VWF levels in cirrhosis may be caused by endothelial dysfunction (27, 28).

Recently, a study in patients with chronic hepatitis C showed that VWF levels are elevated in patients with liver fibrosis. VWF levels increased incrementally with increasing fibrosis stage (29). It is unknown whether this association between VWF and fibrosis is also present in a the general population. Therefore, the aim of the current study was to investigate the association between VWF levels and liver fibrosis – assessed non-invasively using liver stiffness (LS) measurements as proxy – after 10 years in a large, population-based cohort and in a subgroup of participants with steatosis.

MATERIALS AND METHODS

Study population and design

This study was part of the Rotterdam study, a large ongoing prospective population-based cohort study conducted in Rotterdam, The Netherlands (30). For this study, individuals living in Ommoord, a suburb of the city of Rotterdam, aged 55 years and over were asked to participate. This resulted in a response rate of 78%. The initial cohort of the Rotterdam Study (RS-I) started in 1990. As of 2000 a new cohort (RS-II), consisting of participants who had turned 55 years or had moved into the study district, was added to the study population. Participants visit the designated research center every 3-4 years. Each examination cycle consists of an extensive home interview, fasting blood sampling and a variety of physical examinations in the research center. VWF antigen levels were determined in blood samples taken during the third center visit of cohort RS-I (1997-1999) and during the first visit of cohort RS-II (2000-2001). Starting from 2009, LS measurements and abdominal ultrasound were introduced and performed in each examination cycle thereafter. These investigations were performed after obtaining fasting blood samples at the research center. LS measurements and abdominal ultrasound were performed during the fifth and third visit of the RS-I and RS-II cohorts respectively (2009-2012). Median interval between VWF level determination and LS measurement was 10.7 ± 0.4 years (range 8.2-14.8 years).

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical Center and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and permission to obtain information from their treating physicians.

Liver stiffness measurement and abdominal ultrasonography

Presence of liver fibrosis was assessed non-invasively by measuring LS using transient elastography (TE) (Fibroscan®; Echosens™). LS was measured by a single, experienced ultrasonographer. Measurements were performed with the participant lying flat on his/her back with the right arm laying in maximal abduction and in between breathing movements. The failure rate of TE in the present study was 4.0%. LS measurements were considered reliable if 10 valid measurements were obtained with a success rate of at least 60% and an interquartile range (IQR) of less than 30% of the median LS measurement value. Based on these criteria, the unreliability rate in the present study was 22.1%, which is comparable to other studies (31, 32). M or XL-probe was used to obtain LS measurements, according to manufacturer's recommendations. TE was not performed in participants with an intracardiac device, as TE is contraindicated in this setting. An LS cut-off value of 8.0 kPa was used to identify clinically relevant liver fibrosis

(33, 34). A more conservative LS cut-off of ≥9.5 kPa was used to determine presence of severe fibrosis or cirrhosis (35).

Abdominal ultrasonography (Hitachi HI VISION 900) was additionally performed to assess presence of hepatic steatosis, to measure spleen size, and to examine the hepatic parenchyma amongst others. Images were stored digitally and re-evaluated by an expert hepatologist with large experience in abdominal ultrasound. The diagnosis of steatosis was determined by the ultrasonographer according to the Hamaguchi et al. scoring system (36). In all cases, presence of steatosis was reassessed by a hepatologist with vast experience in ultrasonography.

Biochemistry and anthropometry

Fasting venous citrate blood samples were collected at the research center and plasma was stored at -80 ˚C. VWF antigen was determined by an in-house enzyme linked immunosorbent assay (DakoCytomation, Glostrop, Denmark), as discussed in more detail elsewhere (37). Alanine aminotransferase (ALT) and glucose levels were determined using automatic procedures (Roche Diagnostics GmbH, Mannheim, Germany). Diabetes mellitus (DM) was defined as fasting plasma glucose ≥7.0 mmol/L or drug treatment for elevated blood glucose. Hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus (HCV) antibodies were measured using immunoassays (Roche Diagnostics GmbH, Mannheim, Germany) to determine presence of viral hepatitis.

During the home interview, extensive data was obtained on demographics, medical history, comorbidity, alcohol consumption, smoking behavior, and drug use. Excessive alcohol consumption was defined as an intake of more than 14 units of alcohol per week. Trained research nurses performed anthropometric measurements at the research center, from which body mass index (BMI) was calculated as weight (kg) divided by height $(m)^2$.

Genetic analysis

DNA was isolated from whole blood samples and extracted according to standard automated procedures (38). Genotyping was performed in batches using the Illumina Infinium II HumanHap 550K Genotyping Bead-Chip® version 3 (Illumina Inc., San Diego, CA, USA). Imputation was performed using the Markov Chain Haplotyping (MaCH) package (39), using the cohort of the 1000 Genomes Project as reference population (40). A detailed description of these methods has been published elsewhere (41-44). PLINK was used to convert dosage data of two imputed single nucleotide polymorhpisms (SNPs) – rs2726953 and rs868875 – to best guess data (45).

We analyzed ten SNPs known to be associated with VWF levels. These SNPs were identified in two large studies (37, 41). There was no linkage disequilibrium between the ten SNPs included in our analyses. All SNPs were tested for Hardy-Weinberg equilibrium (46).

Statistical analysis

Baseline characteristics are expressed as counts with proportions for categorical variables and as median with IQR or mean with standard deviation for continuous variables. Differences in baseline characteristics were assessed using Chi-squared tests (counts), Student t-tests (means) or Mann-Whitney U tests (medians) respectively. VWF levels and LS measurements were logarithmically transformed prior to all analyses, as both variables were not normally distributed. Linear regression analysis was used to examine the association between VWF levels and LS. Associations between VWF levels and LS ≥8.0 or ≥9.5 kPa were evaluated using logistic regression analyses. In multivariable regression analyses, we adjusted for age, sex, DM, spleen size, ALT, BMI, hepatic steatosis, alcohol consumption, current or former smoking, and presence of HBsAg positivity or anti-HCV positivity at the moment of LS measurement.

Genotypes were coded as 0,1 or 2 based on the number of VWF increasing alleles. The fractional allele count (imputed dosage of the VWF increasing allele) was used in case of imputation. All SNPs were analyzed in an additive genetic regression model. Linear and logistic regression analyses, adjusted for age and sex, were used to assess the association between the VWF-associated SNPs and continuous LS measurements and LS ≥8.0 kPa respectively.

Statistical analyses were performed using SPSS 21 (IBM SPSS Statistics for windows, Version 21.0, Armonk, NY, USA).

RESULTS

Study population

Reliable liver stiffness measurements were available in 1,324 participants. In this group, VWF levels were previously determined in 1,228 participants (92.7%). Characteristics of this cohort are described in Table 6.1. Mean age at time of LS measurement was 74.0 ± 5.6 years and 49.9% was male. Median LS was 5.1 (4.1-6.3) kPa and LS ≥8.0 kPa, suggestive of clinically relevant fibrosis, was present in 113 participants (9.2%). Median VWF level was 1.11 (IQR 0.87-1.43) IU/mL. VWF levels increased with age (β=0.005, p=6.4*10^{-11}). Participants with blood group type non-O had significantly higher VWF levels compared to participants with blood group type O (median VWF level 1.25 (0.99-1.58) IU/mL and 0.93 (0.76-1.14) IU/mL respectively, p<0.001).

Table 6.1 *Baseline characteristics of the study cohort at time of LS measurement*

Characteristic	Total cohort (n=1,228)	Presence of steatosis (n=395)	Absence of steatosis (n=833)	p-value[§]
Age (years)	74.0 ± 5.6	73.1 ± 4.8	74.5 ± 5.9	<0.001
Male sex	613 (49.9)	212 (53.7)	401 (48.1)	0.07
Caucasian ancestry[*]	1069 (96.6)	343 (96.9)	726 (96.4)	0.7
BMI (kg/m^2)	26.7 ± 3.5	28.9 ± 3.4	25.6 ± 3.0	<0.001
Alcohol intake >14 IU/week	143 (11.8)	74 (18.9)	69 (8.4)	<0.001
Current or former smoking	782 (64.2)	271 (69.1)	511 (61.9)	0.01
Diabetes mellitus	143 (11.9)	82 (21.2)	61 (7.5)	<0.001
Viral hepatitis	13 (1.1)	6 (1.5)	7 (0.9)	0.4
Positive HBsAg	1 (0.1)	0	1 (0.1)	1.0
Presence of anti-HCV	12 (1.0)	6 (1.5)	6 (0.7)	0.2
Spleen size (cm)[¥]	9.6 ± 1.3	9.9 ± 1.4	9.5 ± 1.3	<0.001
Liver stiffness measurement (kPa)	5.1 (4.1-6.3)	5.4 (4.4-6.9)	4.9 (4.0-6.1)	<0.001
Fibroscan probe, M	608 (49.5)	135 (34.2)	473 (56.8)	<0.001
ALT (U/L)	18 (14-23)	21 (16-28)	17 (13-21)	<0.001
Blood group type non-O[†]	552 (55.1)	189 (58.7)	363 (53.5)	0.1
Von Willebrand factor level (IU/mL)[‡]	1.11 (0.87-1.43)	1.12 (0.91-1.47)	1.10 (0.86-1.38)	0.1

Values are represented as count (proportion), mean ± standard deviation, or median (interquartile range)
[*] *Data on ethnicity was missing for 121 participants.*
[¥] *Spleen size measurements were missing in 160 participants.*
[†] *Blood group type was missing in 227 participants.*
[‡] *Von Willebrand factor level was measured 10.7 ± 0.4 years before liver stiffness measurement*
[§] *p-value for the comparison of participants with steatosis versus those without steatosis*
Abbreviations: BMI, body mass index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ALT, alanine aminotransferase.

Von Willebrand factor levels, genetic variations and liver stiffness measurements

VWF levels were significantly associated with increasing LS after 10.7 ± 0.4 years (β=0.11 log kPa per log IU/mL, p=4.7*10^{-5}, Figure 6.1). Adjusting for blood group type did not alter this association (β=0.11, p=3.6*10^{-4}). In multivariable analysis, adjusted for factors known to be associated with LS – age, male sex, presence of diabetes mellitus, hepatic steatosis, spleen size, ALT, and current or former smoking – VWF levels were still independently associated with LS (β=0.073 (95%CI 0.019-0.127), p=0.008) (Table 6.2). Increasing VWF levels were also associated with an increased risk of having LS ≥8.0 kPa, suggestive of clinically relevant liver fibrosis (OR 4.85 per log increase in VWF level, 95%CI 1.44-16.31, p=0.01). After adjusting for the abovementioned factors in a multivariable model, this association remained, though it did not reach statistical significance (OR 3.61, 95%CI 0.86-15.16, p=0.08). When applying a LS cut-off of 9.5 kPa, suggestive of presence of severe fibrosis or cirrhosis, we observed an independent association

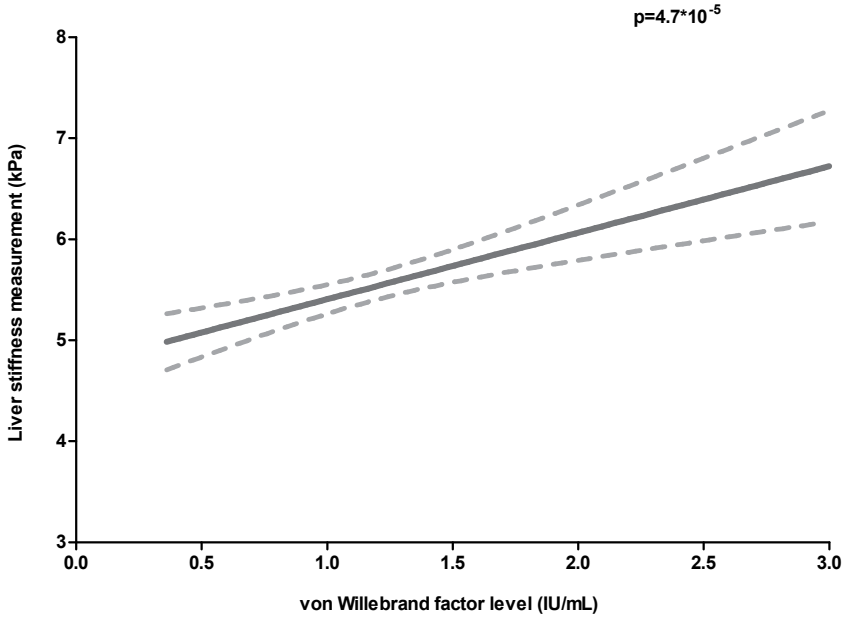

Figure 6.1 *Association between von Willebrand factor Antigen levels (IU/mL) and liver stiffness measurements (kPa) after 10.7 ± 0.4 years (n=1,228). Liver stiffness measurements increase significantly with increasing VWF levels at baseline.*

Table 6.2 *Association between von Willebrand factor levels and liver stiffness measurements in linear multivariable regression analyses (n=1,228)*

Variable	β (95%CI)	p-value
Age (years)	0.006 (0.004-0.008)	<0.001
Male sex	0.062 (0.043-0.081)	<0.001
Diabetes Mellitus	0.033 (0.005-0.060)	0.02
Spleen size (cm)	0.015 (0.008-0.022)	<0.001
ALT (U/L)	0.002 (0.001-0.003)	<0.001
BMI (kg/m^2)	-0.003 (-0.005-<0.001)	0.07
Steatosis	0.038 (0.017-0.058)	<0.001
Alcohol consumption (drinks/week)	<0.001 (-0.002-0.001)	0.5
Current or former smoking	0.020 (0.002-0.039)	0.03
HBsAg or anti-HCV positive	0.015 (-0.076-0.105)	0.8
Von Willebrand factor levels (IU/mL)	0.073 (0.019-0.127)	0.008

Abbreviations: ALT, alanine aminotransferase; BMI, Body Mass Index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus

Table 6.3 *Association between von Willebrand factor level and liver stiffness measurement ≥9.5 kPa in logistic multivariable regression analyses (n=1,228)*

Variable	OR (95%CI)	p-value
Age (years)	1.10 (1.05-1.16)	<0.001
Male sex	1.77 (0.87-3.63)	0.1
Diabetes Mellitus	2.03 (0.94-4.35)	0.07
Spleen size (cm)	1.37 (1.10-1.70)	0.005
ALT (U/L)	1.04 (1.02-1.06)	<0.001
BMI (kg/m^2)	1.01 (0.91-1.12)	0.9
Steatosis	1.19 (0.58-2.41)	0.6
Alcohol consumption (drinks/week)	0.99 (0.94-1.04)	0.8
Current or former smoking	2.12 (0.94-4.79)	0.07
HBsAg or anti-HCV positive	2.61 (0.30-22.69)	0.4
Von Willebrand factor levels (IU/mL)	8.02 (1.09-59.28)	0.04

Abbreviations: ALT, alanine aminotransferase; BMI, Body Mass Index; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus

with VWF levels in both univariable (OR 7.45, 95%CI 1.37-40.44, p=0.02) and multivariable analysis (OR 8.02, 95%CI 1.09-59.28, p=0.04) (Table 6.3). There was no interaction between VWF levels and the other covariables included in the multivariable analyses for the association with LS.

Data on the VWF-associated SNPs was available in 1033 Caucasian participants (84.1% of the total cohort), due to lack of informed consent or non-Caucasian ethnicity in 195 participants. Genotypes and minor allele frequencies of these 10 SNPs are shown in Supplementary Table 6.1. Quality of imputation was high for all SNPs (R^2 ≥0.97) except for rs868875, which had moderate imputation quality (R^2=0.78). One SNP, rs216321, located in the *VWF* gene, was associated with increased LS in an additive linear regression model (β=0.026 log kPa per VWF increasing allele, p=0.02). However, after adjustment for multiple testing, this association was not considered statistically significant. The other VWF-associated SNPs, including the SNP in the *ABO* gene, were not associated with increasing LS (p-values ≥0.07).

Von Willebrand factor levels and liver stiffness measurements in participants with steatosis

Steatosis was detected at abdominal ultrasonography in 395 participants (32.2%). Participants with steatosis were significantly younger, had a higher BMI, had more often an excessive alcohol intake, and were more often current or former smokers compared to participants without steatosis (Table 6.1). In addition, DM, a higher spleen size, higher LS, use of the XL-probe and higher ALT were more often observed in participants with steatosis. VWF levels at baseline did not differ between participants with and without

steatosis after 10.7 ± 0.4 years (median VWF level 1.12 (0.91-1.47) IU/mL versus 1.10 (0.86-1.38) IU/mL respectively, p=0.1). However, presence of steatosis was associated with higher VWF levels at baseline after adjustment for age and sex (β=0.021 log IU/mL, p=0.03). In the subgroup of participants with steatosis, VWF levels were associated with increasing LS in both univariable (β=0.13 log kPa per log IU/mL, p=0.009) and multivariable regression analyses (β=0.11, p=0.04). Participants with steatosis had higher LS in comparison with participants without steatosis for similar VWF levels, when divided into quartiles (p-values 0.01; 0.03; 0.01 and <0.001 for VWF quartiles 1,2,3, and 4 respectively, adjusted for age and sex) (Figure 6.2). LS ≥8.0 kPa was observed in 55 participants (13.9%) with steatosis and 23 participants (5.8%) with steatosis had LS ≥9.5 kPa. We did not observe an association between VWF levels and presence of LS ≥8.0 kPa or ≥9.5 kPa in the subgroup of participants with steatosis (OR 2.22, 95%CI 0.39-12.49, p=0.4 and OR 3.48, 95%CI 0.27-45.51, p=0.3 respectively).

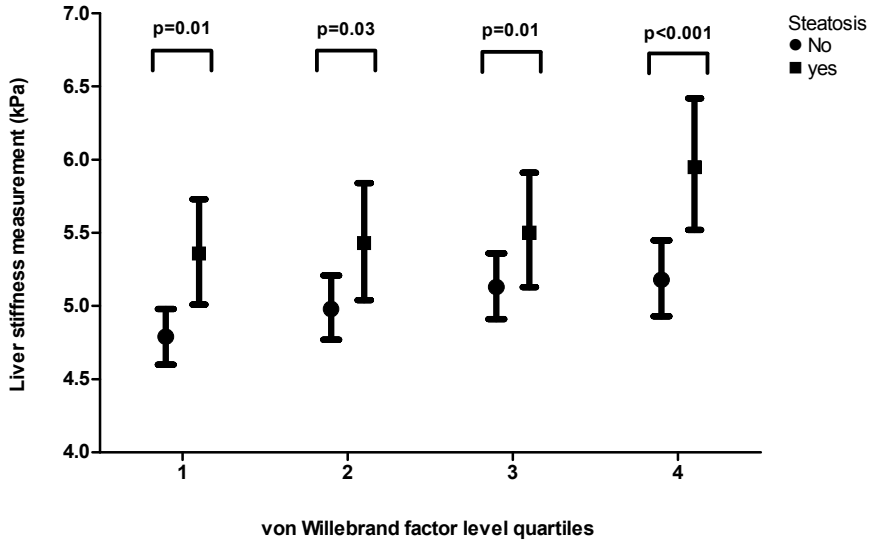

Figure 6.2 *Association between von Willebrand factor Antigen level quartiles and liver stiffness measurements (kPa) after 10.7 ± 0.4 years in participants with and without steatosis respectively. Associations were adjusted for age and sex. Liver stiffness measurements were significantly higher for participants with steatosis for all VWF quartiles.*
VWF quartiles: 1) 0.36-0.87 IU/mL 2) 0.88-1.11 IU/mL 3) 1.12-1.43 IU/mL 4) 1.44-3.03 IU/mL.

DISCUSSION

In this large, population-based study, VWF levels were associated with liver fibrosis – assessed non-invasively using LS measurements as proxy – after 10 years. In addition, we observed an association between VWF levels and LS in a subgroup of participants with steatosis. Participants with steatosis had more severe LS values for similar VWF levels compared to participants without steatosis.

In liver cirrhosis, VWF is a predictor of clinically significant portal hypertension and mortality (23, 24). However, the mechanism resulting in elevated VWF levels in cirrhosis remains to be elucidated (22). It has been hypothesized that this elevation may be caused by endothelial dysfunction (27, 28), since endothelial dysfunction contributes to the development of portal hypertension (25, 26) and VWF is produced by endothelial cells. In addition, the hepatic synthesis of VWF is increased in patients with cirrhosis (47). Cirrhosis is known to be associated with a delicate hemostatic balance, characterized by a decrease in most procoagulant and anticoagulant factors, but with an increase in VWF levels (48). This hemostatic balance may be easily shifted towards a hypercoagulable state. Since VWF is an important promoter of hemostasis, increased VWF levels in cirrhosis might contribute to this hypercoagulable state. It has been demonstrated repeatedly that hypercoagulability is not only a feature of cirrhosis, but also a driver of liver fibrogenesis (4-13). Therefore, there might be a role for VWF in promoting liver fibrosis. This is supported by the previous finding that blood group type non-O, known to be associated with 25% higher VWF levels compared to blood group type O (49, 50), was associated with increased severity of fibrosis in patients with chronic hepatitis C (8). A recent study within the Rotterdam Study cohort on the other hand, did not find an association between blood group type solely and presence of clinically relevant fibrosis. However, the combined presence of blood group type non-O and the factor V Leiden mutation or prothrombin G20210A variant did result in a markedly increased risk of fibrosis in these healthy individuals (7).

Although it is well-established that VWF levels are elevated in patients with cirrhosis, to our knowledge only one recent study has assessed the association between VWF levels and liver fibrosis. This study in patients with chronic hepatitis C found that VWF levels increase with increasing fibrosis stage. The authors concluded that VWF can be used as non-invasive marker to assess presence of significant and advanced liver fibrosis and cirrhosis in patients with hepatitis C (29). Our findings in a large population-based cohort of middle-aged and elderly individuals confirm the association between VWF levels and liver fibrosis, as VWF levels were positively associated with LS measurements after 10 years in the present study. Moreover, VWF levels at baseline were independently associated with an increased risk of LS ≥9.5 kPa, suggesting presence of advanced fibrosis or cirrhosis, 10 years later. In addition, the association between VWF and LS was also observed in a subgroup of participants with steatosis, suggesting this association is irrespective of the etiology of fibrosis.

We did not find an association between SNPs previously demonstrated to influence VWF levels and LS. Also the SNP determining blood group type, the most important genetic determinant of VWF levels (19-21), was not associated with increasing LS. In addition, adjusting for blood group type did not alter the association between VWF levels and LS. These findings are in line with a previous study within this cohort showing a lack of association between blood group type and presence of clinically relevant liver fibrosis (7). Therefore, although the current study shows that VWF levels are associated with liver fibrosis after 10 years, the exact mechanism of this relationship remains to be clarified by future studies.

Strengths of the current study are that we were able to include a large cohort of participants from an ongoing well-described population-based study. In addition, VWF levels, LS measurements and genetic data were available for the vast majority of these participants, enabling us not only to investigate the association between VWF levels and fibrosis, but also to provide more insight in the association between VWF-associated SNPs and fibrosis in the same study. A limitation of the current study is that we did not use liver biopsy, the gold standard, to assess presence of liver fibrosis. Instead, fibrosis was assessed non-invasively in this study, since performing a liver biopsy in healthy participants is not feasible. We assessed fibrosis by using TE to measure LS. TE is a reproducible, non-invasive, rapid and well-established method to assess liver fibrosis (35, 51, 52). TE has been used as phenotype to assess fibrosis in healthy participants in several previous studies (3, 7, 33, 34, 53, 54). In the present study, presence of steatosis was investigated using abdominal ultrasonography. This method allows for accurate identification of steatosis and is the method of choice for screening in population-based settings (55).

In conclusion, VWF levels were associated with liver fibrosis after a mean of 10.7 years in this large population-based cohort and in a subgroup with steatosis. These findings suggest that VWF might be used as marker of preclinical liver fibrosis. As VWF is able to predict mortality and clinical outcomes in patients with cirrhosis (23, 24), future studies should focus on the role of VWF levels in predicting fibrosis progression and clinical outcomes in liver fibrosis.

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Supplementary table 6.1 *Genotype and minor allele frequencies of the single nucleotide polymorphisms (n=1,033)*

SNP	Genotypes	Frequency, n (%)	VWF increasing allele	Allele frequency*
rs9390459	AA	169 (16.4)	G	0.59
	GA	505 (48.9)		
	GG	359 (34.8)		
rs2726953	CC	498 (48.2)	T	0.31
	CT	430 (41.6)		
	TT	105 (10.2)		
rs687621	TT	466 (45.2)	C	0.33
	TC	457 (44.2)		
	CC	109 (10.6)		
rs1063857	TT	455 (44.0)	C	0.34
	TC	445 (43.1)		
	CC	133 (12.9)		
rs4981022	CC	118 (11.4)	T	0.67
	CT	450 (43.6)		
	TT	465 (45.0)		
rs7978987	GG	438 (42.2)	A	0.35
	GA	465 (45.0)		
	AA	130 (12.6)		
rs10133762	GG	342 (33.3)	T	0.42
	GT	497 (48.4)		
	TT	187 (18.2)		
rs868875	GG	42 (4.1)	A	0.76
	GA	408 (39.5)		
	AA	583 (56.4)		
rs216321	TT	8 (0.8)	C	0.92
	TC	154 (15.0)		
	CC	865 (84.2)		
rs2283333	TT	11 (1.1)	C	0.90
	TC	188 (18.2)		
	CC	834 (80.7)		

Abbreviations: SNP, single nucleotide polymorphism; VWF, von Willebrand factor
** Allele frequencies of the von Willebrand factor level increasing allele*

Part III

Etiology and ischemic complications of vascular liver diseases

Chapter 7

7

Somatic Calreticulin mutations in patients with Budd-Chiari syndrome and portal vein thrombosis

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We studied the role of the recently identified CALR mutations in 141 patients with Budd-Chiari Syndrome (BCS) or portal vein thrombosis (PVT) in a large multinational cohort. A CALR mutation was present in one of the 141 patients (0.7%). This patient was previously diagnosed with primary myelofibrosis. This results in CALR positivity in one out of 44 (2.3%) patients with myeloproliferative neoplasm (MPN), and in one of 11 (9.1%) JAK2V617F negative patients diagnosed with MPN. We suggest that analysis of CALR mutations should be performed in JAK2V617F negative BCS and PVT patients.

BCS and non-malignant, non-cirrhotic PVT are rare vascular liver diseases. The etiology of these diseases encompasses both inherited and acquired risk factors, of which MPN are the most common with a prevalence ranging between 20-50% (1-3). Detecting presence of MPN in patients with BCS and PVT is important, given the prognostic and potential therapeutic implications regarding anticoagulant therapy (4, 5). However, diagnosing MPN in patients with BCS and PVT is often difficult as portal hypertension caused by the obstruction of the hepatic veins and/or portal vein can explain splenomegaly, and decreases peripheral blood cell counts through hypersplenism (6). The discovery of the *JAK2V617F* mutation has greatly improved the ability to non-invasively detect MPNs in patients with BCS and PVT (7-9). However, in the absence of the *JAK2V617F* mutation, diagnosing MPN can still be challenging and a bone marrow biopsy remains required in most patients and may still be inconclusive (4, 10). Recently, exome sequencing resulted in the detection of mutations in *CALR* in patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF) lacking *JAK2V617F* and *MPL* mutations. *CALR* mutations were present in 67%-88% of these patients (11, 12). Identification of *CALR* mutations in patients with BCS and PVT without the characteristic blood counts could improve the ability to diagnose ET and PMF. The aim of our study was to determine the prevalence and role of *CALR* mutations in patients with BCS and non-malignant, non-cirrhotic PVT.

In this case-control study, patients and controls were recruited from the European Network for Vascular Disease of the Liver (EN-Vie) study cohort. This study cohort has been described in detail elsewhere (2, 3). For this study, consecutive, incident cases of BCS and non-malignant, non-cirrhotic PVT were enrolled between 2003 and 2005 and prospectively followed in nine European countries. In addition, healthy, unrelated, population-based controls were included. These controls did not have a history of thrombosis and fulfilled the same age criteria as the included patients (13). Blood samples were obtained at diagnosis. DNA was extracted from whole blood according to local standard methods. DNA samples were stored in the Erasmus MC University Medical Center in Rotterdam at -80°C until analysis. Presence of *CALR* mutations was determined with a PCR fragment analysis examining exon 9. Results of this PCR were analyzed using Genemapper 4.0 as previously described (12). MPNs were diagnosed by performing a bone marrow biopsy (in 49% of patients), *JAK2V617F* mutation detection (in 98% of patients), EPO measurements (in 20% of patients), red cell mass measurement (in 11% of patients) and/or spontaneous erythroid colony formation (SECF) testing (in 18% of patients).

DNA samples were available from 77 patients with BCS, 75 patients with PVT and 76 controls. Determination of *CALR* mutation was successful in 92% of all samples, resulting in an inclusion of 70 BCS patients, 71 patients with PVT and 68 controls in the current analysis. Baseline characteristics and underlying etiological factors of this cohort are shown in Table 7.1. Median age of patients with BCS and PVT was 43.1 (31.0-53.4) years and 80 (57%) were female. One or more underlying prothrombotic factor(s) could be identified in 87% of all patients with BCS and PVT. MPN was present in 44 patients (31.2%) of whom 26 (37%) patients with BCS and in 18 (26%) patients with PVT. The median age of patients with MPN was 44.6 (31.1-51.6) years compared to 43.1 (30.2-55.0) years in patients without MPN (p=0.95). There was also no difference in male/female distribution between the patients with and those without MPN (61% and 54% female respectively, p=0.4). Additional prothrombotic risk factors were diagnosed in 32 (73%) of all patients with MPN. Thirty-three of the 44 patients with MPN had a *JAK2V617F* mutation (75%). Of the eleven remaining patients with MPN without *JAK2V617F*, three (27%) had polycythemia vera (PV) (diagnosis based on bone marrow biopsy findings and SECF), two patients (18%) had ET, two (18%) had PMF, two (18%) were unclassifiable and two (18%) had occult MPN, at that time tested by SECF. The diagnosis of MPN was based on the above-mentioned criteria and reviewed by a hematologic expert (FWGL). A *CALR* mutation was present in only one MPN patient (2.3% of all patients with MPN). In the 97 BCS and PVT patients without MPN no *CALR* mutation was detected. PMF or ET was present in 17 patients with BCS and PVT in our cohort (39% of all patients with MPN). Of these 17 patients, 13 (77%) carried a *JAK2V617F*, one (6%) had a *CALR* mutation, and 3 (17%) did not have a known MPN-associated mutation.

The patient with a *CALR* mutation was a 46-year old female Dutch patient with a cardiomyopathy and PMF since 1981, resulting in severe splenomegaly requiring splenectomy in 2003. Pathological examination of the spleen showed extensive extramedullary hematopoiesis with signs of multiple non-recent splenic infarctions. Postoperatively, she was diagnosed with PVT including thrombosis of the splenic vein and superior mesenteric vein. At that time, a bone marrow biopsy revealed end-stage PMF. She did not carry a *JAK2V617F* mutation. The *CALR* mutation detected in this patient was a type 1 mutation, the most common encountered *CALR* mutation, characterized by a 52-bp deletion in exon 9 (11, 12). At the moment of diagnosis of PVT, platelet count in this patient was increased (396*10^9/L), white blood cell count was 6.3*10^9/L, and hemoglobin was 5.8 mmol/L. During follow-up, she presented with several episodes of gastrointestinal bleeding twelve months after diagnosis of PVT, caused by peptic ulcers under treatment of oral anticoagulants and non-steroidal anti-inflammatory drugs and angiodysplasia of the cecum. Fourteen months after diagnosis of PVT, this patient died of progressive multi-organ failure after presenting with gastrointestinal bleeding resulting in circulatory collapse.

Table 7.1 *Baseline characteristics and etiological factors of the study cohort*

	BCS (n=70)	PVT (n=71)
Age, years	36.4 (26.4-50.7)	49.8 (41.6-57.2)
Female sex	41 (58.6)	39 (54.9)
ALT (U/L)	65 (32-169)	40 (24-61)
Platelets (*10^9/L)	224 (126-375)	263 (164-403)
White blood cell count (*10^9/L)	9.7 (6.7-13.6)	9.5 (7.6-12.8)
Hemoglobin (mmol/L)	8.6 (7.5-9.7)	8.2 (7.2-9.3)
Inherited thrombophilia*	15 (21.4)	16 (22.5)
Factor V Leiden mutation	10 (14.3)	4 (5.7)
Prothrombin gene G20210A	2 (2.9)	8 (11.4)
Protein C deficiency	1 (1.5)	1 (1.6)
Protein S deficiency	0	3 (4.9)
Antithrombin deficiency	2 (3.0)	2 (3.3)
Acquired thrombophilia*	54 (77.1)	52 (73.2)
Myeloproliferative neoplasms	26 (37.1)	18 (25.7)
Polycythemia vera	9 (34.6)	3 (16.7)
Essential thrombocytosis	5 (19.2)	7 (38.9)
Primary myelofibrosis	2 (7.7)	3 (16.7)
Unclassifiable	6 (23.1)	4 (22.2)
Occult	4 (15.4)	1 (5.6)
JAK2V617F present	19 (27.1)	14 (19.7)
Antiphospholipid antibody syndrome	21 (30.0)	19 (27.5)
Paroxysmal nocturnal hemoglobinuria	9 (12.9)	0
Hormonal risk factors†	16 (39.0)	18 (46.2)
Non-hematological systemic disorder	8 (11.4)	2 (2.8)
History of thrombosis	14 (20.0)	16 (22.5)
Local risk factor¥	11 (15.7)	19 (26.8)
Single risk factor	24 (34.3)	23 (32.4)
Multiple risk factors	39 (55.7)	37 (52.1)
No risk factor	7 (10.0)	11 (15.5)

Results are expressed as median (interquartile range) for continuous variables and as count (proportion) for categorical variables

* *Patients could have more than one etiological factor simultaneously*

† *Presence of hormonal risk factor was missing in 29 patients with BCS and 32 patients with PVT*

¥ *Local risk factors were defined as presence of an abdominal trauma, abdominal intervention, and/or intra-abdominal infection, i.e. pancreatitis, liver abcess, cholangitis, cholecystitis, intra-abdominal abcess, diverticulitis, appendicitis, gastroenteritis, and/or spontaneous bacterial peritonitis with or without sepsis*

BCS: Budd-Chiari syndrome; PVT: portal vein thrombosis; ALT, alanine aminotransferase

In summary, in this large European cohort of 141 newly diagnosed patients with BCS and non-malignant, non-cirrhotic PVT, a somatic *CALR* mutation was present in only one patient with PVT and in none of the patients with BCS. This resulted in a prevalence of CALR mutations of 0.7% (one of 141) in the total cohort, 2.3% (one of 44) in all patients with MPN, and 9.1% (one of 11) in all patients with MPN without *JAK2V617F*. Importantly, no *CALR* mutations were detected in patients without MPN or controls.

Recently, the association between MPN and somatic mutations in *CALR* was described for the first time (11, 12). *CALR* mutations were present only in PMF and ET lacking *JAK2V617F* and *MPL* mutations. In patients with PMF or ET, the prevalence of *CALR* mutations was reported to be 17-24% (11, 12). In our cohort with only hepatic and/or portal vein thrombosis, the observed prevalence of *CALR* mutations in patients with PMF or ET was considerably lower (6%). This might be attributable to the fact that patients with a *CALR* mutation have a lower risk of thrombosis compared to patients with a *JAK2V617F* mutation. This decreased thrombosis risk may in turn result from the lower hemoglobin and white blood cell counts observed in patients with a *CALR* mutation compared to MPN patients without a *CALR* mutation (11, 12). This finding is in line with previous reports that state that *CALR*-mutant MPN patients do not develop thrombosis as often as patients carrying *JAK2V617F*, resulting in the lower prevalence of *CALR* mutation frequency in our cohort of patients with BCS and PVT as compared to this prevalence in cohorts of patients with MPN (14, 15). No *CALR* mutations were observed in the control group in our study, as was expected based on the results of the study by Nangalia et al (11). Interestingly, *CALR* mutations were also absent in patients with lymphoid cancers or solid tumors in that study, suggesting that *CALR* mutations might be used as a marker to detect presence of MPN in BCS and PVT. Two other studies recently investigated presence of *CALR* mutations in patients with BCS and PVT, with comparable results. Turon et al. found a prevalence of 1.9% *CALR* mutations in a Spanish cohort of patients with BCS and PVT and no *CALR* mutation was present in a cohort of 144 patients with abdominal vein thrombosis, also including renal vein, splenic vein and mesenteric vein thrombosis (16, 17). In the current study, we included 141 consecutive patients with BCS and PVT who were extensively screened for etiological factors and prospectively followed in nine European countries. None of the patients included in the current study was previously described in the other studies assessing presence of *CALR* mutations in BCS and PVT.

In conclusion, *CALR* mutations are rare in patients with BCS and PVT with a prevalence of 0.7% in the total cohort and 2.3% in patients with MPN. This low prevalence is probably due to the relatively lower risk of thrombosis in *CALR*-mutant patients compared to patients with a *JAK2V617F* mutation. Despite this low prevalence, we believe testing for *CALR* mutations should be considered in patients with BCS and PVT who are *JAK2V617F* negative, as diagnosing MPN is often difficult in these patients due to masked blood cell counts because of portal hypertension, occult gastrointesti-

nal bleeds and/or hypersplenism. Screening for somatic *CALR* mutations is an easy to perform diagnostic method that could aid in diagnosing MPN with limited burden for the patient.

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Chapter 8

Genetic variants associated with deep vein thrombosis are not a risk factor for splanchnic vein thrombosis

Manuscript submitted

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ABSTRACT

Background and aims

The etiology of portal vein thrombosis (PVT) and Budd-Chiari syndrome (BCS) shows overlap with that of venous thromboembolism (VTE). The aim of this study was to assess the role of candidate single nucleotide polymorphisms (SNPs), which were recently identified to increase the risk of VTE, as potential risk factors for PVT and BCS.

Methods

Patients were recruited from the European Network for Vascular Disease of the Liver (EN-Vie) study cohort. Genotyping of six SNPs – in *ABO, STXBP5*, *VWF, CYP4V2, GP6,* and *SERPINC1* – was performed using a Taqman assay. Results were validated in a clinical tertiary referral population in three countries.

Results

In this study, 77 patients with PVT, 77 patients with BCS and 81 healthy controls were included. Blood group type non-O, determined by the SNP in *ABO*, was independently associated with an increased risk of PVT compared to both controls (OR 3.1 (95%CI 1.4-6.9), p=0.005) and BCS (OR 3.0 (95%CI 1.4-6.5), p=0.006). No association between blood group non-O and risk of BCS was observed (OR 0.8 (95%CI 0.5-1.8),p=0.8). The association between blood group non-O and PVT was not observed in the validation cohort (OR 1.36 (95%CI 0.77-2.39), p=0.3). None of the other VTE-associated SNPs was associated with an increased risk of PVT or BCS.

Conclusions

None of the six genetic variants associated with VTE was consistently identified as risk factor for PVT or BCS. These findings support the site-specificity of venous thrombosis and suggest that other risk factors are of greater importance in splanchnic vein thrombosis.

INTRODUCTION

Budd-Chiari syndrome (BCS) and non-malignant, non-cirrhotic portal vein thrombosis (PVT) are rare vascular liver diseases, also known as splanchnic vein thrombosis (SVT), characterized by obstruction of the hepatic venous outflow tract and the portal vein, respectively (1, 2). In the pathogenesis of both BCS and PVT, prothrombotic disorders play a pivotal role. These prothrombotic disorders can be subdivided into genetic and acquired risk factors. Together, these risk factors account for approximately 75-85% of the etiology of SVT. As a result, 15-25% of the etiology of these disorders is currently still unknown (3, 4). Among the previously identified prothrombotic risk factors, considerable overlap exists between the etiology of SVT and the more common forms of venous thromboembolism (VTE) as deep venous thrombosis (DVT) and pulmonary embolism (PE). The factor V Leiden mutation, prothrombin G20210A gene variant, protein C deficiency, protein S deficiency and antithrombin deficiency – all well-known genetic prothrombotic risk factors – play an important role in the pathogenesis of both VTE and SVT (3-10).

In 1969, a relationship between ABO blood group type and risk of VTE was described for the first time. Individuals with blood group type non-O, which is associated with higher levels of von Willebrand factor (VWF) and coagulation factor VIII, had a two- to fourfold increased risk of VTE compared to blood group type O (11-13). Combining this increased VTE risk with a blood group type non-O prevalence of 55% in Caucasians makes blood group non-O to be one of the most important genetic risk factors for VTE (14-17).

A recent large meta-analysis of five genome-wide association studies identified eight genes in which single nucleotide polymorphisms (SNPs) were associated with VWF levels (18). One of the described SNPs was located in the *ABO* gene, already known to be associated with VTE (12, 14, 15, 17). In a large case-control study, SNPs in two of the seven other genes associated with VWF and FVIII levels, *STXBP5* and *VWF,* were found to be associated with an increased risk of VTE as well (19).

Besides that, three SNPs in the *CYP4V2*, *GP6*, and *SERPINC1* gene were strongly associated with DVT in a combined analysis of three large case-control study cohorts (20). A meta-analysis of five case-control studies confirmed the role of these three SNPs as risk factors of VTE among Caucasians (21).

All these genetic risk factors for VTE – located in the *ABO*, *STXBP5, VWF*, *CYP4V2*, *GP6*, and *SERPINC1* gene – have not been tested in patients with SVT yet. Based on the fact that other procoagulant factors are shared by both disorders, we hypothesized that these genetic factors would also be important risk factors for SVT. Therefore, the aim of the current study was to elucidate whether these novel candidate SNPs are also risk factors for SVT in the largest study population of patients with BCS and PVT to date.

MATERIAL AND METHODS

Study cohort

For the current study, patients and controls were recruited from the European Network for Vascular Disease of the Liver (EN-Vie) study cohort. This cohort consists of consecutive patients with newly diagnosed non-cirrhotic, non-malignant PVT and BCS. These patients were enrolled between October 2003 and October 2005 and prospectively followed in academic and large regional hospitals in nine European countries. More details regarding the EN-Vie study cohort can be found elsewhere (3, 4). BCS was diagnosed by radiological imaging and defined as a hepatic venous outflow obstruction and its manifestations, regardless of the cause and level of obstruction, ranging from the small hepatic veins to the entrance of the inferior vena cava into the right atrium. PVT was defined as radiological evidence of solid material in the portal vein lumen or its left or right branch. PVT patients with concomitant cirrhosis or abdominal malignancies were excluded. Patients underwent a systematic etiological work-up per protocol. At the moment of diagnosis and at several time-points during follow-up, extensive data was obtained on clinical symptoms, etiological factors, laboratory parameters, and radiological findings. Risk factors for VTE were investigated as reported previously (22, 23).

The EN-Vie study was approved by all national, and if necessary, local ethical committees. All patients and controls agreed to participate in the study by providing written informed consent.

Healthy controls

The control group consists of healthy, unrelated individuals, who were recruited during the EN-Vie study. These controls were usually friends or acquaintances of the patients of the same sex, age, local and ethnic background. Controls were only considered eligible if they did not have a history of thrombosis and were not using anticoagulation.

Genotyping

According to the study protocol, blood samples were drawn from patients and controls for storage of DNA and plasma. DNA was extracted from whole blood according to local standard methods. All EN-Vie samples were stored at the Erasmus MC University Medical Center in Rotterdam, the Netherlands at -80°C until analysis. In the current study, we included all patients and controls of the EN-Vie study cohort of whom an adequate amount of DNA was available at the moment of genotyping. Competitive allele-specific PCR assays (KASP™, LGC genomics, Hoddesdon, Herts, UK) were used to genotype the SNPs. We analyzed six SNPs that were previously identified as risk factors of venous

Table 8.1 *Single nucleotide polymorphisms associated with venous thromboembolism*

SNP	Chromosome (forward strand)	Gene	Minor allele	MAF	Risk allele
rs687289	9:136137106	*ABO*	A	0.37	A
rs1039084	6:147635413	*STXBP5*	A	0.47	A
rs1063857	12:6153514	*VWF*	C	0.38	C
rs13146272	4:187120211	*CYP4V2*	C	0.34	A
rs1613662	19:55536595	*GP6*	G	0.15	A
rs2227589	1:173886216	*SERPINC1*	T	0.09	T

Abbreviations: SNP, Single nucleotide polymorphism; MAF, Minor allele frequency in individuals from European descent (EUR) in the 1000 Genomes project (http://browser.1000genomes.org).

thrombosis (Table 8.1). There was no linkage disequilibrium between these six SNPs (19-21, 24). Genotyping was performed centrally at LGC genomics.

Validation cohort

To validate our findings, we included a cohort of patients diagnosed with BCS or non-cirrhotic, non-malignant PVT as of November 2005, i.e. after the end of inclusion of the EN-Vie cohort. This validation cohort consists of consecutive patients seen during regular hospital care in the 1) Erasmus MC University Medical Center, Rotterdam, the Netherlands, 2) Hopital Beaujon, Clichy, France and the 3) Hospital Clinic, Barcelona, Spain. The etiological work-up was left to the discretion of the treating physician. Baseline characteristics and etiological factors were obtained from medical charts of these patients. Blood group type was retrieved from medical charts or in some cases by contacting the patient or blood transfusion center locally (France). All patients provided written informed consent. For the current study, only patients aged ≥16 years were included.

Statistical analysis

Baseline characteristics are expressed as counts with proportions for categorical variables and as medians with interquartile range (IQR) for continuous variables. Comparison between categorical variables was performed using Pearson Chi-square tests or Fisher's Exact tests. Continuous variables were compared with student t-tests (means) or Mann-Whitney U tests (medians). Call rates and Hardy-Weinberg equilibrium were tested for all SNPs (25). Associations between each of the SNPs and presence of PVT or BCS were assessed by using an additive genetic binary logistic regression model (linear OR per addition of an extra effect allele). The total number of risk-increasing alleles carried was calculated and subsequently compared for patients with BCS, PVT and controls using a binary logistic regression model adjusting for age and sex. A dominant genetic model (non-GG vs GG at rs687289) was applied to assess the association

between blood group type and presence of BCS or PVT in the EN-Vie study group. In multivariable regression analyses, models were adjusted for age, sex, and inherited thrombophilia, i.e. presence of the factor V Leiden mutation, prothrombin G20210A variant, and/or deficiency for protein C, protein S and antithrombin. In addition, models were adjusted for presence of myeloproliferative neoplasms (MPN) in a second multivariable model. To address potential gene-gene and gene-environmental interactions, multivariable logistic regression analyses were also performed taking into account possible interactions between blood group type and either presence of other genetic risk factors for venous thrombosis or presence of MPN. In the validation cohort, binary logistic regression analyses, adjusting for age, sex, and inherited thrombophilia, were used to assess the association between blood group type and presence of PVT or BCS. Statistical tests were two-sided and a p-value <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA).

Table 8.2 *Baseline characteristics of the EN-Vie study cohort*

	EN-Vie study cohort		
	PVT (n=77)	**BCS (n=77)**	**p-value**
Age, years	50.1 (41.9-57.3)	37.1 (26.5-51.1)	<0.001
Female sex	43 (55.8)	46 (59.7)	0.63
ALT (U/L)	43 (24-67)	64 (32-165)	0.03
Platelets (*10^9/L)	258 (174-401)	223 (123-378)	0.19
Hemoglobin (mmol/L)	8.2 (7.2-9.3)	8.5 (7.5-9.8)	0.21
Bilirubin (umol/L)	16.0 (11.3-24.4)	31.0 (17.1-47.9)	0.027
Inherited thrombophilia	17 (22.1)	16 (20.8)	0.84
Protein C deficiency	1 (1.5)	1 (1.4)	1.0
Protein S deficiency	4 (6.0)	0	0.05
Antithrombin deficiency	2 (3.0)	2 (2.7)	1.0
Factor V Leiden mutation	4 (5.3)	11 (14.3)	0.061
Prothrombin gene G20210A	8 (10.5)	2 (2.6)	0.056
Acquired thrombophilia	58 (75.3)	61 (79.2)	0.56
Myeloproliferative neoplasms	20 (26.3)	29 (37.7)	0.13
JAK2V617F positive	15 (20.3)	21 (27.3)	0.31
Antiphospholipid antibodies	21 (28.0)	23 (29.9)	0.80
Paroxysmal nocturnal hemoglobinuria	0	11 (14.3)	0.001
Local risk factor	21 (27.3)	11 (14.3)	0.047
No risk factor	11 (14.3)	7 (9.1)	0.32

Categorical variables are represented as counts (percentage). Continuous variables are expressed as median (interquartile range).
Abbreviations: PVT, portal vein thrombosis; BCS, Budd-Chiari syndrome.

RESULTS

Patient characteristics

For the current study, DNA samples were available from 79 patients with BCS, 80 patients with PVT, and 81 controls of the EN-Vie study cohort. Of these, 77 patients with BCS, 77 patients with PVT and 81 controls were successfully genotyped and included in the current study cohort (overall genotyping success rate 97.9%). Baseline characteristics of the patients with BCS and PVT are described in Table 8.2. Median age of the patients was 43.7 (31.0-54.4) years and 42.2% was male. Inherited thrombophilia was observed in 22.1% of patients with PVT and in 20.8% of patients with BCS, while an acquired prothrombotic risk factor was present in 75.3% and 79.2%, respectively. MPN was diagnosed in 26.3% of patients with PVT and in 37.7% of patients with BCS (p=0.1). In 85.7% of patients with PVT and 90.9% of patients with BCS, at least one pro-thrombotic risk factor could be detected. In the control group, the Factor V Leiden mutation and the prothrombin G20210A variant were present in 3.8% and 2.5%, respectively.

Genetic variations in splanchnic vein thrombosis

Frequencies of genotypes and call rates of the six SNPs are shown in supplementary Table 8.1. All SNPs were in Hardy-Weinberg equilibrium.

Associations between the six SNPs, previously identified as risk factors of VTE, and presence of PVT of BCS in the EN-Vie study cohort, are described in Table 8.3. Only the SNP in the *ABO* gene, rs687289, was associated with an increased risk of PVT com-

Table 8.3 *Associations between venous-thromboembolism associated SNPs and PVT or BCS in the EN-Vie study cohort (n=154 patients and n=81 controls)*

			EN-Vie study cohort			
			PVT (n=77)		BCS (n=77)	
	Risk allele	Gene	OR (95%CI)*	p-value*	OR (95%CI)†	p-value†
rs687289	A	*ABO*	1.86 (1.09-3.18)	0.023	0.88 (0.53-1.46)	0.63
rs1039084	A	*STXBP5*	0.65 (0.40-1.05)	0.081	1.07 (0.69-1.64)	0.78
rs1063857	C	*VWF*	0.98 (0.60-1.61)	0.94	1.22 (0.78-1.91)	0.39
rs13146272	A	*CYP4V2*	1.40 (0.86-2.29)	0.18	1.20 (0.77-1.86)	0.42
rs1613662	A	*GP6*	1.02 (0.57-1.82)	0.95	1.79 (0.97-3.30)	0.063
rs2227589	T	*SERPINC1*	1.59 (0.62-4.06)	0.33	1.98 (0.85-4.61)	0.11

Abbreviations: SNP, single nucleotide polymorphism; BCS, Budd-Chiari syndrome; PVT, portal vein thrombosis.

* *Odds ratio with its 95% confidence interval and p-value are expressed per additional risk allele for comparison of patients with PVT (n=77) with controls (n=81). All analyses were adjusted for age and sex.*

† *Odds ratio with its 95% confidence interval and p-value are expressed per additional risk allele for comparison of patients with BCS (n=77) with controls (n=81). All analyses were adjusted for age and sex.*

pared to controls (OR 1.9 (95% CI 1.1-3.2), p=0.02 per addition of an A allele, adjusted for age and sex). None of the SNPs was associated with an increased risk of BCS compared to controls. We tested the combined effect of the genetic variations by calculating the total number of risk-increasing alleles. The number of risk-increasing alleles per patient or control ranged from 1 to 9. The addition of one risk-increasing allele did not significantly increase the risk of BCS or PVT compared to controls (OR 1.22 (95% CI 0.98-1.51), p=0.077 for BCS and OR 1.12 (95% CI 0.89-1.40), p=0.34 for PVT).

Blood group type non-O in portal vein thrombosis and Budd-Chiari syndrome

In the EN-Vie study cohort, prevalence of blood group type non-O (non-GG at rs687289) was significantly higher in patients with PVT (81.6%) both in comparison with patients with BCS (57.1%, p=0.001) and in comparison with controls (58.8%, p=0.002). In univariable regression analysis, presence of blood group type non-O was associated with a more than threefold increased risk of PVT compared to controls (OR 3.1 (95% CI 1.5-6.5), p=0.002). Patients with blood group type non-O did not have an increased risk of BCS compared to controls (OR 0.8 (95% CI 0.5-1.8), p=0.8). In multivariable regression analysis, additionally adjusting for age, sex, and other genetic risk factors for VTE, presence of blood group type non-O remained significantly associated with a threefold increased risk of PVT compared to controls (OR 3.1 (95% CI 1.4-6.9), p=0.005) (Table 8.4). Presence of other genetic risk factors for VTE, i.e. presence of the factor V Leiden mutation, prothrombin G20210A variant, and/or deficiency for protein C, protein S and antithrombin, was independently associated with an increased risk of both PVT and BCS compared to controls (OR 5.4 (95% CI 1.7-16.9), p=0.004 and OR 4.3 (95% CI 1.5-12.5), p=0.008 respectively), as described previously (3, 4).

Blood group type non-O was also significantly more prevalent in PVT as compared to BCS (OR 3.3 (95% CI 1.6-6.9), p=0.001). In multivariable regression analysis, having blood group non-O remained independently associated with an increased risk

Table 8.4 *Association between blood group type non-O and PVT or BCS compared to healthy controls (n=81) in multivariable analysis in the EN-Vie study cohort*

	EN-Vie study cohort			
	PVT (n=76)		BCS (n=77)	
	OR (95%CI)	p-value	OR (95%CI)	p-value
Age, year	1.08 (1.05-1.10)	<0.001	1.02 (0.99-1.04)	0.16
Female sex	1.00 (0.48-2.08)	1.0	0.89 (0.46-1.73)	0.73
Inherited thrombophilia*	5.37 (1.71-16.9)	0.004	4.28 (1.47-12.5)	0.008
Blood group non-O	3.12 (1.41-6.90)	0.005	0.95 (0.50-1.83)	0.88

* *Presence of other genetic risk factor for venous thrombosis, i.e. presence of the factor V Leiden mutation, prothrombin G20210A variant, protein C deficiency, protein S deficiency and/or antithrombin deficiency*

of PVT compared to BCS (OR 3.0 (95% CI 1.4-6.5), p=0.006). Presence of other genetic risk factors for VTE was not associated with an increased risk of PVT compared to BCS in this model (OR 0.8 (95% CI 0.3-1.9), p=0.6). Additionally adjusting for presence of MPN did not alter the association between blood group type non-O and PVT compared to BCS (OR 2.8 (95% CI 1.3-6.3), p=0.01).

There was no interaction between blood group type and presence of other genetic risk factors for VTE for both the comparison of PVT with controls and PVT versus BCS. Also, we did not observe an interaction between blood group type and presence of MPN for patients with PVT compared to patients with BCS.

Validation cohort

To validate our findings on the role of blood group type non-O in the pathogenesis of SVT, we enrolled a validation cohort of 351 patients diagnosed with BCS or PVT in the tertiary referral clinics of Clichy (France), Barcelona (Spain), and Rotterdam (The Netherlands) between November 2005, the end of inclusion in the EN-Vie study, and 2014. In this cohort, BCS was present in 108 (30.8%) and PVT in 243 (69.2%) patients (Table 8.5). Median age of this cohort was 41.7 (31.8-54.7) years and 46.4% was male. Inherited thrombophilia was observed in 30.3% (64 of 211 tested) of patients with PVT and in

Table 8.5 *Characteristics of the EN-Vie study cohort (n=154) and validation cohort (n=351)*

	PVT (n=320)			BCS (n=185)		
	EN-Vie (n=77)	**Validation (n=243)**	**p-value**	**EN-Vie (n=77)**	**Validation (n=108)**	**p-value**
Age, years	50.1 (41.9-57.3)	44.7 (35.4-57.0)	0.04	37.1 (26.5-51.1)	37.6 (27.0-45.1)	0.5
Female sex	43 (55.8)	112 (46.1)	0.1	46 (59.7)	76 (70.4)	0.1
Protein C deficiency*	1 (1.5)	23 (10.6)	0.02	1 (1.4)	22 (23.4)	<0.001
Protein S deficiency	4 (6.0)	34 (15.3)	0.05	0	15 (16.6)	<0.001
Antithrombin deficiency	2 (3.0)	7 (3.1)	1.0	2 (2.7)	11 (11.3)	0.04
Factor V Leiden mutation	4 (5.3)	7 (3.0)	0.5	11 (14.3)	11 (10.5)	0.4
Prothrombin gene G20210A	8 (10.5)	15 (6.4)	0.2	2 (2.6)	3 (2.8)	1.0
Myeloproliferative neoplasms	20 (26.3)	46 (20.3)	0.4	29 (37.7)	35 (32.7)	0.5

Categorical variables are represented as counts (percentage). Continuous variables are expressed as median (interquartile range).

* *Definitions of presence of pro-thrombotic risk factors were based on local criteria in the validation cohort. In the EN-Vie cohort, all patients were systematically evaluated for etiological factors using a standardized protocol.*

Abbreviations: PVT, portal vein thrombosis; BCS, Budd-Chiari syndrome.

38.9% (37 of 95 tested) of patients with BCS (p=0.14). MPN was diagnosed in 20.3% (46 of 227 tested) and 32.7% (35 of 107 tested) of the patients with PVT and BCS, respectively (p=0.013).

In this validation cohort, blood group type non-O was present in 59.3% of patients with BCS and 65.8% of patients with PVT. Compared to the controls, blood group type non-O was not associated with an increased risk of PVT or BCS in this cohort of patients seen during regular hospital care (OR 1.36 (95% CI 0.77-2.39), p=0.29 and OR 1.01 (95% CI 0.54-1.90), p=0.98 respectively, adjusted for age, sex, and inherited thrombophilia). Presence of blood group type non-O was also not associated with an increased risk of PVT compared to BCS in this validation cohort (OR 1.18 (95% CI 0.69-2.02), p=0.54).

DISCUSSION

In summary, we studied the role of six recently identified SNPs, associated with the risk of common forms of VTE, in the largest prospective multinational cohort of patients with BCS and PVT to date. Interestingly, none of the investigated genetic variations can be considered a risk factor for SVT. Although we observed that presence of blood group type non-O was associated with an increased risk for the development of PVT, but not for BCS in the EN-Vie study cohort, we could not confirm these results in the validation cohort. Our findings support previous hypotheses that thrombosis development is site-specific and depends on a complex interplay between specific local and systemic factors (10).

There is a considerable overlap in etiology between common forms of VTE on one hand, and PVT and BCS on the other hand (10). However, it remains elusive why thrombosis develops in the splanchnic veins in some patients, while the majority of patients with similar risk factors develops a more common form of VTE, i.e. DVT and PE. Likewise, the etiology of BCS and PVT is comparable to a large extent, but there are also some clear differences (10). A hypothesis to explain this phenomenon is that thrombus formation is side-specific. One of the factors that may play a central role in this side-specificity is endothelial cell heterogeneity (26). Endothelial-derived anticoagulants and procoagulants, like VWF, are not evenly expressed in the vasculature resulting in a site-specific equilibrium to balance local hemostasis. This endothelial cell heterogeneity could explain how a systemic imbalance in clotting factors can result in local thrombus formation (26). Endothelial activation has been described previously in PVT and BCS (27). It has been hypothesized that exposure to gut-derived products and vulnerability of the splanchnic veins to activated platelets and high viscosity, characteristic features of MPN, play an important role in the pathogenesis of SVT, while they are not important for thrombus formation at other, non-splanchnic, sites (26). Therefore, it is possible that some risk factors for VTE, including blood group type non-O, are not

associated with an increased risk of SVT, probably because other factors – including MPN – are of more importance in the pathogenesis of SVT.

Blood group type non-O is an important genetic risk factor for common VTE (14-17). The effect of blood group type non-O on VTE risk can be explained by increased VWF and factor VIII levels. Patients with blood group type non-O are known to have 25 percent higher VWF and factor VIII levels than patients with blood group type O, due to VWF glycosylation protecting it from proteolysis and/or clearance (28-31). The increase in VWF, which binds FVIII in the circulation and protects FVIII from degradation, results in higher FVIII levels. Elevated VWF and factor VIII levels on their turn are associated with an increased risk of VTE (11, 13, 32). In the EN-Vie study cohort, the SNP for blood group type non-O was associated with an independently increased risk of PVT. This was not observed in BCS. However, this observation could not be validated in a cohort of patients with BCS or PVT referred for tertiary care in the three largest centers of the original EN-Vie cohort.

The different outcomes in both cohorts may, in part, be due to differences in the studied patient populations. The EN-Vie cohort is a well-described multinational cohort in which all patients were systematically evaluated for etiological factors using a standardized protocol. In the patients in the validation cohort seen during regular hospital care, etiological factors were defined according to local criteria and not all etiological factors have been evaluated, which might have resulted in a selection bias. This accounts in particular for blood group type, as this is not an established risk factor for PVT or BCS.

We did not observe an association between the VTE-associated genetic variations located in the *STXBP5, VWF*, *CYP4V2*, *GP6*, and *SERPINC1* genes and presence of PVT or BCS. It is possible that the sample size of the EN-Vie study cohort (154 patients) was too small to detect an association between these candidate SNPs and PVT, despite the fact that this study was conducted in a large, multinational cohort. In particular, this could have been the case for the SNPs with a small minor allele frequency, like the SNPs in the *GP6* and *SERPINC1* gene, which have been discovered in a very large cohort of over 1,000 patients with VTE (20). Another explanation for the lack of association between the candidate SNPs and PVT and BCS could be that other factors, including local factors and MPN, are of more importance in the etiology of SVT, as discussed before.

The major strength of the current study is that this study was performed in a large, prospective, multinational cohort of newly diagnosed, well-described patients with BCS and PVT and controls. Furthermore, we tried to validate our findings in an extensive cohort of patients diagnosed with SVT in the three largest centers of the EN-Vie study cohort after the EN-Vie inclusion period. Our study also has some limitations that need to be addressed. First, we did not include all patients that were originally included in the EN-Vie study cohort, because DNA was not available anymore of all patients. As this was caused by the fact that DNA was randomly missing in some patients as no DNA was remaining after previous analyses, we believe that no selection bias has occurred

as a result of this. Second, although this study was conducted in the largest multinational cohort of patients with PVT and BCS to date, these diseases are very rare, and therefore absolute numbers of patients might have been too low to detect associations between the candidate VTE-associated SNPs with a small minor allele frequency and SVT. Finally, the control group of the EN-Vie study cohort was also used as reference group for the validation cohort. However, we do not believe this influenced our results, as the distribution of blood group type in this control group (blood group type non-O present in 58.8%) was comparable to that of the general French (58%), Dutch (53%), and Spanish (55%) population (33-35).

In conclusion, none of the six VTE-associated genetic variations located in the *ABO, STXBP5, VWF*, *CYP4V2*, *GP6*, and *SERPINC1* genes was associated with SVT in this large prospective study. Identification of these differences in etiology between SVT and more common forms of VTE may aid in understanding the pathogenesis of venous thrombosis at different sites.

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Supplementary table 8.1 *Frequencies of genotypes and call rates for all single nucleotide polymorphisms in the EN-Vie study cohort (n=235 patients and controls)*

SNP	Genotypes	Frequency, n(%)	MAF (this cohort)	Call rate (%)
rs687289	AA	29 (12.4)	0.39	99.1
	AG	124 (53.2)		
	GG	80 (34.3)		
rs1039084	AA	49 (21.4)	0.43	97.4
	GA	100 (43.7)		
	GG	80 (34.9)		
rs1063857	CC	33 (14.3)	0.36	98.3
	TC	101 (43.7)		
	TT	97 (42.0)		
rs13146272	CC	36 (15.7)	0.36	97.4
	CA	93 (40.6)		
	AA	100 (43.7)		
rs1613662	GG	9 (3.9)	0.19	98.7
	GA	68 (29.3)		
	AA	155 (66.8)		
rs2227589	TT	1 (0.4)	0.087	98.3
	TC	38 (16.5)		
	CC	192 (83.1)		

Call rates were calculated after exclusion of n=5 in whom all genotyping failed, resulting in a remaining cohort of 235 patients. In these 235 patients, in total 35 genotypes were missing in 12 patients.
Abbreviations: SNP, Single nucleotide polymorphism; MAF, minor allele frequency.

Chapter 9

GI ischemia in patients with portal vein thrombosis: A prospective cohort study

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ABSTRACT

Background and aims

Gastrointestinal ischemia is a concerning complication of portal vein thrombosis (PVT). Minimally invasive techniques, such as visible light spectroscopy (VLS), have greatly improved the ability to diagnose gastrointestinal ischemia. The aim of this study was to assess the clinical presentation and characteristics of gastrointestinal ischemia in patients with PVT.

Methods

Patients with non-cirrhotic, non-malignant PVT were included in this prospective cohort study. Clinical symptoms of gastrointestinal ischemia were assessed by a structured questionnaire, VLS, and radiological evaluation of the mesenteric vasculature. VLS measurements were compared to those in patients with cirrhosis and a reference population.

Results

We included 15 patients with chronic PVT and one patient with acute PVT (age 46.1 [IQR 30.9-53.7] years; 44% male). Decreased mucosal oxygenation in at least one location of the gastrointestinal tract was found in 12/16 (75%) patients. Compared to the reference population (median 60.0 [56.2-61.7]), VLS measurements were mostly decreased in the descending duodenum for patients with PVT (median 55.5 [52.3-58.8], *p=0.02*) and patients with cirrhosis (median 52.0 [46.5-54.0], *p=0.003*). Symptoms typical for gastrointestinal ischemia, such as postprandial pain and exercise-induced pain, were reported in 10/16 (63%) patients with PVT. In patients with extension of thrombosis into the superior mesenteric vein and splenic vein and/or presence of hypercoagulability, decreased VLS measurements were observed compared to the historical controls.

Conclusions

In patients with chronic PVT, gastrointestinal ischemia is frequent. VLS enables objective and quantitative determination of gastrointestinal mucosal ischemia. Onset of abdominal symptoms such as postprandial pain should prompt the physician to re-evaluate extent, cause and treatment of PVT.

INTRODUCTION

Portal vein thrombosis (PVT) is an infrequent vascular disorder that often leads to portal hypertension. Etiological factors include systemic and local prothrombotic factors.[1] PVT can be classified as acute or chronic. The latter is characterized by the presence of portal cavernoma, which depends on the duration of existence of the thrombus in the portal vein (PV).[1-3]

Gastrointestinal ischemia is a concerning complication of PVT. It can result in intestinal infarction, a life-threatening complication that often requires immediate surgical intervention.[2] Treatment of acute PVT therefore aims at vascular recanalization, preventing occurrence of gastrointestinal ischemia and portal hypertension.[1] The actual prevalence of gastrointestinal ischemia in patients with PVT is unknown. Studies, mainly conducted in patients with acute PVT, report prevalences of intestinal infarction of 2%-32%, with mortality rates of 0%-20%.[3-9] Regarding patients with chronic PVT, studies report conflicting results. As patients with chronic PVT often have an extensive venous collateral circulation, it is assumed that gastrointestinal ischemia is less likely to occur.[2] However, in a large prospective study in splanchnic vein thrombosis, 26% of patients presented with intestinal infarction. In nearly half of these patients a portal cavernoma was detected, suggesting gastrointestinal ischemia is also a frequent complication in chronic PVT.[4]

Typical complaints suggesting gastrointestinal ischemia are postprandial and/or exercise related abdominal pain, weight loss, and diarrhea. However, some patients present with more atypical symptoms, which limits the diagnosis of gastrointestinal ischemia based on clinical symptoms alone.[10] In addition, clinical signs of gastrointestinal ischemia are often difficult to differentiate from symptoms due to PVT. Also, it has been suggested that signs of ischemia in patients with PVT depend largely on the extent of the occlusion, the size of the vein, and the involvement of the superior mesenteric vein (SMV) and/or splenic vein (SV).[2, 11]

With the development of minimally invasive techniques, such as visible light spectroscopy (VLS), the ability to diagnose gastrointestinal ischemia has greatly improved.[12] VLS enables direct measurement of the adequacy of mucosal perfusion in the gastrointestinal tract.[13] It is a new technique that non-invasively measures capillary hemoglobin oxygen saturation using white light delivered by a fiberoptic probe during gastroscopy.[12, 13] The oxygen saturation reflects the adequacy of mucosal perfusion. Therefore, venous occlusion, hampering oxygenation of the gastrointestinal mucosa, may result in lower mucosal hemoglobin oxygen saturations.[12] VLS is a validated diagnostic method to detect gastrointestinal ischemia with a sensitivity and specificity of 90% and 60%, respectively.[12, 13]

Due to the rarity of the disease and lack of adequate diagnostic tools in the past, most data on gastrointestinal ischemia in patients with PVT stem from retrospective studies in selected patient populations.[3-7] VLS enables us to directly quantify

gastrointestinal ischemia in patients with PVT. Therefore, we performed a prospective cohort study in patients with PVT using VLS, radiological examination, and questionnaires. The objective of this study was to assess the presence, clinical presentation and characteristics of gastrointestinal ischemia in patients with PVT.

MATERIALS AND METHODS

Study population

Patients with non-cirrhotic, non-malignant PVT were studied in this single-center prospective cohort study in a tertiary care center. Patients aged 18 years and over with non-cirrhotic, non-malignant PVT were included in the study from 2009 until 2010. Acute and chronic PVT were defined according to Baveno V criteria as the presence of PVT with or without portal cavernoma and portal hypertension.[14] Patients with isolated SV or SMV thrombosis were excluded. All patients underwent radiological evaluation within two months of the VLS measurements. All patients with PVT received scheduled visits at the out-patient clinic. For this cohort study, we adhered to the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) initiative.[15]

Patients with liver cirrhosis and portal hypertension with patent mesenteric vasculature were included as cirrhotic patient controls. Portal hypertension was defined as: presence of varices and/or splenomegaly and/or ascites and/or hepatic hydrothorax and/or increased hepatic venous pressure gradient (>12mm Hg) AND in the absence of an intrahepatic shunting stent (i.e. TIPS).

For further comparison, VLS measurements of a historical reference population were used. These were participants in the original cohort of van Noord et al., of whom mucosal saturation measurements using VLS were obtained to establish the present cut-off values for gastrointestinal ischemia in the different locations in the gastrointestinal tract. These were consecutive patients referred for evaluation of possible chronic gastrointestinal ischemia who underwent the standard diagnostic work-up for gastrointestinal ischemia consisting of evaluation of symptoms, radiological imaging, and gastrointestinal tonometry in addition to VLS. In all these patients, more common causes of upper gastrointestinal symptoms had been previously excluded. A diagnosis of chronic gastrointestinal ischemia was established if a patient fulfilled two of the three following criteria: (a) distinct clinical presentation including presence of postprandial pain and otherwise unexplained weight loss of >5% of the normal body weight, (b) significant stenosis of >50% of at least one of the mesenteric arteries, and (c) functional signs of mucosal ischemia demonstrated by VLS. None of the historical controls fulfilled the required criteria. The VLS measurements were performed in identical fashion as in the previous study of the same group.[12]

Informed consent was obtained from all patients. Approval for the study was obtained from the Institutional Review Board of the Erasmus MC University Medical Center Rotterdam, the Netherlands. The study was registered at the ISRCTN registry (study ID ISRCTN14235960).

Diagnostic work-up and data collection

All patients with PVT received the standard work-up. This consisted of screening for etiologic factors, laboratory testing, abdominal ultrasound, and gastroscopy.

Standardized abdominal ultrasound was performed to assess the liver parenchyma, PV, hepatic artery, hepatic veins, SV, SMV, and to exclude the presence of cirrhosis and assess features of portal hypertension. Gastroscopy was performed to determine the possible presence of varices.

Use of antithrombotic medication was evaluated for all patients. Antithrombotic medication was initiated at the discretion of the treating physician and/or according to standard care. Time of antithrombotic medication use was assessed from diagnosis of PVT to time of VLS measurements. For each patient, a covered time (in %) of antithrombotic use was calculated by dividing the time (in months) of antithrombotic medication use by time (in months) since diagnosis of PVT.

Assessment of gastrointestinal ischemia

Clinical symptoms of gastrointestinal ischemia were assessed by a structured questionnaire, VLS measurements and radiological evaluation of the mesenteric arteries.

The questionnaire was specifically designed to collect relevant medical and family history, medication use, and gastrointestinal symptoms.[10, 16] To avoid missing data, medical history notes on gastrointestinal symptoms were also included.

Radiological evaluation of the mesenteric vasculature was performed by CT- A, or MR-angiography (MR-A), with a maximum slice thickness of 3mm. Site and extension of the PVT, as well as involvement of the SMV and SV, were also evaluated. Stenosis of the mesenteric arteries of ≥70% of vessel lumen was considered significant.

Mucosal saturation measurements

Gastroscopy was performed in all patients. VLS measurements were performed during gastroscopy as described before.[12, 17] In short, cleansing of remnant bile, fluids or food remnants was done prior to measuring along with administration of butylscopolamine 20 mg intravenously to halt upper gastrointestinal motility. Measurements were performed at three specific locations: descending duodenum, duodenal bulb and gastric antrum. We averaged three repeated readings per location, with every measurement within 5% variation of panel read-out once a stable reading was obtained. This

was considered the most accurate reflection of the mucosal saturation at that location. The sites of the VLS measurements per location were standardized in the way that in every patient the same anatomical regions were used. Peripheral oxygenation was kept above 94%. Mucosal ischemia was defined as: mucosal saturation <63%, <62% and < 58%, for measurements in the gastric antrum, duodenal bulb, and descending duodenum, respectively.[12] The physician performing the VLS measurements was not blinded to the patient characteristics. However, he was not the treating physician of the patient and was therefore not familiar with the specifics of the radiological findings of these patients. At time of inclusion, only two physicians in our center were specialized in performing the VLS measurements. The second physician was trained by the other physician, limiting a possible inter-observer variability.

Statistical analysis

Baseline characteristics were calculated using descriptive statistics. Data were expressed as mean and standard deviation (SD), median and interquartile range (IQR) or count and percentage (%), when appropriate. Differences in VLS measurements per location in the GI-tract between the different groups were assessed with linear regression analysis. Statistical analysis was performed using SPSS 21.0 program (SPSS Inc., Chicago, IL, USA). A two-sided p-value of <0.05 was considered statistically significant.

RESULTS

Study population

In this prospective cohort study, we included 16 patients with PVT (age 46.1 [30.9-53.7] years; seven patients were male). Baseline characteristics of these patients, at the moment of VLS measurement, are displayed in Table 9.1. At diagnosis, five patients had an acute PVT and in 11 patients a portal cavernoma was observed. At the moment of VLS measurement, 15 patients (94%) had chronic PVT.

In 14 patients (87%), at least one prothrombotic risk factor was present, including an inherited hypercoagulable risk factor in six patients of whom three with a factor V Leiden mutation. A local risk factor, such as inflammatory bowel disease or pancreatitis, was found in four patients.

Treatment with heparin followed by oral anticoagulants (OAC) was administered in 12 patients (75%). In nine of these patients (75%), anticoagulants were started at diagnosis and was continued at least until the moment of VLS measurement (covered time 99-100%). In two of these 12 patients, administration of OAC was delayed by 6 and 9 months respectively, which resulted in treatment with anticoagulants with a covered time <99-100%. In the remaining patient, OAC was directly administered, but

discontinued early because of an upper gastrointestinal bleeding, resulting in a covered time of 16.7%. The absolute length and percentage covered time of anticoagulation use is further described in Table 9.1. In two patients, acetylsalicylic acid was started because of presence of a myeloproliferative neoplasm (MPN). Before administering oral anticoagulants, thrombolysis was unsuccessfully attempted in one patient. All patients who initially presented with an acute PVT were treated with OAC from diagnosis until at least the moment of VLS measurement.

To determine whether the VLS measurements in patients with PVT are affected only by presence and extent of the portal and mesenteric venous thrombosis or also by portal hypertension, we included a cirrhotic patient control group consisting of five patients (age 61.0 [57.5-66.1] years (*p=0.02* compared to patients with PVT), three patients were male (*p=0.2*)) with liver cirrhosis without PVT. All of these patients had Child-Pugh C cirrhosis and were screened for liver transplantation. In these five patients, liver cirrhosis was caused by hepatitis B virus infection (n=2), hepatitis C virus infection (n=2) or primary sclerosing cholangitis (n=1). All patients in the cirrhotic patient control group had oesophageal varices. There were no liver function abnormalities in all patients with PVT.

Clinical features of gastrointestinal ischemia

Abdominal pain was reported by ten out of 16 (63%) patients with PVT (see Table 9.2). Postprandial pain, typical for gastrointestinal ischemia, was reported by nine out of 16 (56%). Furthermore, exercise-induced pain and weight loss (median 5.0 [2.0-6.7] kg/month) were reported in eight (50%) and seven (44%) patients with PVT. None of the cirrhotic patient controls reported any presence of postprandial abdominal or exercise-induced pain. Of the 11 PVT patients with extension into the SMV and SV, eight reported postprandial or exercise-induced abdominal pain. None of the PVT patients without involvement of the SMV and SV reported abdominal pain.

Abdominal pain was the presenting symptom and reason for referral in the vast majority of the 29 historical controls (38% male (*p=0.09* and *p=0.09* compared to patients with PVT and cirrhotic patient controls respectively), age 56.5 [43.9-66.4] years (*p=0.7* and *p=0.4* respectively)). However, the occurrence of significant weight loss (median 0.6 [0-1.1] kg/month) and loose stools (28%) was significantly lower in this group compared to patients with PVT (*p=0.001* and *p=0.002*, respectively). Mesenteric arterial stenosis was ruled out in the patients with PVT and the cirrhotic patient controls. Four historical controls were referred because of a stenosis of the mesenteric arteries, however the diagnosis of gastrointestinal ischemia was excluded during the diagnostic work-up.

Table 9.1 *Baseline characteristics*

			PVT										
ID	**Age (yrs)**	**Sex**	**Site***	**Presentation†**	**Underlying liver disease**	**Degree of occlusion**	**Extrahepatic involvement**	**Hypercoagulability‡**	**Jak2 mutation**	**MPN§**	**Anticoagulant time (%)‖/ length (mo)¶**	**Antiplatelets**	**Varices◊**
1	30	F	3	Chronic	None	Complete	SMV+SV	-	+	NOS	0/0	-	+
2	38	M	2a	Chronic	None	Complete	SMV+SV	-	-	-	38.5/6	-	+
3	48	F	1	Chronic	None	Complete	SMV	FVL	-	-	100/66	-	+
4	34	F	3	Chronic	None	Partial	SMV	APA	na	na	99/55	-	-
5	53	F	2b	Acute	None	Partial	SMV	na	na	na	100/62	-	-
6	62	M	3	Chronic	None	Complete	None	-	+	PV	100/26	+	+
7	26	F	1	Chronic	None	Complete	None	FVL	na	na	0/0	-	-
8	53	F	3	Chronic	None	Complete	SMV+SV	FVIII	+	ET	100/59	-	+
9	54	M	3	Chronic	None	Complete	SMV+SV	PC	-	na	16.7/1	-	+
10	30	F	2a	Chronic	None	Partial	SMV	-	-	-	100/8	-	-
11	48	M	3	Chronic	None	Complete	SMV+SV	-	-	-	100/23	-	-
12	43	F	3	Chronic	None	Complete	SMV+SV	-	+	PV	0/0	+	+
13	53	F	3	Chronic	None	Complete	SMV+SV	-	+	ET	100/57	-	-
14	20	M	3	Chronic	None	Complete	None	-	-	-	0/0	-	+
15	19	M	3	Chronic	None	Complete	None	-	-	-	100/31	-	-
16	54	M	3	Chronic	None	Complete	SMV+SV	FVL	+	ET and PV	20.8/22	-	+
17	61	M	none	none	Cirrhosis	none	none	na	na	na	-	-	+
18	55	M	none	none	Cirrhosis	none	none	na	na	na	-	-	+
19	59	F	none	none	Cirrhosis	none	none	na	na	-	-	-	+
20	64	M	none	none	Cirrhosis	none	none	na	na	na	-	-	+
21	67	F	none	none	Cirrhosis	none	none	na	na	na	-	-	+

Table 9.1 *Continued*
Abbreviations: PVT, portal vein thrombosis; PS, platelets/spleen; MPN, myeloproliferative neoplasm; SMV, superior mesenteric vein; SV, splenic vein; FVL, factor V Leiden; APA, antiphospholipid antibodies; na, not available; FVIII, factor VIII; PC, protein C deficiency; NOS, not otherwise specified; PV, polycythemia vera; ET, essential thrombocytosis.
[*] *Site of thrombotic involvement of the portal vein. Type 1=thrombosis of the trunk; type 2a=thrombosis of one branch; type 2b=both branches; type 3=thrombosis of both portal trunk and branches.*
[†] *Presentation at moment of VLS measurement, as diagnosed by radiological imaging (CT or abdominal ultrasound).*
[‡] *One patient (ID 4) had elevated antiphospholipid (anti-cardiolipin) antibodies and ID 8 had elevated levels of factor VIII. Three patients had an FVL mutation.*
[§] *MPN NOS: an MPN is present, but the type could not be classified into more detail by histological examination of bone marrow.*
[‖] *Amount of time on anticoagulants (oral anticoagulants and/or heparin) from diagnosis until VLS measurement divided by total amount of time from diagnosis until VLS measurement. All patients had oesophageal varices, except for ID 2, who was diagnosed with duodenal varices. ID 1 had both gastric (GOV-1) and oesophageal varices.*
[◊] *PS ratio: platelet (n/mm3) divided by spleen size (mm); cut-off ≤ 909*

Table 9.2 *Clinical symptoms and risk factors for gastrointestinal ischemia at time of VLS measurements*

	Cases n=16	**Cirrhotic patient controls n=5**	**Historical controls n=29**
Abdominal pain	10 (63)	0	28 (97)
- Postprandial pain	9 (56)	0	15 (52)
- Exercise-induced pain	8 (50)	0	13 (45)
Weight loss	7 (44)	1(20)	19 (66)
Weight loss (kg/month)[*]	5 [2.0-6.7]	-	0.6 [0-1.1]
Loose stools	12 (75)	3 (60)	8 (28)
BMI (kg/m^2)[*]	25.4 [21.9-26.8]	28.4 [24.0-33.5]	20.9 [19.6-23.9]
Coronary and/or peripheral arterial disease	0	1 (20)	7 (24)
Family history of CVD[†]	4(27)	5 (100)	12 (44)
DM type II	1 (6)	2 (40)	3 (10)
Hypertension[‡]	9 (56)	1 (20)	10 (35)
Hypercholesterolemia[§]	4 (27)	1 (25)	6 (21)
Smoking	3 (19)	1 (20)	13 (46)
Mesenteric arterial stenosis[‖]	0	0	4 (14)

Values represent number of patients (percentage %), unless otherwise specified.
Abbreviations: BMI, Body Mass Index; CVD, cardiovascular disease; DM, diabetes mellitus.
[*] *Median, interquartile range.*
[†] *Positive family history is defined as first-degree relatives with history of coronary and/or peripheral arterial disease. Family history of cardiovascular disease was missing in one patient with PVT.*
[‡] *Hypertension is defined as systolic blood pressure of >140mm Hg and/or diastolic blood pressure of ≥90mm Hg or use of anti-hypertensive medication.*
[§] *Hypercholesterolemia is defined as serum LDL cholesterol ≥190 mg/dl or use of statins. Serum LDL-cholesterol was missing in one cirrhotic patient control.*
[‖] *Mesenteric arterial stenosis is defined as a significant stenosis of ≥70% of the vessel lumen of the celiac trunk (CT), superior mesentery artery (SMA) or inferior mesentery artery (IMA) on CT-angiography with maximum slice thickness of 3mm.*

Mucosal saturation measurements

VLS measurements were performed in all patients with PVT and in the five patients with severe cirrhosis and portal hypertension. VLS measurements of the historical controls were used as reference population. Decreased VLS measurements in at least one location of the gastrointestinal tract were found in 12 out of 16 (75%) patients with PVT. Compared to the reference population (median 60.0 [56.2-61.7]), mucosal saturation measurements were mostly decreased in the descending duodenum for both patients with PVT (median 55.5 [52.3-58.8], *p=0.02*) and patients with cirrhosis (median 52.0 [46.5-54.0], *p=0.003*) (see Figure 9.1). The median VLS measurement of patients with PVT (median 62.0 [60.0-65.8]) and cirrhosis (median 57.0 [55.0-60.0]) were also significantly lower in the antrum compared to the historic reference population (median 65.9 [64.2-69.3], *p=0.01* and *p<0.001* respectively). There was a significant difference in median VLS measurements in the duodenal bulb between patients with cirrhosis and portal hypertension (median 58.0 [50.5-61.0]) compared to the historic reference population (median 62.9 [59.2-66.9], *p=0.02*), but not between patients with PVT (median 60.5 [59.3-64.0]) and the reference population (*p=0.5*)). There seemed to be an association between presence of postprandial abdominal pain and/or exercise-induced ab-

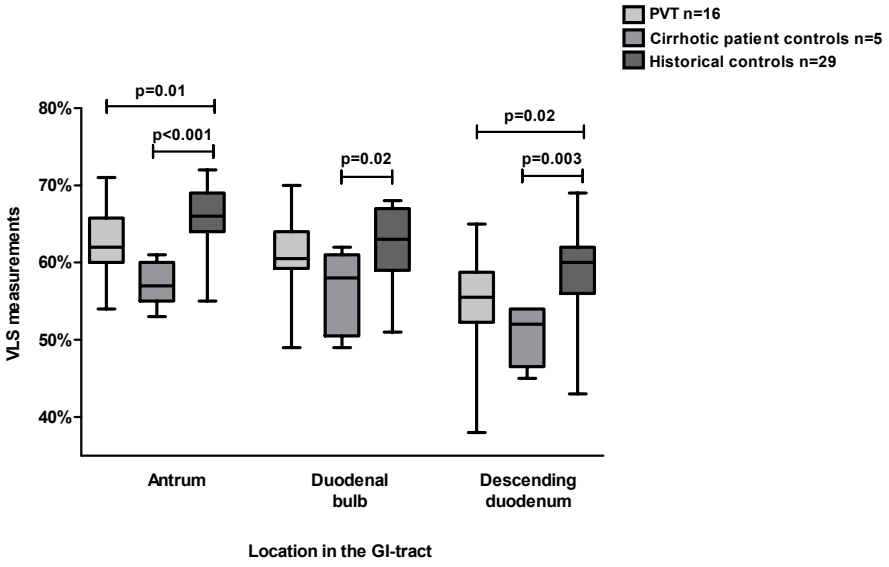

Figure 9.1 *Visible light spectroscopy (VLS) measurements in patients with portal vein thrombosis (PVT), cirrhotic patient controls and in the reference population. VLS measurements performed at three locations in the gastrointestinal tract for all three groups of patients. VLS measurements were significantly decreased for patients with PVT compared to the reference population in the antrum and descending duodenum, but not in the duodenal bulb. Compared to the reference population, patients with portal hypertension had decreased VLS measurements in all locations, indicating mucosal ischemia. Boxes represent medians with interquartile range, whiskers extend to the most and least extreme scores respectively.*

dominal pain and decreased VLS measurements in the descending duodenum. Patients with abdominal pain tended to have lower VLS measurements than patients without abdominal pain (median 54.0 [48.8-57.3] vs median 58.5 [53.8-60.5], *p=0.09*). Of the ten patients with PVT with postprandial abdominal pain and/or exercise-induced abdominal pain, nine (90%) had decreased VLS measurements, of which six at more than one location in the gastrointestinal tract.

Characteristics of ischemic colitis

Colonoscopy was performed in three out of 16 patients with PVT at the moment of VLS measurements. Two patients had abnormal findings. In one patient, signs of ulceration and colitis were seen in the descending colon attributed to ischemia, which was confirmed by histological tissue evaluation. This patient (ID 2) was diagnosed with a complete occlusion of a side branch of the PV, SMV, and SV. This patient also had decreased VLS measurements in the descending duodenum. In the second patient (ID 3), colonoscopy revealed mucosal edema and erythema in the distal colon and sigmoid, suggestive for colitis due to congestion. This patient had a complete occlusion of the PV and SMV, and decreased VLS measurements of the duodenum. None of the cirrhotic patients had signs of ischemic colitis during colonoscopy.

Factors associated with VLS measurements

Patients with an extension of the thrombus into the SMV and/or SV had significantly lower VLS measurements in the antrum and descending duodenum compared to the reference population, indicating mucosal ischemia (*p=0.01* and *p=0.009*, respectively) (see Figure 9.2). There was no difference in VLS measurements between patients with PVT without extension compared to the historical controls. Furthermore, no difference in VLS measurements was observed in patients with extension of the PVT into the SMV and/or SV compared to patients without extension of thrombus outside the PV.

Patients who were treated with OAC had higher VLS measurements compared to those who were not or partly (covered time <99%) treated, (median 58.0 [53.5-59.5] vs 53.0 [49.0-57.0], *p=0.096* in the descending duodenum) (see Figure 9.3). Patients who were not or partly treated with OAC had significantly lower VLS measurements in the antrum and descending duodenum compared to the reference population, indicating mucosal ischemia (*p=0.007* and *p=0.003*, respectively). The use of OAC in patients with PVT results in a relative risk difference of 19% in decrease of VLS measurements. Hence, the number needed to treat with anticoagulants to prevent decreased VLS measurements in one patient is 5.3.

Abdominal pain was reported in a lower proportion of patients using OAC compared to patients without anticoagulation, but this difference was not statistically significant (56% vs 71%, *p=0.6*).

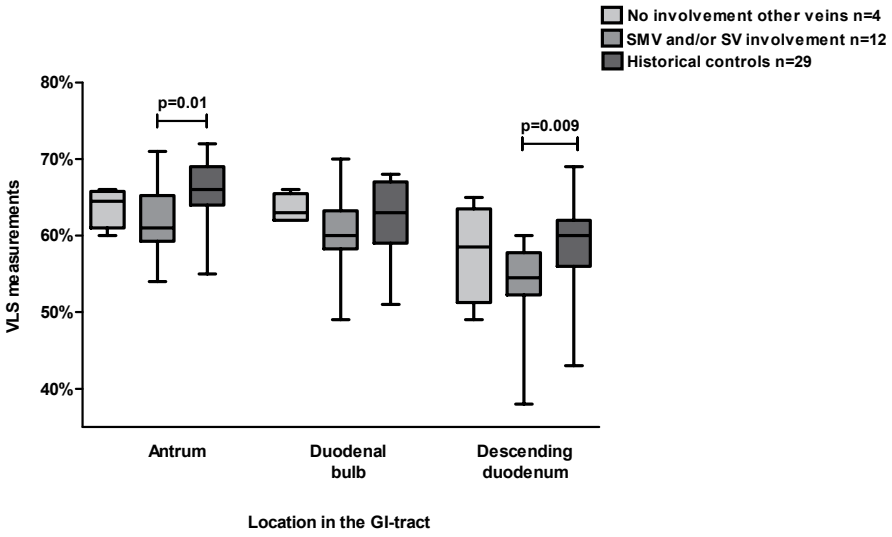

Figure 9.2 *VLS measurements and extent of venous thrombosis. Patients with PVT with an extension of the thrombus into the superior mesenteric vein (SMV) and/or splenic vein (SV) had significantly lower VLS measurements in the antrum and descending duodenum compared to the reference population, indicating mucosal ischemia. Boxes represent medians with interquartile range, whiskers extend to the most and least extreme scores respectively.*

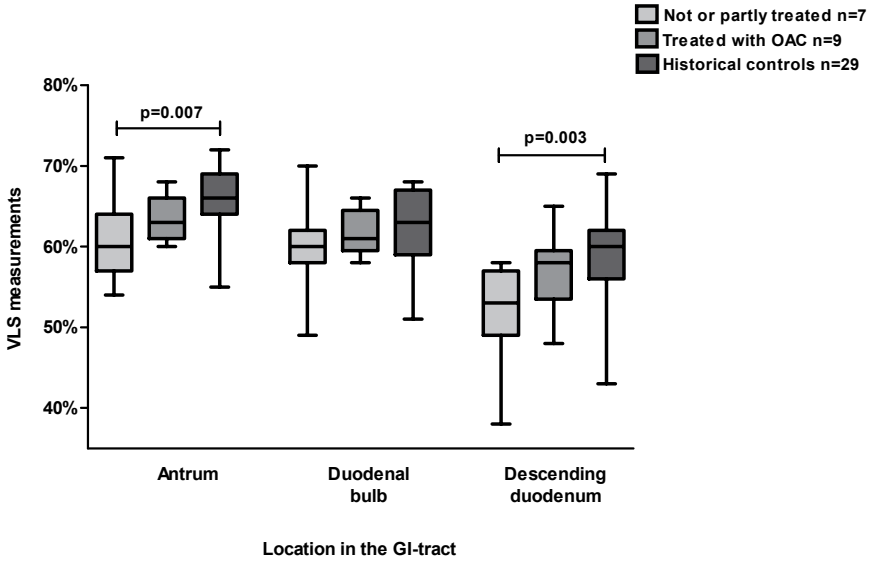

Figure 9.3 *VLS measurements in relation to use of antithrombotic therapy. Patients who were not or partly treated with oral anticoagulation (OAC) had significantly lower VLS measurements in the antrum and descending duodenum compared to the reference population, indicating mucosal ischemia. Boxes represent medians with interquartile range, whiskers extent to the most and least extreme scores respectively.*

Presence of an inherited hypercoagulable risk factor resulted in decreased VLS measurements at all locations in three out of six (50%) patients. In two of the three remaining patients with an inherited risk factor, decreased measurements in the descending duodenum – and antrum in one patient – were observed. One patient with an inherited risk factor did not have decreased VLS measurements, however this patient had an incomplete occlusion of the PV. Patients with a known inherited hypercoagulable risk factor more often reported abdominal pain typical for gastrointestinal ischemia compared to patients without an inherited hypercoagulable risk factor (83% vs 44%, *p=0.3*).

Follow-up after VLS measurements

After a median follow-up time of 51 (11-59) months, 15 of the 16 patients with PVT were still alive. One patient developed multi-organ failure due to renal insufficiency and died of pneumonia and sepsis.

Ischemic events occurred in two patients. One patient (ID 13) presented with an acute small bowel ileus at time of diagnosis of PVT, which was treated conservatively. Four months after diagnosis, due to ongoing symptoms, an explorative laparotomy was performed which showed a complete stenosis of the jejunum as well as thrombosis of the draining mesenteric veins. A side-to-side jejuno-jejunostomy was performed proximally of the stenosis. The second patient (ID 2) presented with melena one month after the VLS measurements. Distal enteroscopy showed signs of ischemic colitis for which the patient was treated conservatively. In both patients decreased VLS measurements were observed in at least one location of the gastrointestinal tract.

Gastrointestinal bleeding events occurred in three patients and were all related to variceal bleeding (ID 1, 2, and 9). One patient did not use OAC at time of bleeding. However, in the fourth year of follow-up, the patient developed deep vein thrombosis (DVT), for which OAC treatment was initiated. Two patients were using OAC at time of bleeding. In one of these patients, OAC had to be discontinued, which resulted in a DVT five months later.

DISCUSSION

Gastrointestinal ischemia is a concerning complication of PVT. The occurrence and risks of gastrointestinal ischemia have in particular been acknowledged in patients with acute PVT.[1-3, 5, 6] The clinical spectrum of gastrointestinal ischemia in patients with chronic PVT is hitherto unknown. Therefore, we performed a unique prospective study in a well-characterized cohort of PVT patients in which clinical symptoms for gastrointestinal ischemia were assessed using a structured questionnaire, imaging of the mesenteric vasculature and VLS measurements. The latter enabled us to objectively and

quantitatively determine the presence and extent of gastrointestinal mucosal ischemia. Patients presenting with non-malignant, non-cirrhotic PVT are quite rare, but we were able to include 15 patients meeting our inclusion criteria during a period of 24 months. The rarity of the disease made it difficult to compose a homogenous group. Therefore, we included patients with both acute and chronic PVT, patients on anticoagulation versus no anticoagulation, and complete versus partial occlusion of PV.

Nevertheless, this is one of the few studies with prospective data on the occurrence of gastrointestinal ischemia in a population of patients with PVT. In our study, 15 out of 16 patients were known with chronic PVT at time of the VLS measurements. We found that postprandial abdominal pain and exercise-induced pain are common in patients with chronic PVT and that mucosal ischemia is often present in these patients, especially in the antrum and descending duodenum. We also found that patients with a thrombus extending into the SMV and/or SV more often report abdominal pain than patients without extension of the thrombus. Also, patients with extension of the thrombus into the SMV and/or SV had significantly lower VLS measurements in the antrum and descending duodenum compared to the reference population.

The findings presented in this study warrant caution in the patient with PVT: onset of abdominal symptoms such as postprandial pain should prompt the physician to re-evaluate extent, cause and treatment of PVT. For instance, one should be vigilant of a possible thrombus extension into the SMV, as this is associated with worse outcome.[18] The results of our study show that involvement of the SMV and/or SV in patients with PVT can be detected by VLS measurements, however this was not the case in patients with PVT without thrombus extension. This suggests that mucosal ischemia could be more prevalent in patients with PVT with thrombus extension. Also, one should be aware of presence of a systemic hypercoagulable state, as this is associated with thrombosis of the smaller venules. Hypercoagulability results in thrombosis beginning in the intramural venules, venous arcades, and vasa recta, often without involvement of the larger venous vessels. Occlusion of these small veins, easily missed on conventional CT-A or MR-A,[19] results in compromised venous drainage and is associated with an increased risk of bowel infarction.[2, 11, 20] Indeed, we found decreased VLS measurements in five out of six patients with an inherited hypercoagulable risk factor. Finally, we observed that VLS measurements were higher in patients treated with OAC compared to patients who were not or only partly treated, although this difference was not statistically significant, suggesting that there might be a positive effect of OAC on gastrointestinal ischemia in patients with chronic PVT. The reported NNT needs to be interpreted with caution given it is based on a very limited number of patients. Combined with the demonstrated beneficial effects of anticoagulation in chronic PVT in previous studies, showing a reduction in thrombotic events and splanchnic venous infarction without increasing the risk of gastrointestinal bleeding, it is tempting to advocate for a more prominent role of OAC in the treatment of patients with chronic PVT.[21, 22]

In order to extrapolate our findings into daily practice, it is important to address a few issues. First, patients with chronic PVT by definition have portal hypertension leading to venous congestion, which may result in decreased VLS measurements. This might be attributed to the presence of portal hypertension, since decreased VLS measurements were also observed in patients with cirrhosis. Furthermore, patients with cirrhosis had even lower VLS measurements than patients with PVT. Gastrointestinal ischemia in patients with portal hypertension might be attributed to the substantial circulatory imbalance between vasoactive mediators, such as endothelin and nitric oxide synthetase, due to the affected liver parenchyma in cirrhosis that may lead to vascular dysfunction in the gastric antrum.[23, 24] Nonetheless, intestinal infarction is not a common complication of cirrhotic portal hypertension. This suggests compensatory mechanisms in these patients, which are lacking in patients with non-cirrhotic portal hypertension such as PVT. However, an in-depth studiy of the differences between cirrhotic and non-cirrhotic portal hypertension was beyond the scope of this study.

Second, we used historical controls as a reference group for VLS measurements as concurrent controls were not available. These controls were derived from the study by van Noord et al.[12] The VLS measurements in this group were performed in the same fashion as in the patients with PVT and cirrhotic patient controls, using the same protocol of measuring, devices, and equipment. Nevertheless, we cannot exclude the possibility that the inclusion of historical controls, instead of concurrent controls, might have led to a selection bias. Therefore, caution is needed in the interpretation of these results.

Finally, cardiovascular risk factors for atherosclerosis, especially DM type II and obesity, are associated with occurrence of PVT and intestinal infarction.[20, 25, 26] In our study, only one patient had diabetes. However, given the limited number of patients, we cannot exclude the possibility that cardiovascular risk profile is associated with PVT-related gastrointestinal ischemia.

To our knowledge, this is the first study which quantitatively assessed the presence of gastrointestinal ischemia in patients with PVT. VLS measures capillary hemoglobin oxygen saturation and is a validated diagnostic method with a high sensitivity to assess the presence of gastrointestinal ischemia. The established cut-off values for each of the specific sites of the mesenteric tract were previously calculated by van Noord et al. These cut-off values were based on a trainee data set obtained from patients diagnosed with gastrointestinal ischemia using gastric tonometry and the values were additionally validated in a confirmation cohort.[12] Events that decrease the delivery of oxygen to the mesenteric mucosa (i.e. concomitant cardiopulmonary diseases) will result in lower mucosal hemoglobin oxygen saturations. Therefore, peripheral oxygen saturation and heart rate are continuously monitored during VLS measurements. In order to minimize the effect of modifying factors such as concomitant cardiopulmonary diseases, peripheral saturation is kept above 94% by administering oxygen (FiO_2 21%), if necessary. Factors that may affect VLS measurements are luminal spasms and bile

acid remnants. Therefore, butylscopolamine is administered intravenously prior to the start of VLS measurements and these measurements are performed after irrigation of the target area. There is no difference in VLS measurements between younger and older patients, as VLS measurements are known to be affected only by the above mentioned factors and by presence of a significant stenosis of the mesenteric vasculature. As VLS measurements were performed at one time point only, it would be interesting to study the time course of gastrointestinal ischemia in patients with PVT, for instance before and after initiation of anticoagulation. Nonetheless, we were able to collect follow-up data in all patients, enabling us to assess occurrence of bleeding events, recurrent thrombosis, ischemic complications, and mortality.

In future studies, it would be interesting to perform colonoscopy in all patients with PVT to assess ischemic changes in the colonic mucosa. Because bloody diarrhoea is only rarely observed in gastrointestinal ischemic patients, colonoscopy is now only included in our standard work up of the patient in case of lower GI symptoms. Furthermore, we believe it would be interesting to quantify the potential presence of ischemia in the colon by colonoscopy and also by VLS in future studies. However, currently VLS measurements are only validated in the upper GI tract and have not yet been validated in the colon.

In addition, future studies should look into interactions between the factors associated with VLS measurements. Unfortunately, we were not able to look into this further due to the limited number of patients included in our cohort.

In conclusion, gastrointestinal ischemia in patients with chronic PVT is more frequently encountered than previously assumed. Albeit in a small cohort, mucosal ischemia was detected in 75% of patients with PVT. Furthermore, two patients experienced an ischemic event. To increase generalizability, future studies assessing patients with PVT should put more focus on the diagnosis and treatment of gastrointestinal ischemia.

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Part IV

Summary, general discussion and appendices

Chapter 10

10

Summary and general discussion

This thesis aimed to provide more insight in the role of thrombophilia and genetic variations in the pathogenesis and outcome of liver disease. The first part of the thesis focused on the prevalence and factors associated with liver fibrosis in the general population. In part two of this thesis, we addressed the association between thrombophilia and liver fibrogenesis. Finally, in the third part, the role of new genetic risk factors in SVT and the occurrence of gastrointestinal ischemia in portal vein thrombosis were discussed. In the current chapter, the main findings of our studies will be summarized and discussed. Furthermore, directions for future research will be presented.

PREVALENCE AND RISK FACTORS OF LIVER FIBROSIS IN THE GENERAL POPULATION

Worldwide, each year over 1.7 million persons die due to cirrhosis and/or liver cancer (1). It is estimated that approximately 29 million persons are currently suffering from chronic liver disease in the European Union (2). In the next years, the burden of chronic liver diseases is projected to increase due to an expected, ongoing, increment in the number of persons suffering from non-alcoholic fatty liver disease (NAFLD). This increase in prevalence of NAFLD will parallel the rise in the number of individuals affected by obesity and diabetes mellitus. As individuals with NAFLD, particularly those with concomitant diabetes mellitus, are at risk of progression to non-alcoholic steatohepatitis (NASH) and advanced fibrosis, the number of individuals with chronic liver injury will rise significantly in the years to come (3, 4).

The exact number of individuals suffering from chronic liver disease is not well known. For decades, it was not feasible to study the presence of liver fibrosis in healthy individuals, due to the lack of non-invasive tools to detect liver fibrosis. As a result, the prevalence of liver fibrosis in the general population was unknown. The introduction of transient elastography (TE) (Fibroscan®, Echosens™, France) enabled non-invasive, fast and reproducible assessment of fibrosis, paving the way for studies investigating fibrosis without the morbidity and mortality associated with liver biopsy (5-8). In **chapter 2**, we investigated the distribution of and risk factors associated with clinically relevant liver fibrosis, using TE, in a large population-based cohort of Caucasian individuals. This study, as well as the studies described in chapter 3, 5, and 6 of this thesis, were part of the Rotterdam Study, a large, ongoing, prospective population-based cohort study. The main objective of the Rotterdam Study is to investigate the prevalence and incidence of and risk factors for chronic diseases in the elderly (9). For this purpose, all individuals living in Ommoord, a suburb of the city of Rotterdam, aged 55 years and older were asked to participate at the start of this study in 1990. Of all invitees, 78% (7,983/10,275) agreed to participate in the study. In 2000, a second cohort was added to the Rotterdam Study including participants who had turned 55 years of age or had moved into the study district. A third cohort consisting of participants of 45 years and over was added to the

study in 2006. Each participant visits the designated research center every 3-5 years. During each visit, a uniform and extensive evaluation is conducted in all participants, including a home interview and a range of physical examinations. As of the most recent research center visit of all three cohorts, abdominal ultrasonography and a liver stiffness (LS) measurement were added to this evaluation for all participants.

We found that clinically relevant liver fibrosis, defined by a LS measurement of ≥8.0 kPa, was present in 5.6% of 3,041 consecutive participants that visited the research center between January 2011 and September 2013. Higher age, presence of diabetes mellitus and/or steatosis, higher ALT, larger spleen size, current or former smoking, and positive viral serology for hepatitis B and/or C were all independent risk factors of clinically relevant fibrosis. These results were comparable to the results of two smaller previously reported studies assessing liver fibrosis by means of TE in healthy volunteers attending a free medical check-up in France and in a community-based study in Hong Kong Chinese subjects (10, 11). Taking the identified risk factors of fibrosis into account, we calculated the predicted probability of fibrosis for all participants. This probability increased with age and with the presence of steatosis or diabetes mellitus. Participants with both steatosis and diabetes mellitus had the highest probability of fibrosis, irrespective of age. In this subgroup, the overall probability of clinically relevant fibrosis was 17.2% (12.5-23.4%). These findings highlight the impact of obesity and diabetes mellitus – modifiable risk factors – on fibrogenesis in the general population. Next to that, the observation that probability of fibrosis did not increase with age in participants with concurrent steatosis and diabetes mellitus implicates that the relative burden of these risk factors is the highest for the youngest participants in our cohort, aged 50-60 years. In the context of an ageing population with an increasing prevalence of obesity and diabetes mellitus, these findings suggest that liver fibrosis and its complications will become a more prominent public health issue in the nearby future. Our results therefore advocate the importance of targeting obesity and insulin resistance in order to prevent liver fibrosis.

Genetics in liver fibrosis

Recent technical advancements and the unravelling of the human genome have enabled hypothesis-free testing of common variants or single nucleotide polymorphisms (SNPs) throughout the genome for an association with complex traits and diseases. Since then, several genome-wide association studies (GWAS) have been performed in at-risk populations, including patients with viral hepatitis, alcoholic liver disease, cholestatic liver diseases, gallstones, and NAFLD (12-22). In addition, multiple candidate SNP studies have been performed in patients with chronic liver injury. Several of these studies identified SNPs predisposing to liver fibrosis in at-risk populations (13, 16, 17, 20, 23-25). However, it is unknown whether these SNPs affect fibrogenesis by interacting with the agents causing chronic liver injury, for instance alcohol or viral hepatitis,

or whether these SNPs inflict fibrogenesis independently of other factors. Therefore, we studied the association between these fibrosis-associated SNPs and LS in the Rotterdam Study in **chapter 3**. We found that two linked SNPs in the interferon gamma receptor 2 (*IFNGR2*) gene, *rs9976971* and *rs2284553,* were independently associated with increasing LS in this otherwise healthy Caucasian population. Exclusion of participants with positive serology for hepatitis B and/or C and those with an excessive alcohol intake did not alter these results, suggesting that the SNPs in the *IFNGR2* gene affect fibrogenesis independently of presence of chronic liver injury. The association between *IFNGR2* and LS was also observed in a subgroup of participants with NAFLD, as a third SNP in the *IFNGR2* gene, *rs9808753,* was independently associated with LS in this subgroup. In addition, we discovered that the association between these three *IFNGR2* SNPs and fibrosis was significantly modified by body mass index. The association between the *IFNGR2* SNPs and fibrosis was stronger for overweight (for *rs9808753*) and obese (for *rs9976971* and *rs2284553*) participants compared to the total cohort. This finding might be explained by the presence of a systemic, chronic, pro-inflammatory state in obesity, which on its turn could reinforce the effect of interferon gamma on liver fibrosis development (26, 27). Future studies should aim at exploring the mechanism by which *IFNGR2* influences fibrogenesis, as this mechanism may give clues for the development of interferon gamma-based antifibrotic therapy.

THROMBOPHILIA IN LIVER FIBROGENESIS

Liver fibrosis is a multifactorial disorder. Besides chronic liver injury and genetic susceptibility, other factors, including hypercoagulability, are considered important in its pathogenesis. The first evidence for a role of hypercoagulability in fibrogenesis came from the observation that patients with concurrent chronic hepatitis C and haemophilia were slow progressors of liver disease (28, 29). Later, several studies showed that presence of prothrombotic mutations – including the factor V Leiden polymorphism, prothrombin G20210A variant, and blood group type non-O – increased development and/or progression of fibrosis in patients with chronic viral hepatitis and NAFLD (30-36). In **chapter 4**, we summarized the available evidence on the effect of anticoagulant drugs on fibrogenesis. Overall, studies suggest that administration of anticoagulant drugs could prevent fibrogenesis and possibly also reverse already existing fibrosis (37-43). In addition, we described the hypotheses postulated in literature regarding the mechanism by which hypercoagulability could influence liver fibrogenesis, in which thrombin is a key player.

To further explore the role of thrombophilia in liver fibrogenesis, we studied the association between common prothrombotic genetic risk factors and liver fibrosis in participants of the Rotterdam Study cohort in **chapter 5**. Presence of the factor V Leiden mutation or prothrombin G20210A gene variant was associated with a twofold-increased

risk of clinically relevant fibrosis in this Caucasian population-based study cohort. This association was independent of other risk factors for liver fibrosis, including presence of steatosis, diabetes mellitus, smoking, age, ALT level, and alcohol intake. Combined presence of blood group type non-O and the factor V Leiden mutation or prothrombin G20210A gene variant resulted in an even higher risk of fibrosis. Participants with both blood group type non-O and the factor V Leiden mutation or prothrombin G20210A gene variant had a predicted probability of 14.3% of having fibrosis, which was significantly higher than the probability for those with blood group type O and/or those without the factor V Leiden mutation or prothrombin G20210A gene variant. These results may be explained by the fact that both the factor V Leiden mutation and blood group type non-O reduce sensitivity for activated protein C, causing an additional increase in procoagulant capacity if both factors are present (44-46). We concluded that these findings suggest that thrombophilia is an important factor in liver fibrogenesis in the general population. Therefore, one may suggest screening for common prothrombotic genetic risk factors in individuals with liver fibrosis of unknown etiology, as these individuals could potentially benefit from anticoagulant therapy.

Von Willebrand factor (VWF) is a key player in hemostasis, as it is involved in platelet adhesion, thrombus formation and binding of clotting factor VIII, thereby preventing its clearance (47, 48). Previous studies have shown that high levels of VWF are associated with an increased risk of both venous and arterial thrombosis (49-51). It is known that VWF levels are increased in patients with cirrhosis. In these patients, VWF levels correlate with the hepatic venous pressure gradient and can even predict clinical outcomes (52, 53). In **chapter 6**, we showed that increased VWF levels were associated with liver fibrosis after 10 years in the general population and in a subgroup of individuals with steatosis. Recently, a study in patients with hepatitis C showed that VWF levels increase with increasing fibrosis stage (54). However, the nature of the relationship between VWF levels and fibrosis and cirrhosis remains to be elucidated. Future studies are therefore warranted unravelling the mechanism behind the association of VWF levels with fibrosis and cirrhosis. Next to that, as our study suggests that VWF might be used as marker of preclinical liver fibrosis in the general population, future studies should assess the role of VWF levels in the prediction of development and progression of fibrosis. Finally, it remains to be determined whether VWF levels can also be used as predictor of clinical outcomes in individuals with fibrosis.

ETIOLOGY AND ISCHEMIC COMPLICATIONS OF VASCULAR LIVER DISEASES

Budd-Chiari syndrome (BCS) and non-malignant, non-cirrhotic portal vein thrombosis (PVT), collectively called splanchnic vein thrombosis (SVT), are rare forms of venous thrombosis of the hepatic venous outflow tract and the portal vein respectively. SVT is associated with considerable morbidity and mortality (55, 56). However, due to the low

prevalence of these disorders, few prospective studies have been performed assessing their etiology, diagnosis, treatment and prognosis. In chapter 4, we describe the role of anticoagulant therapy in the treatment of vascular liver diseases, including BCS and PVT, based on currently available data.

Like in common venous thrombosis, systemic prothrombotic risk factors are pivotal in the etiology of SVT. Within this group of risk factors, genetic and acquired conditions are distinguished. Myeloproliferative neoplasms (MPNs) are the most common acquired risk factor for SVT with a prevalence of 20-50% (57, 58). Diagnosing MPNs in patients with SVT is important, given the prognostic and therapeutic implications regarding anticoagulant therapy (59, 60). However, detecting presence of MPNs is often challenging in clinical practice as portal hypertension resulting from the obstruction of the hepatic veins and/or portal vein causes hypersplenism and hemodilution. These conditions may conceal the characteristic peripheral blood cell changes associated with MPN, such as thrombocytosis or polycythemia (61). The discovery of the *JAK2V617F* mutation has greatly improved the ability to non-invasively detect MPNs in patients with SVT (62-64). However, diagnosing MPNs remains difficult in the absence of the *JAK2V617F* mutation (59, 65). Recently, exome sequencing resulted in the detection of somatic mutations in *CALR* in patients with MPN lacking *JAK2V617F* and *MPL* mutations (66, 67). We hypothesized therefore that identification of *CALR* mutations in patients with SVT could improve the ability to diagnose MPNs in these patients. In **chapter 7**, we evaluated the prevalence and role of *CALR* mutations in patients with SVT. This study was based on data from the European Network for Vascular Disorders of the Liver (EN-Vie). For the EN-Vie study cohort, patients with BCS and non-malignant, non-cirrhotic PVT were prospectively enrolled from nine European countries. Data on etiology, clinical presentation, diagnosis and follow-up was systematically collected for all patients. In addition, DNA and plasma was stored for future analyses (57, 58). Using these DNA-samples, we found that *CALR* mutations are rare in patients with SVT, as only one patient had a *CALR* mutation in our cohort. As a result, prevalence of *CALR* mutations was 0.7% in the total cohort and 2.3% in patients with MPN. Our findings were confirmed by other studies, the reported prevalence of *CALR* mutations in patients with SVT ranged between 0 and 2% (68-72). This low prevalence might be attributable to the fact that patients with a *CALR* mutation have a lower risk of thrombosis compared to patients with a *JAK2V617F* mutation. This could be explained by the presence of lower hemoglobin and white blood cell counts in patients with a *CALR* mutation compared to MPN patients without a *CALR* mutation (66, 67). Nonetheless, we believe that testing for *CALR* mutations should be performed in patients with *JAK2V617F* negative SVT, as screening for somatic *CALR* mutations is an easy to perform diagnostic method that could aid in the challenging diagnosis of MPN with limited burden for the patient. The recently published consensus guidelines from the Baveno VI faculty also recommend screening for presence of *CALR* mutations in *JAK2V617F* negative SVT patients (73).

Despite the fact that multiple genetic and acquired risk factors have been identified, in 15-25% of patients with SVT no etiologic factor can be detected (57, 58), underlining the need for studies investigating new risk factors. Given the fact that many risk factors of common venous thrombosis are also recognized as etiologic factors in SVT, we investigated the role of novel candidate SNPs, known to increase the risk of common venous thrombosis, as risk factors for SVT in **chapter 8**. We genotyped six SNPs – in the *ABO, STXBP5*, *VWF, CYP4V2, GP6,* and *SERPINC1* gene – known to increase the risk of common venous thrombosis using DNA samples from patients and controls of the EN-Vie study cohort (74-77). In this cohort, the SNP determining blood group type was associated with an increased risk of PVT, but not BCS. However, this finding could not be confirmed in a validation cohort consisting of patients diagnosed with SVT in three large tertiary referral clinics in France, Spain and the Netherlands. The other five SNPs were not associated with an increased risk of either PVT or BCS in the EN-Vie cohort. Therefore, none of the six SNPs can be considered a risk factor for BCS or PVT. These findings suggest that other local and systemic risk factors are of more importance in the etiology of SVT. The identification of these differences in etiology between SVT and common venous thrombosis contributes to a better understanding of the pathogenesis of venous thrombosis at different sites and supports the site-specificity of venous thrombosis.

Gastrointestinal ischemia is considered a concerning adverse event of PVT, as it can result in intestinal infarction, a life-threatening condition (78). However, the prevalence and characteristics of gastrointestinal ischemia in PVT are not well known. The reported prevalence of gastrointestinal ischemia in patients with acute PVT ranges between 2% and 32%, with mortality rates of 0-20% (58, 79-84). In chronic PVT, studies are more scarce and report conflicting results. Some assume that ischemia is unlikely to occur in these patients due to presence of a collateral circulation, while others did find a portal cavernoma in nearly half of the SVT patients presenting with ischemia (78, 80). The paucity of data on the occurrence of ischemia in PVT results, at least in part, from a lack of diagnostic tools. As clinical symptoms of ischemia are often atypical and comparable to the clinical findings in patients with PVT, it is difficult to diagnose ischemia based on clinical symptoms solely. The introduction of visible light spectroscopy (VLS) offers a new minimally invasive tool in diagnosing gastrointestinal ischemia in PVT (85). VLS enables objective and direct quantification of ischemia in the gastrointestinal tract, by measuring the adequacy of mucosal perfusion during gastroscopy (85, 86). Therefore, we prospectively determined the presence and characteristics of gastrointestinal ischemia in patients with PVT using VLS in **chapter 9**. Gastrointestinal ischemia was frequent in these patients with mainly chronic PVT, as a decreased mucosal oxygenation was found in 75% of patients in at least 1 location of the gastrointestinal tract. VLS measurements were mostly decreased in the descending duodenum. Symptoms typical for ischemia, such as postprandial pain and exercise-induced pain, were present in 63% of patients. We found that extension of thrombus into the superior mesenteric

vein and splenic vein and/or presence of hypercoagulability were associated with lower VLS measurements in patients with PVT compared to historical controls. Next to that, VLS measurements were higher in patients treated with oral anticoagulation than in those who were not or only partly treated, but this difference did not reach statistical significance. However, this finding suggests that there might be a positive effect of oral anticoagulation on the occurrence of ischemia in patients with chronic PVT. Confirmation of these results in larger studies is warranted, but our findings suggest that onset of abdominal symptoms, such as postprandial pain, should prompt the physician to re-evaluate extent, cause, and treatment of PVT.

DIRECTIONS FOR FUTURE RESEARCH

As described in this thesis, liver fibrosis affects approximately 1 in 18 older Caucasian adults in Rotterdam. In particular, individuals with steatosis and diabetes mellitus are at risk, as their probability of fibrosis is as high as 17%. Given the current increase in prevalence of steatosis and diabetes mellitus in the Western world, the prevalence of NAFLD-induced fibrosis is expected to increase and become a major public health problem (4). Wide implementation of prevention programs targeting obesity, insulin resistance, and its complications is therefore urgently needed. Lifestyle interventions, most importantly diet and exercise, are the cornerstone of the management of NAFLD, but adherence to these lifestyle measures is often challenging (4). A recent trial in patients with coronary heart disease showed that frequent semipersonalized test messages improved cardiovascular disease risk factors, including BMI, smoking and physical activity (87). Investigating the efficacy of such lifestyle-focused support programs might prove to be beneficial in improving the adherence to lifestyle interventions in patients with NAFLD. Despite multiple studies, no drug has yet been approved for treatment of these patients, limiting other therapeutic options. Studies evaluating mortality rates associated with fibrosis in the general population will aid in assessing the impact of this public health issue.

During the last decade, there has been an exponential increase in the number of studies investigating the role of genetics in various complex diseases, including liver diseases. However, the currently available studies only represent the first steps in unravelling the complex interplay between genetic variations and liver diseases. Therefore, many promising fields remain to be explored. With respect to liver fibrosis, several SNPs have currently been identified as risk factors of fibrosis (13, 16, 17, 20, 23-25). It would be very interesting to perform a GWAS within the Rotterdam Study to identify genetic variants contributing to the development of liver fibrosis in the general population. In addition, as techniques in mapping the human genome are rapidly evolving, it is now possible to sequence exomes and even whole genomes. Therefore, these new techniques will offer more possibilities to identify loci associated with various liver diseases in the years to come.

The observation that administration of low molecular weight heparin reduced the incidence of liver decompensation and improved survival in cirrhosis (43) warrants further investigation of the potential beneficial role of anticoagulant drugs in these patients in larger, double-blind trials. These studies should also include an objective assessment of portal hypertension. Additional research questions that remain to be answered include the effect of other anticoagulant drugs, including new oral anticoagulants (NOACs), and the optimal treatment dose and duration. Furthermore, as studies also showed a potential beneficial effect of anticoagulant drugs on liver fibrosis (37-42), it would be desirable to study the effect of anticoagulant medication on fibrosis in larger populations, including at-risk groups and population-based studies.

In patients with PVT, long-term anticoagulant therapy is currently recommended only in patients with a persistent prothrombotic condition, recurrent thrombosis or intestinal infarction (73). Prospective studies assessing risks and benefits of anticoagulant therapy in patients with chronic non-malignant, non-cirrhotic PVT without these conditions are warranted, as the role of anticoagulant treatment in the management of these patients is not known. In recent years, two new types of direct acting oral anticoagulants were introduced: direct thrombin inhibitors and factor Xa inhibitors. A major advantage of these new drugs is that they do not require laboratory monitoring, have fewer drug-drug and drug-food interactions, and have a wider therapeutic range. In a large meta-analysis in almost 28,000 participants, these NOACs were as effective as standard anticoagulation in the treatment of deep venous thrombosis (88). Although an increased risk of gastrointestinal bleeding has been described in patients treated with NOACs (89), studies in patients with venous thromboembolism reported a comparable or even decreased risk of bleeding associated with NOACs when compared to the standard treatment with vitamin K antagonists (88, 90, 91). Use of these NOACs in patients with common venous thrombosis is currently implemented in clinical practice. It is important to determine the place of these new oral anticoagulants in the treatment of patients with SVT. Therefore, trials assessing the efficacy and safety of these new oral anticoagulants in comparison to standard anticoagulation should be initiated in patients with SVT.

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Chapter 11

11

Samenvatting en discussie

Het doel van dit proefschrift was om meer inzicht te geven in de rol van trombofilie en genetische varianten in de pathogenese en uitkomst van leverziekten. Het eerste deel van het proefschrift focuste zich op de prevalentie en factoren geassocieerd met lever fibrose in de algemene bevolking. In deel twee van dit proefschrift hebben we de associatie tussen trombofilie en lever fibrogenese behandeld. Tot slot werden in het derde deel de rol van nieuwe genetische risicofactoren in splanchnische veneuze trombose (SVT) en het voorkomen van gastro-intestinale ischemie bij vena portae trombose besproken. In dit hoofdstuk zullen de belangrijkste bevindingen van onze studies worden samengevat en bediscussieerd. Verder zullen suggesties voor toekomstig onderzoek worden gepresenteerd.

PREVALENTIE EN RISICOFACTOREN VAN LEVER FIBROSE IN DE ALGEMENE BEVOLKING

Wereldwijd sterven jaarlijks 1.7 miljoen personen aan de gevolgen van cirrose en/of leverkanker (1). Naar schatting lijden ongeveer 29 miljoen personen in de Europese Unie aan een chronische leverziekte (2). De verwachting is dat de ziektelast ten gevolge van chronische leverziekten de komende jaren zal toenemen door een verdere stijging van het aantal personen met niet-alcoholische vetleverziekte ('non-alcoholic fatty liver disease', NAFLD). Deze stijging van de prevalentie van NAFLD zal parallel lopen aan de toename in het aantal personen met obesitas en diabetes mellitus. Het aantal personen met een chronische leverziekte zal significant toenemen in de komende jaren, omdat personen met NAFLD, in het bijzonder degenen die tevens diabetes mellitus hebben, risico lopen op progressie naar niet-alcoholische steatohepatitis (NASH) en gevorderde fibrose (3, 4).

Het is niet precies bekend hoeveel personen aan een chronische leverziekte lijden. Het was lange tijd niet mogelijk om gezonde personen te onderzoeken op de aanwezigheid van lever fibrose, vanwege het ontbreken van niet-invasieve methoden voor het opsporen van fibrose. Daardoor was de prevalentie van lever fibrose in de algemene bevolking onbekend. De introductie van transiente elastografie ('transient elastography' (TE) (Fibroscan®, Echosens™, France) maakte het mogelijk om aanwezigheid van fibrose te onderzoeken op een niet-invasieve, snelle en reproduceerbare manier. Hierdoor ontstond de mogelijkheid om studies naar fibrose op te zetten zonder de morbiditeit en mortaliteit geassocieerd met lever biopsie (5-8). In **hoofdstuk 2** onderzochten we de verdeling van- en risicofactoren geassocieerd met klinisch relevante lever fibrose, onderzocht met TE, in een groot bevolkingsonderzoek onder Kaukasische personen. Deze studie en de studies beschreven in hoofdstuk 3, 5 en 6 van dit proefschrift waren onderdeel van het Erasmus Rotterdam Gezondheid Onderzoek (ERGO), ook wel de 'Rotterdam Study' genoemd. Het ERGO is een groot, longitudinaal, nog steeds lopend bevolkingsonderzoek. Het belangrijkste doel van het ERGO is het onder-

zoeken van de prevalentie, incidentie en risicofactoren van chronische ziekten bij ouderen (9). Met dit doel werd bij de start van het ERGO aan alle inwoners van Ommoord, een wijk in Rotterdam, van 55 jaar en ouder gevraagd om deel te nemen aan dit bevolkingsonderzoek. Van alle genodigden, besloot 78% (7,983/10,275) om deel te nemen. In 2000 werd een tweede cohort toegevoegd aan het ERGO. Dit tweede cohort bestond uit personen die 55 jaar of ouder waren geworden of waren verhuisd naar Ommoord sinds de start van het eerste ERGO-cohort. Een derde cohort, bestaande uit deelnemers van 45 jaar en ouder, werd toegevoegd aan het onderzoek in 2006. Iedere deelnemer bezoekt het onderzoekscentrum van ERGO elke 3-5 jaar. Tijdens ieder bezoek wordt een uniform en uitgebreide evaluatie uitgevoerd bij alle deelnemers, bestaande uit een thuisinterview en een aantal onderzoeken. Sinds het meest recente bezoek aan het onderzoekscentrum wordt bij alle deelnemers van de drie cohorten ook een echo van de buik en een meting van de leverstijfheid (LS) verricht.

Wij zagen dat klinisch relevante lever fibrose, gedefinieerd als een LS meting van ≥8.0 kPa, aanwezig was in 5.6% van 3041 opeenvolgende deelnemers die het onderzoekscentrum bezochten tussen januari 2011 en september 2013. Een hogere leeftijd, aanwezigheid van diabetes mellitus en/of steatose, hoger ALT, grotere miltdiameter, roken en aanwezigheid van virusserologie voor hepatitis B en/of C waren onafhankelijke risicofactoren voor het hebben van klinisch relevante fibrose. Deze resultaten waren vergelijkbaar met de resultaten van twee kleinere, eerder gepubliceerde studies, waarin lever fibrose werd onderzocht door middel van TE in gezonde vrijwilligers die meededen aan en gratis medische controle in Frankrijk en in een studie met personen uit de Hong Kong Chinese bevolking (10, 11). Voor alle deelnemers in onze studie hebben we de voorspelde waarschijnlijkheid van het hebben van fibrose berekend met inachtneming van de risicofactoren voor fibrose. Deze waarschijnlijkheid nam toe met de leeftijd en met de aanwezigheid van steatose of diabetes mellitus. Deelnemers met zowel steatose als diabetes mellitus hadden de hoogste kans om fibrose te hebben, ongeacht hun leeftijd. De waarschijnlijkheid van het hebben van klinisch relevante fibrose was 17.2% (12.5-23.4%) in deze subgroep van ons cohort. Deze bevindingen onderstrepen de impact van obesitas en diabetes mellitus op fibrogenese in de algemene bevolking. De observatie dat de waarschijnlijkheid van fibrose niet toenam met de leeftijd in deelnemers met zowel steatose als diabetes mellitus impliceert bovendien dat de gevolgen van deze risicofactoren relatief het grootst zijn voor de jongste deelnemers in ons cohort, dat wil zeggen de deelnemers tussen 50 en 60 jaar oud. In het licht van de huidige vergrijzing van de samenleving met daarnaast een stijgende prevalentie van obesitas en diabetes mellitus suggereren deze bevindingen dat lever fibrose en de bijbehorende complicaties een prominenter gezondheidsprobleem voor de samenleving zullen worden in de nabije toekomst. Onze resultaten pleiten daarom voor de aanpak van obesitas en insuline resistentie met het doel lever fibrose te voorkomen.

Genetica in lever fibrose

Recente technologische ontwikkelingen en de ontrafeling van het menselijk genoom hebben het mogelijk gemaakt om zonder hypothese te testen of veelvoorkomende veranderingen van een enkele nucleotide ('single nucleotide polymorphism' (SNP)) in het genoom geassocieerd zijn met het voorkomen van complexe aandoeningen en ziektes. Sindsdien zijn verschillende genoom-brede associatie studies ('genome-wide association studies' (GWAS) verricht in risicopopulaties, zoals patiënten met virale hepatitis, alcoholische leverziekte, cholestatische leverziekten, galstenen en NAFLD (12-22). Daarnaast zijn er verscheidene kandidaat SNP studies uitgevoerd in patiënten met chronische leverschade. In verschillende van deze studies werden SNPs geïdentificeerd die zorgden voor een genetische aanleg voor lever fibrose in risicopopulaties (13, 16, 17, 20, 23-25). Het is echter niet bekend of deze SNPs het ontwikkelen van fibrose beïnvloeden door een interactie met de factoren die chronische leverschade veroorzaken, zoals alcohol en virale hepatitis, of dat deze SNPs de ontwikkeling van fibrose onafhankelijk van andere factoren stimuleren. Daarom hebben wij de associatie tussen deze fibrose-gerelateerde SNPs en LS onderzocht in het ERGO in **hoofdstuk 3.** We vonden dat 2 gelinkte SNPs in het interferon gamma receptor 2 (*IFNGR2*) gen, *rs9976971* en *rs2284553,* onafhankelijk waren geassocieerd met een stijgende LS in deze verder gezonde Kaukasische bevolking. Exclusie van deelnemers met positieve virusserologie voor hepatitis B en/of C en van deelnemers met excessief alcoholgebruik veranderde de resultaten niet. Dit suggereert dat de SNPs in het *IFNGR2* gen fibrogenese onafhankelijk van de aanwezigheid van chronische leverschade beïnvloeden. De associatie tussen *IFNGR2* en LS werd ook gezien in een subgroep van deelnemers met NAFLD. In deze subgroep was namelijk een derde SNP in het *IFNGR2* gen, *rs9808753,* onafhankelijk geassocieerd met LS. We ontdekten ook dat de associatie tussen deze drie *IFNGR2* SNPs en fibrose significant werd veranderd door de body mass index (BMI). De associatie tussen de *IFNGR2* SNPs en fibrose was sterker voor deelnemers met overgewicht (voor *rs9808753*) en deelnemers met obesitas (voor *rs9976971* en *rs2284553*) vergeleken met het totale cohort. Deze bevinding zou verklaard kunnen worden door de aanwezigheid van een systemische, chronische, pro-inflammatoire status in obesitas, die op haar beurt het effect van interferon gamma op de ontwikkeling van lever fibrose zou kunnen versterken (26, 27). Toekomstige studies zouden zich moeten richten op het ontdekken van het mechanisme waarmee *IFNGR2* fibrogenese beïnvloedt, aangezien dit mechanisme aangrijpingspunten voor de ontwikkeling van interferon gamma-gebaseerde antifibrotische therapie zou kunnen geven.

TROMBOFILIE IN LEVER FIBROGENESE

Lever fibrose is een multifactoriële aandoening. Naast chronische leverschade en genetische aanleg spelen andere factoren, zoals hypercoagulabiliteit, een belangrijke rol in

de pathogenese. De eerste aanwijzing dat hypercoagulabiliteit een rol speelt in fibrogenese was de observatie dat patiënten die zowel chronische hepatitis C als hemofilie hadden een trage progressie hadden van hun leverziekte (28, 29). Verschillende studies toonden later dat aanwezigheid van protrombotische mutaties – waaronder het factor V Leiden polymorfisme, protrombine G20210A variant en bloed groep type non-O – de ontwikkeling en/of progressie van fibrose versnelden in patiënten met chronische virale hepatitis en NAFLD (30-36). In **hoofdstuk 4** hebben we een overzicht gegeven van het beschikbare bewijs over het effect van anticoagulantia op fibrogenese. Samenvattend suggereren studies dat het geven van anticoagulantia fibrogenese zou kunnen voorkomen en reeds gevormde fibrose zou kunnen verminderen (37-43). Daarnaast hebben we in dit hoofdstuk de in de literatuur gepostuleerde hypothese over het mechanisme waarmee hypercoagulabiliteit lever fibrogenese beïnvloedt beschreven. Trombine speelt een essentiële rol in deze hypothese.

Om de invloed van trombofilie op lever fibrogenese verder te bestuderen, hebben we de associatie tussen veelvoorkomende protrombotische genetische risicofactoren en lever fibrose in deelnemers van het ERGO cohort onderzocht in **hoofdstuk 5**. Het hebben van een factor V Leiden mutatie or protrombine G20210A gen variant was geassocieerd met een twee keer verhoogd risico op klinisch relevante lever fibrose in dit Kaukasische cohort bestaande uit personen uit de algemene bevolking. Deze associatie was onafhankelijk van andere risicofactoren voor lever fibrose, waaronder aanwezigheid van steatose, diabetes mellitus, roken, leeftijd, ALT waarde en alcohollinname. Gecombineerde aanwezigheid van bloed groep type non-O en het factor V Leiden polymorfisme of protrombine G20210A gen variant leidde tot een nog hoger risico op fibrose. Deelnemers met zowel bloed groep type non-O als het factor V Leiden polymorfisme of de protrombine G20210A gen variant hadden een voorspelde waarschijnlijkheid van 14.3% op het hebben van fibrose. Deze kans was significant hoger dan de kans voor deelnemers met bloed groep type O en/of deelnemers zonder het factor V Leiden polymorfisme of de protrombine G20210A gen variant. Deze resultaten zouden mogelijk kunnen worden verklaard door het feit dat zowel het factor V Leiden polymorfisme als bloed groep type non-O de gevoeligheid voor geactiveerd proteïne C verminderen. Hierdoor ontstaat een extra verhoogde procoagulante capaciteit wanneer beide factoren aanwezig zijn (44-46).

Wij concludeerden dat deze resultaten suggereren dat trombofilie een belangrijke factor is in lever fibrogenese in de algemene bevolking. Screening op aanwezigheid van veelvoorkomende protrombotische genetische risicofactoren zou daarom aangeraden kunnen worden in personen met lever fibrose van onbekende origine, aangezien deze personen potentieel voordeel zouden kunnen hebben van behandeling met anticoagulantia.

Von Willebrand factor (VWF) is een essentiële factor in hemostase, aangezien het betrokken is bij de adhesie van trombocyten, trombusvorming en binding van stollingsfactor VIII, waarmee het zijn klaring voorkomt (47, 48). Voorgaande studies hebben

laten zien dat hoge VWF waarden geassocieerd zijn met een verhoogd risico op zowel veneuze als arteriële trombose (49-51). Het is bekend dat VWF waarden verhoogd zijn in patiënten met cirrose. In deze patiënten correleren VWF waarden met de hepatische veneuze drukgradiënt en kunnen ze zelfs klinische uitkomsten voorspellen (52, 53). In **hoofdstuk 6** toonden we dat verhoogde VWF waarden geassocieerd zijn met de aanwezigheid van lever fibrose na 10 jaar in de algemene bevolking en in een subgroep van personen met steatose. Een recente studie in patiënten met hepatitis C liet zien dat VWF waarden stegen met een toenemend fibrosestadium (54). De aard van de relatie tussen VWF en fibrose en cirrose is echter nog onbekend. Daarom zijn toekomstige studies nodig die het mechanisme achter deze associatie onderzoeken. Omdat onze studie suggereert dat VWF gebruikt zou kunnen worden als mogelijke marker van preklinische lever fibrose in de algemene bevolking zijn er ook studies nodig die zich richten op de rol van VWF waarden in de predictie van ontwikkeling en progressie van fibrose. Tot slot blijft het onopgehelderd of VWF waarden ook gebruikt kunnen worden om klinische uitkomsten in personen met fibrose te voorspellen.

ETIOLOGIE EN ISCHEMISCHE COMPLICATIES VAN VASCULAIRE LEVERZIEKTEN

Budd-Chiari syndroom (BCS) en niet-maligne, niet-cirrotische vena portae trombose (PVT), samen SVT genoemd, zijn zeldzame vormen van veneuze trombose van respectievelijk de afvoerende bloedvaten van de lever en de vena portae. SVT is geassocieerd met een aanzienlijke morbiditeit en mortaliteit (55, 56). Door de lage prevalentie zijn er echter weinig prospectieve studies naar de etiologie, diagnose, behandeling en prognose van deze aandoeningen gedaan. In hoofdstuk 4 hebben we op basis van de beschikbare literatuur een overzicht gegeven van de rol van anticoagulantia in de behandeling van vasculaire leverziekten, waaronder BCS en PVT.

Systemische protrombotische risicofactoren zijn cruciaal in de etiologie van SVT, net zoals bij de meer gebruikelijke vormen van veneuze trombose. Binnen deze groep risicofactoren worden genetische en verworven aandoeningen onderscheiden. Myeloproliferatieve neoplasieën (MPNs) zijn de meest voorkomende verworven risicofactor voor SVT, met een prevalentie van 20-50% (57, 58). Het diagnosticeren van MPNs in patiënten met SVT is belangrijk, gezien de prognose en de implicaties voor de behandeling met anticoagulantia (59, 60). Het is echter lastig om aanwezigheid van MPNs vast te stellen in de klinische praktijk, aangezien portale hypertensie veroorzaakt door obstructie van de hepatische venen en/of vena portae leidt tot hypersplenisme en hemodilutie. Deze omstandigheden kunnen de karakteristieke veranderingen van MPNs in het perifere bloed, zoals trombocytose en polycytemie, verhullen (61). De ontdekking van de *JAK2V617F* mutatie heeft tot een grote verbetering geleid in het niet-invasief diagnosticeren van MPNs in patiënten met SVT (62-64). Het blijft echter lastig om

MPNs te diagnosticeren in de afwezigheid van de *JAK2V617F* mutatie (59, 65). Exome sequencing heeft recent geresulteerd in de ontdekking van somatische mutaties in *CALR* in patiënten met MPN zonder de *JAK2V617F* en *MPL* mutaties (66, 67). Wij hebben daarop de hypothese opgesteld dat identificatie van *CALR* mutaties in patiënten met SVT het diagnosticeren van MPNs in deze patiënten zou verbeteren. In **hoofdstuk 7** hebben we de prevalentie en rol van *CALR* mutaties in patiënten met SVT geëvalueerd. Deze studie was gebaseerd op data van een uniek patiëntencohort dat het resultaat is van een Europees samenwerkingsverband (European Network for Vascular Disorders of the Liver (EN-Vie)). Voor het EN-Vie studiecohort werden patiënten met BCS en niet-maligne, niet-cirrotische PVT geïncludeerd afkomstig uit negen Europese landen. Van alle patiënten werd systemisch data verzameld over de etiologie, klinische presentatie, diagnose en follow-up. Daarnaast werd DNA en plasma opgeslagen voor toekomstige analyses (57, 58). Gebruikmakend van deze DNA-monsters vonden we dat *CALR* mutaties zeldzaam zijn in patiënten met SVT, aangezien slechts één patiënt in ons cohort een *CALR* mutatie had. Als gevolg hiervan was de prevalentie van *CALR* mutaties 0.7% in het totale cohort en 2.3% in de patiënten met MPN. Onze bevindingen werden bevestigd door andere studies, de beschreven prevalentie van *CALR* mutaties in patiënten met SVT varieert tussen 0 en 2% (68-72). Deze lage prevalentie zou verklaard kunnen worden door het feit dat patiënten met een *CALR* mutatie een lager risico op trombose hebben dan patiënten met een *JAK2V617F* mutatie. Dit zou kunnen komen door de aanwezigheid van een lager hemoglobine gehalte en een kleiner aantal witte bloedcellen in patiënten met een *CALR* mutatie vergeleken met MPN patiënten zonder een *CALR* mutatie (66, 67). Desalniettemin geloven we dat men zou moeten testen op de aanwezigheid van *CALR* mutaties in patiënten met SVT zonder de *JAK2V617F* mutatie, aangezien screening naar somatische *CALR* mutaties een eenvoudig diagnosticum is dat zou kunnen helpen in de lastige diagnose van MPN met een beperkte belasting voor de patiënt. Ook de recent gepubliceerde richtlijnen van de Baveno VI expert groep adviseren om te screenen op aanwezigheid van *CALR* mutaties in *JAK2V617F* negatieve SVT patiënten (73).

Ondanks het feit dat meerdere genetische en verworven risicofactoren voor SVT bekend zijn, kan in 15-25% van de patiënten met SVT geen etiologische factor worden ontdekt (57, 58). Daarom zijn studies nodig gericht op het ontdekken van nieuwe risicofactoren. Gezien het feit dat veel risicofactoren voor de meer gebruikelijke vormen van veneuze trombose ook een rol spelen bij de etiologie van SVT, hebben we onderzocht of nieuwe kandidaat SNPs, waarvan we weten dat ze het risico op de meer gebruikelijke vormen van veneuze trombose verhogen, een risicofactor zijn voor SVT in **hoofdstuk 8**. We hebben zes SNPs – in het *ABO, STXBP5*, *VWF, CYP4V2, GP6 en SERPINC1* gen – onderzocht waarvan we weten dat ze het risico op de meer gebruikelijke vormen van veneuze trombose verhogen. Hiervoor hebben we DNA monsters gebruikt van patiënten en controles van het EN-Vie studie cohort (74-77). In dit cohort was de SNP die het bloed groep type bepaalt geassocieerd met een verhoogd risico op PVT, maar niet

op BCS. Deze bevinding kon echter niet worden gevalideerd in een cohort bestaande uit patiënten gediagnosticeerd met SVT in drie grote tertiaire verwijzingscentra in Frankrijk, Spanje en Nederland. De andere vijf SNPs waren niet geassocieerd met een verhoogd risico op PVT of BCS in het EN-Vie cohort. Geen van de zes SNPs kan daarom worden beschouwd als een risicofactor voor BCS of PVT. Deze resultaten suggereren dat andere lokale en systemische risicofactoren een grotere rol spelen in de etiologie van SVT. Het ontdekken van deze verschillen in de etiologie van SVT en de meer gebruikelijke vormen van veneuze trombose zorgt voor meer inzicht in de pathogenese van veneuze trombose op verschillende plaatsen in het lichaam en ondersteunt de locatieafhankelijkheid van veneuze trombose.

Gastro-intestinale ischemie wordt beschouwd als een ernstig gevolg van PVT, omdat het kan resulteren in intestinale infarcering, een levensbedreigende aandoening (78). De prevalentie en karakteristieken van gastro-intestinale ischemie in PVT zijn echter niet goed bekend. De beschreven prevalentie van gastro-intestinale ischemie in patiënten met acute PVT varieert tussen 2% en 32% met een mortaliteit van 0-20% (58, 79-84). Er zijn nauwelijks studies naar ischemie in patiënten met chronische PVT en de studies die beschikbaar zijn, rapporteren conflicterende resultaten. Sommige stellen dat het optreden van ischemie onwaarschijnlijk is bij deze patiënten vanwege de aanwezigheid van een collaterale circulatie, terwijl anderen een portaal cavernoom vonden in bijna de helft van de patiënten met SVT die zich presenteerden met ischemie (78, 80). Het gebrek aan data over het voorkomen van ischemie in PVT komt, in ieder geval deels, door de afwezigheid van goede diagnostische methodes. Het is lastig om de diagnose ischemie te stellen gebaseerd op alleen klinische symptomen, aangezien deze vaak atypisch zijn en vergelijkbaar met de klinische bevindingen bij patiënten met PVT. De introductie van zichtbaar licht spectroscopie ('visible light spectroscopy', VLS) biedt een nieuwe, minimaal invasieve methode om gastro-intestinale ischemie te diagnosticeren in PVT (85). Met behulp van VLS kan een objectieve en directe kwantificatie van ischemie in het maagdarmkanaal worden verkregen, door de mucosale perfusie te meten tijdens gastroscopie (85, 86). Daarom hebben wij prospectief de aanwezigheid en karakteristieken van gastro-intestinale ischemie in patiënten met PVT bepaald met behulp van VLS in **hoofdstuk 9**. Gastro-intestinale ischemie kwam frequent voor in deze patiënten met voornamelijk chronische PVT. In 75% van de patiënten werd een verlaagde mucosale oxygenatie gemeten op minimaal 1 plaats in het maagdarmkanaal. VLS metingen waren voornamelijk verlaagd in het pars descendens van het duodenum. Typische symptomen van ischemie, zoals postprandiale pijn en inspanningsgerelateerde pijn waren aanwezig in 63% van de patiënten. Uitbreiding van de trombose naar de vena mesenterica superior en de vena lienalis en/of aanwezigheid van hypercoagulabiliteit waren geassocieerd met lagere VLS metingen in patiënten met PVT vergeleken met historische controles. Daarnaast zagen we dat VLS metingen hoger waren in patiënten die behandeld werden met orale anticoagulantia vergeleken met patiënten die niet of slechts gedeeltelijk behandeld werden, dit verschil was echter niet

statistisch significant. Deze bevinding suggereert echter wel dat het gebruik van orale anticoagulantia een positief effect zou kunnen hebben op het optreden van ischemie in patiënten met chronische PVT. Bevestiging van deze resultaten in grotere studies is noodzakelijk, maar onze bevindingen suggereren dat het optreden van abdominale symptomen, zoals postprandiale pijn, zouden moeten leiden tot herbeoordeling van de uitbreiding, oorzaak en behandeling van PVT.

AANBEVELINGEN VOOR TOEKOMSTIG ONDERZOEK

Lever fibrose komt voor bij ongeveer 1 op de 18 oudere Kaukasische volwassenen in Rotterdam, zoals beschreven in dit proefschrift. Voornamelijk personen met steatose en diabetes mellitus lopen een verhoogd risico, hun kans op fibrose is zelfs 17%. Gezien de huidige stijging van de prevalentie van steatose en diabetes mellitus in de Westerse wereld, is de verwachting dat het aantal personen met fibrose op basis van NAFLD zal toenemen en belangrijke gevolgen voor de volksgezondheid zal hebben (4). Brede implementatie van programma's gericht op de preventie van obesitas, insulineresistentie en hun complicaties is daarom hard nodig. Leefstijlinterventies, waarvan afvallen en lichamelijke inspanning het meest belangrijk zijn, zijn de hoeksteen van de behandeling van NAFLD, maar het volhouden van deze leefstijlmaatregelen blijkt in de praktijk vaak lastig (4). Een recente trial in patiënten met hart- en vaatziekten liet zien dat het regelmatig sturen van semi-gepersonaliseerde tekstberichten cardiovasculaire risicofactoren, zoals BMI, roken en de hoeveelheid lichamelijke inspanning, kan verbeteren (87). Dergelijke ondersteunende programma's zouden ook gunstige effecten kunnen hebben op het volhouden van leefstijlveranderingen in patiënten met NAFLD. Onderzoek naar het effect van deze programma's in patiënten met NAFLD is daarom gewenst. Op dit moment is er nog geen medicament goedgekeurd voor de behandeling van deze groep patiënten, ondanks verschillende studies naar mogelijke medicamenten, waardoor andere behandelopties beperkt zijn. Studies gericht op het bepalen van de mortaliteit veroorzaakt door fibrose in de algemene bevolking zullen helpen in het bepalen van de impact van dit volksgezondheidsprobleem.

Gedurende het laatste decennium was er een exponentiele toename van het aantal studies gericht op de rol van genetica in verschillende complexe ziekten, waaronder leverziekten. Het huidige aanbod aan studies vormt echter slechts de eerste stap in de ontrafeling van de complexe samenhang tussen genetische varianten en leverziekten. Verschillende veelbelovende onderzoeksrichtingen zijn daarom nog onontgonnen. Op dit moment zijn verschillende SNPs geïdentificeerd als risicofactor voor fibrose (13, 16, 17, 20, 23-25). Het zou erg interessant zijn om een GWAS in het ERGO te verrichten om genetische varianten die bijdragen aan de ontwikkeling van lever fibrose in de algemene bevolking te identificeren. Bovendien is het door de snel-

le ontwikkeling van technieken nu mogelijk om sequenties van exomen en zelfs hele menselijke genomen te bepalen. Deze nieuwe technieken zullen de komende jaren nog meer mogelijkheden bieden om loci te identificeren die geassocieerd zijn met diverse leverziekten.

De observatie dat het geven van laag moleculair gewicht heparine de incidentie van lever decompensatie en de overleving in patiënten met cirrose verbetert (43) behoeft verder onderzoek in grote, dubbelblinde trials gericht op de potentieel gunstige rol van anticoagulantia in deze patiënten. In deze studies zou ook een objectieve bepaling van portale hypertensie verricht moeten worden. Aanvullende onderzoeksvragen die nog beantwoord zouden moeten worden, omvatten onder andere het effect van andere anticoagulantia, waaronder nieuwe orale anticoagulantia (NOACs) en de optimale behandeldosis en –duur. Bovendien zou het ook wenselijk zijn om het effect van anticoagulantia op lever fibrose in verschillende populaties – waaronder risicogroepen en de algemene bevolking – te onderzoeken, omdat eerdere studies ook een potentieel gunstig effect van anticoagulantia op fibrose hebben laten zien (37-42).

In patiënten met PVT wordt op dit moment alleen langdurige behandeling met anticoagulantia aangeraden indien er sprake is van een persisterende protrombotische aandoening, terugkerende trombose of een intestinaal infarct (73). Prospectieve studies naar de risico's en voordelen van behandeling met anticoagulantia in patiënten met chronische niet-maligne, niet-cirrotische PVT zonder deze aandoeningen zijn nodig, aangezien de rol van anticoagulantia in de behandeling van deze patiënten onbekend is. In de laatste jaren zijn er twee types direct werkende orale anticoagulantia geïntroduceerd: directe trombineremmers en factor Xa-remmers. Een groot voordeel van deze medicijnen is dat laboratoriumcontrole niet nodig is. Daarnaast hebben ze minder interacties met andere medicijnen en voeding en hebben ze een grotere therapeutische breedte. In een grote meta-analyse van bijna 28.000 deelnemers waren NOACs net zo effectief als standaard anticoagulantia in de behandeling van diep veneuze trombose (88). Ondanks het feit dat een verhoogd risico op gastro-intestinale bloedingen is beschreven in patiënten die behandeld worden met NOACs (89), laten studies in patiënten met veneuze trombo-embolieën een vergelijkbaar of zelfs verlaagd risico op bloedingen geassocieerd met NOACs zien vergeleken met de standaardbehandeling met vitamine K antagonisten (88, 90, 91). Het gebruik van NOACs in patiënten met veelvoorkomende vormen van veneuze trombose wordt op dit moment geïmplementeerd in de klinische praktijk. Het is belangrijk om de plaats van deze nieuwe orale anticoagulantia in de behandeling van patiënten met SVT te bepalen. Trials gericht op het vergelijken van de effectiviteit en veiligheid van deze nieuwe orale anticoagulantia met de standaard anticoagulantia zouden daarom moeten worden geïnitieerd in patiënten met SVT.

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Chapter 12

12

Appendices

ABBREVIATIONS

ALP	alkaline phosphatise
ALT	alanine aminotransferase
APA	antiphospholipid antibody
APC	activated protein C
APTT	activated partial thromboplastin time
AST	aspartate aminotransferase
BCS	Budd-Chiari syndrome
BMI	body mass index
BP	blood pressure
CT-A	CT-angiography
DM	diabetes mellitus
DVT	deep venous thrombosis
EN-Vie	European network for vascular disease of the liver
ET	essential thrombocythemia
FPG	fasting plasma glucose
FVL	factor V Leiden
FVIII	factor VIII
GGT	gamma glutamyl transferase
GWAS	genome-wide association studies
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
HDL-C	high-density lipoprotein cholesterol
HIV	human immunodeficiency virus
HSC	hepatic stellate cells
HWE	Hardy-Weinberg equilibrium
HOMA-IR	homeostasis model assessment of insulin resistance
IFNGR2	interferon gamma receptor 2
IL28B	interleukin 28B
INCPH	idiopathic noncirrhotic portal hypertension
INR	international normalized ratio
IQR	interquartile range
IU	international units
LMWH	low molecular weight heparin
LS(M)	liver stiffness (measurement)
MPN	myeloproliferative neoplasm
MR-A	MR-angiography
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NOAC	new oral anticoagulant

OAC	oral anticoagulation
PE	pulmonary embolism
PAR	protease activated receptor
PMF	primary myelofibrosis
PNPLA3	patatin-like phospholipase domain-containing protein 3
PT	prothrombin time
PV	polycythemia vera
PVT	portal vein thrombosis
SD	standard deviation
SECF	spontaneous erythroid colony formation
SNP	single nucleotide polymorphism
SOS	sinusoidal obstruction syndrome
SMV	superior mesenteric vein
SV	splenic vein
SVT	splanchnic vein thrombosis
ULN	upper limit of normal
TE	transient elastography.
TIPS	transjugular intrahepatic portosystemic shunt
VKA	vitamin K antagonists
VLS	visible light spectroscopy
VTE	venous thromboembolism
VWF	von Willebrand factor

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2. **Elisabeth P.C. Plompen**, Sarwa Darwish Murad, Bettina E. Hansen, Daan W. Loth, Jeoffrey N.L. Schouten, Pavel Taimr, Moniek P.M. de Maat, Albert Hofman, André G. Uitterlinden, Bruno H. Stricker, Harry L.A. Janssen, Frank W.G. Leebeek, *Is von Willebrand factor level a predictive marker of liver fibrosis in the general population? The Rotterdam Study,* manuscript submitted
3. Arjola Bano*, Layal Chaker*, **Elisabeth P.C. Plompen**, Albert Hofman, Abbas Dehghan, Oscar H. Franco, Harry L.A. Janssen, Sarwa Darwish Murad, Robin P. Peeters, *Thyroid function and the risk of non-alcoholic fatty liver disease: The Rotterdam Study*, manuscript submitted
4. Willem P. Brouwer, Adriaan J. van der Meer, André Boonstra, **Elisabeth P.C. Plompen**, Suzan D. Pas, Robert J. de Knegt, Rob A. de Man, Fiebo J.W. ten Kate, Harry L.A. Janssen, Bettina E. Hansen, *Prediction of long-term clinical outcome in a diverse chronic hepatitis B population: role of the PAGE-B score,* manuscript submitted
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9. **Elisabeth P.C. Plompen,** Peter JM Valk, Isabel Chu, Sarwa Darwish Murad, Aurelie Plessier, Fanny Turon, Jonel Trebicka, Massimo Primignani, Juan-Carlos Garcia-Pagan, Dominique C. Valla, Harry L.A. Janssen, Frank W.G. Leebeek, *Somatic Calreticulin mutations in patients with Budd-Chiari syndrome and portal vein thrombosis*, Haematologica 2015 Jun; 100(6):e226-8
10. **Elisabeth P.C. Plompen**, Bettina E. Hansen, Jeoffrey N.L. Schouten, Sarwa Darwish Murad, Daan W. Loth, Willem P. Brouwer, Aaron Isaacs, Pavel Taimr, Albert Hofman, Cornelia M. van Duijn, André G. Uitterlinden, Bruno H. Ch. Stricker, Frank W.G. Leebeek, Harry L.A. Janssen; *Gene variants in the interferon gamma receptor 2 gene are associated with liver fibrosis in the general population: Results from the Rotterdam Study*, Gut. 2015 Apr;64(4):692-4
11. **E.P.C. Plompen**, J.N.L. Schouten, H.L.A. Janssen; *Role of anticoagulant therapy in liver disease*, Hepatology International 2013 Jun; 7(2):369-76
12. **E.P.C. Plompen**, R.P.L.M. Hoogma en R.G.W. Nijman; *Verhoogde waarden van troponine in de zwangerschap: goede parameter voor myocardschade of fysiologisch fenomeen?*, Nederlands tijdschrift voor obstetrie en gynaecologie 2012 mei; 128

PHD PORTFOLIO

Name PhD student:	Elisabeth P.C. Plompen
PhD period:	October 2011-February 2016
Erasmus MC department:	Gastroenterology and Hepatology
Promotoren:	Prof. dr. H.L.A. Janssen and Prof. dr. F.W.G. Leebeek

Courses and workshops

	Year	Workload
Genetics for dummies, Molecular medicine postgraduate school, Rotterdam	2011	8 hours
SNP course, Molecular medicine postgraduate school / Netherlands institute for Health Sciences (NIHES), Rotterdam	2011	40 hours
Introduction course on statistics & survival analysis, Molecular medicine postgraduate school, Rotterdam	2011	8 hours
Biostatistics for clinicians, Netherlands institute for Health Sciences (NIHES), Rotterdam	2012	40 hours
Regression analysis for clinicians, Netherlands institute for Health Sciences (NIHES), Rotterdam	2012	40 hours
Browsing genes and genomes with UCSC, Molecular medicine postgraduate school, Rotterdam	2012	8 hours
BROK-cursus, Consultatiecentrum Patiëntgebonden onderzoek (CPO), Erasmus MC, Rotterdam. Certificate Good Clinical Practice obtained	2012	24 hours
Abdominal ultrasonography course, Dutch liver week 2012, Rotterdam	2012	10 hours
Intensive course "English for Professionals", Oxford, United Kingdom	2012	40 hours
Genome wide association analysis, Netherlands institute for Health Sciences (NIHES), Rotterdam	2012	40 hours
Integrity in scientific research, Dept. of Medical ethics and philosophy, Erasmus MC, Rotterdam	2012	16 hours
Methodology of clinical research and preparation of grant applications Consultatiecentrum Patiëntgebonden onderzoek (CPO), Erasmus MC, Rotterdam	2013	8 hours
Training "omgaan met groepen", Erasmus MC, Rotterdam	2013	4 hours
Biomedical English writing and communication, Erasmus MC, Rotterdam	2013	40 hours
Medical Business masterclass, Amsterdam, The Netherlands	2015	12 hours

Oral presentations

	Year	Workload
Von Willebrand factor levels are independently associated with liver stiffness: results of a population-based study. Twice annual meeting of the Netherlands association of Hepatology, Zeist, The Netherlands	2012	12 hours

Gene variants in the interferon gamma receptor 2 gene are associated with liver stiffness in the general population: Results of a population-based study. Twice annual meeting of the Netherlands association of Hepatology, Veldhoven, The Netherlands	2013	12 hours
Genetics in liver disease, 11th Post-AASLD symposium, Rotterdam, The Netherlands	2013	24 hours
Prothrombotic genetic risk factors are associated with liver stiffness in the general population: results from the Rotterdam Study. 49th Annual meeting of the European Association of the Study of the Liver (EASL), London, United Kingdom	2014	36 hours
Blood group non-O is a risk factor for portal vein thrombosis. Twice annual meeting of the Netherlands association of Hepatology, Veldhoven, The Netherlands	2014	12 hours
Factors associated with and prevalence of liver fibrosis in a general elderly population: results from the Rotterdam Study. 65th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America	2014	36 hours
Low prevalence of positive viral serology for HBV and HCV among a general Dutch elderly population: results from the Rotterdam Study. Early morning workshop "Public Health and viral hepatitis: what can we do to reduce the future burden of disease?" 50th Annual meeting of the European Association of the Study of the Liver (EASL), Vienna, Austria	2015	24 hours

Poster presentations

	Year	Workload
Genetic risk factors and history of venous thromboembolism in relation to liver fibrosis in the elderly. 63th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America	2012	12 hours
Von Willebrand factor levels are independently associated with liver stiffness: results of a population-based study. 63th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America	2012	12 hours
Von Willebrand factor levels and variations in the von Willebrand factor gene independently influence liver stiffness: the Rotterdam study. 48th Annual meeting of the European Association of the Study of the Liver (EASL), Amsterdam, The Netherlands. ***Abstract rated top 10% of all abstracts***	2013	12 hours
Gene variants in the interferon gamma receptor 2 gene are associated with liver stiffness in the general population: Results of a population-based study. 64th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Washington DC, United States of America. ***Presidential poster of distinction, abstract rated top 10% of all abstracts***	2013	12 hours
Gastrointestinal ischemia in patients with acute and chronic portal vein thrombosis. 49th Annual meeting of the European Association of the Study of the Liver (EASL), London, United Kingdom	2014	12 hours
Gastrointestinal ischemia in patients with acute and chronic portal vein thrombosis. Digestive Disease Week 2014, Chicago, IL, United States of America	2014	12 hours
Blood group non-O is a risk factor for portal vein thrombosis. 65th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America	2014	12 hours

Low prevalence of positive viral serology for HBV and HCV among a general Dutch elderly population: results from the Rotterdam Study. 50th Annual meeting of the European Association of the Study of the Liver (EASL), Vienna, Austria. ***Abstract rated top 10% of all abstracts***	2015	12 hours

Attended (inter)national conferences

	Year	Workload
47th Annual meeting of the European Association of the Study of the Liver (EASL), Barcelona, Spain	2012	28 hours
Monothematic conference on vascular liver diseases, European Association of the Study of the Liver (EASL), Tallinn, Estonia	2012	16 hours
Twice annual meeting of the Netherlands association of Hepatology, Zeist, The Netherlands	2012	12 hours
63th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America	2012	28 hours
48th Annual meeting of the European Association of the Study of the Liver (EASL), Amsterdam, The Netherlands	2013	28 hours
5th International congress on coagulopathy in liver disease, Padua, Italy	2013	16 hours
Twice annual meeting of the Netherlands association of Hepatology, Veldhoven, The Netherlands	2013	12 hours
64th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Washington DC, United States of America	2013	28 hours
49th Annual meeting of the European Association of the Study of the Liver (EASL), London, United Kingdom	2014	28 hours
Twice annual meeting of the Netherlands association of Hepatology, Veldhoven, The Netherlands	2014	12 hours
65th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America	2014	28 hours
50th Annual meeting of the European Association of the Study of the Liver (EASL), Vienna, Austria	2015	28 hours
66th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America	2015	28 hours

Awards

	Year
Registration bursary from the European Association of the Study of the Liver (EASL) awarded for the best abstracts by young investigators	2013
Full bursary from the European Association of the Study of the Liver (EASL) for the best abstracts by young investigators	2014
Young investigators award from the American Association for the Study of Liver Diseases (AASLD) for the best abstracts by young investigators	2014
Full bursary from the European Association of the Study of the Liver (EASL) for the best abstracts by young investigators	2015

Attended seminars

	Year	Workload
26th Erasmus Liver day. Rotterdam, The Netherlands	2011	6 hours
9th Post-AASLD symposium. Rotterdam, The Netherlands	2011	2 hours
27th Erasmus Liver day. Rotterdam, The Netherlands	2012	6 hours
10th Post-AASLD symposium. Rotterdam, The Netherlands	2012	2 hours
28th Erasmus Liver day. Rotterdam, The Netherlands	2013	6 hours
11th Post-AASLD symposium. Rotterdam, The Netherlands	2013	2 hours
5th Lagerhuisdebat Hepatitis B and C. Utrecht, The Netherlands	2013	2 hours
1st Pancreasdag. Utrecht, The Netherlands	2014	6 hours

Memberships

Netherlands Association of Gastroenterology (NVGE)

Netherlands Association of Hepatology (NVH)

European Association of the Study of the Liver (EASL)

American Association for the Study of Liver Diseases (AASLD)

Reviewing for scientific journals

New England Journal of Medicine, Hepatology, Scientific reports, Acta Gastroenterologica Belgica

Educational activities and lecturing

	Year	Workload
Budd-Chiari syndrome and portal vein thrombosis, 2nd year curriculum Medicine, Erasmus University Rotterdam, Rotterdam, The Netherlands	2013	6 hours
Tutoring first year students curriculum Medicine, Erasmus University Rotterdam, Rotterdam, The Netherlands	2013-2014	40 hours
Budd-Chiari syndrome and portal vein thrombosis, 2nd year curriculum Medicine, Erasmus University Rotterdam, Rotterdam, The Netherlands	2014	6 hours
Tutoring introduction clinical practice for first year students curriculum Medicine, Erasmus University Rotterdam, Rotterdam, The Netherlands	2014	10 hours
Genetics in liver disease, Cursus klinische hepatologie, Dutch liver week, Amsterdam, The Netherlands	2014	24 hours
Budd-Chiari syndrome and portal vein thrombosis, 2nd year curriculum Medicine, Erasmus University Rotterdam, Rotterdam, The Netherlands	2015	6 hours

Abdominal ultrasonography and transient elastography, Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands	2015	6 hours
Moderator oral session on imaging and noninvasive markers of liver disease, 66th Annual meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America	2015	6 hours

DANKWOORD

Met het schrijven van dit dankwoord sluit ik een geweldige periode van 4 jaar af. Vooraf had ik niet verwacht dat ik de kans zou krijgen om met zoveel verschillende mensen te mogen samenwerken. Ik heb dit als een groot voorrecht ervaren. Ik wil iedereen die op welke manier dan ook heeft bijgedragen aan de totstandkoming van dit proefschrift heel erg bedanken. Een aantal van hen wil ik graag in het bijzonder noemen.

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Lisanne
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ABOUT THE AUTHOR

Elisabeth (Lisanne) PC Plompen was born on June 10th 1986 in Bergen op Zoom and grew up in Ossendrecht, the Netherlands. She attended Gymnasium Juvenaat in Bergen op Zoom, where she graduated in 2004 with cum laude honors. She started Medical School at the University of Leiden in the same year. During the bachelor phase of this study, she conducted extra-curricular research at the department of Thrombosis and Hemostasis at the Leiden University Medical Center. She investigated bleeding complications associated with low molecular weight heparin use in patients with venous thromboembolism in daily clinical practice in three hospitals in the Leiden region. In 2011, she obtained her bachelor's degree and qualification as a Medical Doctor, both with cum laude honors. Before starting the work described in this thesis, she worked a few months as a resident not in training (ANIOS) at the department of Internal Medicine of the Bronovo Hospital, The Hague. In October 2011, she started her PhD trajectory as described in this thesis at the department of Gastroenterology and Hepatology of the Erasmus MC University Medical Center under supervision of Prof. dr. Harry L.A. Janssen and Prof. dr. Frank W.G. Leebeek. As of July 2015, she started with her two-year Internal Medicine residency at Reinier de Graaf Gasthuis in Delft (program director dr. H. Boom) as part of the formal postgraduate training in Gastroenterology and Hepatology. Hereafter, she will continue her training in Gastroenterology and Hepatology, both at Reinier de Graaf Gasthuis (program director dr. J.T. Brouwer) and Erasmus MC University Medical Center Rotterdam (program director Prof. dr. R.A. de Man).